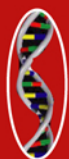


Food Science and Technology



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Phytochemicals and Human Health

Pharmacological and Molecular Aspects

A Tribute to Late Professor
Bimal Kumar Bachhawat



Akhlaq A. Farooqui
Tahira Farooqui
Editors



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PHYTOCHEMICALS AND HUMAN HEALTH: PHARMACOLOGICAL AND MOLECULAR ASPECTS

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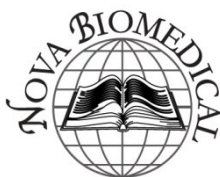
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FOOD SCIENCE AND TECHNOLOGY

**PHYTOCHEMICALS AND HUMAN
HEALTH: PHARMACOLOGICAL
AND MOLECULAR ASPECTS**

**AKHLAQ A. FAROOQUI
AND
TAHIRA FAROOQUI
EDITORS**



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Dedication



A Tribute to the Late Professor Bimal Kumar Bachhawat (1925 – 1996)

This book is dedicated to the memory of late Professor Bimal Kumar Bachhawat, an outstanding scientist, leader, organizer, and teacher, who nurtured generations of biochemists in India. He will be missed by the international biochemistry community.

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Foreword

BIMAL KUMAR BACHHAWAT, Ph.D.

Department of Biochemistry, University of Delhi, India

Late Professor B.K. Bachhawat was born on 16th August, 1925, in Bengal. After receiving his Master's degree in Applied Chemistry from Calcutta, India, he came to University of Illinois, USA, and received his Ph. D with Dr. Carl Vestling in 1953. He worked as Research Associate and then accepted a position as Assistant Professor in University of Pennsylvania and University of Michigan, Ann Arbor, USA, respectively, till 1957. He returned to India, joined as Assistant Professor, became full Professor, and then Chief of Neurochemistry laboratory, Christian Medical College, Vellore. Late Professor Bachhawat was responsible for establishing the neurochemistry laboratory and an active research program in the area of Neurochemistry and glycobiology in Vellore. In 1976, he joined Indian Institute of Chemical Biology as Director and continued till 1985. In September 1985, he joined as Professor and Head, Department of Biochemistry, University of Delhi South Campus, New Delhi. He became Dean, Faculty of Interdisciplinary and Applied Sciences, University of Delhi South Campus from 1988-1990. From 1990 till 1995 he continued as Bhatnagar Fellow (Council for Scientific and Industrial Research) in the Department of Biochemistry, University of Delhi South Campus. He was elected to all of the scientific academies in India (such as Fellow of National Academy of Sciences, Indian Academy of Sciences and Indian National Science Academy). He received numerous national awards and honors, including the Shanti Swarup Bhatnagar award (1962), the Golden Jubilee Medal (1976) in India. He was elected President of Federation of Asian and Oceanian Biochemistry (1983-1985), Indian Society of Biological Chemists (1970-1972 and 1990-1994); and National Organizing Committee, International Union of Biochemistry and Molecular Biology (1994).

Late Professor Bachhawat was a leader, organizer, and teacher and who nurtured a generation of biochemists not only in India, but around the world. He was a man of many admirable qualities and was known for his keen interest in others. Late Professor Bachhawat made several scientific advancements in biochemistry. He made a pioneering contribution to the understanding of ketone body formation in mammals at the University of Michigan. At Christian Medical College, Vellore, he studied complex and difficult problems of glycolipids, glycosaminoglycans and glycoproteins in neural development and neurological disorders. Late Professor Bachhawat and his colleagues discovered that metachromatic leukodystrophy is a glycolipid storage disease that is caused by the deficiency of arylsulfatase A. This

pathbreaking study set the stage for the elucidation of enzymic defects in other glycolipid storage disorders, including Gaucher's disease and Tay-Sachs disease. He pioneered the development of carbohydrate-bearing liposomes for the site-specific delivery of drugs and enzymes to diseased organs. He was also involved in the development of liposomal formulations for treating systemic fungal infections.

He mentored many graduate students and post-doctoral fellows. He was well known for having his trainees to be independent. He was always ready to listen to them and guide them. Students trained by him are now making excellent research/teaching/writing contributions to their respective fields. Late Professor Bimal Kumar Bachhawat will always be remembered for his simplicity, positive attitude, warm and generous nature, and scientific guidance to his students. Because of his contribution and guidance, Professor Bachhawat will always be remembered and missed by his students, colleagues and the international biochemistry community.

Akhlaq A. Farooqui
Tahira Farooqui

Preface

Phytochemicals (flavonoids, carotenoids, allicin, polyphenols, hydrolyzable tannins, lignans, and phytosterols) provide flavor and color to vegetables, fruits, and herbs. Health protecting effects of phytochemicals are due to antiinflammatory, antioxidant, antiviral, antibacterial, anticarcinogenic, antiproliferative, hypocholesterolemic, and cellular repair properties. These effects are mediated through the regulation of various transcription factors, growth factors, inflammatory cytokines, protein kinases, and other enzymes. Regular consumption of colored fruit and vegetables is associated with reduced risks of cancer, cardiovascular disease, stroke, Alzheimer disease, cataracts, osteoporosis, diabetes, and some of the functional declines associated with aging. It is becoming increasingly evident that antioxidant and antiinflammatory properties of phytochemicals mitigate the damaging effect of oxidative stress. In recent years, there have been considerable developments in defining biological activities of phytochemicals at the molecular level. The effects of phytochemicals can be conducive, additive, synergistic, and antagonistic. Through these properties, phytochemicals modulate cellular differentiation, proliferation, oxidative stress, inflammation, and apoptosis. Other effects of phytochemicals include alterations in immune function and modulation of enzymic activities. Among phytochemicals, carotenoids, flavonoids, and polyphenols produce antioxidant effects and are involved in the scavenging of reactive oxygen species (ROS), singlet molecular oxygen, and peroxy radicals generated during lipid peroxidation. Cancer-preventive activities of carotenoids, flavonoids and polyphenols not only involve antioxidant effects, but also the induction and stimulation of intercellular communication via gap junctions, which play important roles in the regulation of cell growth, differentiation and apoptosis. Gap junctional communication between cells may be another way by which phytochemicals retard the growth of cancer and provide protection against cancer development.

Information on the effects of phytochemical on human health is scattered throughout the literature in the form of original papers, and reviews. We have decided to provide readers with a comprehensive and cutting edge description of metabolism of molecular mechanism associated with the beneficial effects of phytochemical in chronic human diseases in a manner that is useful not only to students and teachers but also to researchers and physicians. This book has 19 chapters. The first chapter provides an introduction to the structure activity relationship of phenolic antioxidants. Chapters 2, 3, 4, and 5 describe beneficial effects of flavonoids and polyphenols in reducing damage caused by reactive oxygen species and neuroinflammation. In these chapters, molecular mechanisms associated with antioxidant and antiinflammatory activities of phytochemicals are discussed. This is followed by chapter 6,

which provides the possible molecular mechanism of antiaging herbs, such as *L. barbarum*. Chapters 7 and 8 present beneficial effects of neem and propolis on human health. Chapters 9, 10, and 11 discuss health benefits of phytochemicals found in virgin olive oil, mulberry and grapes. Chapter 12 presents readers with beneficial effects of Indian herbs for the treatment of diabetes. Chapters 13, 14, and 15 describe the molecular mechanism involved in the beneficial effects of tumeric (curcumin) on human health. Chapters 16 and 17 provide information on molecular mechanism associated with health benefits of garlic. Finally, Chapters 18 and 19 focus on the biological activity and mechanism of action of plant polyphenols and perspective on the importance of phytochemicals in diet and direction for future research on phytotherapeutics. These topics fall in a fast-paced research area related to cell death that provides opportunities for target-based therapeutic intervention using phytochemicals. This book can be used as supplement text for a range of phytotherapeutics courses. Clinicians and pharmacologists will find this book useful for understanding molecular aspects of phytochemical in chronic human diseases.

We have tried to ensure uniformity in mode of presentation as well as a logical progression of subject from one topic to another and our authors have provided extensive bibliography. For the sake of simplicity and uniformity a large number of figures with chemical structures of phytochemicals used for the treatment of chronic diseases and signal transduction pathways diagrams are also included. We hope that our attempt to integrate and consolidate the knowledge on the molecular aspects of phytochemicals will provide the basis of more dramatic advances and developments on molecular mechanisms associated with beneficial effects of phytochemicals in chronic human diseases.

Akhlaq A. Farooqui
Tahira Farooqui

Chapter I

Phenolic Antioxidants of Natural Origin - Structure Activity Relationship and their Beneficial Effects on Human Health

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Lipid Chemistry Department, Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

Abstract

97 phenolic antioxidants, AH (80 individual compounds and 17 binary mixtures of two antioxidants) and extracts from natural sources (17) were chosen for a comparable analysis of their radical scavenging (H donating) and chain breaking (antioxidant) activities. They were separated in the following groups: Group A: Benzoic acids (5), Group B: Cinnamic acids and derivatives (18), Group C: Standard antioxidants (10): butylated hydroxytoluene, hydroquinone, DL- α -tocopherol, tertbutylated hydroquinone, eugenol, *iso*-eugenol, resveratrol, chroman C1, rosmarinic acid, *p*-methoxyphenol; Group D: Flavonoids and related compounds (17), Group E: Hydroxy chalcones (8), Group F: Simple hydroxy coumarins (11), Group G: Complex bis-coumarins (5), Group H: Hydrolyzable tannins (6), Group I: Extracts from medicinal plants and spices (17), Group J: Extracts from propolis (4), Group K: Binary mixtures of antioxidants (17). This comparable study illustrates a new aspect to the antioxidative and health promoting properties of extracts and individual compounds on the base of correlations between experimental (antiradical and antioxidant) and predictable (theoretical) activities which were summarized and discussed. The following models: a) model 1 – a DPPH assay used for determination of the radical scavenging capacity ($AH + DPPH\cdot \rightarrow A\cdot + DPPH-H$); b)

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model 2 - chemiluminescence of a model substrate RH (cumene or diphenylmethane) used for determination of the rate constant of reaction with model peroxy radicals ($AH + RO_2\bullet \rightarrow ROOH + A\bullet$); c) model 3 – lipid autoxidation used for determination of the chain-breaking antioxidant efficiency and reactivity ($AH + LO_2\bullet \rightarrow LOOH + A\bullet$; $A\bullet + LH (+O_2) \rightarrow AH + LO_2\bullet$); and d) model 4 – theoretical methods used for predicting the activity (predictable activity) were applied to explain the structure - activity relationships of phenolic antioxidants of natural origin. The highest lipid oxidation stability was found for antioxidants with a catecholic structure and for their binary mixtures with DL- α -tocopherol, as a result of synergism between them. Antiradical and antioxidant properties of different extracts from medicinal plants: *Tribulus terrestris*, *Carthamus lanatus* L. (*Asteraceae*), *Sideritis* (*Labiatae*), Marigold (*Calendula officinalis*), Karanja (*Pongamia glabra*) and of spices: Black Pepper (*Pepper Nigrum* L.), Red Chili, Sesame lignans (*Sesamum Indicum*) as well as from propolis of Europe, Brazil, El Salvador and Venezuela also were compared and discussed.

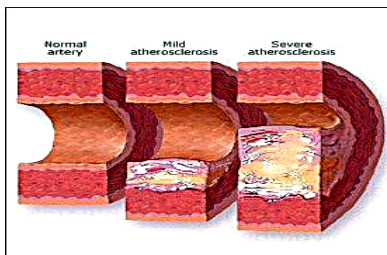
1. Introduction

1.1. Oxidative Stress

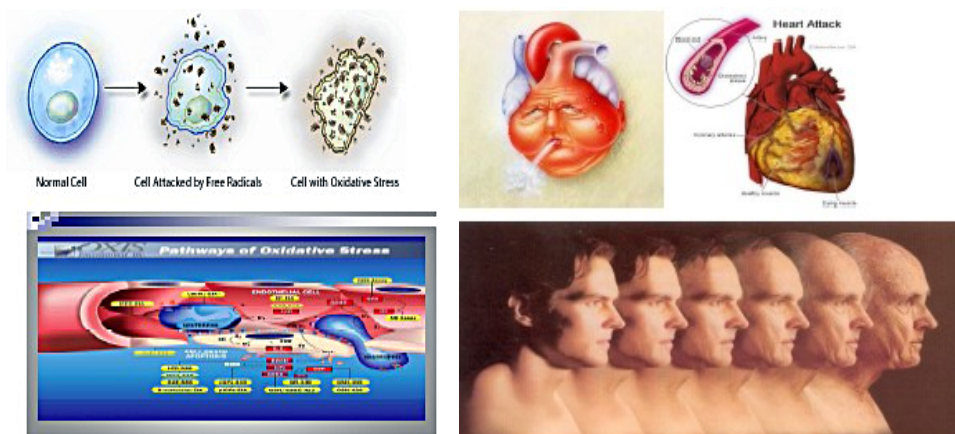
The human body is constantly subjected to a significant oxidative stress as a result of the misbalance between antioxidative protective systems and the formation of strong oxidizing substances, including free radicals. The stress can damage DNA, proteins, lipids and carbohydrates and could cause negative effect to intracellular signal transmission. The induction of free radicals formation in the hydrophobic parts of the biological membranes initiates radical disintegration of the hydrocarbon “tails” of the lipids. This process is known as lipid oxidation [1-5].

1.2. Free Radical Formation and Human Health

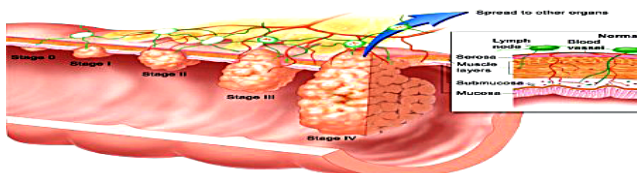
Free radicals are responsible for the pathogenesis of a wide range of diseases. The most serious and difficult to treat health problems are such as cancer and cardiovascular diseases. They also cause asthma, arthritis, inflammations, neurodegenerative disorders, Parkinson's disease and dementia. We would like to focus your attention not on the various specific causes for the different diseases but to the fact, that in all cases of diseases a change of the rate of peroxide oxidation is observed.



It has been found that in the first stage of the development of atherosclerosis the system works in its normal regime. The introduction of antioxidants in the affected body normalizes not only the peroxide oxidation, but also the lipid content [2,4].



Aging is a complex combination of deleterious free radical reactions, which affect cells and tissues.



Antioxidants used in oncology are effective: a) in the first stages as mono-therapy with antioxidants at high concentrations and b) at the last stages mainly as additives in the complex tumor therapy - the antioxidant is in low concentrations [2].

In this respect the medical treatment of most of them includes formulations based on a combination of traditional drugs with targeted functionality and different antioxidants.

1.3. Phenolic Antioxidants

Plant (poly) phenolics are multifunctional antioxidants and act as: reducing agents (free radical terminators), metal chelators, and singlet oxygen quenchers. The activity of antioxidants depends on complex factors including: the nature of antioxidants, the conditions of oxidation, the properties of the oxidizing substrate and the stage of oxidation [6-10]. The problem of antioxidant capacity of food has at least two sides: a) the antioxidant potential, which is determined by the antioxidant composition and antioxidative properties of constituents and is the subject of food chemistry, and b) the biological effects that depend, among other things, on bioavailability of antioxidants, and is a medico-biological problem [3,5,10]. The inherent compositional and structural complexity of real foods means that systematic studies of lipid oxidation must first be carried out in model systems [11-15]. These systems should have the basic feature of the real system but have well-determined compositions and structural properties. Structure-activity relationship studies using theoretical methods are gaining interest among scientists for prediction or elucidation of differences in the activity of series of molecules [16-22].

The aim of this study is to present a comparable analysis of antioxidant's activity as radical scavengers and chain – breaking antioxidants as well as their structure - activity relationships using various experimental and theoretical methods. For that reason 97 phenolic antioxidants, AH (80 individual compounds and 17 binary mixtures of two antioxidants) and extracts from natural sources (17) (Tables 1 and 2) were chosen for a comparable analysis of their radical scavenging (H donating) and chain breaking (antioxidant) activities. Correlations between experimental (model 1 - DPPH assay; model 2 – chemiluminescence method; and model 3 - lipid autoxidation) and theoretical (model 4 - QSAR) methods applied to explain the structure - activity relationships of antioxidants and obtained data are summarized and discussed.

2. Experimental

2.1. Model 1: Estimation of Radical Scavenging Activity (%RSA) by Rapid DPPH Radical Test which Gives Information about the H - Donating Capacity of the Phenolic Compounds towards Free (DPPH) Radicals

Radical scavenging activity (RSA) of phenolic compounds (AH) and extracts under study was determined on the base of two approaches: A) Quantitative TLC DPPH rapid test [23-25], separating tested compounds and extracts into three main groups: with strong activity (+++), their white-yellowish spots appeared immediately after spraying; with moderate activity (++) , their spots appeared after 10 min after spraying; and with weak or no activity (+/-), they didn't show spots 10 min after spraying (for experimental details please see Refs. 23,25). B) The second approach is the qualitative (%RSA) DPPH test based on the decrease after addition of the AH in the absorbance at 516-517nm of the DPPH radical solution in ethanol or acetone [5,26-28] measured in a glass cuvette (1cm long), automatically. Different concentrations (expressed as the number of antioxidant moles per mole of DPPH ($[AH]/[DPPH] = 0.15$ and 0.25) were used and %RSA of antioxidants under study was calculated from the absorbance at the start and after 10 and 20 min reaction time and expressed as $\%RSA_{exp} = [Abs_{516nm(t=0)} - Abs_{516nm(t=20)} \times 100 / Abs_{516nm(t=0)}]$ [22,26]. Absorbance values were corrected for radical decay using blank solutions. All tests were performed in triplicate at 25°C.

2.2. Model 2: Estimation of the Rate Constant of Antioxidant with Peroxide Radicals (k_A) Using Kinetic Chemiluminescence (CL) Method [29-31] – Provides Information about the Antioxidant Efficiency (k_A) and Stoichiometry (n)

The absolute value of the rate constant of the inhibitor reactions with peroxy radicals (k_A) can be determined only by using this highly sensitive CL method (the kinetic model is presented on Table 3).

Table 1.

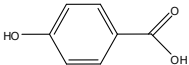
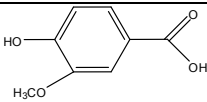
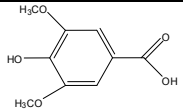
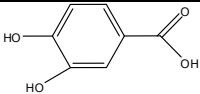
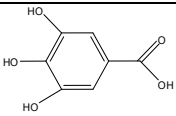
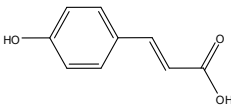
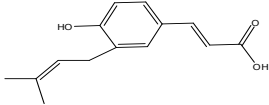
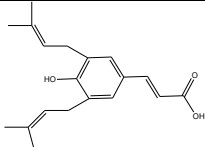
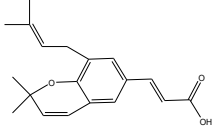
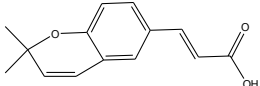
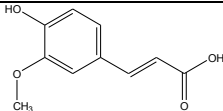
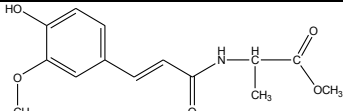
No	Antioxidants	Structures	Group	Method	Activity	References
I.	Benzoic acids		A			
1	p-Hydroxy-benzoic acid (p-HBA)		A	DPPH CL Lipid AO	Weak Weak Weak	3,4,9 31,60 31,60
2	Vanillic acid (VanA)		A	DPPH CL Lipid AO	Weak Weak Weak	3,4,9 31,60 31,6,63
3	Syringic acid (SyrA)		A	DPPH CL Lipid AO	Moderate Moderate Moderate	3,4,9 31,60 31,60,63
4	3,4-Dihydroxybenzoic acid (DHBA)		A	DPPH CL Lipid AO	Strong Strong Strong	3,4,9 31,60 31,60
5	Galic acid (GalA)		A	DPPH CL Lipid AO BDE(O-H)	Strong Strong Strong Strong	3,9,36 36 36 34
II.	Cinnamic acid derivatives		B			
6	p-Coumaric acid (p-CumA)		B	DPPH CL Lipid AO TOPS-MODE BDE(O-H)	Weak Weak Weak Weak Weak	3,4,5,15,26,29 29,31,60 5,15,29,31,47,60,63,66 5,15,39 29,45

Table 1. (Continued)

No	Antioxidants	Structures	Group	Method	Activity	References
7	3-Prenyl-4-hydroxy-cinnamic acid (PHC)		B	Lipid AO TOPS-MODE	Moderate Weak	5,15,36,47 5,15,36,47
8	3,5-Diprenyl-4-hydroxy-cinnamic acid (DPHC)		B	Lipid AO TOPS-MODE	Moderate Moderate	5,15,36,47 5,15,36,47
9	2,2-Dimethyl-6-carboxyethenyl-8-prenyl-2H-benzopiran (DCEPB) DPHC2		B	Lipid AO TOPS-MODE	No No	47 5,15
10	2,2-Dimethyl-6-carboxyethenyl-2H-benzopiran (DMCB) PHC2		B	Lipid AO TOPS-MODE	No No	5,15,47 5,15,47
11	Ferulic acid (FA)		B	DPPH CL Lipid AO TOPS-MODE BDE(O-H)	Weak Weak Weak Weak Weak	4,5,15,26,29,63 29,31 5,15,36,47,29, 63,66 5,36,39 29,45
12	N-(trans-feruloyl)-alanine-t-butyl ester (N1)		B	Lipid AO TOPS-MODE	Moderate Moderate	5,15, 36,66 5,15,36

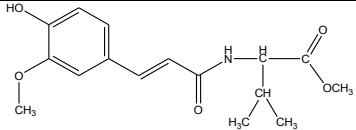
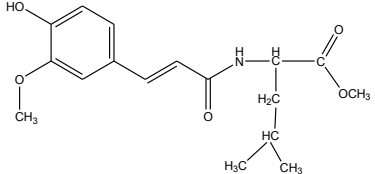
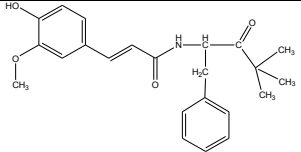
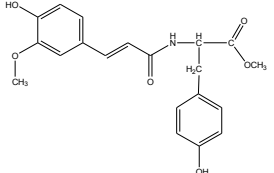
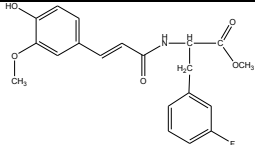
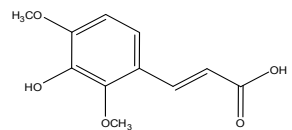
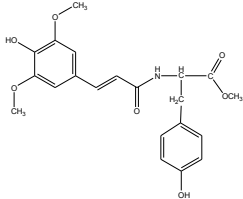
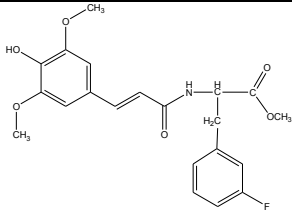
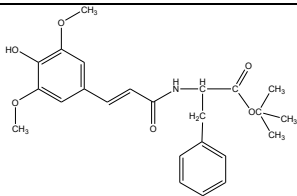
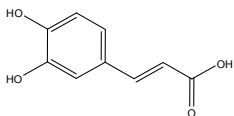
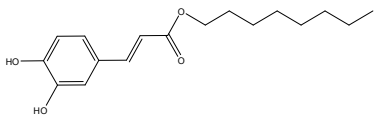
No	Antioxidants	Structures	Group	Method	Activity	References
13	N-(trans-feruloyl)-valine methyl ester (N2)		B	Lipid AO TOPS-MODE	Moderate Moderate	5,15,36,66 5,15,36
14	N-(trans-feruloyl)-leucine methyl ester (N3)		B	Lipid AO TOPS-MODE	Moderate Moderate	5,15,36,66 5,15,36
15	N-(trans-feruloyl)-phenylalanine t-butyl ester (N4)		B	Lipid AO TOPS-MODE	Moderate Moderate	5,15,36,66 5,15,36
16	N-(trans-feruloyl)-tyrosine methyl ester (N5)		B	Lipid AO TOPS-MODE	Moderate Moderate	5,15,36,66 5,15,36
17	N-(trans-feruloyl)-m-fluorophenylalanine methyl ester (N6)		B	Lipid AO TOPS-MODE	Moderate Moderate	5,15,36,66 5,15,36
18	Sinapic acid (SA)		B	DPPH CL Lipid AO TOPS-MODE BDE(O-H)	Moderate Moderate Moderate Moderate	4,5,15,16,26,29 29,31 5,15, 31,47,63,66 5,15,42 29, 45

Table 1. (Continued)

No	Antioxidants	Structures	Group	Method	Activity	References
19	N-(trans-sinapoyl)-tyrosine methyl ester (N7)		B	Lipid AO TOPS-MODE	Moderate Moderate	5,15,36,66 5,15,36
20	N-(trans-sinapoyl)-m-fluorophenylalanine methyl ester (N8)		B	Lipid AO TOPS-MODE	Moderate Moderate	5,15,36,66 5,15,36
21	N-(trans-sinapoyl)-phenylalanine t-butyl ester (N9)		B	Lipid AO TOPS-MODE	Moderate Moderate	5,15,36,66 5,15,36
22	Caffeic acid (CA)		B	DPPH CL Lipid AO TOPS-MODE BDE(O-H)	Strong Strong Strong Strong Strong	4,5,15,16,26,29,44,50 29,30,31 5,31,47, 48,63,66 5,15,42 29,30,34,45
23	Octyl caffeate (OC)		B	Lipid AO TOPS-MODE	Moderate Moderate	36,48 5,36
III.	Standard antioxidants		C			

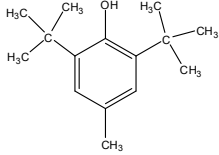
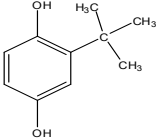
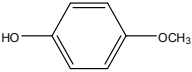
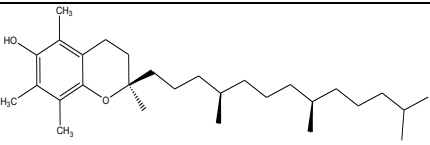
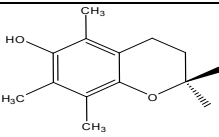
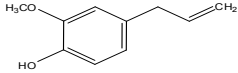
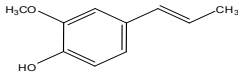
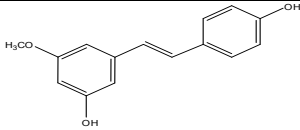
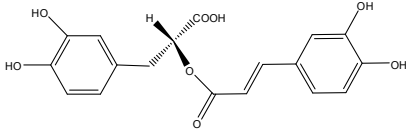
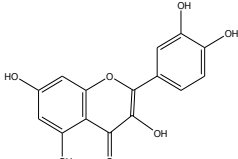
No	Antioxidants	Structures	Group	Method	Activity	References
24	Butylated hydroxy-toluene (BHT)		C	DPPH CL Lipid AO TOPS-MODE BDE(O-H)	Weak Moderate Moderate Weak Moderate	26,28,36 29,30,60 5, 31,36,47,51,60,63 5,36,47,42,51 34
25	Tert-Butyl-Hydroquinone (TBHQ)		C	DPPH Lipid AO BDE(O-H)	Strong Strong Strong	3,26,36 36 34
26	Hydroquinone (HQ)		C	CL Lipid AO TOPS-MODE BDE(O-H)	Moderate Moderate Weak Moderate	30 5,36,47,51,63 5,36,47,51 34
27	p-Methoxy-phenol (p-MeOH)		C	CL Lipid AO BDE(O-H)	Moderate Weak Moderate	11, 31 13,52 34
28	DL-alpha-Tocopherol (TOH)		C	DPPH CL Lipid AO TOPS-MODE BDE(O-H)	Strong Strong Strong Moderate Strong	26,36 30, 31 5,36,47,51,63 5,6 38
29	ChromanC1 (CrC1)		C	CL Lipid AO BDE(O-H)	Strong Strong Strong	29,30,31 2, 31,36 34

Table 1. (Continued)

No	Antioxidants	Structures	Group	Method	Activity	References
30	Eugenol (4-Allyl-2-methoxyphenol) (Eu)		C	DPPH CL Lipid AO BDE(O-H)	Moderate Moderate Moderate Moderate	44,26,28 11,31 35 16, 34
31	Iso-eugenol (Iso-Eu)		C	DPPH CL Lipid AO BDE(O-H)	Moderate Moderate Moderate Moderate	44,28 11,31 35, 16, 38
32	Resveratrol (RS)		C	DPPH Lipid AO BDE(O-H)	Moderate Moderate Moderate	3,7 63,69,71 34
33	Rosmarinic acid		C	DPPH Lipid AO BCBT BDE(O-H)	Strong Moderate Strong Strong	3,56 35, 56 3, 56 34
IV.	Flavonoids		D			
34	Quercetin (Qu)		D	DPPH CL Lipid AO TOPS-MODE BDE(O-H)	Strong Strong Strong Strong Strong	30,41,46,53,67,68 30 5,15,36,41 5,15,36,41 5,15,34,36,41,68,71

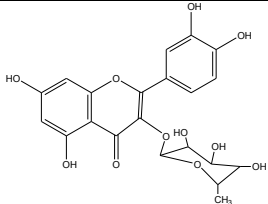
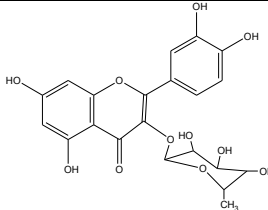
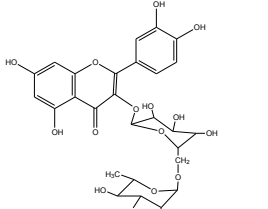
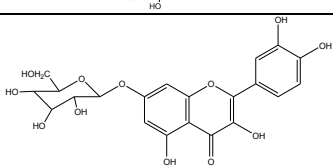
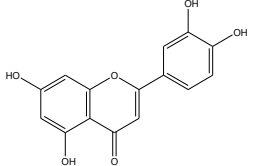
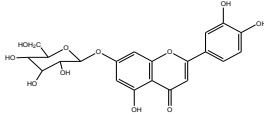
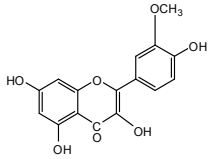
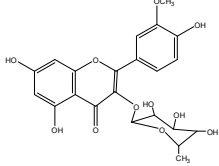
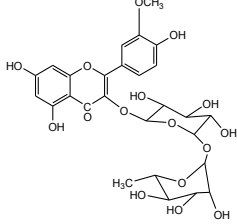
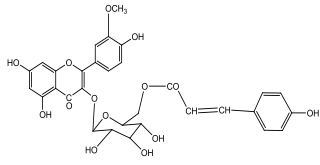
No	Antioxidants	Structures	Group	Method	Activity	References
35	Quercitrin (Qu-3-Glu)		D	DPPH CL Lipid AO TOPS-MODE BDE(O-H)	Strong Strong Moderate Strong Strong	41,50,53,68 30 5,15,36,41 5,15,36,41 5,15,34,36,41,68,71
36	Quercetin-3-O-Rhamnoside (Qu-3-Rhm)		D	CL Lipid AO TOPS-MODE BDE(O-H)	Strong Moderate Strong Strong	30 5,15,36,41 5,15,36,41 5,15,34,36,41
37	Rutin (Ru)		D	DPPH CL Lipid AO TOPS-MODE BDE(O-H)	Strong Strong Moderate Strong Strong	5,36,41,46,53 30,68 5,15,36,41 5,15,36,41 5,15,34,36,41,68,71
38	Quercimetrin (Qu-7)		D	DPPH CL Lipid AO TOPS-MODE BDE(O-H)	Strong Strong Moderate Strong Strong	41,53 30 5,15,36,41 5,15,36,41 5,15,34,36,41
39	Luteolin (Lu)		D	DPPH CL Lipid AO TOPS-MODE BDE(O-H)	Strong Strong Moderate Strong Strong	50,53,68 30 5,15,36,41 5,15,36,41 5,15,34,36,41,68,71

Table 1. (Continued)

No	Antioxidants	Structures	Group	Method	Activity	References
40	Luteolin-7-O-Glucoside (Lu-7)		D	DPPH CL Lipid AO TOPS-MODE BDE(O-H)	Strong Strong Moderate Strong Strong	53 30 5,15,36,41 5,15,36,41 5,15,34,36,41,71
41	Isorhamnetin (Isorh)		D	DPPH CL Lipid AO TOPS-MODE BDE(O-H)	Strong Strong Moderate Strong Strong	5,15,36,41,50,67 30 36 5,15,36,41 5,6,21, 38,53,71
42	Isorhamnetin-3-O-Glucoside (Isorh-3-O-Glu)		D	DPPH BDE(O-H)	Weak Weak	15,36,50,53 15,36
43	Isorhamnetin-3-O-Rutinoside (Isorh-3-O-Rut)		D	DPPH BDE(O-H)	Weak Weak	5,15,36,41 5,15,36,41
44	Isorhamnetin-3-O-Cumaroyl-Glucoside (Isorh-3_O-Cum-Glu)		D	DPPH BDE(O-H)	Weak Weak	5,15,36,41 5,15,36,41

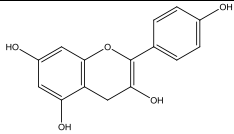
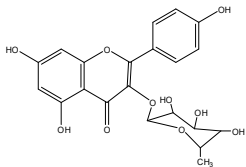
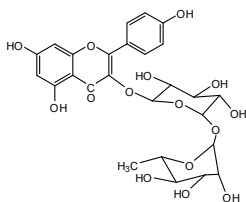
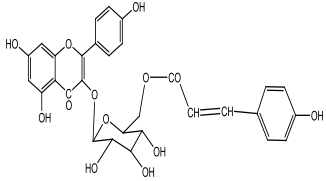
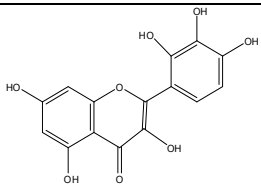
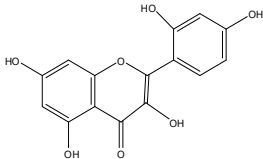
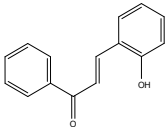
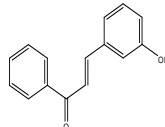
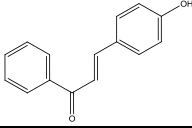
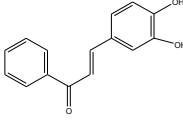
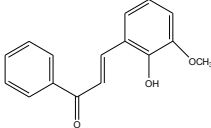
No	Antioxidants	Structures	Group	Method	Activity	References
45	Kaempferol (Kf)		D	DPPH CL Lipid AO TOPS-MODE BDE(O-H)	Moderate Moderate Weak Moderate Weak?	3,5,15,26,36,41,68 5,15,30,36,41 15, 36 5,15,36,41 5,15,34,36,41,71
46	Kaempferol-3-O-Glucoside (Kf-3-O-Glu)		D	DPPH CL Lipid AO BDE(O-H)	Weak Weak Weak Weak?	5,15,36,41 5,15,36,41 15,36 5,15,36,41
47	Kaempferol-3-O-Rutinoside (Kf-3-O-Rut)		D	DPPH Lipid AO BDE(O-H)	Weak Weak Weak	5,15,36,41 15,36 5,15,36,41
48	Kaempferol-3-O-Cumaroyl-Glucoside (Kf-3-O-Cum-Glu)		D	DPPH Lipid AO BDE(O-H)	Weak Weak Weak	5,15,36,41 15,36 5,15,36,41
49	Myricetin (Myr)		D	DPPH Lipid AO BDE(O-H)	Weak Weak Weak	3,15,36 9,15,36, 70 6,34,71

Table 1. (Continued)

No	Antioxidants	Structures	Group	Method	Activity	References
50	Morin (Mo)		D	DPPH Lipid AO BDE(O-H)	Weak Weak Weak	3 73 34,71
V.	Hydroxy Chalcones		E			
51	2-Hydroxy-chalcone (Ch1)		E	DPPH CL Lipid AO TOPS-MODE BDE(O-H)	Weak Weak Weak Weak Weak	5,15,29,36 5,15,29,36 5,15,29,36 5,15,29,36 5,15,29,36
52	3-Hydroxy-chalcone (Ch2)		E	DPPH CL Lipid AO TOPS-MODE BDE(O-H)	Weak Weak Weak Weak Weak	5,15,29,36 5,15,29,36 5,15,29,36 5,15,29,36 5,15,29,36
53	4-Hydroxy-chalcone (Ch3)		E	DPPH CL Lipid AO TOPS-MODE BDE(O-H)	Moderate Moderate Moderate Moderate Moderate	5,15,29,36 5,15,29,36 5,15,29,36 5,15,29,36 5,15,29,36
54	3,4-Dihydroxy-chalcone (Ch4)		E	DPPH CL Lipid AO TOPS-MODE BDE(O-H)	Strong Strong Strong Strong Strong	5,15,29,36 5,15,29,36 5,15,29,36 5,15,29,36 5,15,29,36
55	2-Hydroxy-3-methoxy-chalcone (Ch5)		E	DPPH CL Lipid AO TOPS-MODE BDE(O-H)	Weak Weak Weak Weak Weak	5,15,29,36 5,15,29,36 5,15,29,36 5,15,29,36 5,15,29,36

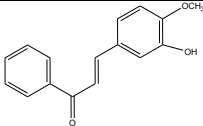
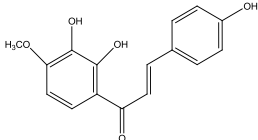
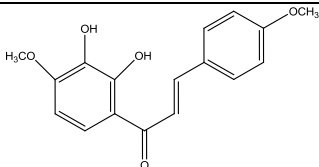
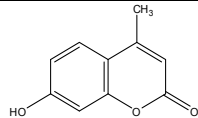
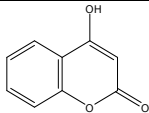
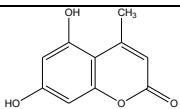
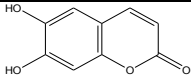
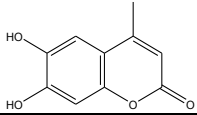
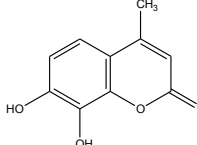
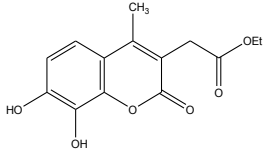
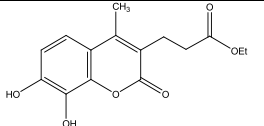
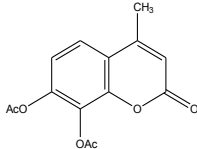
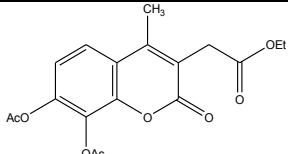
No	Antioxidants	Structures	Group	Method	Activity	References
56	3-Hydroxy-4-methoxy-chalcone (Ch6)		E	DPPH CL Lipid AO TOPS-MODE BDE(O-H)	Weak Weak Weak Weak Weak	5,15,29,36 5,15,29,36 5,15,29,36 5,15,29,36 5,15,29,36
57	2',3'-Dihydroxy-4,4'-dimethoxy chalcones (Ch7)		E	DPPH CL Lipid Oxid DFTcalc	Moderate Moderate Moderate Moderate	5,8,36 5,8,36 5,8,36 5,8,15,36
58	2',3',4'-Trihydroxy-4-methoxy chalcones (Ch8)		E	DPPH CL Lipid Oxid DFTcalc	Weak Weak Weak Weak	5,8,36 5,8,36 5,8,36 5,8,15,36
VI.	Simple Hydroxy-coumarins with anti cancer activity		F			
59	7-Hydroxy-4-methyl-coumarin (Cum1)		F	DPPH Lipid AO BDE(O-H)	Weak Weak Weak	5,15,25,36 5,15,25,36 15,25,36,57
60	4-Hydroxy-coumarin (Cum2)		F	DPPH Lipid AO BDE(O-H)	Weak Weak Weak	15,36, 15,23,36 15,23,36
61	5,7-Dihydroxy-4-methyl-coumarin (Cum3)		F	DPPH Lipid AO BDE(O-H)	Weak Weak Weak	5,15,25,36 5, 15,25,36 15, 25,36
62	6,7-Dihydroxy-coumarin (Cum4)		F	DPPH Lipid AO BDE(O-H)	Strong Strong Strong	5,15,25,36 5, 15,25,36 15, 25,36,

Table 1. (Continued)

No	Antioxidants	Structures	Group	Method	Activity	References
63	6,7-Dihydroxy-4-methyl-coumarin (Cum5)		F	DPPH Lipid AO BDE(O-H)	Strong Strong Strong	5,15,25,36 5, 15,25,36 15, 25,36,
64	7,8-Dihydroxy-4-methyl-coumarin (Cum6)		F	DPPH Lipid AO BDE(O-H)	Strong Strong Strong	5,15,25,36 5,15,25,36 15,17,25,36,
65	Ethyl 2-(7,8-dihydroxy-4-methyl-coumarin)-3-acetate (Cum7)		F	DPPH Lipid AO BDE(O-H)	Strong Strong Strong	5,25,15,36, 5,25,15,36 15,25,36
66	Ethyl 3-(7,8-dihydroxy-4-methyl-2-oxo-2H-chromen-3-yl) propanoate (Cum8)		F	DPPH Lipid AO BDE(O-H)	Strong Strong Strong	15,25,36 15,25,36 15,25,36
67	7,8-Diacetoxy-4-methyl-coumarin (Cum9)		F	DPPH Lipid AO	Weak Weak	15,25,36 15,25,36 ,
68	Ethyl 2-(7,8-diacetoxy-4-methyl-coumarin)-3-acetate (Cum10)		F	DPPH Lipid AO	Weak Weak	15,25,36 15,25,36

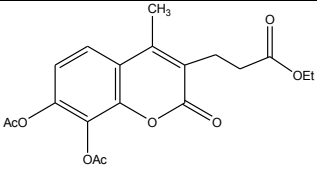
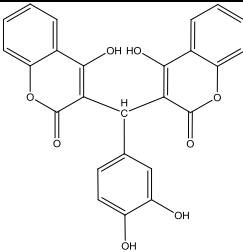
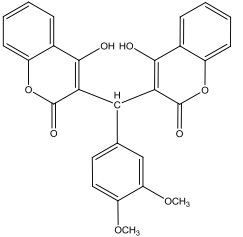
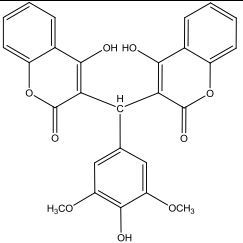
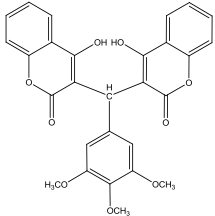
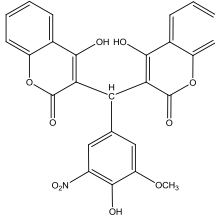
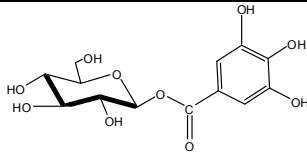
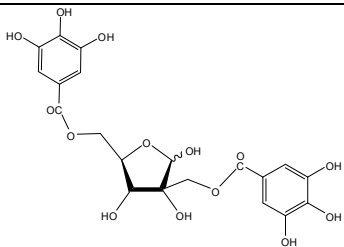
No	Antioxidants	Structures	Group	Method	Activity	References
69	3-(3-ethoxy-3-oxopropyl)-4-methyl-2-oxo-2H-chromene-7,8-diyl diacetate (Cum11)		F	DPPH Lipid AO	Weak Weak	15,25,36 15,25,36
VII.	Bis-coumarins with anti HIV effect		G			
70	3,3'-((3,4-Dihydroxyphenyl) methylene)bis (4-hydroxy-2H-chromen-2-one) (BisCum1)		G	DPPH Lipid AO	Strong Strong	15,23,36 15,23,36 ,
71	3,3'-((3,4-Dimethoxyphenyl) methylene) bis (4-hydroxy-2H-chromen-2-one) (BisCum2)		G	DPPH Lipid AO	No No	15,23,36 15,23,36
72	3,3'-((4-Hydroxy-3,5-dimethoxyphenyl) methylene)bis(4-hydroxy-2H-chromen-2-one) (BisCum3)		G	DPPH Lipid AO	Moderate Moderate	15,23,36 15,23,36

Table 1. (Continued)

No	Antioxidants	Structures	Group	Method	Activity	References
73	3,3'-((3,4,5- Trimethoxyphenyl) methylene)bis (4-hydroxy-2H-chromen-2-one) (BisCum4)		G	DPPH Lipid AO	No No	15,23,36 15,23,36
74	3,3'-((4-Hydroxy-3-methoxy-5-nitrophenyl)methylene)bis(4-hydroxy-2H-chromen-2-one) (BisCum5)		G	DPPH Lipid AO	Weak Weak	15,23,36 15,23,36
VIII.	Tannins		H	Methods	Results	References
75	Glucogallin (GG)		H	Pulse radiolysis EPR spectrosc DFTcalc	Rate constants Spectrum Hiperfine coupling constants	42 42 42
76	Hammamelitannin (HT)		H	Pulse radiolysis EPR spectrosc DFTcalc	Rate constants Spectrum Hiperfine coupling constants	42 42 42

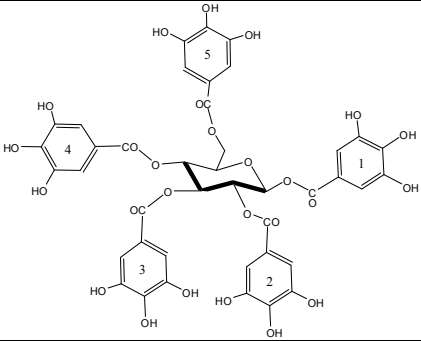
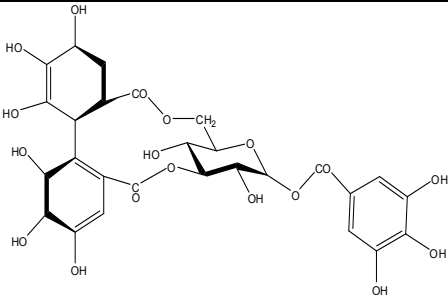
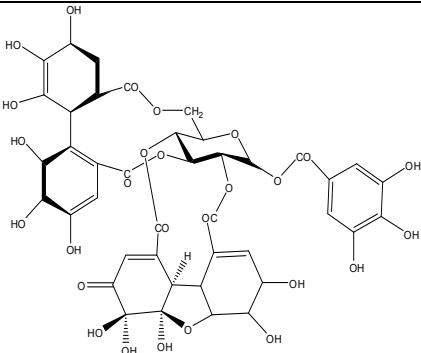
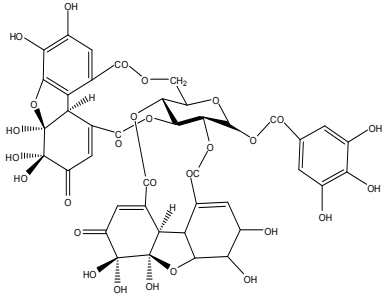
No	Antioxidants	Structures	Group	Method	Activity	References
77	Pentagalloylglucose (PGG)		H	Pulse radiolysis EPR spectrosc DFTcalc	Rate constants Spectrum Hiperfine coupling constants	42 42 42
78	Corilagin (Cor) 1,3,6,-trigalloyl glucose		H	Pulse radiolysis EPR spectrosc DFTcalc	Rate constants Spectrum Hiperfine couplong constants	42 42 42
79	Geranin (Ger)		H	Pulse radiolysis EPR spectrosc DFTcalc	Rate constants Spectrum Hiperfine coupling constants	42 42 42

Table 1. (Continued)

No	Antioxidants	Structures	Group	Method	Activity	References
80	Amarin (Am)		H	Pulse radiolysis EPR spectrosc DFTcalc	Rate constants Spectrum Hiperfine coupling constants	42 42 42
IX.	Extracts from medicinal plants and spices	Constituents	I	Methods	Activity	References
IX.1	Tribulus terrestris	Flavonoids: Qu, Qu-3-Glu, Ru, Isorh, Isorh-3-Glu, Isorh-3-Rut, Isorh-3-Cum-Glu, Kf, Kf-3-Glu, Kf-3-Rut, Kf-3-Cum-Glu	I	DPPH CL	Strong Strong	36, 41 36,41
IX.2	Carthamus lanatus l.	Flavonoids: Qu, Qu-7-Glu, Qu-3-Rhm, Ru, Lu, Lu-7-Glu	I	DPPH	Strong	36,53
IX.2.1.	Total EtOH extract			Lipid AO	Strong	36,53
IX.2.2.	BuOH extract			Lipid AO	Moderate	36,53
IX.2.3.	MeOH extract			Lipid AO	Moderate	36,53
IX.2.4.	50%MeOHextract			Lipid AO	Weak	36,53
IX.3	Sideritis species (Labiatae)	Main constituents: Terpenoids, flavonoids Rosmarinic acid	I	DPPH BCBT Lipid AO	Strong Moderate Moderate	56 56 56
	S scardica - MeOH, EtOAc, BuOH, hot H2O extracts			DPPH BCBT	Moderate Strong	56 56
	S scardica-t-BuMeOH extract			DPPH BCBT	Moderate No	56 56
	S scardica – H2O extract			DPPH BCBT	Moderate No	56 56
IX.4.	Sideritis species (Labiatae)					
	S syriaca - MeOH, EtOAc, BuOH, hot H2O			DPPH	Strong	56

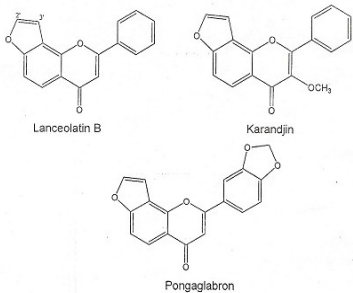
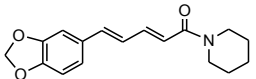
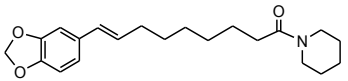
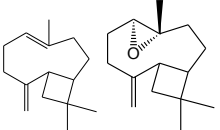
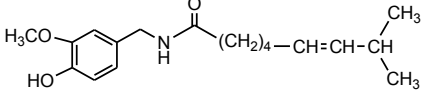
No	Antioxidants	Structures	Group	Method	Activity	References
	extracts			BCBT Lipid AO	Moderate Moderate	56 56
	S syriaca-t-BuMeOH			DPPH BCBT	Moderate Moderate	56 56
	S syriaca – H2O extract			DPPH BCBT	Moderate No	56 56
IX.5.	Sideritis species (Labiatae)			DPPH BCBT Lipid AO	Strong Moderate Moderate	56 56 56,
	S montana- MeOH, EtOAc, BuOH, hot H2O extracts			DPPH BCBT	Moderate Moderate	56 56
	S montana-t-BuMeOH extract			DPPH BCBT	Strong Moderate	56 56
IX.6	Marigold (Calendula officinalis)	Main constituents: Carotenoids; beta-carotene, gamma-carotene, flavoxanthin, lycopene, rubixanthin, lutein	I	DPPH Lipid AO	Weak Weak	36, 57 36,57
IX.7	Karanja (Pongamia glabra)	 Lanceolatin B Karandjin Pongaglabron	I	DPPH Lipid AO	Moderate Moderate	36, 57 36,57

Table 1. (Continued)

No	Antioxidants	Structures	Group	Method	Activity	References
IX.8.	Indian black pepper-(Pepper nigrum L.) Oleoresin-ethylenedichloride extract	Main constituents: Piperine  piperolein B 	I	DPPH DMDP ABTS Lipid AO	Strong Strong Strong Strong	36, 57 36, 57 36, 57 36, 57
IX.9	Etnanol/methanol extract	-		DPPH DMDP ABTS Lipid AO	Strong Strong Strong Strong	36, 57 36, 57 36, 57 36, 57
IX.10.	Hexane extract	 Caryophyllene Caryophyllene oxide caryophyllene (35.2%), caryophyllene oxide (10.6%), copaene (4.3%), δ-elemene (1.8%), β-elemene (1.4%), γ-amorphene (4.7%), piperine (6.3%), and piperolein B (0.5%).		DPPH Lipid AO	Strong Strong	36,57 36,57
IX.11.	Acetone extract	The same constituents as hexane extract, but piperine (59.2%) и caryophyllene (12.3%).		DPPH Lipid AO	Strong Moderate	36,57 36,57
IX.12.	Indian red chilli Oleoresin - ethylenedichloride extract	Main constituents: Capsaicin 	I	DPPH DMDP ABTS Lipid AO	Strong Strong Strong Strong	36, 57 36,57 36,57 36,57
IX.13	Etnanol/methanol extracts			DPPH DMDP	Strong Strong	36, 57 36,57

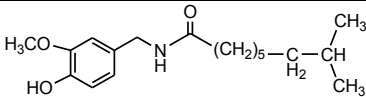
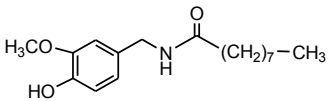
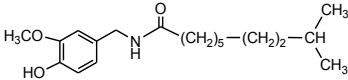
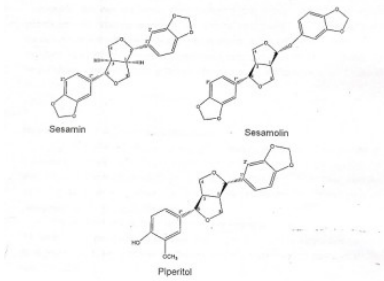
No	Antioxidants	Structures	Group	Method	Activity	References
IX.14	Hexane fraction	 Dihydrocapsaicin		ABTS Lipid AO	Strong Strong	36,57 36,57
		 Nonivamide		DPPH Lipid AO	Strong Strong	36,57 36,57
IX.15	Acetone fraction	 Homodihydrocapsaicin Vitamin E		DPPH Lipid AO	Strong Strong	36,57 36,57
IX.16	Indian sesame lignans from Sesamum indicum	 Sesamin Sesamol Piperitol	I	DPPH Lipid AO	Moderate Moderate	36, 57 36,57
IX.17	Indian rice bran oil and methanolic extract	triacylglycerols, diacylglycerols, sterols, sterol and wax esters, lipid zone containing γ -oryzanol and other important components, hydrocarbons, traces of mono - acylglycerols and polar lipids	I	DPPH Lipid A	Moderate Strong	36,57 36,57
X	Extracts from propolis		J			
X.1.	Propolis from Brazil – Total EtOH extract	Main constituents: PHC, PHC2, DPHC, DPHC2	J	Lipid AO	Strong	47,59
X.2.	Propolis from Venezuela – total EtOH extracts (1-4)		J	Lipid AO	Moderate	58,59
X.3.	Propolis from El Salvador	Hydroxy-chalcones: Ch7 and Ch8	J	DPPH CL Lipid AO BDE(O-H)	Moderate Moderate Moderate Weak	8,59 8,59 8,59 8,59
X.4.	Propolis from Europe	Flavonoids	J	Lipid AO	Strong	59,72

Table 2. Inhibiting efficiency of various binary mixtures of two (1 and 2) antioxidants in equimolar concentrations (0.1mM-1.0mM) and 1:1 ratio, during TGSO^a (80°C), TGSO^b (100°C) and TGL^c (100°C) autoxidation

No	Binary mixtures (1:1)	[AH] mM	IP ₁₊₂ h	IP ₁ h	IP ₂ h	Synergism (%) Additivism Antagonism	Lipid substrate	Refs.
1	Quercetin + Luteolin (Qu + Lu) ₁ (Qu + Lu) ₅	0.1	7.5±0.4	9.9±0.9	2.2±0.2	Antagonism	TGSO ^a	5,53
		0.5	12.3±0.5	24.5±0.6	6.3±0.4	Antagonism	TGSO ^a	5,53
2	Quercetin +Rutin (Qu + Ru) ₁ (Qu + Ru) ₅	0.1	8.3±0.8	9.9±0.9	2.7±0.2	Antagonism	TGSO ^a	5,53
		0.5	21.5±0.6	24.5±0.6	2.8±0.2	Antagonism	TGSO ^a	5,53
3	Qu-7Glu +Lu-7Glu (Qu-7 + Lu-7) ₅ (Qu-7 + Lu-7) ₁₀	0.5	2.0±0.2	1.5±0.2	1.8±0.2	Antagonism	TGSO ^a	5,53
		1.0	2.1±0.2	1.0±0.2	1.4±0.2	Antagonism	TGSO ^a	5,53
4	Quercetin + α -Tocopherol (Qu + α -TOH) ₁	0.1	29.7±1.5	9.9±0.9	10.5±0.9	Synergism (46%)	TGSO ^a	5,53
5	Rutin + α -Tocopherol (Ru + α -TOH) ₁	0.1	24.9±1.5	2.7±0.2	10.5±0.9	Synergism (89%)	TGSO ^a	5,53
6	Caffeic acid + α -Tocopherol (CA + α -TOH) ₁	0.1	20.4±1.5	9.8±0.4	10.5±0.9	Additivism	TGSO ^a	6,24
7	Sinapic acid + α -Tocopherol (SA + α -TOH) ₁	0.1	16.1±1.5	5.3±0.4	10.5±0.9	Additivism	TGSO ^a	6,24
8	Butylated hydroxytoluene + α -Tocopherol (BHT + α -TOH) ₁	0.1	21.5±1.5	7.5±0.4	10.5±0.9	Synergism (19%)	TGSO ^a	6, 15
9	Tert-butylated hydroquinone + α -Tocopherol (TBHQ + α -TOH) ₁	0.1	26.1±1.5	7.9±0.4	10.5±0.9	Synergism (42%)	TGSO ^a	6, 15
10	7-OH-Cumarin + α -TOH (Cum ₁ + α -TOH) ₁	0.1	11.8±0.9	1.5±0.2	10.5±0.9	Additivism	TGSO ^a	6,24
11	7,8-diOH-Cumarin + α -TOH (Cum ₆ + α -TOH) ₁	0.1	14.2±0.9	2.0±0.2	10.5±0.9	Synergism (14%)	TGSO ^a	5,27
12	6,7-diOH-Cumarin + α -TOH (Cum ₄ + α -TOH) ₁	0.1	12.7±0.9	7.1±0.8	10.5±0.9	Antagonism	TGSO ^a	5,25
13	BisCum1 + α -TOH (BisCum1 + α -TOH) ₁	0.1	12.6±0.9	7.9±0.8	10.5±0.9	Antagonism	TGSO ^a	6,24
14	BisCum3 + α -TOH (BisCum3 + α -TOH) ₁	0.1	6.1±0.5	2.2±0.2	10.5±0.9	Antagonism	TGSO ^a	6,24
15	Sinapic acid + α -TOH (SA + α -TOH) ₁ *	0.1	45.0±1.0	8.5±0.8	21.0±1.5	Synergism (52%)	TGL ^c	5,36

No	Binary mixtures (1:1)	[AH] mM	IP ₁₊₂ h	IP ₁ h	IP ₂ h	Synergism (%) Additivism Antagonism	Lipid substrate	Refs.
16	Resveratrol + α -TOH (RS + α -TOH) ₅	0.5	7.2 $\pm$ 0.8	2.2 $\pm$ 0.2	7.6 $\pm$ 0.8	Antagonism	TGSO ^b	69
17	Myricetin + α -TOH (Myr + α -TOH) ₁	0.1	10.5 $\pm$ 0.9	4.7 $\pm$ 0.3	3.2 $\pm$ 0.3	Synergism (33%)	TGSO ^b	70
	(Myr + α -TOH) ₃	0.3	20.5 $\pm$ 1.5	8.9 $\pm$ 0.8	5.5 $\pm$ 0.8	Synergism (42%)	TGSO ^b	70
	(Myr + α -TOH) ₆	0.6	31.1 $\pm$ 1.5	16.3 $\pm$ 0.9	7.4 $\pm$ 0.8	Synergism (14%)	TGSO ^b	70

%Synergism = $[\text{IP}_{1+2} - (\text{IP}_1 + \text{IP}_2) / (\text{IP}_1 + \text{IP}_2)] \times 100, \%$.

Table 3. Kinetic models and their characteristics

Kinetic Models and Characteristics	Model 2	Model 3
Name	Kinetic CL method	Lipid autoxidation
Initiator	AIBN	Without an initiator
Oxidizing substrate	Cumene/ DPM	TGSO kinetically pure
Experimental conditions	t=60°C/ t=50°C; homogeneous solution	t=80°C; homogeneous solution
<i>Kinetic scheme of oxidation</i>	<i>Initiated oxidation</i>	<i>Autoxidation</i>
<i>In absence of the antioxidant</i>	<i>Non-inhibited process</i>	<i>Non-inhibited process</i>
Chain generation	AIBN + RH(O ₂) $\rightarrow$ RO ₂ $\cdot$ (R _{IN})	LH + O ₂ (Y) $\rightarrow$ LO ₂ $\cdot$ (R _{IN})
Chain propagation	RO ₂ $\cdot$ +RH (+O ₂) $\rightarrow$ ROOH + RO ₂ $\cdot$ (kp)	LO ₂ $\cdot$ +LH (+O ₂) $\rightarrow$ LOOH + LO ₂ $\cdot$ (kp)
Chain termination	RO ₂ $\cdot$ + RO ₂ $\cdot$ $\rightarrow$ P* $\rightarrow$ P + hv (kt)	LO ₂ $\cdot$ + LO ₂ $\cdot$ $\rightarrow$ P (kt)
Chain branching 1		LOOH (+O ₂) $\rightarrow$ δ_1 LO ₂ $\cdot$ + P1 (k _{b1})
Chain branching 2		LOOH + LH (+O ₂) $\rightarrow$ δ_2 LO ₂ $\cdot$ + P2 (k _{b2})
Chain branching 3		LOOH + LOOH (+O ₂) $\rightarrow$ δ_3 LO ₂ $\cdot$ + P3 (k _{b3})
<i>In presence of the antioxidant</i>	<i>Inhibited process</i>	<i>Inhibited process</i>
Inhibition1	RO ₂ $\cdot$ + AH $\rightarrow$ ROOH + A $\cdot$ (k _A)	LO ₂ $\cdot$ + AH $\rightarrow$ LOOH + A $\cdot$ (k _A)
Inhibition 2	RO ₂ $\cdot$ + A $\cdot$ $\rightarrow$ ROO-A (k _{A'})	LO ₂ $\cdot$ + A $\cdot$ $\rightarrow$ LOO-A (k _{A'})
Inhibition 3 (Recombination)	A $\cdot$ + A $\cdot$ $\rightarrow$ A-A (k _R)	A $\cdot$ + A $\cdot$ $\rightarrow$ A-A (k _R)
Inhibition 4 (Disproportionation)	A $\cdot$ + A $\cdot$ $\rightarrow$ AH + Q (k _D)	A $\cdot$ + A $\cdot$ $\rightarrow$ AH + Q (k _D)
<i>Side reactions</i>		
<i>of phenolic antioxidant</i>	AH + ROOH $\rightarrow$ δ_1 RO ₂ $\cdot$ + P _A	AH + LOOH $\rightarrow$ δ_1 LO ₂ $\cdot$ + P _A
<i>of phenoxyl radical</i>	A $\cdot$ + ROOH $\rightarrow$ RO ₂ $\cdot$ + AH (k _{-A})	A $\cdot$ + LOOH $\rightarrow$ LO ₂ $\cdot$ + AH (k _{-A})
	A $\cdot$ + O ₂ $\rightarrow$ AO ₂ $\cdot$	A $\cdot$ + O ₂ $\rightarrow$ AO ₂ $\cdot$
	A $\cdot$ + RH (+O ₂) $\rightarrow$ AH + RO ₂ $\cdot$	A $\cdot$ + LH (+O ₂) $\rightarrow$ AH + LO ₂ $\cdot$
	A $\cdot$ + ROOH (+O ₂) $\rightarrow$ AH + RO ₂ $\cdot$	A $\cdot$ + LOOH (+O ₂) $\rightarrow$ AH + LO ₂ $\cdot$

Table 3. (Continued)

<i>Rate of initiation (R_i)</i>	Constant and well-controlled	Accelerated during the process
<i>Rate of oxidation (R_0) and (R_A)</i>	Constant and well-controlled	Accelerated during the process
Rate of non-inhibited oxidation (R_0)	$R_0=k_p [RH](R_{IN}/k_t)^{0.5}$	$R_0=k_p [LH](R_{IN}/k_t)^{0.5}$
Rate of inhibited oxidation (R_A)	$R_A=k_p[RH]R_{IN}/nk_A[AH]_0$	$R_A=k_p[LH]R_{IN}/nk_A[AH]_0$
<i>Oxidation chain length (v_0) and (v_A)</i>		
Chain length of non-inhibited oxidation (v_0)	$v_0= R_0/R_{IN}$	$v_0= R_0/R_{IN}$
Chain length of inhibited oxidation (v_A)	$v_A= R_A/R_{IN}$	$v_A= R_A/R_{IN}$
<i>Inhibition degree (ID)</i>	$ID = v_0/v_A$	$ID = R_0/R_A$
<i>Induction period (IP)</i>	$IP=n[AH]_0/R_{IN}$	$IP=n[AH]_0/R_{IN}$
<i>Antioxidant efficiency</i>	k_A	$RAE=(IP_A-IP_0)/IP_0$

The reaction between phenolic antioxidant and peroxy radicals ($AH + RO_2\bullet \rightarrow ROOH + A\bullet$) is known as the key reaction of the inhibited oxidation process. In CL experiments, the duration of induction period (IP) is equal to the time from the moment of the inhibitors' injection to 50% restoration of the CL intensity (for experimental details see [30, 31]). The kinetics of CL was studied by monitoring the autoxidation of cumene (60°C) and/or diphenyl methane (DPM) at 50°C under air atmosphere. The rate of free radical generation (rate of initiation, R_{IN}) was determined from the induction period, IP, caused by adding the standard inhibitor CrC₁ at known concentrations, $[CrC_1]_0$: $R_{IN}=2[CrC_1]_0/IP$.

2.3. Model 3: Estimation of the Chain - Breaking Antioxidant Activity of the Phenolic Compounds during Lipid Autoxidation – Provides Information about the Efficiency (RAE) and Reactivity (ID) of the Phenolic Antioxidants to Inhibit the Lipid Oxidation [5,15]

Triacylglycerols of commercially available sunflower oil (TGSO) were cleaned from pro- and antioxidants by adsorption chromatography and stored under nitrogen at -20°C. Lipid samples (kinetically pure TGSO) containing various inhibitors were prepared directly before use. Aliquots of the antioxidant solutions in purified acetone (or methanol and 1-2 droplets of DMSO depending on the solubility) were added to the lipid sample. Solvents were removed in nitrogen flow (99.99%). Fatty acid composition of the lipid sample (in wt%): C_{16:0}- 6.7 %, C_{18:0}- 3.6 %, C_{18:1}- 25.1 %, C_{18:2}- 63.7%, C_{20:0}- 0.2 %, C_{22:0}- 0.7 %) was determined by GC analysis of the methyl esters of the total fatty acids obtained according to Christie [32] with GC-FID Hewlett-Packard 5890 equipment (Hewlett-Packard GmbH, Austria) and a capillary column HP INNOWAX (polyethylene glycol mobile phase, Agilent Technologies, USA) 30 m x 0.25mm x 0.25mm. Lipid autoxidation was carried out at 80°C (±0.2) by blowing air through the samples (2.0 mL) in the dark at a rate of 100 mL min⁻¹ (kinetic regime of oxidation). The oxidation process was monitored by withdrawing samples at various time intervals and determining the primary oxidation products (hydroperoxides) as peroxide value (PV) [33]. All kinetic data were calculated as the mean result of two or three independent experiments and were processed using the computer programs Origin 6.1 and Microsoft Excel-97. The standard deviation (SD) for different mean values of induction period (IP) was (in h): IP=2.0, SD=0.2; IP=5.0, SD=0.3; IP=15.0, SD=1.0; IP=25, SD=1.5; IP=50.0, SD=3.0. The SD of PV determination (in meq kg⁻¹), according to the modified iodometric method for different mean values of PV, was: PV=12.0, SD=1.0; PV=30.0, SD=2.0; PV=70.0, SD=5.0; PV=150.0, SD=10; PV=250.0, SD=20. The R_A and R_C were quite constant varying by less than 2% [5,10].

The following main antioxidative properties, characterizing the kinetics of lipid autoxidation in absence and in presence of different additives were determined and compared [5,15]:

- *Relative antioxidant efficiency (RAE)*, expresses the relative increase in the oxidation stability of the sample in the presence of antioxidant, taking into account the oxidizability of the control sample, i.e. $RAE = (\Delta IP/IP_C) = (IP_A - IP_C)/IP_C$. IP_C and IP_A represent the induction periods in absence and in presence of an antioxidant resp.

- *Inhibition degree (ID)*, is a measure of the antioxidant reactivity or strength, e.g. how many times the antioxidant shortens the oxidation chain length, i.e. $ID = R_C/R_A$. R_C and R_A – the initial oxidation rate in absence and in presence of an antioxidant resp. *Synergism, additivism, and antagonism of binary mixtures* [34]
- *Synergism* – is observed when the inhibiting effect of the binary mixtures (IP_{1+2}) is higher than the sum of the induction periods of the individual phenolic antioxidants ($IP_1 + IP_2$) i.e. $IP_{1+2} > IP_1 + IP_2$. The percent of the synergism is presented by the following formulae [35]:

$$\% \text{ Synergism} = \{[IP_{1+2} - (IP_1 + IP_2)] / (IP_1 + IP_2)\} \times 100, \%$$

- *Additivism* - is observed when the inhibiting effect of the binary mixtures (IP_{1+2}) is equal to the sum of the induction periods of the phenolic antioxidants alone ($IP_1 + IP_2$) i.e. $IP_{1+2} = IP_1 + IP_2$.
- *Antagonism* - is observed when the inhibiting effect of the binary mixtures (IP_{1+2}) is lower than the sum of the induction periods of the individual phenolic antioxidants ($IP_1 + IP_2$) i.e. $IP_{1+2} < IP_1 + IP_2$

2.4. Model 4: Theoretical Methods (Predictable Activity)[18-22, 36-40]

2.4.1. Statistical Analysis – Gives Information about the Correlation between Experimental and Theoretical (Calculated / Predictable) Radical Scavenging Activity of Flavonoids and Related Compounds [5,18,37,41]

A correlation between the antiradical activity with various physic - chemical parameters of flavonoids, presented as theoretical values i.e. QSAR is based on the statistical analysis of Amic *et al.* [37] considering the well-known fact that the position of phenolic groups in flavonoids could be more important for the radical scavenging activity than the number of the phenolic groups. Thus the indicator variable I is defined as the sum of the following molecular features: i) the presence of 2,3-double bond ($I = 1$) or (ii) two of 3,5,7-OH groups ($I=1$) or (iii) two of 3',4',5'-OH groups ($I=1$) or (iv) in the case of absence of the above situations ($I = 0$): $\%RSA_{calc} = 3.954 + 75.950 \cdot I_{3',4'\text{-di-OH or } 3\text{-OH}} + 8.499 \cdot I_{5\text{-OH}}$; ($I = 1$ for 3',4'-di-OH and/or 3-OH) and $I = 1$ for 5-OH)

2.4.2. TOPS - MODE QSAR Approach

TOPS - MODE is the acronym of TOPological Substructural MOlecular Design. The philosophy of this approach consists of deriving linear quantitative structure-activity relationship models using moments of the bond matrix. These models contain encrypted structural information. The key advantage of the TOPS-MODE approach is that the developed encrypted models may be transformed into quantitative contributions of the different bonds in the molecule. These bond contributions can then be used in formulating rules regarding the influence of key features of the molecule in a mechanism of action. The TOPS-MODE descriptors account for hydrophobicity/polarity, electronic and steric features of molecules on the basis of bond weights. The bond weights used in this work describe the n-octanol/water partition coefficient (H), polar surface, polarizability, Gasteiger-Marsilli

charges, van der Waals atomic radii and molar refraction. Selected phenolic compounds differ significantly with their antioxidant activity and hydrophilic-lipophilic balance was tested using this TOPS – MODE QSAR approach according to Estrada [38,39].

2.4.3. Quantum Chemical Calculations – Determination of the O-H Bond Dissociation Energy

The most important factor has been considered for the characterization of free radical scavenging activity of antioxidants, for instance, the difference in the heat of formation (ΔH_f) of the antioxidant molecule and its corresponding radical i.e. the O-H bond dissociation energy (BDE_{O-H}). Theoretical hyperfine coupling constants were found by using quantum-chemical calculations. Due to the large size of the antioxidant molecules, semi empirical quantum-chemical methods have been employed in most cases, DFT calculations (B3LYP, and basic sets 6-31* and 6-31**) also were applied [5, 15,17, 21, 22, 29, 34, 43-46].

3. Results and Discussions

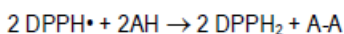
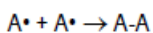
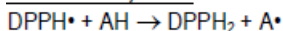
Tables 1 and 2 present structures of all individual phenolic antioxidants in this study, which were separated into 8 main groups, based on their structural characteristics. Different extracts from medicinal plants and spices as well as from propolis of different geographic areas and binary mixtures of two phenolic antioxidants were also included.

Model 1 – Comparable analysis of radical scavenging activity with quantitative DPPH TLC rapid test demonstrated, that this approach is very useful for rapid determination of active compounds and also to separation into three main groups (with strong, moderate and weak radical scavenging activity). All extracts from spices and medicinal plants were testing by this approach and a lot of individual phenolic compounds, especially the new hydroxyl-coumarins, complex bis-coumarins, etc. Comparison of the qualitative determination of %RSA of compounds tested with results from TLC DPPH rapid test demonstrated excellent agreement - the same separation into the main three groups of activity from qualitative DPPH test as with the quantitative DPPH test was obtained. This result is very important, because we may apply this rapid TLC DPPH test when we have a lot of new compounds or extracts for separation from them the active ones. The benefit of this approach is the much faster and not needed the UV/VIS spectrophotometer. The qualitative approach separated all studied compounds and extracts by using these criteria: a) strong activity (%RSA > 70) these are compounds with a catecholic structure (e.g. Qu, Qu-3Gu, Qu-3-Rhm, Ru, CA, Ch4); b) moderate activity (%RSA = 30-70) these are mono-phenolic antioxidants with electron donating substituent in *ortho* position (e.g. α -TOH, SA, FA) and with a free 3-OH phenolic group of flavonoids (Kf, Isrh) and c) weak activity (%RSA < 20) there are mono phenols without an *ortho* substituent (e.g. p-CumA, Ch1, Ch2, Ch3,) and 3-O-Glycosides of Kf and Isorh (Kf-3Glu, Isrh-3-Glu, Kf-3-Rut, Isrh-3-Rut, Kf-3-Cum-Glu, Isrh-3-Cum-Glu). From this comparable analysis could be concluded that all phenolic compounds with catecholic moieties demonstrate much higher activity as radical scavengers than those of mono phenols. The highest RSA of quercetin and its derivatives is due to reaction of bimolecular recombination with disproportionation of the semiquinone radicals formed from them (Qu,

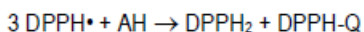
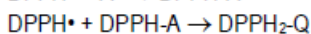
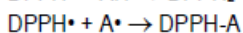
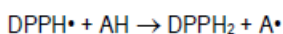
Qu-7-Glu, Qu-3Glu, Qu-3-Rhm, Ru), which results in regeneration of initial antioxidant molecule and also in formation of *ortho*- and *para*- quinone [5,15, 29,41,43-48].

The stoichiometry (*n*) of reaction between AH and DPPH[•], i.e. how many radicals (DPPH) were scavenged by one molecule of the antioxidant (AH), depends on antioxidant's structure and mechanism. The following stoichiometries are possible:

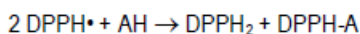
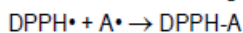
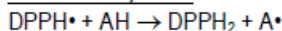
Stoichiometry *n* = 1



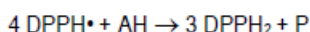
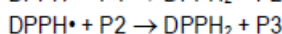
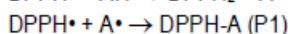
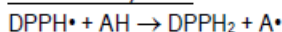
Stoichiometry *n* = 3



Stoichiometry *n* = 2



Stoichiometry *n* = 4



These mechanisms of action of AH with DPPH[•] explain the higher stoichiometry (*n*=3.6) for Qu than the calculated theoretical value of 2 and for Kf and Isorh – higher than 1 (*n*=1,5 and 1,6 resp.) [5, 15, 49, 50].

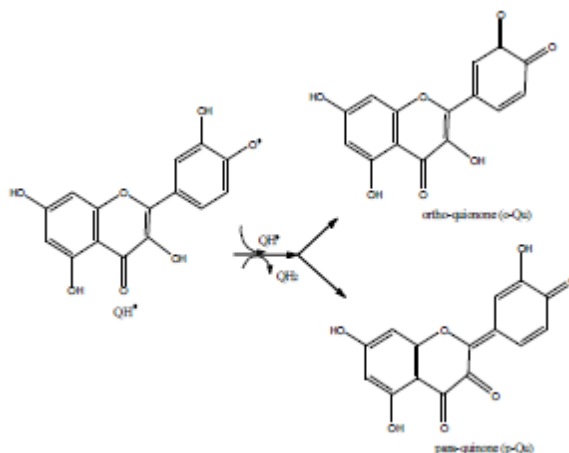
Model 2 –The oxidation mechanism of model hydrocarbons RH (cumene or DPM), initiated by a free radical initiator azo-bis-isobutyronitril (AIBN) in absence (non-inhibited) and in presence (inhibited) of antioxidants (AH), may be described with the following kinetic scheme (see Table 3). The results obtained [5,29-31] by both kinetic models (cumene and DPM) demonstrated that the antioxidant activity (determined as a rate constant of the reaction with peroxy radicals, *k_A*) decreases in the following sequence: (*Cumene*): α-TOH (1.3 10⁵) = CrC1 (1.2 10⁵) >> CA (8.3 10⁴) > SA (2.5 10⁴) > Ch7 (1.5 10⁴) ≥ BHT (1.4 10⁴) = Ru (1.4 10⁴) = Ch8 (1.3 10⁴) > FA (0.8 10⁴) > p-CumA (0.7 10⁴) and (*DPM*): Lu (2.2 10⁷) = Qu (2.1 10⁷) > Ch4 (1.3 10⁷) > α-TOH (8.5 10⁶) > CrC1 (7.6 10⁶) > CA (4.6 10⁶) > Kf (1.0 10⁶) > Ch3 (1.1 10⁵) > Ch1 (3.9 10⁴) > FA (3.6 10⁴) = BHT (3.6 10⁴) > p-CumA (2.2 10⁴) > Ch2 (1.1 10⁴) > Ch1 (9.6 10³) = Ch5 (9.6 10³) = Ch6 (9.6 10³). Obtained *k_A* for the same phenolic antioxidant is much lower in case of cumene, due to the lower rate constant of cumene peroxy radicals' termination (*k_t*) [12, 30, 31, 34]. In case of DPM as oxidizing substrate the most powerful antioxidants are those with a catecholic structure of the phenolic groups: Qu, Lu, CA, Ch4 (*k_A* ≈ 10⁷), and also α-TOH and Kf (*k_A* ≈ 10⁶) and Ch3 (*k_A* ≈ 10⁵). In case of cumene as oxidizing substrate – the most powerful antioxidant is α-TOH (*k_A* ≈ 10⁵), but its value is much lower than this determined with DPM. CA, Ru, BHT, SA demonstrated a moderate activity (*k_A* ≈ 10⁴) in case of cumene oxidizing substrate. It could be concluded that only the antioxidants with weak and moderate rate constants of reactions with peroxy radicals should be studied using a cumene as oxidizing substrate. In case of DPM as oxidizing substrate higher values of *k_A* were obtained for the antioxidants with moderate and weak activity (see data for cumene of the same antioxidants). The antioxidants with highest activity, *k_A*, demonstrated almost 2 - fold lower values with cumene than with DPM, which makes DPM much better substrate for studying the powerful antioxidants [5,30,31].

Model 3 – Lipid autoxidation is a radical process involved in a chain reaction including induction, propagation and termination steps [5,10,15,35]. During the induction period various lipid peroxy radicals are formed. These highly reactive chemical species produce hydroperoxides (LOOH) during the propagation stage. Termination consists of association of two radicals together to form more stable products. The basic kinetic scheme of lipid (LH) autoxidation in absence (non-inhibited oxidation) and in presence (inhibited oxidation) of mono-phenolic (AH) or bi- and poly-phenolic (QH₂) antioxidants, is presented in Table 3. It has been established that antioxidant efficiency (RAE) and inhibition degree (ID) of the tested compounds at concentration of 0.1mM decrease in the following sequences:

RAE: Ch4 (10.7) > CA (8.8) > Qu (6.6) > BisCum1(5.1) > α -TOH (4.5) > Cum₅ (3.7) > HQ (3.3) > SA (3.2) > Cum₄ (2.7) > PHC (2.6) > N9 (2.0) > Lu (1.7) > BHT(1.5) > N8 (1.4) > N4 (1.3) > (Qu-7) (1.2) = DPHC (1.2) = N7 (1.2) > Ru (1.1) = N3 (1.1) = N1 (1.1) > N5 (0.8) = N2 (0.8) > N6 (0.7) = BisCum3 (0.7) = FA (0.7) > Cum₇ (0.5) = (Lu-7) (0.5) > Cum₆ (0.3) > Cum₃ (0.2) > Ch3 (0.1) = Ch5 (0.1) = (Qu-3) (0.1) = Ch2 (0) = Ch1 (0) = Ch6 (0) = Cum₁ (0) = Cum₂(0) = Cum₈ (0) = Cum₉ (0) = p-CumA (0);

ID: Ch4 (25) > Qu (22.5) > CA (11.0) > Ru (8.0) > BisCum1 (7.5) > Cum₅(7.0) = Cum₄ (7.0) > N3 (6.8) > N4 (6.4) = N6 (6.4) > α -TOH (6.0) = N9 (6.0) > Cum₇ (5.6) = Lu (5.5) > PHC (4.9) > N1(4.6) > N7 (4.4) > N2 (3.5) > DPHC (3.4) > HQ (3.3) > N8 (3.2) > N5 (3.1) = BisCum3 (3.1) > SA (2.9) > (Qu-7) (2.2) > FA (1.9) > BHT (1.7) > Ch3 (1.6) > Cum₆ (1.3) = (Lu-7) (1.3) > Ch5 (1.2) > Cum₃ (1.1) = Cum₁ (1.1) = Cum₉ (1.1) = Cum₁ (1.0) = Cum₈ (1.0) = p-CumA (1.0) > Ch6 (0.9) = BisCum2 (0.8) = BisCum4 (0.8) = BisCum5 (0.8) = Ch2 (0.8) > = Ch1 (0.8) > (Qu-3) (0.5).

It can be seen from presented data, that the antioxidants Ch4, Qu, CA, Cum₅, Cum₅, BisCum1 and Ru (all of them are with a catecholic moiety) demonstrate strongest antioxidant efficiency (RAE) and highest values of their inhibition degree (ID). The latter can be explained with the possibility of those biphenolic groups to produce semiquinone radicals (QH•) during the reaction between bi- and poly-phenolic antioxidants with a catecholic structure (QH₂) and lipid peroxy radicals (LO₂•): QH₂ + LO₂• → QH• + LOOH. Semiquinone radicals recombine with the highest rate constant (almost 10⁹ M⁻¹s⁻¹, [11,15,34,42,52,53]) by two possible reaction mechanisms: 1) bimolecular recombination with dimerization of semiquinone radicals (path A) 2QH• → QH-QH and 2) bimolecular recombination with homo-disproportionation of semiquinone radicals (path B) 2QH• → QH₂ + Q. Path B is very important, due to the possibility to regenerate initial molecule of phenolic compounds. In case of flavonoid compounds (QH₂) the *ortho*- and *para*-flavonoid quinones are formed and initial molecule of QH₂ is regenerated during this reaction of homo-disproportionation of two QH• as shown in Scheme 1:



Scheme 1. Path B. Bimolecular recombination with disproportionation of semiquinone radicals from quercetin (QH•) and formation of ortho-quinones (o-Qu) or para-quinones (p-Qu).

It was found that following new sequences for RAE and ID decrease for 1.0mM of AH:

RAE: Qu (38.5) > CA (33.0) > BisCum1 (24.4) > Cum₅ (15.9) > N9 (14.5) > N7 (14.1) > N8 (14.0) > SA (13.6) > Cum₄ (11.8) > BisCum3 (7.1) > Cum₇ (4.4) > Cum₆ (3.9) = Lu (3.8) > Qu-3-Rhm (2.2) > FA (2.0) = Cum₃ (2.4) > Ru (1.9) > BisCum2 (1.0) = BisCum5 (1.0) = p-CumA (1.0) > BisCum4 (0.6) > Cum₂ (0.5) > Cum₈ (0.3) = Qu-7-Glu (0.2) > Lu-7-Glu (0.1)= Cum₁ (0.5) = Cum₉ (0).

ID: Qu (80) > Cum₅ (56.0) > CA (42) > BisCum1 (30.0) > Cum₄ (28) > BisCum3 (10) > N7 (9.6) > Lu (8.6) > SA (8.2) > N8 (8.1) = N9 (8.1) > Cum₆ (7.0) > Cum₇ (6.2) > Lu-7-Glu (5.5) > Cum₃ (4.3) = Ru (4.2) > BisCum4 (3.8) > Qu-3-Rhm (3.6) > BisCum5 (3.3) > FA (3.1) > p-CumA (2.3) = BisCum2 (2.0) > Cum₈ (1.6) > Qu-7 (1.2) > Cum₁(1.0) = Cum₉ (1.0).

In order to study the possible synergism between two phenolic antioxidants, various binary mixtures of two antioxidants were tested and compared: a) binary mixtures of two antioxidants without α -tocopherol; and b) binary mixtures with α -tocopherol (Qu+ α -TOH, Ru+ α -TOH, Cum₁+ α -TOH, Cum₂+ α -TOH, Cum₃+ α -TOH, BisCum1+ α -TOH, BisCum2+ α -TOH, CA + α -TOH, SA + α -TOH, BHT+ α -TOH, TBHQ+ α -TOH). Table 2 presents the synergism and/or additivism/antagonism obtained. It is shown that only binary mixtures with α -TOH demonstrate synergism. Most of binary mixtures without α -TOH do not show any synergism, additivism even an antagonism between them may be observed. The following new sequences of % synergism and of the inhibiting activity (IP_{I+2}) are: (Ru+ α -TOH)₁ > (SA+ α -TOH)₁ > (Qu+ α -TOH)₁ > (TBHQ + α -TOH)₁ > (Myr+ α -TOH)₁ > (BHT + α -TOH)₁ > (Cum₄+ α -TOH)₁ = (Myr+ α -TOH)₆

This synergism of binary mixtures with α -TOH may be explained taking into account that the catecholic moiety of the caffeic acid, flavonoid and coumarine molecules allows formation of semiquinone radicals. These semiquinone radicals may regenerate the initial antioxidant molecule during reaction of bimolecular recombination with homo- and cross-disproportionation of semiquinone radicals [5,15,52]:

- a) *Homo-disproportionation reaction*: $2\text{QH}\cdot \rightarrow \text{QH}_2 + \text{Q}$ regeneration of QH_2 (Flavonoid or coumarine) and
 $2\alpha\text{-TO}\cdot \rightarrow \alpha\text{-TOH} + \alpha\text{-T=O}$ regeneration of $\alpha\text{-TOH}$
- b) *Cross-disproportionation reaction*: $\text{QH}\cdot + \alpha\text{-TO}\cdot \rightarrow \text{QH}_2 + \alpha\text{-T=O}$ regeneration of QH_2 and
 $\alpha\text{-TO}\cdot + \text{QH}\cdot \rightarrow \alpha\text{-TOH} + \text{Q}$ regeneration of $\alpha\text{-TOH}$

These reactions demonstrate that during oxidation process initial molecules of flavonoids and $\alpha\text{-TOH}$ are regenerated by different mechanisms and that makes these binary mixtures the most powerful antioxidant compositions. It must be noted that $\alpha\text{-TOH}$ regeneration is evidently responsible for the highest % synergism obtained. For binary mixtures Qu+Lu, Qu+Ru, and Qu-Lu at the same equimolar (1:1) concentrations (0.1mM and 0.5 mM) neither synergism, nor additive effect ($\text{IP}_{1+2} = \text{IP}_1 + \text{IP}_2$) was observed [5,53]. In this case only reactions of homo-disproportionation are possible and only the initial molecule of the corresponding flavonoid antioxidants is regenerated. Evidently, lack of $\alpha\text{-tocopherol}$ molecule during the lipid autoxidation is a reason that no synergisms were found in these binary systems.

Model 4a - QSAR Statistical Analysys

The relationship between the structure and radical scavenging activity of flavonoids (aglycones, glucosides, rutinoides and p-coumaroyl-glucosides) has been studied. The detailed statistical analysis of theoretically calculated (%RSAcalc) and the experimentally obtained (%RSAexp) values for the radical scavenging activities of all studied flavonoids is presented in Refs. 5,37,41. The experimental data are presented as %RSA obtained by using rapid DPPH radical test and from paper of Amic *et al* [37], who calculated the theoretical values of %RSAcalc using the statistical model: $\% \text{RSAcalc} = 3.954 + 75.950 \text{I}_{3',4'\text{-diOH or } 3\text{-OH}} + 8.499 \text{I}_{5\text{-OH}}$. This model divides the compounds into two main groups [37,53]: i) Active - %RSAcalc = 88.4% - for all flavonoids with catecholic structure in ring B and free 3-OH and 5-OH groups, i.e. for these compounds I =1 (for 5 flavonoids: Qu, Qu-3-Glu, Ru, Kf and Isrh); ii) Inactive - %RSA calc =12.45%. This value is found only for flavonoid compounds without catecholic structure and without free 3-OH (for 6 compounds: Kf-3-Glu, Kf-3-Rut, Kf-3-Cum-Glu, Isrh-3-Glu, Isrh-3-Rut, Isrh-3-Cum-Glu). A good correlation between experimental and theoretical RSA has been found. New data obtained are in agreement with those published earlier about structure-radical scavenging activity relationships [49, 50].

Model 4b – TOPS - MODE QSAR

Phenolic compounds with a wide range of antioxidant activity (presented as inhibition degree, ID) were chosen for TOPS-MODE QSAR study. ID parameter is of great importance for the practice, because it demonstrates the possibility of the antioxidant molecule to reduce

significantly the length of lipid autoxidation chain, and to terminate the oxidation process. In this comparable analysis flavonoids, cinnamic acid derivatives, standard antioxidants and chalcones were included with a wide range of their inhibiting degree values and then separated into three main groups: Group 1 – compounds with the highest values of their inhibition degree, strong activity, i.e. $ID > 8.0$. Group 2 – compounds with moderate values of their inhibition degree, moderate activity, i.e. $8.0 > ID > 3.0$. Group 3 – compounds with the lowest values of their inhibition degree, weak activity or inactive - $3.0 > ID$. The obtained theoretical model demonstrates an excellent agreement between experimental and predictable inhibiting degree of the studied compounds using the following theoretical parameters of the model: a catecholic moiety, dipole moment component and sterical effect component. The classification percent correct for the very active compounds is 100%; percent correct for the moderate active group is 92.7%; percent correct for the weakly active/inactive group is 83.3% and percent correct total is 89.65% [5,15].

Model 4c - Quantum Chemical Calculations

The highest radical-scavenging activity of quercetin derivatives correlates with quantum-chemical calculations [17,21,22,29,43-45], indicating that in case of catecholic structure of ring B the rest of molecule doesn't affect its ability to scavenge free radicals. The latter can be explained with possible formation of *ortho* - quinone as a result of homo-disproportionation reaction of semiquinone radicals, formed in the ring B. The important role of the catecholic structure in ring B of a flavonol was demonstrated in case of kaempferol and isorhamnetin, which antiradical capacities are significantly lower (no formation of *o*-quinone is possible) than those for quercetin and its 3-O-glycosides. It has been demonstrated that the substitution of 3-OH hydrogen with sugar moiety (glucose, rutinose and/or *p*-coumaroyl-glucose) in case of kaempferol and isorhamnetin, leads to a strong decrease of their antiradical activity. The poor radical-scavenging activities of these compounds could be explained by the substitution of the 3- and 3'-hydroxyl groups by sugar and OCH₃ or H groups. Thus, these compounds cannot be oxidized to *o*- and *p*-quinones. 3-OH group fixes B ring by a hydrogen bond formation with 4-keto group of ring C and when flavonoid radical is formed, the electron delocalization to the whole molecule is possible. As a result higher stability of radical formed was observed and also higher antiradical activity of Qu than those of Qu-3, Ru and Lu. In case of Qu-3-Glu, Ru, and Lu the formation of semiquinone radicals in B ring is possible. However, lack of 3-OH group (Lu) or glycoside moiety (Qu-3-Glu and Ru) makes electron delocalization impossible, because ring B is twisted [45]. As a result their chain-breaking antioxidant activity decreases. In case of Kf and Isorh without a catecholic structure (FIOH) in B ring only formation the *para*-flavonoid quinone (*p*-Qu) is possible in the reaction of homo-disproportionation of the flavonoid radicals ($2FIO\bullet \rightarrow FIOH + FI=O$). For that reason the substitution of the 3-OH group with a glycoside moiety strongly decreases their antioxidant activity [5, 15, 51]. We have used a semi empirical PM3 and PM6 methods as most reliable for molecules with filled non-bonding p-orbitals on adjacent atoms [5,15, 29]. The following sequence of $\Delta H(O-H)$ (kcal/mol) was found for the chalcones: Ch3 (81.42) $\approx$ CA (81.46) < FA (83.91) < Ch1 (86.93) $\approx$ Ch2 (86.95) < p-CumA (87.29). The main conclusion of this comparable analysis is that both compounds with the catecholic structure in B ring (CA and Ch4) demonstrate the strongest activity assessed by all methods applied -

experimental (lipid autoxidation; chemiluminescence method; DPPH test and PM3 calculations). Chalcone Ch2 (3-hydroxy-chalcone) without a catecholic structure and *ortho* substituent showed the lowest activity by all methods applied. However Ch3 (4-hydroxy-chalcone) has a moderate activity against peroxy radicals (CL method).

DFT calculations were applied to prove the structure of phenoxyl radicals from methoxyphenols [16] and chalcones [8] as well as aroxyl radicals from hydrolyzable tannins [42] formed during the oxidation process on the base of theoretical hyperfine coupling constants obtained. Comparable analysis of experimental EPR spectrum and simulated EPR spectrum with theoretical hyperfine coupling constants of probable aroxyl radicals formed demonstrated an excellent agreement and prove the structure of radicals formed [16,42].

Comparison of Experimental and Theoretical Parameters

Main structural fragments of phenolic antioxidants and their experimental (antiradical and chain-breaking antioxidant) activity and theoretical (predictable) activity were compared using different methods: (DPPH test, %RSAexp), CL experiments (k_A), lipid autoxidation (RAE, ID), statistical analysis (%RSAcalc), TOPS-MODE QSAR (ID predictable) and quantum chemical calculations (bond dissociation energy). All compounds were separated in three main groups: a) with strong activity; b) with moderate activity; and c) with weak activity. A good correlation between %RSAexp and H-donating ability could be seen, calculated as %RSAcalc and with H bond dissociation energy as D_{O-H} [5, 18,29, 41]. Best correlation has been found between experimental inhibition degrees with the predictable values found by using TOPS-MODE QSAR approach. There is a good correlation also between the absolute rate constants of antioxidants with peroxy radical, determined using CL approach with the H-bond donating ability and with antioxidant efficiency during lipid autoxidation.

4. Phenolic Antioxidants of Natural Origin and their Effect of Human Health

4.1. Extracts from Medicinal Plants and Spices

Plants, due to their inability to avoiding detrimental conditions by removing themselves from the source of the danger, have evolved a whole battery of protective measures. One of the unavoidable adverse conditions is oxidative stress, against which numerous antioxidative active components exist in plants. Animals and humans, in their diet, ingest these ingredients and thus partake in the plant antioxidant defence system.

The plant *Tribulus terrestris* is used in traditional medicine of India, China and Bulgaria for treatment of various diseases. The saponin fraction of this plant finds application in the contemporary medicine as a component of drugs effective in treating impotence and cardiovascular diseases [74]. It is also included in many dietary supplements with claim of a general stimulating action on motor activity, much tone and restorative tonic for vigor [75]. The cytotoxic activity of the plant has been also reported [76]. The saponin fraction is a rich

source of steroidal saponins and flavonoids [77,78]. The following flavonoids were isolated: Qu, Qu-3-Glu, Ru, Isorh, Isorh-3-glu, Isorh-3-rut, Isorh-3-Cum-Glu, kf, Kf-3-Glu, Kf-3-Rut, Kf-3-Cum-Glu (for abbreviation see Table 1). New coumarin-glucosides of kaempferol (Kf-3-Cum-Glu) and of isorhamnetin (Isorh-3-Cum-Glu) were found in the saponine fraction from the first time [41] and they showed a moderate radical scavenging activity.

Shaer *et al.* [79] recommended *Carthamus lanatus* L. (Asteraceae) as an important flavonoid bearing plant of phyto-pharmaceutical importance. From aerial parts of *Carthamus lanatus* grown in Bulgaria twelve bioactive phenolic compounds were isolated [80]. Antioxidative behavior of extracts, fractions and individual flavonoid constituents (quercetin, Qu, luteolin, Lu, quercitrin, Qu-3-Rhm, rutin, Ru, quercimetrin, Qu-7-Glu, and luteolin 7-*O*-glucoside, Lu-7-glu) (Table 1) from this plant were presented and compared with the inhibiting activities of known phenolic antioxidants. The comparable kinetic analysis at the same kinetic model for different phenolic antioxidants allowed us to compare their antioxidative properties and to make conclusions about their structure-activity relationship.

Plants belonging to the genus *Sideritis* (*Labiatae*) are well known in Bulgarian and other folk medicines for treating various infections, inflammations, etc. [56]. The presence of terpenoids, essential oils and flavonoids has been established. From some species a coumarins, sesamin and phenylpropanoid glycosides have been isolated. The radical scavenging activity and antioxidant activity of plant samples from several species and populations of the genus *Sideritis* (*Labiatae*) grown in Bulgaria (*S scardica*, *S syriaca*, and *S montana*) extracted with different solvents were determined by the beta-carotene bleaching test (BCBT), DPPH test and static headspace gas chromatography (HS-GC). Their activity was compared with those of known and standard antioxidants like rosmarinic acid and BHT. The pure reference compounds were applied in a ten-time lower concentration than the plant extracts. The highest antioxidant activity in the BCBT, close to that of BHT, was observed for the more apolar extracts. The inhibitory effect on beta-carotene bleaching of the polar extracts and rosmarinic acid was much lower than that of BHT. The inhibition of hexanal formation in bulk safflower oil by most of *S scardica* and *S syriaca* extracts was as effective as BHT but less so than rosmarinic acid. *S Montana* extracts showed weak antioxidant or even pro-oxidant properties. Extracts from butanol and from ethyl acetate and the total methanol extracts from all *Sideritis* plants studied showed a strong radical scavenging activity against DPPH radical, close to that of rosmarinic acid. The antioxidant activity of *Sideritis* extracts was attributed to the presence of flavonoids and phenylpropanoid glycosides [56].

The genus *Piper* belongs to the Piperaceae and has over 700 species distributed and *Piper* species have high commercial, economical and medicinal importance. Plants belonging to the genus *Piper* are required in the Indian Ayurvedic system of medicine for their medicinal properties and in folklore medicine in Latin America and West Indies [36, 55, 57]. Black pepper (*Piper Nigrum*) is one of the most widely used among spices. It is valued for its distinct biting quality attributed to the alkaloid, piperine. Black pepper is used not only in human dietaries but also for a variety of other purposes such as medicinal, as a preservative, and in perfumery. Many physiological effects of black pepper, its extracts, or its major active principle, piperine, have been reported in recent decades. Dietary piperine, by favorably stimulating the digestive enzymes of pancreas, enhances the digestive capacity and significantly reduces the gastrointestinal food transit time. Piperine has been demonstrated *in vitro* studies to protect against oxidative damage by inhibiting or quenching free radicals and reactive oxygen species. Black pepper or piperine treatment has also been evidenced to lower

lipid peroxidation *in vivo* and beneficially influence cellular thiol status, antioxidant molecules and antioxidant enzymes in a number of experimental situations of oxidative stress. The most far-reaching attribute of piperine has been its inhibitory influence on enzymatic drug biotransforming reactions in the liver. It strongly inhibits hepatic and intestinal aryl hydrocarbon hydroxylase and UDP-glucuronyl transferase. Piperine has been documented to enhance the bioavailability of a number of therapeutic drugs as well as phytochemicals by this very property. Piperine's bioavailability enhancing property is also partly attributed to increased absorption as a result of its effect on the ultrastructure of intestinal brush border. Although initially there were a few controversial reports regarding its safety as a food additive, such evidence has been questionable, and later studies have established the safety of black pepper or its active principle, piperine, in several animal studies. Piperine, while it is non-genotoxic, has in fact been found to possess anti-mutagenic and anti-tumor influences.

Chili is one of the important spices used in varieties of Indian dishes for imparting color, flavor and pungency to the food. Chilies contain Vitamin A, Vitamin C and capsaicin which are good antioxidants and anti-inflammatory agents. Antioxidants can also boost immune system. The antioxidants present in the chili scavenges the free radicals and are able to be used as effective and powerful chain breaking antioxidants [55,57].

It is amazing that propolis samples from different geographic and plant origin, of different chemical composition, all possess pronounced antioxidant properties [8,47,58,59]. The reason is the fact, that bees collect propolis from materials that lay on the plant surface and they are a barrier, protecting plant tissues from infestation by bacteria, fungi and viruses, but also from oxidative damage by reactive oxygen species. Thus, in the process of evolution the antioxidant properties of these surface materials have offered a clear advantage and were genetically preserved and it is not surprising that whatever its origin and chemistry may be, propolis always contains effective natural antioxidants. It is clear that propolis extracts and individual constituents may be used as effective bioantioxidants which will allow to reduce the coronary risk of human and to ensure the higher protective effect in foods. The beneficial effects of antioxidant activity of propolis against oxidation stress and cancer, i.e. the important role of propolis on human health have been proven by experimental, chemical and epidemiological data.

4.2. Disease Prevention

The antioxidant activity of polyphenols offers many health benefits. However, in some cases the impact of phenolic compounds in the diet may not be related to their antioxidant activity. For example, phenols might exert effects within the gastrointestinal tract. Such effects could include binding of iron, scavenging reactive nitrogen, chlorine, and oxygen species, and inhibition of cyclooxygenases and lipoxygenases [82].

Resveratrol presents in grapes and wines and has been shown to have cancer chemopreventive activity in the three major stages of carcinogenesis: promotion, initiation, and progression [3,4,9]. The effect of resveratrol on factors implicated in the development of coronary heart disease-human platelet aggregation and the synthesis of eicosanoids (lipids) from arachidonate by platelets – were investigated and compared with the actions of other wine phenolics. Resveratrol and quercetin demonstrated a dose-dependent inhibition of platelet aggregation, whereas the other compounds tested were inactive. Resveratrol also

inhibited the synthesis of the eicosanoids in a dose-dependent manner, whereas the other phenolics were less effective or not effective at all. Removing the alcohol from the wine did not diminish the effect of platelet aggregation [83,84].

A rich dietary intake of fruits and vegetables has constitutently been associated with a reduced risk of various cancers including those of the lung, breast, prostate, and colon [3,4,9]. It is unknown which bioactive compound or compounds in plant foods provide these protective effects, but flavonoids have been of special interest. There are numerous animal model studies that suggested that flavonoids influence important cellular mechanisms related to carcinogenesis, including cell cycle control and apoptosis. There is substantial evidence that flavonoids, especially quercetin reduce the risk of lung cancer.

Green and black teas are sources of different polyphenolic compounds. Consumption of tea and its polyphenolic constituents offers protection against skin cancer induced by either chemical carcinogenesis or ultraviolet radiation in mice. Tea consumption also provides protection against cancers induced by chemical carcinogens that involve the lung, forestomach, esophagus, duodenum, pancreas, liver, colon, and skin in mice, rats, and hamsters. As was shown for resveratrol from grapes, polyphenols from green tea do not only have anticarcinogenic properties, but also protect against coronary heart disease and atherosclerosis, as well as inflammation [3,4,9].

Ellagitannins are dietary polyphenols containing ellagic acid subunits that are thought to act as cancer chemo-preventive agents. Thus, they may have properties that contribute to health benefits in humans. Little is known, however, of their metabolic fate. Since epidemiological studies have suggested a correlation between high flavonoids consumption and decreased risk of cancer, cardiovascular disease, and other age-related diseases, enhancing flavonoids biosynthesis in crops may result in foods with benefits to human health [3,4,9].

Plant phenolic compounds have also been suggested to provide a means for preventing the adverse effects that fungal toxins (mycotoxins) have on human health as well as serving in their detoxification [9].

Conclusion

The radical scavenging activity (%RSA) differs significantly from the chain breaking antioxidant activity. RSA towards DPPH radical gives information only about the H-donating capacity of the studied compounds and some preliminary information for their possibility to be used as antioxidants. This comparable study showed a good correlation between experimental and theoretical/predictable RSA. Antioxidant activity is capacity of the compound to short the oxidation chain length as a result of its reaction with peroxy radicals. For that reason we mean as antioxidant activity the chain breaking activity of the compounds. This comparable study showed an excellent correlation between experimental inhibiting degree of phenolic compounds and their predictable activity by using TOPS-MODE QSAR approach. Obtained theoretical model demonstrated an excellent agreement between experimental and predictable inhibiting degree of the studied compounds using following theoretical parameters of the model: a catecholic moiety, dipole moment component and steric effect component. It has been demonstrated that phenolic compounds with catecholic

moiety are the most powerful scavengers of free radicals and they may be used as effective chain breaking antioxidants. The highest antiradical and antioxidant activity of phenolic antioxidants with catecholic moiety is explained by possible mechanism of homo-disproportionation of their semiquinone radicals formed. Thus regeneration of the antioxidant molecule during the oxidation process is possible. It has been found that the rest of the antioxidant molecule is not of significance for the radical scavenging activity of phenolic antioxidants with a catecholic moiety. This comparable analysis demonstrates also that the activity of the antioxidant depends significantly not only on its structural characteristics, but also on the properties of the substrate being oxidized and the experimental conditions applied. Structural characteristics of the complex system: oxidizing substrate – antioxidant must be considered. The synergism has been found only for the binary mixtures of antioxidants with catecholic structure and tocopherol in equimolar concentrations. On the basis of this comparable analysis the most effective individual antioxidants and binary mixtures are proposed for highest and optimal lipid oxidation stability.

The nature creates a molecular diversity under the principles of usability and economy that selects only those structures that are necessary for some specific functions. However, this process of natural molecular evolution takes thousands of years in producing this limited but effective molecular diversity. We can learn from the nature about what kind of molecular structures are useful, for one practical purpose. Then we can use rational approaches to generate a great diversity from these structures in a fast and efficient way. The relation of structure to antiradical activity of individual compounds from plant extracts was studied by using theoretical models and compared with that of known natural antioxidants. It is of great importance, that individual constituents of different plant sources are mainly phenolic compounds and allow covering a wide range of hydrophilic-lipophylic balance. For that reason effective synthesis of new bio-antioxidants will be developed.

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Chapter II

Flavonoids and Brain Health

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A. Introduction

Representing one of the most important lifestyle factors, diet can strongly influence the incidence and onset of cardiovascular disease and neurodegenerative disorders. Evidence suggests that flavonoids are highly effective in reversing age-related declines in neuro-cognitive performance through their ability to interact with the cellular and molecular architecture of the brain responsible for memory and by reducing neuronal loss due to neurodegenerative processes. In particular, they may increase the number of, and strength of, connections between neurons, via their specific interactions with the ERK and Akt pathways, leading to an increase in neurotrophins such as BDNF. Concurrently, their effects on the peripheral and cerebral vascular system may also lead to enhancements in cognitive performance through increased brain blood flow and an ability to initiate neurogenesis in the hippocampus. Finally, they have also been shown to reduce neuronal damage and losses induced by various neurotoxic species and neuroinflammation. Together, these processes act to maintain the number and quality of synaptic connections in the brain, a factor known to be essential for efficient long term potentiation (LTP), synaptic plasticity and ultimately the efficient working of memory. This chapter will highlight the neuroprotective mechanisms of flavonoids through their ability to interact with neuronal signalling pathways, an ability to suppress neuroinflammation, their potential to promote memory, learning and cognitive function and their impact on the vascular system.

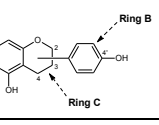
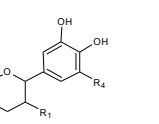
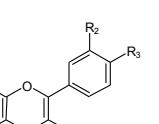
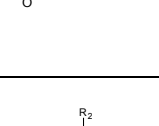
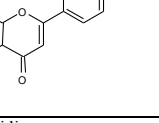
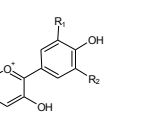
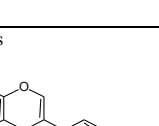
B. Synthesis, Source and Structure of Flavonoids

Flavonoids are polyphenolic compounds comprising 15 carbons, with two aromatic rings connected by a three carbon bridge ($C_6-C_3-C_6$) and are synthesised in plants from the reaction of a chalcone precursor with three molecules of malonyl-CoA. Under the action of the enzymes chalcone synthase and chalcone flavanone isomerase, the chalcone precursor is isomerized into a flavanone [37, 186]. Hydroxylation in position 3 of C-ring allows the differentiation of flavanonols from flavanones since they share a similar structure based on the 2,3-dihydro-2-phenylchromen-4-one skeleton (Table 1). From these central intermediates, the pathway diverges into several side branches, each resulting in a different class of flavonoids. Flavonoids share a common feature which consist of two aromatic carbon rings, benzopyran (A and C rings) and benzene (B ring), and may be divided in various subgroups based on the degree of the oxidation of the C-ring, the hydroxylation pattern of the ring structure and the substitution of the 3-position (Table 1). The main dietary groups of flavonoids are 1) flavones (e.g. apigenin, luteolin), which are found in parsley and celery. Hydroxylation on position 3 of the flavone structure gives rise to the 3-hydroxyflavones also known as the 2) flavonols (e.g. kaempferol, quercetin), which are found in onions, leeks, broccoli; 3) isoflavones (e.g. daidzein, genistein), which are mainly found in soy and soy products. These compounds have a large structural variability and more than 600 isoflavones have been identified to date and are classified according to oxidation level of the central pyran ring (Table 1); 4) flavanones/flavanonols (e.g. hesperetin, naringenin/astilbin, engeletin), which are mainly found in citrus fruit, herbs (oregano) and wine; 5) flavanols (e.g. (+)-catechin, (-)-epicatechin, epigallocatechin, epigallocatechin gallate (EGCG), which are abundant in green tea, red wine, chocolate. Flavanols are found both as monomers and oligomers referred to as condensed tannins or proanthocyanidins. Variations in their structures lie in the hydroxylation pattern of the B ring and the presence of gallic acid in position 3. The lack of a double bond at the 2-3 position and the presence of a 3-hydroxyl group on the C ring create two centres of asymmetry; 6) anthocyanidins (e.g. pelargonidin, cyanidin, malvidin), whose sources include red wine and berry fruits. These compounds exist as glycosides in plants, are water-soluble and appear red or blue according to pH. Individual anthocyanins arise from the variation in number and arrangement of the hydroxyl and methoxy groups around the 3 rings (Table 1).

C. Flavonoid Bioavailability and Access to the Brain

Many studies have reported the bioavailability of flavonoids in the systemic circulation [32, 104-105, 185], however little is known about their uptake within the brain. In order to understand whether these phenolic compounds could affect signal transduction pathways and neuro-hormetic mechanisms, it is crucial to ascertain their presence within the cerebral tissue.

Table 1. Structures and occurrences of the main classes of flavonoids

Classes and structures	Names and substitution patterns				Dietary Source	Content (mg/kg) or (mg/L) fresh weight
General structure 						
Flavanols 	Catechin Epicatechin EGC ECG EGCG	R1 OH OH OH gallate gallate	R4 H H OH H OH		Chocolate, Green tea, Beans, Black tea Apricot, Cherry, Grape, Peach, Blackberry, Apple, Red wine, Cider	500-1000 300-500 10-300
Flavanols 	Kaempferol Quercetin Myricetin Isorhamnetin	R2 H OH OH OCH3	R3 H H OH H		Onion, Kale Leek, cherry tomato, broccoli, blueberry blackcurrant, Apricot Apple, Green Bean, Black grape, Tomato, Black tea, Green tea	300-1200 50-300 0-50
flavones 	Chrysin Luteolin Apigenin	R2 H OH H	R3 H H OH		Parsley Celery Capsicum pepper	200-2000 20-200 1-20
Anthocyanidins 	Pelargonidin Cyanidin Delphinidin Peonidin Petunidin Malvidin	R1 H OH OH OCH3 OCH3 OCH3	R2 H H OH H OH OCH3		Aubergine, Blackberry, Blackcurrant, Blueberry, Black grape, Cherry Rhubarb Strawberry, red wine, plumb, red cabbage	1000-5000 1000-2000 10-1000
Isoflavones 	Genistein Daidzein	R2 OH OH	R3 OH H		Soya flour Soya beans, Miso, Tofu, Tempeh, Soya milk	1000-2000 10-1000
Flavanones/Flavanonols 	Naringenin Hesperetin Taxifolin Astilbin Engeletin	R1 H H OH O-rhamnosyl O-rhamnosyl	R2 OH OCH3 OH OH OH	R3 OH H OH OH OH	Orange, orange juiceGrapefruit, Grapefruit juice Lemon juice Wine	200-1000 100-700 50-300 1-15

In most cases, following oral ingestion and during absorption flavonoids are extensively metabolised. For example, the majority of flavonoid glycosides and aglycones present in plant-derived foods, are extensively conjugated and metabolised during absorption (reviewed in [146, 151]). In the upper gastrointestinal tract, dietary polyphenols are substrates for a number of enzymes, such as phase I enzymes (hydrolysing and oxidizing) and phase II enzymes (conjugating and detoxifying). During the transfer across the small intestine, and again in the liver, they are transformed into glucuronides, sulphates and *O*-methylated forms [149-150]. Further transformations occur in the colon, where the enzymes of the gut microflora act to metabolise flavonoids to simple phenolics acids, which are absorbed and further metabolized in the liver [32]. In addition, flavonoids may undergo at least three types of intracellular metabolism: 1) Oxidative metabolism, 2) P450-related metabolism and 3) Conjugation with thiols, particularly GSH [154]. The brain is protected by a structurally unique endothelial barrier: the blood-brain barrier (BBB), which differs from other vascular barriers in its physical characteristics including the nature and number of transporters it possesses [120]. In order for flavonoids to access the brain, they must first cross the physical filter, which controls the entry of xenobiotics into the brain. Flavanones such as hesperetin, naringenin and their *in vivo* metabolites, along with some dietary anthocyanins, cyanidin-3-rutinoside and pelargonidin-3-glucoside, have been shown to traverse the BBB in relevant *in vitro* and *in situ* models [191]. Their degree of BBB penetration is dependent on compound lipophilicity [190], meaning that less polar *O*-methylated metabolites may be capable to greater brain uptake than the more polar flavonoid glucuronides. However, evidence exists to suggest that certain drug glucuronides may cross the BBB [1] and exert pharmacological effects [87, 158], suggesting that there may be a specific uptake mechanism for glucuronides *in vivo*. Their brain entry may also depend on their interactions with specific efflux transporters expressed in the BBB, such as P-glycoprotein [99] which appears to be responsible for the differences between naringenin and quercetin flux into the brain *in situ* [191]. In animals, flavanones have been found to enter the brain following their intravenous administration [122], whilst epigallocatechin gallate [161], epicatechin [2] and anthocyanins [41, 164] are found in the brain after their oral administration. Furthermore, several anthocyanins have been identified in different regions of the rat [121] and pig brains [80, 110] of blueberry fed animals, with 11 intact anthocyanins found in the cortex and cerebellum. A recent investigation also reported anthocyanin presence within rat brains fed with a blackberry anthocyanin-enriched diet for 15 days. A total amount of 0.25 nmol.g⁻¹ of anthocyanins, including blackberry anthocyanins and peonidin 3-O-glucoside, were found in the cerebral tissue of these animals [164]. These results indicate that flavonoids transverse the BBB and are able to localize in the brain, suggesting that they are candidates for direct neuroprotective and neuromodulatory actions.

D. The Impact of Flavonoid on Memory, Learning and Neuro-Cognitive Performance

There is a growing interest in the potential of phytochemicals to improve memory, learning and general cognitive ability [145-146]. A recent prospective study aimed at examining flavonoid intake in relation to cognitive function and decline, has provided strong

evidence that dietary flavonoid intake is associated with better cognitive evolution, i.e. the preservation of cognitive performance with ageing [94]. In this PAQUID study (Personnes Agées QUID), a total of 1,640 subjects (aged 65 years or older) free from dementia at baseline and with reliable dietary assessment data were examined for their cognitive performance (Mini-Mental State Examination, Benton's Visual Retention Test, "Isaacs" Set Test) four times over a 10-year period. After adjustment for age, sex, and educational level, flavonoid intake was found to be associated with significantly better cognitive performance at baseline and with a significantly better evolution of the performance over time. In particular, subjects included in the two highest quartiles of flavonoid intake had better cognitive evolution than subjects in the lowest quartile and after 10 years follow-up, subjects with the lowest flavonoid intake had lost on average 2.1 points on the Mini-Mental State Examination, whereas subjects with the highest quartile had lost 1.2 points. Such data provides a strong indication that regular flavonoid consumption may have a positive effect on neuro-cognitive performance as we age.

There has been much interest in the neuro-cognitive effects of soy isoflavones, primarily in post-menopausal women [14, 44, 92, 181]. Isoflavone supplementation has been observed to have a favourable effect on cognitive function [22], particularly verbal memory, in postmenopausal women [86] and a 6 and 12 week supplementation was observed to have a positive effect of frontal lobe function [43]. Furthermore, animal studies have also indicated that isoflavones are capable of improving cognitive function [93, 101, 119]. However, there is still uncertainty regarding their effects as some large intervention trials have reported that isoflavone supplementation does not lead to cognitive improvements [48]. The rationale behind the potential of isoflavones to exert positive effects on cognitive function is believed to lie primarily in their potential to mimic the actions and functions of oestrogens in the brain [14]. For example, postmenopausal women who undertake oestrogen-replacement therapy have a significantly lower risk for the onset of Alzheimer's disease than women who do not [64]. They may also be effective by affecting the synthesis of acetylcholine and neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in hippocampus and frontal cortex [117-118].

There is also extensive evidence that berries, in particular blueberries, are effective at reversing age-related deficits in motor function and spatial working memory [10, 21, 78-79, 130, 182]. In addition to spatial memory, blueberry supplementation has been shown to improve 'object recognition memory' [54] and 'inhibitory fear conditioning learning' [10, 130]. Blueberry appears to have a pronounced effect on short-term memory [130] and has also been shown to improve long-term reference memory following 8 weeks of supplementation. [21]. Tests using a radial arm maze have supported these findings and have provided further evidence for the efficacy of blueberries [182]. Indeed, these have shown that improvements in spatial memory may emerge within 3 weeks, the equivalent of about 3 years in humans. The beneficial effects of flavonoid-rich foods and beverages on psychomotor activity in older animals have also been reported [78, 143]. In addition to those with berries, animal studies with tea [24] and pomegranate juice [62], or pure flavonols such as quercetin, rutin [128] or fisetin [103] have provided further evidence that dietary flavonoids are beneficial in reversing the course of neuronal and behavioural aging.

The flavonoid-rich plant extract, *Ginkgo Biloba* has also been shown to induce positive effects on memory, learning and concentration [28-29, 35]. *Ginkgo Biloba* has a prominent effect on brain activity and short-term memory in animals and humans suffering from

cognitive impairment [74, 141, 187] and promotes spatial learning in aged rodents [68, 160, 177, 187]. Furthermore, *Ginkgo Biloba* promotes inhibitory avoidance conditioning in rats with high-dose intake leading to short-term, but not long-term, passive avoidance learning in senescent mice [160, 165]. However, the pharmacological mechanisms by which *Ginkgo Biloba* promotes cognitive effects are unclear, with its ability to elicit a reduction in levels of ROS [114-115], to increase cerebral blood flow [51], to modulate brain fluidity [160], to interact with the muscarinic cholinergic system [27] and to protect the striatal dopaminergic system [129] all being suggested as possible mechanisms of brain action.

E. Mechanisms Underpinning the Neuroprotective Effects of Flavonoids

1. Antioxidant Effects

Historically the beneficial effects of flavonoids were believed to be due to their antioxidant capacity. As a consequence, their ability to act as hydrogen donating species was used to explain their protective effects against oxidative stress-associated diseases [132]. For example, flavonoids have been shown to act as classical electron (or hydrogen) donating antioxidants [65], as scavengers of reactive species [53, 56] and as inhibitors of low-density lipoprotein [188] and DNA oxidation [39]. Furthermore, flavonoids have also been shown to have the capacity to increase plasma total antioxidant capacity [133] and to decrease specific markers of oxidative stress, such as F2-isoprostanes [113] and lymphocyte 8-hydroxy-2'-deoxyguanosine levels [67]. However, although the brain is potentially susceptible to oxidative injury, due to high levels of oxidisable lipids and low levels of antioxidant protection, the bioavailability of flavonoids in the brain [2] suggests that their antioxidant activity would be negligible relative to that of ascorbic acid and α -tocopherol, which are present at much higher concentrations [57]. Ultimately, a precise understanding of whether or not flavonoids do influence oxidative stress in the brain will be wholly dependent on the physiological relevance of the markers of oxidative stress measured [55]. However, it appears that the effects of flavonoids may also be mediated by their interactions with specific neuronal intracellular signalling cascades, such as the mitogen activated protein kinase (MAP kinase) signalling pathway and the phosphoinositide 3-kinase (PI3 kinase/Akt) signalling cascade [84, 145, 148, 183].

2. Interactions with the Signalling Cascades

There is strong evidence that flavonoids may exert beneficial effects on memory via an ability to modulate the cellular and molecular architecture involved in the processes of memory [38, 145-147] (Figure 1). The concentrations of flavonoids in the brain are thought to be sufficiently high to exert pharmacological activity at receptors, kinases and transcription factors. Although the precise site of their interaction with signalling pathways is unclear, evidence indicates that they are capable of acting in a number of ways: 1) by binding to ATP sites on enzymes and receptors; 2) by modulating the activity of kinases directly, i.e.

MAPKKK, MAPKK or MAPK; 3) by affecting the function of important phosphatases, which act in opposition to kinases; 4) by preserving Ca^{2+} homeostasis, thereby preventing Ca^{2+} -dependent activation of kinases in neurons; and 5) by modulating signalling cascades lying downstream of kinases, i.e. transcription factor activation and binding to promoter sequences [145, 148]. The effects of flavonoids on neuronal signalling pathways are highly concentration-dependent and are likely to be related to their ability to exert high affinity receptor agonist-like actions at low concentrations (low to mid nanomolar) and direct enzyme inhibition at higher concentrations (high nonomolar to micromolar) [142, 147]. Potential flavonoid-binding sites on neurons include adenosine [4], GABA_A [77, 173] and testosterone receptors [75] and a specific brain plasma membrane binding site for polyphenols has been proposed [3]. By affecting such pathways they have the potential to induce new protein synthesis in neurons and thus an ability to induce morphological changes which have a direct influence on memory acquisition, consolidation and storage. Alternatively, their well establisd effects on the vascular system may also induce increases in cerebral blood flow capable of impacting on acute cognitive performance, or may lead to an increase hippocampal vascularisation capable of inducing new neuronal growth.

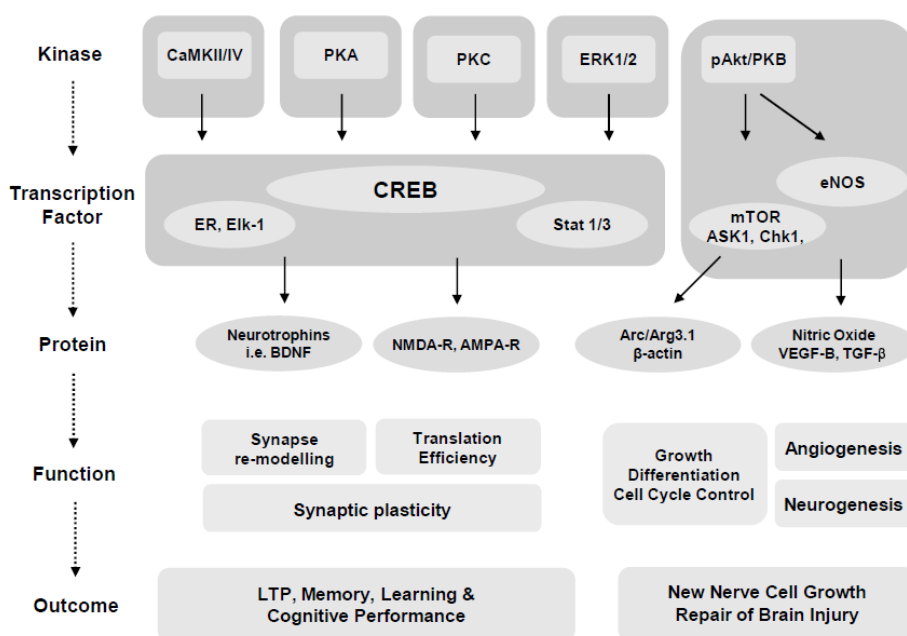


Figure 1. Signalling pathways underlying neuronal survival and cognitive performance. Flavonoids activate ERK-CREB pathway and the PI3 kinase-mTOR cascade leading to changes in synaptic plasticity. They are also capable of influencing neurogenesis through the activation of PI3 kinase-Akt-eNOS.

3. Stimulation of Synaptic Plasticity

Long-term potentiation (LTP) is widely considered to be one of the major mechanisms by which the brain ‘acquires’ and ‘stores’ information [58]. LTP refers to a persistent increase in the chemical strength of a synapse that can last from minutes to several days. LTP is known

to contribute to ‘synaptic plasticity’ or the increased strength of the connection, or synapse, between two neurons, a process thought to underlie memory [19, 112]. Studies into human mental retardation syndromes have led to new insights into the molecular underpinnings of human cognitive processing [69, 179, 184] and have indicated that both short-term and long-term memory is controlled at the molecular level [20, 34, 166]. Whereas short-term memory involves covalent modifications of pre-existing proteins, long-term memory requires the synthesis of new mRNAs and proteins [17, 81, 108, 144]. The activation of various signalling pathways have been linked with the control of de novo protein synthesis in the context of LTP, synaptic plasticity and memory: (i) cAMP-dependent protein kinase (protein kinase A) [82], (ii) protein kinase B (PKB) or Akt [7, 125], (iii) protein kinase C (PKC) [124], (iv) calcium-calmodulin kinase IV (CaMKIV) [5], and (v) extracellular signal regulated kinase (ERK) [163, 180]. The activation of all five pathways converge to signal to the cAMP-response element-binding protein (CREB), a transcription factor which binds to the promoter regions of many genes associated with synapse re-modelling, increases in neuronal spine density and synaptic plasticity [9, 61, 71, 162] (Figure 1). Changes in spine density, morphology and motility have been shown to occur with paradigms that induce synaptic, as well as altered sensory experience, and lead to alterations in synaptic connectivity and strength between neuronal partners, affecting the efficacy of synaptic communication [9].

There is much evidence to support the actions of nanomolar concentrations of flavonoids, in particular flavanols and flavanones, on the ERK pathway [6, 139, 170], which appear to be mediated by interactions with MEK1 and MEK2, and potentially membrane receptors [100, 137]. Indeed, the flavone backbone (2-phenyl-1,4-benzopyrone) has close structural homology to specific pharmacological modulators of ERK signalling, such as PD98059 (2'-amino-3'-methoxyflavone). Fisetin, a flavonoid found in strawberries, has been shown to improve long-term potentiation and to enhance object recognition in mice by a mechanism dependent on the activation of ERK and CREB [84]. Similarly, the flavanol (-)-epicatechin (0.1 and 0.3 nM) induces both ERK1/2 and CREB activation in cortical neurons and subsequently increases CREB regulated gene expression [103], whilst nanomolar concentrations of quercetin are effective at enhancing CREB activation [136]. Blueberry (2% w/w diet; 12 weeks)-induced improvements in memory have been shown to be mediated by increases in the phosphorylation state of ERK1/2, but not via the activation of calcium calmodulin kinase (CaMKIV) or protein kinase A [130]. Other flavonoids have also been found to influence the ERK pathway, with the citrus flavanone hesperetin capable to activating ERK1/2 signalling in cortical neurons at nanomolar concentrations [139] and flavanols such as ECGC restoring both protein kinase C and ERK1/2 activities in 6-hydroxydopamine treated and serum deprived neurons [97, 155]. Furthermore, this ability to activate the ERK pathway is not restricted to neurons and has also been observed in fibroblasts exposed to nanomolar concentrations of epicatechin [131].

CREB activation downstream of ERK appears critical in the induction of long-lasting changes in synaptic plasticity and memory [72, 126-127] and disruption of CREB activity specifically blocks the formation of long-term memory [70], whereas agents that increase the amount or activity of CREB accelerate the process [15]. CREB is known to be a critical transcription factor linking the actions of neurotrophins, such as BDNF, to neuronal survival, differentiation, and synaptic function [31, 45, 167]. Consequently, the central role of CREB in these processes has led to considerable interest in identifying safe effective agents that may enhance the activity of CREB in specific regions of the brain, as these may lead to an

improvement in memory [15]. Recent studies have shown that spatial memory performance in rats supplemented with blueberry, correlates well with the activation of cAMP-response element-binding protein (CREB) and with increases in both pro- and mature levels of hippocampal brain-derived neurotrophic factor (BDNF) [130]. Regulation of BDNF is interesting as this neurotrophin has been linked with the control of synaptic plasticity and long-term memory [46] and decreases in BDNF and pro-BDNF have been reported in Alzheimer's disease [16, 123]. Furthermore, a polymorphism that replaces valine for methionine at position 66 of the pro-domain of BDNF is associated with memory defects and abnormal hippocampal function in humans [40].

Blueberry flavonoid-induced activation of CREB and BDNF expression has also been shown to lead to the activation of the PI3 kinase/Akt signalling pathway [130], via the binding of BDNF to pre- or post-synaptic TrkB receptors. In addition, flavonoids have long been known to modulate PI3K, via direct interactions with its ATP binding site [124]. Indeed, a number of studies have demonstrated that the structure of flavonoids determines whether or not they act as potent inhibitors of PI3K [52, 142]. One of the most selective PI3K inhibitors available, LY294002, was modelled on the structure of quercetin [42, 109, 147]. Quercetin and some of its *in vivo* metabolites have been shown to inhibit pro-survival Akt/PKB signalling pathways by a mechanism of action consistent with quercetin and its metabolites acting at and inhibiting PI3K activity [136]. However, other flavonoids such as the citrus flavanone hesperetin (0.1 and 0.3 nM) cause the activation of Akt/PKB and the inhibition of pro-apoptotic proteins such as apoptosis signal-regulating kinase 1 (ASK1), Bad, caspase-9 and caspase-3 in cortical neurons [170]. The activation of Akt by flavonoids in hippocampal neurons may trigger the activation of the mTOR (the mammalian target of rapamycin) pathway and the increased translation of specific mRNA subpopulations [172], including the activity-regulated cytoskeletal-associated protein (Arc/Arg3.1) [130]. Arc is known to be important in LTP and has been proposed to be under regulatory control of both BDNF [135] and the ERK signalling [189]. Increased Arc expression may facilitate changes in synaptic strength, and the induction of morphological changes, such as that observed when small spines are converted into large mushroom-shaped spines through a mechanism dependent on actin polymerization [174]. In support of this, studies have indicated that changes in neuronal morphology occur in response to flavonoid supplementation [59] and that certain flavonoids can influence neuronal dendrite outgrowth *in vitro* [95].

4. Flavonoid-Induced Changes in Vascular Function

Dementia is a serious degenerative disease effecting predominantly elderly people with the two most common forms of this illness being Alzheimer's and vascular dementia. The factors affecting dementia are age, hypertension, arteriosclerosis, diabetes mellitus, smoking, atrial fibrillation and those with the ApoE4 genotype [18]. There is evidence to suggest that flavonoids may be capable of preventing many forms of cerebrovascular disease, including those associated with stroke and dementia [30, 33]. There is powerful evidence for the beneficial effects of flavonoids on peripheral [138] and cerebrovascular [36, 49] blood flow and these vascular effects are potentially significant as increased cerebrovascular function is known to facilitate adult neurogenesis in the hippocampus [50]. Indeed, new hippocampal cells are clustered near blood vessels, proliferate in response to vascular growth factors and

may influence memory [116] (Figure 1). As well as new neuronal growth, increases in neuronal spine density and morphology are considered vital for learning and memory [60]. Changes in spine density, morphology and motility have been shown to occur with paradigms that induce synaptic, as well as altered sensory experience, and lead to alterations in synaptic connectivity and strength between neuronal partners, affecting the efficacy of synaptic communication (Figure 1). These events are mediated at the cellular and molecular level and are strongly correlated with memory and learning.

Efficient cerebral blood flow is also vital for optimal brain function, with several studies indicating that there is a decrease in cerebral blood flow (CBF) in patients with dementia [111, 134]. Brain imaging techniques, such as 'functional magnetic resonance imaging' (fMRI) and 'trans-cranial Doppler ultrasound' (TCD) has shown that there is a correlation between CBF and cognitive function in humans. For example, cerebral blood flow velocity is significantly lower in patients with Alzheimer disease and low CBF is also associated with incipient markers of dementia. In contrast, non demented subjects with higher CBF were less likely to develop dementia. Flavonoids have been shown to exert a positive effect on cerebral blood flow (CBF) in humans [47, 49]. After consumption of a flavanol-rich cocoa drink, the 'flow oxygenation level dependent' (BOLD)-fMRI showed an increase in blood flow in certain regions of the brain, along with a modification of the BOLD response to task switching. Furthermore, 'arterial spin-labelling sequence magnetic resonance imaging' (ASL-MRI) [178] also indicated that cocoa flavanols increase CBF up to a maximum of two hours after ingestion of the flavanol-rich drink. In support of these findings, an increase in cerebral blood flow through the middle cerebral artery has been reported after the consumption of flavanol-rich cocoa using TCD [47].

5. Protection against Neuronal Injury Induced by Neurotoxins

Neurodegeneration in Parkinson's, Alzheimer's, and other neurodegenerative diseases appears to be triggered by multi-factorial events including neuroinflammation, glutamatergic excitotoxicity, increases in iron and/or depletion of endogenous antioxidants [11, 76, 159]. There is a growing body of evidence to suggest that flavonoids may be able to counteract the neuronal injury underlying these disorders [106, 145, 148]. For example, a Ginkgo biloba extract has been shown to protect hippocampal neurons from nitric oxide- and beta-amyloid-induced neurotoxicity [102] and studies have demonstrated that the consumption of green tea may have beneficial effect in reducing the risk of Parkinson's disease [25]. In agreement with the latter study, tea extracts and (–)-epigallocatechin-3-gallate (EGCG) have also been shown to attenuate 6-hydroxydopamine-induced toxicity [97], to protect against hippocampal injury during transient global ischemia [91] and to prevent nigral damage induced by MPTP [96].

The death of nigral neurons in Parkinson's disease is thought to involve the formation of the endogenous neurotoxin, 5-S-cysteinyl-dopamine [152-153] (Figure 2). Recent investigations have shown that 5-S-cysteinyl-catecholamine conjugates possess strong neurotoxicity and initiate a sustained increase in intracellular reactive oxygen species (ROS) in neurons leading to DNA oxidation, caspase-3 activation and delayed neuronal death [63, 157] (Figure 2). Such adducts may be generated by reactive species [169] and have been observed to be elevated in the human substantia nigra of patients who died of Parkinson's disease [152], suggesting that such species may be potential endogenous nigral

toxins. However, 5-S-cysteinyl-dopamine-induced neuronal injury is counteracted by nanomolar concentrations of various flavonoids including pelargonidin, quercetin, hesperetin, caffeic acid, the 4'-O-Me derivatives of catechin and epicatechin [169]. Furthermore, in presence of the flavanol, (+)-catechin, tyrosinase-induced formation of 5-S-cysteinyl-dopamine was inhibited by a mechanism linked to the capacity of catechin to undergo tyrosinase-induced oxidation to yield cysteinyl-catechin adducts [171]. In contrast, the inhibition afforded by flavanones, such as hesperetin, was not accompanied with the formation of cysteinyl-hesperetin adducts, indicating that it may inhibit via direct interaction with tyrosinase [171].

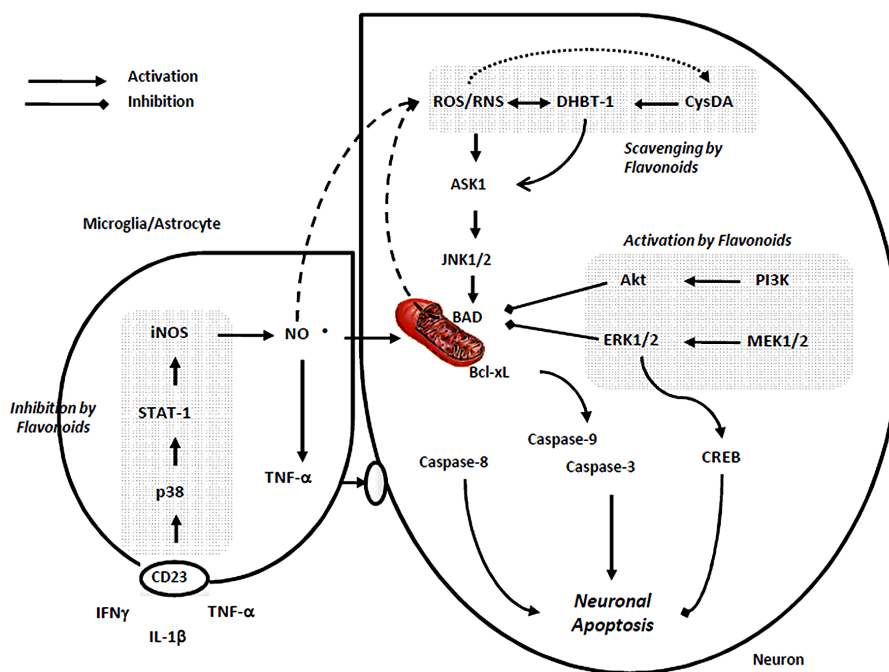


Figure 2. The cellular mechanisms by which flavonoids and their metabolites protect against neuroinflammation and neuronal injury induced by 5-S-CysDA, DHBT-1 and related ROS. Flavonoids inhibit the p38 pathway glia cells leading to a reduction in iNOS expression and NO• release. In neurons, they scavenge neurotoxic species and induce pro-survival signalling pathways, such as ERK1/2 and PI3-kinase/Akt, leading to an inhibition of neuronal apoptosis.

Reactive oxygen and nitrogen species have also been proposed to play a role in the pathology of many neurodegenerative diseases [76] (Figure 2). There is abundant evidence that flavonoids are effective in blocking this oxidant-induced neuronal injury, although their potential to do so is thought not to rely on direct radical or oxidant scavenging activity [148, 151, 156]. Instead, they are believed to act by modulating a number of protein kinase and lipid kinase signalling cascades, such as the PI3 kinase (PI3K)/Akt, tyrosine kinase, protein kinase C (PKC) and mitogen-activated protein kinase (MAP kinase) signalling pathways [148, 183]. Inhibitory or stimulatory actions at these pathways are likely to profoundly affect neuronal function by altering the phosphorylation state of target molecules, leading to changes in caspase activity and/or by gene expression [183]. For example, flavonoids have been observed to block oxidative-induced neuronal damage by preventing the activation of

caspase-3, providing evidence in support of their potent anti-apoptotic action [139-140, 156]. The flavanols epicatechin and 3'-O-methyl-epicatechin also protect neurons against oxidative damage via a mechanism involving the suppression of JNK, and downstream partners, c-jun and pro-caspase-3 [139]. Flavanones, such as hesperetin and its metabolite, 5-nitro-hesperetin, have been observed to inhibit oxidant-induced neuronal apoptosis via a mechanism involving the activation/phosphorylation of signalling proteins important in the pro-survival pathways [170]. Similarly, the flavone, baicalein, has been shown to significantly inhibit 6-hydroxydopamine-induced JNK activation and neuronal cell death and quercetin may suppress JNK activity and apoptosis induced by hydrogen peroxide [73, 175], 4-hydroxy-2-nonenal [168] and tumour necrosis factor-alpha (TNF-alpha) [83].

6. Inhibition of Neuroinflammation

Neuroinflammatory processes in the CNS are believed to play a crucial role in the development of neurodegenerative diseases such as Alzheimer's and Parkinson's disease [66] as well as with neuronal injury associated with stroke [193]. Glial cell (microglia and astrocyte) activation leads to the production of cytokines and other inflammatory mediators which may contribute to the apoptotic cell death of neurons observed in many neurodegenerative diseases. In particular, increases in cytokine production (interleukin-1 β , IL-1 β ; tumor necrosis factor-alpha, TNF- α) [85], inducible nitric oxide synthase (iNOS) and nitric oxide (NO $\bullet$), and increased NADPH oxidase activation [8] all contribute to glial-induced neuronal death (Figure 2). These events are controlled by MAPK signalling which mediate both the transcriptional and post-transcriptional regulation of iNOS and cytokines in activated microglia and astrocytes [12-13, 107]. Whilst ibuprofen, a non-steroidal anti-inflammatory drug, has been shown to delay the onset of neurodegenerative disorders, such as Parkinson disease [23], the majority of existing drug therapies for neurodegenerative disorders has failed to prevent the underlying degeneration of neurons. Consequently, there is a desire to develop alternative strategies capable of preventing the progressive neuronal loss resulting from neuroinflammation.

Flavonoid-rich blueberry extracts have been observed to inhibit NO $\bullet$, IL-1 β and TNF- α production in activated microglial cells [88-89], whilst the flavonol quercetin [26], the flavones wogonin and baicalein [90], the flavanols catechin and epigallocatechin gallate (EGCG) [98], and the isoflavone genistein [176] have all been shown to attenuate microglia and/or astrocyte mediated neuroinflammation via mechanisms that include inhibition of: 1) iNOS and cyclooxygenase (COX-2) expression, 2) NO $\bullet$ production, 3) cytokine release, and 4) NADPH oxidase activation and subsequent reactive oxygen species generation, in astrocytes and microglia. Flavonoids may exert these effects via direct modulation of protein and lipid kinase signalling pathways [145, 148, 183], for example via the inhibition of MAPK signalling cascades, such as p38 or ERK1/2 which regulate both iNOS and TNF- α expression in activated glial cells [13] (Figure 2). In this respect, fisetin inhibits p38 MAP kinase phosphorylation in LPS-stimulated BV-2 microglial cells [192] and the flavone luteolin inhibits IL-6 production in activated microglia via inhibition of the JNK signalling pathway [2345]. The effects of flavonoids on these kinases may influence downstream pro-inflammatory transcription factors important in iNOS transcription. One of these, nuclear factor-Kappa B (NF- κ B), responds to p38 signalling and is involved in iNOS induction [12],

suggesting that there is interplay between signalling pathways, transcription factors and cytokine production in determining the neuroinflammatory response in the CNS. However, flavonoids have also been shown to prevent transcription factor activation, with the flavonol quercetin able to suppress NF- κ B, signal transducer and activator of transcription-1 (STAT-1) and activating protein-1 (AP-1) activation in LPS- and IFN- γ -activated microglial cells [26].

Summary

The actions of dietary flavonoids on cognition appear to involve a number of effects within the brain, including a potential to protect neurons against injury induced by neurotoxins and neuroinflammation, a potential to activate synaptic signalling and an ability to improve cerebrovascular blood flow. These effects appear to be underpinned by an ability to interact with neuronal signalling cascades in the brain, which leads a number of responses, including the inhibition of apoptosis triggered by neurotoxic species, the promotion of neuronal survival and differentiation, and an enhancement of peripheral and cerebral blood perfusion. With regards to the latter, future studies investigating whether such changes in blood flow are also capable of inducing angiogenesis and new nerve cell growth in the hippocampus would be of interest. Furthermore, the modulation of neuronal signalling and the protection against neuronal losses induced by flavonoids suggest that optimal maintenance of brain morphology may underpin their effects of cognition. If so, this is particularly relevant as this innate brain structure is known to deteriorate with ageing, with neuronal populations or synaptic connections lost over time, leaving the system less efficient in the processing and storage of sensory information.

The consumption of flavonoid-rich foods, such as berries or cocoa, throughout life may have the potential to limit or even reverse age-dependent deteriorations in memory and cognition. However, there are a number of questions still to be resolved. Most notably, at present there is no data in support of a causal relationship between the consumption of flavonoids and behavioural outcomes. In order to make such relationships, future intervention studies will be required to utilise better characterised intervention materials, more appropriate controls and more rigorous clinical outcomes. Whilst cognitive behavioural testing in humans and animals provides an appropriate way of assessing function, *in vivo* structural and dynamic quantitative assessments will ultimately be required to provide hard evidence of effects in the brain. For example, it would be highly advantageous to directly link behavioural responses to changes in hippocampal volume and density, changes in neural stem cell and progenitor cells and alterations in brain blood flow using MRI and fMRI techniques. Functional MRI measures may be used to assess changes in blood flow that underlie improved cognitive functioning as a result of flavonoid supplementation. In addition, such haemodynamic changes may be further compared to changes in grey matter density and to biomarkers of neural stem and progenitor cells using proton nuclear magnetic resonance spectroscopy (H-MRS). Such an approach will be essential to provide links between flavonoid intake and brain function in a mechanistic, dynamic and quantitative way. Taking such an approach one may also be able to assess other factors relating to intake such as what timeframe is required to gain maximum beneficial effects, which flavonoids are most effective in inducing these changes and in which doses? Due to the intense interest in the

development of drugs capable of enhancing brain function, flavonoids may represent important precursor molecules in the quest to develop of a new generation of brain enhancing drugs.

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Chapter III

Anti-Inflammatory Potential of Flavonoids

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Abstract

Background: Flavonoids comprise a large group of plant constituents and more than 5,000 chemical varieties have been reported to date. Certain flavonoids are known to possess anti-inflammatory activity in vitro and in vivo.

Materials and Methods: In this chapter, the pivotal anti-inflammatory action mechanisms of several classes of flavonoids are discussed. In addition, the anti-inflammatory activity of flavonoids in animal models of inflammation is summarized. Furthermore, the anti-inflammatory activities of some natural biflavonoids are described. Finally, human clinical trials of flavonoid therapy are discussed.

Results: Although not fully understood, several action mechanisms have been proposed to explain the in vivo anti-inflammatory action of flavonoids. The antioxidative action and inhibition of eicosanoid metabolizing enzymes are well-known cellular action mechanisms. Indeed, many flavonoids have been reported to regulate the expression of pro- and/or anti-inflammatory molecules depending on their chemical structures. Proinflammatory genes affected by flavonoids include cyclooxygenase-2, inducible nitric oxide synthase, interleukins, tumor necrosis factor, matrix metalloproteinases and inflammatory chemokines. These proinflammatory molecules are down-regulated through the action of flavonoids along several signaling pathways. The signal transduction pathways interfered with by flavonoids include various protein kinases such as the mitogen-activated protein kinase pathway, activation of the transcription factors including nuclear transcription factor- κ B and activator protein-1. Recent studies have also demonstrated that some flavonoids enhance their antioxidative action by inducing detoxifying enzymes including superoxide dismutase. This action is primarily mediated by activating redox-sensitive transcription factors such as nuclear factor erythroid 2-

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related factor. Through all of these pathways, flavonoids are believed to show anti-inflammatory activity. Therefore, flavonoids are thought to be natural anti-inflammatory agents having multiple action mechanisms. All of these actions of flavonoids may contribute to the anti-inflammatory properties of many nutraceutical and traditional herbal drugs that contain flavonoids as major ingredients. Although there has been little evidence of anti-inflammatory flavonoid therapy in response to pharmacological treatment in human clinical trials to date, several clinical studies have shown that flavonoids have the potential for treatment of chronic inflammatory disorders.

Conclusion: Nutritional ingestion of flavonoids may be beneficial for reducing symptoms of inflammatory diseases. In pharmacological treatment, flavonoids have strong potential for treating chronic inflammatory disorders for long-term use, while they may not be adequate for treating acute inflammatory disorders due to their low potencies. Through continual investigation to identify more potent and comparable flavonoids, new anti-inflammatory flavonoid therapy will be successfully launched, especially for the treatment of chronic inflammatory disorders.

Abbreviations

COX	cyclooxygenase;
LOX	lipoxygenase;
iNOS	inducible nitric oxide synthase;
PLA ₂	phospholipase A ₂ ;
TNF	tumor necrosis factor;
LPS	lipopolysaccharide;
MMP	matrix metalloproteinase;
HO-1	heme oxygenase-1;
TPA	tetrahydrodecanylphorbol 13-acetate;
TLR	toll-like receptor;
MAPK	mitogen-activated protein kinase;
ERK	extracellular signal-related kinase;
JNK	c-Jun amino-terminal kinase;
PI3K	phosphatidylinositol 3-kinase;
Akt	protein kinase B;
IKK	IκB-kinase;
NF-κB	nuclear transcription factor-κB;
AP-1	activator protein-1;
TBK1	TANK binding kinase 1;
Jak	Janus kinase;
STAT	signal transduction and activator of transcription;
Nrf2	nuclear factor erythroid 2-related factor 2;
Keap1	Kelch-like erythroid CNC homologue (ECH)-associated protein 1;
CREB	cAMP response element binding protein;
C/EBP	CCAAT enhancer binding protein;
IRF	interferon regulatory factor;
EGCG	(-)-epigallocatechin-3-gallate;

1. Introduction

Flavonoids (Figure 1) from plants are benzo- γ -pyrone derivatives that can mainly be classified as chalcones, flavans, flavones, isoflavones, flavonols and polymeric flavonoids such as anthocyanins and biflavonoids. Chalcones are open chain flavonoids. Catechins including (-)-epigallocatechin-3-gallate (EGCG) are classified as flavane-3-ols. Flavanones are closed chain flavonoids without a C-2,3 double bond such as naringenin. Closed chain flavonoids having a C-2,3-double bond are flavones, isoflavones and flavonols. Flavonols have a C-3 OH group. The most abundant natural flavonoids are apigenin, luteolin (flavones), genistein and daidzein (isoflavones), and kaempferol and quercetin (flavonols). These groups of flavonoids possess significant pharmacological activities. Many flavonoids are flavonoid glycosides, which contain sugar(s) attached to the flavonoid aglycone molecules. While some flavonoid glycosides do not show considerable anti-inflammatory activity *in vitro* due to their inability to penetrate cells, most show similar *in vivo* anti-inflammatory activity to their aglycones following oral administration. The bioavailability of flavonoid glycosides is similar to or even higher than the corresponding flavonoid aglycones. Flavonoids possess various biological/pharmacological activities including antimicrobial activity, antiviral activity, anticancer activity, anti-inflammatory activity and anti-allergic activity [1]. In particular, certain flavonoids show considerable anti-inflammatory activity *in vitro* and *in vivo*.

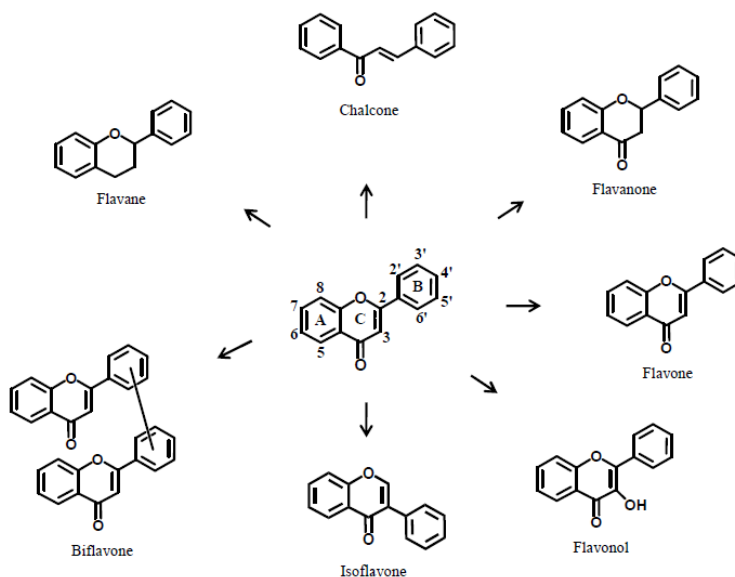


Figure 1. Major classes of naturally-occurring flavonoids.

2. Anti-Inflammatory Action of Flavonoids in Animal Models of Inflammation

Some selected studies of anti-inflammatory activities of various flavonoids in animal models (*in vivo*) are summarized in Table 1. The anti-inflammation of flavonoids by oral

administration is presented here since flavonoids are supposed to be orally administered to humans. Investigations conducted up to 1984 are described in a review conducted by Gabor [2]. The anti-inflammatory activity of flavonoids by topical application is summarized in a previously published review [3].

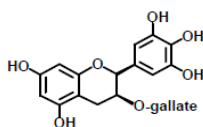
As Gabor [2] summarized, many different types of flavonoids exert anti-inflammatory activity in animal models of inflammation. They showed more or less anti-inflammatory activity in vivo. A study conducted by Kudo et al. [4] demonstrated that flavones from *Scutellaria radix* such as baicalin and wogonin (Figure 2) showed anti-inflammatory activity against acute as well as chronic inflammation, suggesting that they could be useful for the treatment of chronic inflammatory disorders such as rheumatoid arthritis. Many nonsteroidal anti-inflammatory drugs have been successfully used for the treatment of acute inflammatory diseases; however, there is still a need for new anti-inflammatory drugs for the treatment of chronic disorders. Flavonoids may be safely used for long-term anti-inflammatory drugs for chronic inflammatory diseases because they are of natural origin and have long been consumed as parts of the diet.

Our experiments also demonstrated that many flavones/flavonols isolated from various plants showed meaningful and significant activity following oral administration [5, 6]. Additionally, structure activity analysis has shown that the C-2,3-double bond is important and that flavonoid glycosides are also active when administered orally. However, their potencies may not be strong enough to warrant a clinical trial. Similarly, it has been shown that the antiedematous activity of luteolin and quercetin was not strong following oral administration [7]. Indeed, the anti-inflammatory activity of flavonoids via the oral route of administration may be limited by low bioavailability and rapid metabolism.

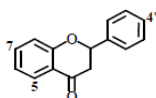
Quercetin is a well-known flavonoid that exerts anti-inflammatory activity in several animal models of inflammation. However, it should be noted that long-term quercetin ingestion may be harmful to the kidneys [8], and that these effects occurred through the activation of activator protein-1 (AP-1). These results are correlated with the findings of a clinical study of quercetin treatment administered via i.v. route [9].

It was recently reported that hesperidin administered orally inhibited rat adjuvant-induced arthritis (AIA) [10]. Similarly, α -glucosylhesperidin was found to inhibit mouse collagen-induced arthritis (CIA) via oral treatment. These findings indicate that flavonoids have anti-inflammatory potential for the treatment of chronic inflammatory disorders. Importantly, Geraets et al. [11] found that fisetin strongly inhibited lipopolysaccharide (LPS)-induced pulmonary inflammation and the expression of proinflammatory molecules such as IL-1 and IL-6 in the lung.

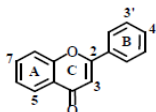
As stated above, many flavonoid derivatives were examined for their anti-inflammatory activity in a variety of animal models of inflammation. The results of these studies demonstrated that flavonoids exert considerable anti-inflammatory activity. Specifically, luteolin, wogonin and quercetin showed high anti-inflammatory activity in several different models of inflammation. However, it is thought that flavonoids generally do not show high enough anti-inflammatory activity to warrant a clinical trial when administered orally. This may be due to their low intrinsic potency compared to clinically used anti-inflammatory drugs,



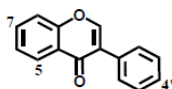
epigallocatechin-3-gallate (EGCG)



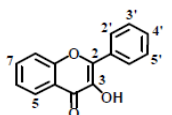
	5	7	4'
Flavanone	H	H	H
naringenin	OH	OH	OH



	5	7	8	3'	4'
Flavone	H	H	H	H	H
chrysin	OH	OH	H	H	H
apigenin	OH	OH	H	H	OH
luteolin	OH	OH	H	OH	OH
wogonin	OH	OH	OCH ₃	H	H



	5	7	4'
Isoflavone	H	H	H
genistein	OH	OH	H
daidzein	H	OH	OH



	5	7	2'	3'	4'	5'
Flavonol	H	H	H	H	H	H
galangin	OH	OH	H	H	H	H
kaempferol	OH	OH	H	H	OH	H
quercetin	OH	OH	H	OH	OH	H
morin	OH	OH	OH	H	OH	H
myricetin	OH	OH	H	OH	OH	OH

Figure 2. The representative flavonoids mentioned in this study.

and low bioavailability. As many studies have found, most flavonoids show low bioavailability and rapid metabolism to inactivate compounds when administered orally. To overcome this situation and to examine the drug potential, a variety of flavonoids have been synthesized. For example, hydroxyl-, methoxyl-, allyl- and halogenated-derivatives of flavones have been synthesized in our laboratory and their pharmacological activities are currently being evaluated [24-26]. Nevertheless, the continual search for new flavonoid molecules having high anti-inflammatory activity is needed. And the anti-inflammatory

activity of new flavonoids should be compared with those of luteolin or wogonin, which are the most potent flavonoids that have been identified to date.

Table 1. Anti-inflammatory activity of flavonoids in animal models of inflammation

Compounds	Dose (mg/kg)	In vivo models	References
Brazilin, haematoxylin, hypolaetin-8-glycoside, apigenin dimethylether	(p.o.)	CGN-induced edema, AIA, writhing, etc.	[2]
Taxifolin, gossypin, nepitrin, diosmin, fisetin, sophoricoside, catechin, epicatechin	(i.p.)	CGN-induced edema, AIA, writhing, etc.	[2]
Scutellaria extract, wogonin, baicalein, baicalin	100 (p.o.)	CGN-induced paw edema, AIA (rat)	[4]
Many flavones and flavonols	100 (p.o.)	Croton oil-, AA-induced ear edema (mouse)	[5]
Many flavonoid glycosides	100 (p.o.)	Croton oil-, AA-induced ear edema (mouse)	[6]
Quercetagenin, hispidulin, scutellarein	150 (p.o.)	CGN-induced paw edema (mouse)	[12]
Luteolin-7-O-rutinoside	100, 300 (p.o.)	Allergy model (rat) (antiallergic effect)	[13]
Baicalein (but not baicalin or wogonin)	20/day (10 days)	DSS-induced colitis (mouse)	[14]
Apigenin	50 (p.o.)	LPS (i.p.)-induced inflammation (mouse)	[15]
Luteolin, apigenin	1/mouse (p.o.)	TNF- α production (mouse)	[7]
Luteolin, quercetin	1 $\times$ 2/mouse (p.o.)	TPA-induced ear edema (mouse)	[7]
Wogonin	20/day (2 weeks) (p.o.)	DSS-induced colitis (rat)	[16]
Kaempferol-3,7-dirhamnoside, quercetin-3,7-dirhamnoside	50 (p.o.)	CGN-induced paw edema, writhing (mouse)	[17]
Luteolin	10, 50 (p.o.)	CGN-paw edema, cotton pellet granuloma (mouse)	[18]
Kaempferol, kaempferol-3-O-glucoside	50, 100 (p.o.)	CGN-paw edema, xylene-ear edema, writhing (mouse)	[19]
Quercetin and isoquercitrin	10(q)/day, 15(i)/day (5 days) (p.o.)	Allergic asthma (mouse)	[20]
Quercetin	5-50 (p.o.)	No effect on LPS-induced fever (rat)	[21]
Pectolarigenin, pectolarin	4-100 (p.o.)	CGN-paw edema (mouse)	[22]
Hesperidin	40-160 (p.o.)	AIA (rat)	[10]
α -Glucosylhesperidin	10/week(p.o.)	CIA (mouse)	[23]
Fisetin, flavone, triclin	22-30 (p.o.)	LPS-induced pulmonary inflammation (mouse)	[11]

CGN: carrageenan, AIA: adjuvant-induced arthritis, AA: arachidonic acid, CIA: collagen-induced arthritis, DSS: dextran sulfate sodium.

3. Cellular Action Mechanisms: Effects on Transcriptional Expression of Proinflammatory Molecules

At least three different mechanisms of action are believed to be involved in the anti-inflammatory action of flavonoids. The classical mechanism is the anti-oxidative action of flavonoids. Many flavonoids have been shown to possess considerable anti-oxidative activity, as discussed below. Another mechanism of flavonoids is their effect on arachidonic acid (AA) metabolizing enzymes such as PLA₂s, COXs and LOXs.

Table 2. Proinflammatory molecules down-regulated by flavonoid treatment

Compounds	Target cells and agonists		Molecules down-regulated	References
Apigenin, baicalein, baicalin, quercetin, kaempferol, wogonin, EGCG, genistein, nobiletin, galangin, tectorigenin, oroxylin A, isoliquiritigenin, amoradin, luteolin, delphinidin, chrysin, myricetin, ayanin, phloretin, santin, ermanin, centaureidin, 5,3'-diOH-4'-methoxy-7-methoxy-carbonylflavonol, 7-OH-flavonol, eryodictyol etc.	RAW 264.7, peritoneal macrophages, human chondrocytes, J774A.1, rat C6 glial cells, HT-29 colon cancer cells, A549, synovial fibroblasts, HUVEC, gingival fibroblasts, ARPE-19, Caco-2, lymphocytes, keratinocytes, HaCaT, rat pancreatic islets, BV-2, hepatocytes, HT29, EL4 (By LPS, IFN- γ , IL-1 β , TNF- α , TPA, KLH, A23187, hypoxia)		COX-2, iNOS, IL-1 β , IL-4, IL-6, IL-8, IFN- γ , TNF- α , MCP-1, MIP-1, VEGF, ICAM-1, VCAM-1	[28,29]
Scutellarin	rats	ischemia	VFGF, β FGF, iNOS	[36]
Baicalein	rats	LPS	TNF- α , iNOS	[37]
Baicalin	rats	aging	COX-2, iNOS, HO-1	[32]
Apigenin, chrysin, galangin, kaempferol, etc.	human aortic endothelial cells	TNF- α	E-selectin, ICAM-1	[38]
Apigenin	mouse bone marrow-derived dendrite cells	LPS	IL-12	[39]
Quercetin	human airway epithelial cells	TNF- α	IL-8, MCP-1	[40]
Baicalin	human peritoneal ligament cells	IL-1 β	RANKL, COX-2	[41]
Wogonin	human aortic smooth muscle cells	TNF- α	MMP-9	[42]
Fisetin	human mast cells	TPA plus A23187	TNF- α , IL-1 β , IL-4, -6, -8	[43]
Baicalein	HUVEC	strain	ET-1	[44]
Wogonin	HUVEC	LPS	VEGF, VEGFR-2, IL-6, sIL-6R α	[45]
Silibinin	MCF-7	TPA	MMP-9	[46]
Daizhein, genistein, isorhamnetin, kaempferol, quercetin, naringenin, anthocyanin	J774	LPS	iNOS	[33]
Isoliquiritigenin	RAW 264.7	LPS	iNOS, COX-2, TNF- α , IL-6	[34]

HUVEC: human umbilical vein endothelial cells.

The most important cellular mechanism of action of flavonoids is the transcriptional regulation of pro-inflammatory molecules including COX-2, iNOS, ILs, TNF- α , chemokines

and adhesion molecules. For this regulation, flavonoids influence signal transducing molecules such as MAPKs and transcriptional factors including NF- κ B and AP-1.

In 1999, our group demonstrated that various flavonoids are iNOS down-regulators in RAW 264.7 macrophages. A C-2,3-double bond was found to be most important in this pharmacological activity, as in flavones and flavonols [27]. This demonstrates the ability of flavonoids to function as a transcriptional modulator of proinflammatory molecules. Table 2 shows selected studies demonstrating the down-regulation of proinflammatory molecule expression in various cells following inflammatory insults. The studies showing down-regulating capacity of proinflammatory molecules carried out up to 2004 are referred to the previous reviews [28, 29]. Some flavonoids such as luteolin, wogonin and baicalein certainly possess down-regulating ability of proinflammatory molecules expression.

Pergola et al. [30] showed that the anthocyanin fraction of blackberry extract and its major component, cyanidin-3-*O*-glucoside, inhibited NO production by iNOS from LPS-induced J774 cells, and that this effect occurred in part by down-regulation, and in part through direct inhibition. The iNOS down-regulation of these compounds was achieved via inhibition of NF- κ B activation mediated by inhibition of I κ B α degradation and/or inhibition of ERK1/2 activation. Conversely, a previous study demonstrated that cyanidin-3-*O*-glucoside induced eNOS expression and NO production in bovine vascular endothelial cells [31]. Based on the results of these studies, it is possible that the same flavonoid might affect the NOS system oppositely depending on NOS isozymes.

Kim et al. [32] found that orally-administered baicalin reduced the expression of COX-2, iNOS and heme oxygenase-1 (HO-1) in aged rats. Because these proinflammatory molecules (COX-2 and iNOS) may be regarded as biomarkers of aging, their study indicated that there is the potential for the use of flavonoids in anti-aging therapy. Apart from their cancer prevention effects that occur via direct COX-2 inhibition, flavonoids may inhibit inflammatory aging processes by slowing the inflammatory reaction, leading to the prevention of cancer.

Similar findings were reported by Hamalainen et al. [33], who demonstrated that flavonoids including genistein, kaempferol, quercetin and naringenin suppressed iNOS induction in J774 macrophages. In addition, isoliquilitinigenin (chalcone) inhibited the expression of iNOS, COX-2, TNF- α and IL-6 in RAW 264.7 macrophages [34].

It is not surprising that many varieties of flavonoids have the potential for regulation of the expression of many proinflammatory molecules. Some of these properties were also verified by in vivo experiments. Thus, it is strongly suggested that one of the main anti-inflammatory action mechanisms of flavonoids is regulation of proinflammatory molecule expression in vitro and in vivo.

Based on the aforementioned studies, it is now clear that certain flavonoids affect the transcriptional expression of proinflammatory molecules. It is significant to mention that one particular flavonoid may act on multiple points in a cellular system. Some flavonoids even affect particular proinflammatory molecules at multiple points. For example, wogonin inhibits COX-2 enzyme activity as well as COX-2 expression [35]. Certain flavonoids affect one particular pro-inflammatory molecule in a completely opposite fashion. Flavonoids act in cell type-specific and agonist type-specific ways for the transcriptional regulation of proinflammatory molecules.

4. Cellular Mechanisms of Modulating Transcriptional Regulation

The transcriptional factors of NF- κ B and AP-1 are deeply involved in the expression of many inflammatory-, allergy- and carcinogenesis-related events. These transcription factors are activated by the action of various protein kinases including MAPKs. Therefore, interference with these signaling pathways may lead to anti-inflammation, antiallergic response and anticancer activity.

There have been various studies conducted to demonstrate the regulatory mechanisms of flavonoids on signal transduction pathways and nuclear transcription factors. For example, quercetin has been reported to attenuate JNK and AP-1 activation, resulting in the down-regulation of TNF- α - or TPA-induced ICAM-1 expression [47], while the same compound was recently found to inhibit iNOS expression by inhibiting NF- κ B activation, but not by JNK inhibition [48]. iNOS down-regulation via inhibition of NF- κ B activation by quercetin was also observed in rat hepatocytes [49]. Quercetin affects other kinases or transcription factors and inhibits ERK activation [50]. Thus, the cellular targets of quercetin are cell type- and agonistic-specific.

The action mechanisms of other flavonoids also show this type of specificity. Luteolin and apigenin inhibited the NF- κ B pathway in macrophages, leading to blockage of LPS-induced iNOS and COX-2 expression and the release of TNF- α and IL-6 [51, 52]. It has also been demonstrated that luteolin inhibited IL-8 production in HT29 cells by inhibiting p38 MAPK, ERK, I κ B degradation and NF- κ B activation [53]. On the other hand, luteolin inhibited IL-4 expression of basophils stimulated with A23187 and TPA via the inhibition of c-jun and DNA binding of AP-1, but not through inhibition of the MAPK pathway [54]. The similar results were found in our experiment [55]. Apigenin, wogonin, kaempferol and quercetin inhibited MMP-1 production by TPA-treated human dermal fibroblasts via inhibition of AP-1 activation. However, the inhibitory mechanisms were different depending on the chemical structures of the flavonoids. Quercetin interferes with ERK and p38 MAPK activation, while kaempferol inhibits JNK and p38 MAPK activation. Interestingly, flavones (apigenin and wogonin) did not affect the activation of these major MAPKs, although they certainly inhibited AP-1 activation [55]. Therefore, it is repeatedly found that certain flavonoids inhibit the activation of different transcription factors such as NF- κ B or AP-1 depending on the cells and the stimuli.

Some other transcription factors and signaling protein kinases have also been shown to be affected by flavonoid treatment. Genistein (isoflavone) inhibited iNOS expression by inhibiting NF- κ B and C/EBP β activation of LPS-induced RAW 264.7 cells, whereas kaempferol and quercetin (flavonol) inhibited ERK and AP-1 activation [50]. It should be noted that apigenin inhibited COX-2 expression by TPA-treated HaCaT cells via inhibition of Akt kinase (PKB) activation, but not by inhibiting the MAPK pathway [56], and that it inhibited nuclear factor for activated T-cells (NF-AT) DNA binding in activated T-cells [57]. These results have also shown that the same flavonoid affects different cellular machineries depending on the cell type.

Wogonin (flavones) is one of the most potent flavonoids that down-regulates COX-2 and iNOS expression from LPS-induced RAW cells [27, 35]. It has been shown that the action of wogonin occurs through inhibition of AP-1 and NF- κ B [58, 59]. Structurally-similar baicalein

(flavone) also inhibited iNOS expression of LPS-treated rat primary microglia, BV-2 and RAW 264.7 cells, and these effects occurred primarily through inhibition of the DNA binding activity of NF-IL6, but not through inhibition of NF- κ B and AP-1 activation [60].

Table 3. Transcription factors and protein kinases modulated by flavonoid treatment

Transcription factors inhibited	Flavonoids	Protein kinases affected
NF- κ B	apigenin, genistein, kaempferol, quercetin, EGCG	IKK
	Wogonin, oroxylin A, genistein, apigenin, kaempferol, luteolin, quercetin, myricetin, eupatilin-3-O-galactose, catechin, epicatechin, naringenin, daidzein, isorhamnetin, flavone ^{a)}	
	quercetin	ERK, p38
	luteolin	Akt [74], p38, ERK
	delphinidin	ERK, p38, JNK
	baicalin	NIK, IKK, ERK, p38 [32]
	quercetin	PI3K, Akt [40]
	fisetin	p38, ERK, JNK [43]
	apigenin	p38, JNK [75]
	isoliquiritigenin	IKK, ERK, p38 [34]
	quercetin	p38, JNK
	wogonin	ERK, JNK
	quercetin, kaempferol	ERK
	keampferol, chrysin, apigenin, luteolin, eupatilin-3-O-galactoside	
AP-1	delphinidin	ERK, p38, JNK
	baicalein	ERK [44]
	luteolin	Akt [76]
	silibinin	ERK, JNK [46]
	wogonin	JNK [77]
	luteolin	
	quercetin	[63, 64]
CREB	genistein, kaempferol, quercetin, daidzein	[33]
	EGCG	[68, 69]
	apigenin, luteolin	[65]
	myricetin, delphinidin	[71]
	naringenin	[66]
	EGCG	[70]
STAT1	silibinin	[72]
	luteolin, diosmin	[67]
	baicalein	[78]
C/EBP	genistein	
	delphinidin	
	various flavonoids, negletin, chalcone	[79]
NF-AT	apigenin	
	baicalein	
NF-IL-6	quercetin, phloretin	
	IRF-1	
IRF-3	luteolin	[73]

^{a)}No inhibition points described, For the flavonoids and inhibition points which do not have reference, please refer to the review [29] and the references therein.

One of the most abundant tea polyphenols, EGCG, is known to modulate the MAPK pathway and activation of transcription factors. This area is well summarized in a recent review conducted by Santagelo et al. [61]. EGCG primarily inhibits NF- κ B activation by

interfering with IKK. And EGCG was found to inhibit p38 MAPK activation among the MAPK family.

It was recently demonstrated that STAT-1 is an important target for anti-inflammatory agents [62]. In relation to this proinflammatory molecule, Muthian and Bright [63] showed that the anti-inflammatory activity of quercetin occurred via blockage of IL-12 signaling through the Jak/STAT pathway in T lymphocytes. Quercetin was also found to inhibit iNOS expression of BV-2 microglia via blocking STAT1 activation [64], and it has been shown that genistein, kaempferol, quercetin and diadzein inhibited STAT1 in activated macrophages [33]. Apigenin and luteolin have also been found to inhibit STAT1-induced CD40 expression in microglia [65]. Naringenin is another flavonoid that inhibits STAT activation in glial cells [66]. Specifically, luteolin and diosmin inhibited Jak2/STAT3 signaling to reduce IL-6/MIA associated autism in vitro and in vivo [67]. Moreover, it has been shown that the inhibition of STAT was involved in the action of flavonoids, although they are not directly related with anti-inflammatory therapy. For example, EGCG [68-70], myricetin, robinetin, delphinidin [71] and silibinin [72] were found to inhibit STAT activation in the various cells.

It is important to note that luteolin was found to inhibit innate immunity. Lee et al. [73] demonstrated that luteolin inhibited IRF3 and NF- κ B activation by toll-like receptor (TLR) 3 and 4 agonists, down-regulating TNF- α , IL-6, IL-12, interferon-inducible protein-10, IFN, CXCL9 and IL-27 in macrophages. Importantly, luteolin inhibited TBK1-kinase activity, and this inhibition was also observed in response to treatment with quercetin, chrysin and eriodictyol.

Table 3 summarizes some selected studies demonstrating the modulation of nuclear transcription factor activation and various protein kinases including MAPKs, IKK and Akt.

5. Cellular Action Mechanisms: Anti-Oxidative Pathway

Anti-oxidative capacity is a well-known property of flavonoids. In the classical view, flavonoids scavenge peroxides as well as radicals. The reactive oxidative metabolites produced by inflammatory reaction attack target cell molecules, deleterious the inflammatory process. Thus, it is important to protect the target cell from damage, which can be achieved by inhibiting the formation of these reactive metabolites or by enhancing their elimination. The anti-oxidative nature of flavonoids may reduce the amount of reactive species by directly interacting with them (scavenging). In addition, recent investigations have demonstrated that some flavonoids enhance the elimination of reactive metabolites by inducing phase II enzymes, which catalyze the formation of conjugated compounds with various molecules in the body such as glutathione. These detoxifying enzymes include glutathione-transferase, glutathione peroxidase and superoxide dismutase. These enzymes are known to be induced by the activation of transcription factor, nuclear factor erythroid 2 related factor 2 (Nrf2). In the normal state, Nrf2 is combined with Keap1 in the cytosol [80]; however, under oxidative stress, ROS triggers various protein kinases that phosphorylate Keap1, which results in Nrf2 being translocated to nucleus and binding with antioxidant response element (ARE) to produce anti-oxidative enzymes.

Some flavonoids show their anti-oxidative effects by enhancing the production of these anti-oxidative enzymes. For example, EGCG has been shown to activate Nrf2, leading to the production of anti-oxidative enzymes [81]. Therefore, it is suggested that EGCG inhibits NF- κ B activation by inhibiting corresponding kinases in certain cells against inflammatory insults, while the same compound may enhance Nrf2 activation, leading to an antioxidative effect in cells like liver and skin.

Quercetin is another flavonoid with Nrf2 activating properties [82], and its effects may be mediated by heme oxygenase-1 (HO-1) induction through MAPK activation [83]. However, it is still not clearly understood whether other flavonoids possess similar properties, and additional research is needed to elucidate the entire mechanism associated with the antioxidative effects of flavonoids, including cross-talk with the inflammatory pathway.

6. Biflavonoids as anti-Inflammatory Drug Candidates

Biflavonoids are essentially flavonoid-flavonoid dimers with various chemical structures. Many different flavonoid dimer combinations including two different types of bond connections between the flavonoids (C-C bond or C-O-C bond) are possible. Amentoflavone and ginkgetin (Figure 3) are the most common biflavonoids. Some biflavonoids affect the functions of inflammatory/allergic effector cells such as mast cells and lymphocytes, suggesting that certain biflavonoids are possible therapeutic agents against allergic and deleterious autoimmune disorders such as rheumatoid arthritis and lupus erythematosus. Recently, we published a review paper summarizing anti-inflammatory research regarding natural and synthetic biflavonoids [84]. Here, some interesting properties of anti-inflammatory biflavonoids are described to emphasize their possible use as new anti-inflammatory agents.

Certain biflavonoids affect AA metabolizing enzymes. For example, ochnaflavone, morelloflavone and several other biflavones were found to inhibit secretory phospholipase A₂ (sPLA₂-IIA) [85,86]. Later, ginkgetin was found to inhibit cytosolic PLA₂ (cPLA₂) [87]. In addition, some biflavonoids inhibit COXs. For example, amentoflavone has been shown to be a potent COX-1 inhibitor comparable to indomethacin [88, 89]. A biflavone structurally-similar to amentoflavone, tetrahydroamentoflavone, was also demonstrated to be a weak COX-1/COX-2 inhibitor [90]. On the other hand, biflavonoids are not effective LOX inhibitors.

Similar to flavonoid monomers, one important anti-inflammatory mechanism of biflavonoids is transcriptional regulation of proinflammatory molecules. The biflavonoids, bilobetin and ginkgetin, were initially found to suppress inducible nitric oxide synthase (iNOS) and COX-2 expression in LPS-treated RAW 264.7 cells [91] and amentoflavone inhibited NF- κ B activation [92]. Several biflavones including ginkgetin, isoginkgetin, bilobetin and ochnaflavone down-regulated iNOS expression in LPS-induced RAW 264.7 cells, whereas amentoflavone did not [93]. Moreover, ginkgetin was shown to inhibit COX-2 induction in LPS-treated RAW cells [94].

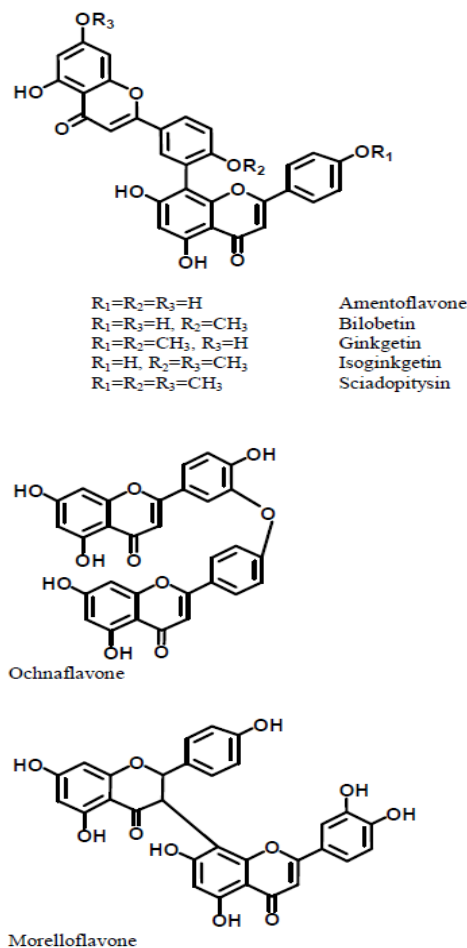


Figure 3. The representative naturally-occurring biflavonoids.

Topical treatment with ginkgetin also reduced COX-2 induction in TPA-treated mouse skin [95]. Other biflavonoids showed similar properties and were also found to affect transcription factor activation. For example, ochnaflavone was found to inhibit iNOS expression by LPS-treated RAW 264.7 cells mainly by blocking ERK and NF- κ B activation [96]. In addition, isoginkgetin was reported to inhibit MMP-9 induction by blocking PI3K/Akt activation in HT1080 human fibrosarcoma cells [97]. Recently, sumaflavone and amentoflavone showed significant MMP-1 inhibitory activity in primary human dermal fibroblasts following UV irradiation [98].

As mentioned above, there have been continual findings of the regulatory effects of biflavonoids on proinflammatory gene expression, and it is evident that certain biflavonoids down-regulate the expression of proinflammatory molecules by regulating signal transduction pathways in addition to their direct inhibitory actions on PLA₂ and/or COXs.

It is important to note that there is no data available regarding the absorption and distribution of biflavonoids in animals and humans. Morelloflavone and tetra-

ydroamentoflavone showed *in vivo* anti-inflammatory activity following oral administration [85, 89]. However, in our experiments, oral treatment produced much lower or no activity, indicating that the oral bioavailability of biflavonoids may be very low. It has been speculated that orally administered biflavonoids may be rapidly degraded in the gastro-intestinal tract. Conversely, intraperitoneal administration resulted in higher anti-inflammatory activity, and topical treatment also yielded favorable results [95, 99]. Several synthetic approaches also recently yielded new biflavonoid molecules with anti-inflammatory potential that exhibit phospholipase A₂ and COX-2 inhibitory activity [100, 101]. Although the bioavailability needs be improved, some biflavonoids certainly have the potential for use as new anti-inflammatory agents.

7. Anti-Inflammatory Flavonoids: Human Clinical Trials

There have been several pharmacokinetic studies of flavonoid ingestion as a dietary food or supplement. In addition, many clinical trials have been conducted to evaluate the use of flavonoids or flavonoid-like molecules for anticancer therapy. For example, human clinical studies of isoflavones such as genistein have been conducted to determine their effectiveness against breast cancer and prostate cancer. Furthermore, a phase II clinical trial was conducted to determine the effectiveness of soy milk for the treatment of prostate cancer. Clinical studies of flavopiridol as an anticancer therapy have also been conducted. In contrast, only a few clinical investigations of flavonoids have been conducted to determine if they are useful as anti-inflammatory drugs. Even fewer clinical trials of pure flavonoid compounds for the development of new anti-inflammatory drugs have been carried out.

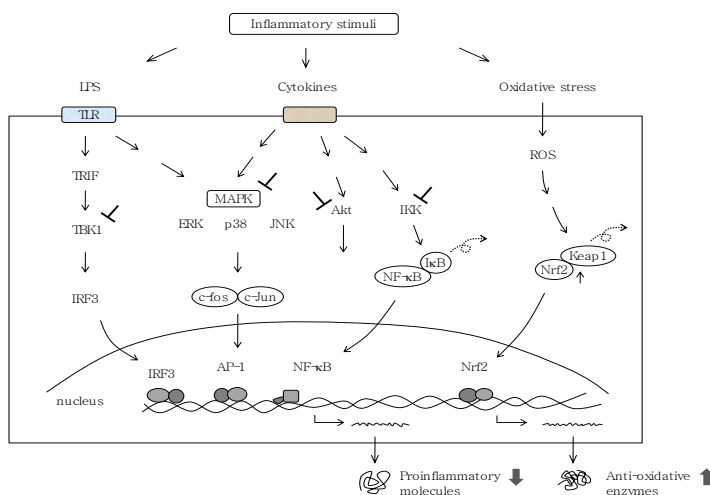
Gugler et al. [102] evaluated the pharmacokinetics of quercetin administered by the i.v. and p.o. routes. As expected, they found that the bioavailability of oral quercetin treatment was low. In a phase I trial in which quercetin was administered by i.v. injection, lymphocyte tyrosine kinase was inhibited, but renal toxicity was observed [9]. Conquer et al. [103] found that quercetin ingestion did not affect risk factors for heart disease. Additionally, they found that the oxidative state was not altered by quercetin, which is similar to the results of a study conducted by Edwards et al. [104]. McNulty et al. [105] also reported that quercetin ingestion did not influence the oxidative state or inflammatory state of normal healthy volunteers. Moreover, a diet including high levels of quercetin did not inhibit COX-2 expression by lymphocytes [106]. Negative effects of orally administered quercetin were also demonstrated by Boots et al. [107]. Conversely, quercetin, naringenin and hesperetin diets lowered the incidence of asthma [108].

To date, there has been little evidence of anti-inflammatory effects of orally administered flavonoid treatment in humans. Indeed, only a few studies have suggested that flavonoids may be effective against chronic inflammatory disorders. Specifically, Kometani et al. [109] showed some evidence of the effectiveness of treatment by the oral administration of the flavonoid congener, α -glucosylhesperidin, against human RA. In addition, a recent clinical trial claimed that ingestion of flavocoxid (90% baicalein + catechins) had effects equivalent to naproxen for management of the signs and symptoms of osteoarthritis of the knee [110]. With the exception of quercetin, which can induce renal toxicity, oral flavonoid treatment

appears to be safe. More phase II and III clinical trials may demonstrate the effectiveness of flavonoids against inflammatory disorders, especially chronic inflammatory disorders.

8. Perspective

Many flavonoids exert anti-inflammatory activity *in vitro* and *in vivo*. They show anti-inflammatory activity primarily by regulating the expression of proinflammatory molecules (Scheme I). Although nutritional intake of flavonoids as part of food may help to reduce certain inflammatory conditions, successful anti-inflammatory therapy using flavonoids by pharmacological treatment has been rare to date. The continual search for more potent flavonoids from plants and from the synthetic approach should be conducted and more clinical trials are needed, especially for chronic inflammatory disorders, to enable the first anti-inflammatory flavonoid therapy.



Scheme 1. Modulation of proinflammatory gene expression by flavonoids Flavonoids modulate the signal transduction pathways at various points. Inhibition (⊥), activation (↑), down-regulation (↓), up-regulation (↑).

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Chapter IV

Chemoprevention with Phytochemicals Targeting Inflammation-Associated Enzymes

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Abstract

Background: Inflammation is involved in the processes of many, if not all, pathological diseases, including cancer, diabetes, and arteriosclerosis. Biological crosstalk among cells with different phenotypes produces versatile proteins and small molecules with pro-inflammatory properties, thus generating a complex inflammatory condition. Immune cells including neutrophils and macrophages are central players in the production of these molecules via multistep mechanisms. On the other hand, there is ample evidence showing that certain natural products, especially phytochemicals, possess pronounced anti-inflammatory activities and some act on immune cells by targeting pro-inflammatory mediators, which results in mitigation of inflammation.

Materials and Methods: The molecular mechanisms underlying production of the pro-inflammatory mediators cyclooxygenase (COX), tumor necrosis factor (TNF)- α , the ligand of the receptor activator of nuclear factor (NF)- κ B (RANKL), and TNF-related apoptosis-inducing ligand (TRAIL), as well as their signaling pathways are described. Moreover, phytochemicals shown to disrupt the production and/or signaling pathways of these molecules are highlighted, with plausible molecular mechanisms presented.

Results: Various inflammatory stimuli activate corresponding cell surface receptors for initiating signal transduction pathways involved with numerous protein kinases including mitogen-activate protein kinases (MAPKs), which leads to concomitant and concerted activation of several key transcription factors that target the expression of proinflammatory mediator genes. In addition to this conventional transcriptional

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mechanism for the *de novo* synthesis of a protein, recent studies have provided solid evidence showing that synthesis of a number of inflammatory and oncogenic molecules is amplified by post-transcriptional, translational, and post-translational mechanisms. These alternative regulation mechanisms appear to be indispensable for their rapid production in transient or sustained manners, which is situation-dependent, and essential for homeostasis and environmental adaptation of host cells. Meanwhile, the molecular mechanisms and targets of phytochemicals are elusive as compared with those of synthetic drugs, e.g., non-steroidal anti-inflammatory drugs, which are rationally designed to target COX. Nonetheless, our continuous efforts to dissect the modes of actions of natural products have partially revealed some unique mechanisms. For example, 1'-acetoxychavicol acetate (ACA) and zerumbone, major components of the subtropical zingiberaceous plants *Alpinia galanga* and *Zingiber zerumbet*, respectively, were found to attenuate lipopolysaccharide (LPS)-induced COX-2 protein expression in macrophages and prevented chemical carcinogenesis in several rodent models. Interestingly, ACA also abrogated the activation of extracellular signal-regulated protein kinase (ERK)1/2 and c-Jun N-terminal kinase (JNK)1/2, while zerumbone did not have those effects, but rather promoted mRNA degradation of COX-2 presumably by disrupting the p38 MAPK pathway. In addition, our recent *in vitro* data showed that zerumbone forms covalent bindings to several proteins, which determines the stability of pro-inflammatory mRNAs.

Conclusion: The present review partially reveals molecular mechanisms by which phytochemicals modulate the production of pro-inflammatory mediators, which may be useful for understanding their anti-inflammatory activities in cellular, rodent, and human models. However, the current mechanistic data appear to be inadequate and unsatisfactory to begin clinical studies. Nevertheless, discovery of their binding proteins may provide breakthrough findings to achieve this goal.

Abbreviations

Akt	protein kinase B
ARE	AU-rich element
ADAM	a disintegrin and metalloproteinase
ACA	1'-acetoxychavicol acetate
C/EBP	CCAAT enhancer-binding protein
COX-2	cyclooxygenase
CREB	cAMP-responsive element-binding protein
CR	conserved region
DISC	death inducing signal complex
DR	death receptor
EGCG	(-)-epigallocatechin-3-gallate
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FADD	FAS-associated death domain
13-HOA	(+/-)-13-hydroxy-10-oxo- <i>trans</i> -11-octadecenoic acid
HuR	Hu antigen R
IFN	interferon
IκB	inhibitor of NFκB
IKK	IκB kinase

IL	interleukin
IRF	IFN regulatory factor
ITC	isothiocyanate
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
MAPK	mitogen-activate protein kinases
MK-2	MAPK-activated protein kinase 2
MMP	matrix metalloproteinase
NFAT	nuclear factor of activated T-cells
NSAID	nonsteroidal anti-inflammatory drug
OPG	osteoprotegerin
PEITC	phenethyl isothiocyanate
PDCD4	programmed cell death 4
PKC	phosphoinositide-dependent kinase
PKA	protein kinase A
PG	prostaglandin
PI3K	phosphatidylinositol 3-kinase
PPAR	peroxisome proliferator-activating receptor
RANK	receptor activator of NF- κ B
RANKL	ligand of the receptor activator of NF- κ B
ROS	reactive oxygen species
TACE	TNF- α -converting enzyme
TIMP	tissue inhibitor of matrix metalloproteinase
TLR	toll-like receptor
TNF	tumor necrosis factor
TNFR	TNF receptor
TPA	12- <i>O</i> -tetradecanoylphorbol 13-acetate
TRADD	TNFR1-associated death domain protein
TRAF	TNF- α -associated factor
TTP	tristetraprolin
TRAIL	TNF-related apoptosis-inducing ligand
UTR	untranslated region

1. Introduction

Inflammation has both homeostatic and pathophysiological roles that are related to the onset of numerous diseases, including vascular atherosclerosis, diabetes, obesity, inflammatory bowel diseases, and sepsis. Furthermore, a considerable proportion of chronic inflammatory diseases displays an overlap with the development of oncogenesis. For example, there are close relationships between Crohn's disease and colorectal cancer, reflux esophagitis and esophageal carcinoma, and hepatitis and hepatocellular carcinoma [1]. Thus, regulation of inflammatory conditions with phytochemicals may open wide opportunities for health promotion and disease prevention.

Understanding of the cellular and molecular mechanisms underlying the biological and biochemical processes of inflammation is essential to discover or identify phytochemicals with anti-inflammatory properties. In the process of non-pathologic inflammation, e.g., wound healing, platelets release biochemical mediators that tightly regulate vascular permeability and recruit fibrinogen, leading to the formation of fibrin clots. These activities also induce the production of platelet-derived growth factor, transforming growth factor- β , and interleukin (IL)-1 β , which lead to activation of stromal cells that are responsible for the release of a cocktail of proteases, such as the matrix metalloproteinases (MMPs) superfamily that can virtually degrade the extracellular matrix. Concurrently, neutrophils and monocytes are matured and recruited, then infiltrate inflamed tissue as part of the innate immune machinery. Those are well-known biological sources of reactive oxygen species (ROS), prostaglandins (PGs), inflammatory cytokines, and chemokines, as well as others. In addition, PGE₂ has been demonstrated to induce angiogenesis and plays some notable roles in the growth of fibroblasts, and endothelial and epithelial cells, while cytokines and chemokines are also released into the bloodstream and circulate for further amplification of the immune system. Importantly, the above-noted biological phenomena progress in a concerted fashion toward re-epithelialization and wound healing. It should also be pointed out that most of those processes are shared in pathogenic conditions, and the determinant that differentiates pathological from homeostatic inflammation lies in the fact that they are sustained and exaggerated in a dysregulated manner. Therefore, anti-inflammatory drugs or phytochemicals may have unavoidable side-effects that are related to dysregulation and/or decline of the immune system.

2. Pro-Inflammatory Mediators

The molecular mechanisms involved in the production of pro-inflammatory mediators are multistep and complex processes, while it is also important to note that they are largely different, depending upon each molecule. Nonetheless, it may be helpful to present a rough picture showing how inflammatory stimuli produce pro-inflammatory mediators within a cell. As depicted in Figure 1, endogenous or exogenous stimuli are bound to their own receptors for activating the signaling cascade, which consists of numerous protein kinases and other signal molecules, leading to expressions of pro-inflammatory genes mediated by several transcription factors. As described below, most, if not all, mRNAs of pro-inflammatory genes are regulated at post-transcriptional levels. In addition, some precursor proteins are shed to generate biologically active proteins that are released from cells to exert their biological functions via autocrine or paracrine manners.

2.1. Cyclooxygenases

PGH₂ synthase, more frequently referred to as cyclooxygenase (COX), donates two oxygen molecules to arachidonic acid for generating PGG₂ by peroxidation and then PGH₂ by reduction. These sequential conversions are the initial steps for production of a variety of prostanoids that play versatile roles in both physiological and pathological conditions. It is

well established that this key enzyme in the process of inflammation is the molecular target for analgesic and anti-inflammatory remedies, including nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin. NSAIDs have received considerable attention for their ability to not only mitigate inflammatory responses, but also potentially prevent cancer in humans.

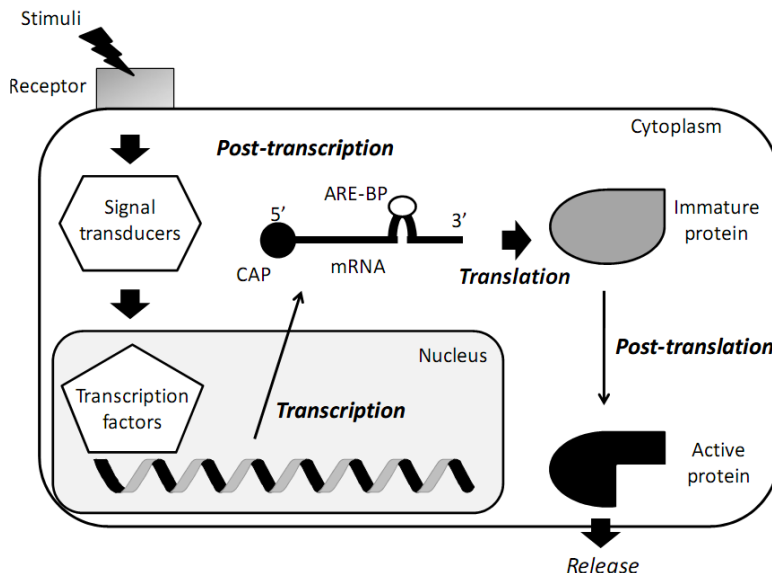


Figure 1. General scheme for molecular events involved in the production of pro-inflammatory proteins. Endogenous or exogenous stimuli are bound to their corresponding receptors for activating the cascade of signal transducing pathways, leading to transactivation of pro-inflammatory genes through several transcription factors. It is well known that most, if not all, mRNAs of pro-inflammatory genes are regulated by post-transcriptional mechanisms, in which ARE-BPs play pivotal roles (see Figure 2). Following translation, a protease sheds a precursor protein to generate a biologically active protein, which is released from the cell and exerts its function via an autocrine or paracrine manner. ARE-BP, AU-rich element-binding protein.

The COX enzyme consists of at least two isoforms, COX-1 and COX-2, the latter of which has been cloned as a *v-src*-inducible gene [2]. At 22 kilobases (kb), the human *COX-1* gene is much larger than *COX-2* (8.3 kb), whereas the size of the COX proteins are nearly identical at approximately 70 kDa. In humans, the expression of COX-1 is constitutive in many normal tissues at relatively stable levels and is believed to play some house-keeping functions such as the production of PG precursors for thromboxane in platelets [3], which is vital for regulation of blood flow. This is a reasonable mechanism, since platelets, which have no nuclei, are unable to synthesize inducible enzymes. In the kidney, COX-1 has an essential role of generating vasodilating PGs that maintain renal plasma flow at a normal level and also determines the glomerular filtration rate during systemic vasoconstriction conditions. In contrast to COX-1, COX-2 protein is only slightly expressed in most normal mammalian tissues in response to physical, chemical, and biological stimuli, including UV light exposure, dioxin, and lipopolysaccharide insult. In the last two decades, COX-2 has received the attention of numerous researchers for the relevance of its expression to pathogenesis [4]. In particular, there is ample evidence of the involvement of COX-2 expression in carcinogenesis in many different target organs. Recent research evidence does not support the notion that COX-1 plays house-keeping functions and is only important for homeostasis, or that

inducible COX-2 alone is a remarkable target for anti-inflammation and anti-carcinogenesis strategies. Rather, the activities of these individual COX isoforms induce different signaling pathways in a concerted fashion, thereby creating various PGs profiles that are related to disease onset [5].

COX-2 mRNA expression is regulated by at least three distinguishable stages in a complex manner. The earliest induction mechanism is related to the finding that COX-2 mRNA contains the AU-rich element (ARE) in its 3'-untranslated region (UTR), which has some critical roles in the stability of its mRNA [6] (Figure 2). Several reports of different cell types have shown that activation of p38 mitogen-activated protein kinase (MAPK) leads to stabilization of COX-2 mRNA. Also, a substrate for p38 MAPK, i.e., MAPK-activated protein kinase 2 (MK-2), phosphorylates certain candidate proteins such as HSP27 [7], heterogeneous nuclear ribonucleoprotein A0 [8], and Hu antigen R (HuR) [9], which bind to AREs, thereby contributing to rapid synthesis of COX-2 protein. In addition, Sully *et al.* identified several proteins that target the conserved region 1 (CR 1) located at 3'-UTR, including AUF-1 [AU-rich element/poly(U)-binding/degradation factor-1], AUF-2, tristetraprolin (TTP), HuR, and far-upstream-sequence-element-binding protein 1 [10]. However, they concluded that CR-1-mediated COX-2 mRNA decay has no correlation with the binding of those proteins and that another unidentified process may play a critical role in the instability of COX-2 mRNA.

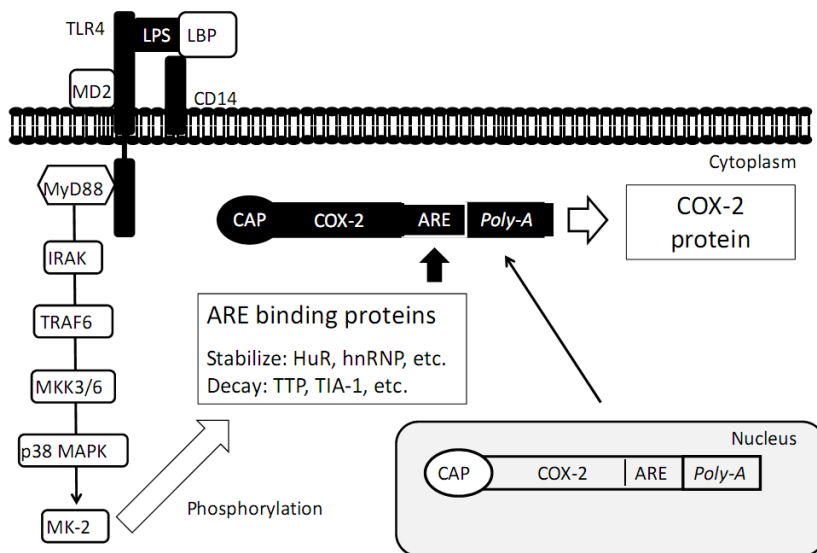


Figure 2. Post-transcriptional regulation of COX-2 mRNA in LPS-challenged macrophages. LPS is bound to its receptor TLR4 and this signal is mediated through adaptor proteins such as Myd88, leading to activation of the p38 MAPK pathway. Subsequently phosphorylation of ARE-BPs such as HuR determine the lifespan of COX-2 mRNA in a positive or negative manner. TLR, toll-like receptor; LPS, lipopolysaccharide; LPB, LPS-binding protein; MD2, myeloid differentiation factor; IRAK, interleukin-1 receptor-associated kinase; TRAF, TNF- α -associated factor; MKK, MAPK kinase; MAPK, mitogen-activated protein kinase; MK, MAPK-activated kinase; COX, cyclooxygenase; ARE-BP, AU-rich element-binding protein; HuR, Hu antigen R; hnRNP, heterogeneous nuclear ribonucleoprotein; TTP, tristetraproline.

The next stage for COX-2 expression takes place at two different transcriptional levels. In macrophages, lipopolysaccharide (LPS)-triggered induction and activation of signal transduction pathways lead to transcriptional activation of the COX-2 gene. The toll-like receptor (TLR) family, particularly TLR4, is now recognized as the central receptor for LPS [11]. TLR4 has several associated proteins, such as MD2 and Myd88, which are needed for full activation. Subsequently, they stimulate some protein kinases, including phosphatidylinositol 3-kinase (PI3K) and phosphoinositide-dependent kinase. The phosphorylated protein kinase B (Akt) in turn activates I κ B kinase (IKK), which is composed of the α , β , and γ subunits, and phosphorylates I κ B protein [12], leading to degradation of this protein. Since I κ B is a suppressive protein that binds to the nuclear factor (NF) κ B transcription factor in a normal state, LPS-induced I κ B degradation results in activation of this particular transcription factor for COX-2 mRNA induction. It is essential to understand that NF κ B-targeted genes include the I κ B gene, and this negative feedback mechanism causes NF κ B-induced COX-2 expression to be intermediate and transient.

For full induction of COX-2, several NF κ B-targeted genes play a major role, which is recognized as the third stage. Two of these genes are CCAAT enhancer-binding protein beta (C/EBP β) and delta (C/EBP δ), which form β/β homodimer and β/δ heterodimer, both of which play crucial roles in late and continuous COX-2 expression [13]. In parallel, COX-2-generated PGE₂ binds to its receptors for activation of adenylyl cyclase and thereby increases the level of intracellular cAMP, leading to activation of protein kinase A (PKA), which is responsible for the transcriptional activity of cAMP-responsive element-binding protein (CREB) [14]. Collectively, these form a positive feedback loop in PKA-mediated COX-2 expression.

2.2. Tnf- α

Accumulated evidence has revealed that the TNF superfamily of proteins consists of 19 members and their 29 receptors. While serving as homeostatic molecules responsible for immune responses, host defense, wound healing, and morphogenesis, as well as other processes, these bioactive proteins also play critical roles in numerous inflammatory diseases, including tumorigenesis, septic shock, bone resorption, diabetes, and arthritis. Thus, these proteins are known as 'double-edged swords.' On one hand, an early study by Suganuma *et al.* [15] reported that tumor onset was delayed 4 weeks in TNF(-/-) mice treated with 7,12-dimethylbenz[a]anthracene plus 12-*O*-tetradecanoylphorbol 13-acetate (TPA), while the time to development of small tumors in 100% of the mice was 9 weeks later than that seen in TNF(+/-) CD-1 mice. Those results demonstrated that this cytokine plays key roles in the tumor promotion stage in mouse skin and subsequent studies supported their hypothesis [16, 17]. On the other hand, long-term TNF- α blockade may result in decay and dysregulation of the tumor surveillance system [18]. These contrasting results may be associated with differences in the experimental conditions used, including the biological system for tumor development.

The production of TNF- α is regulated in a multiplex and complex manner (Figure 3). This is reasonable based on the biological necessity that it should be produced in a rapid and well-organized fashion in response to endogenous and exogenous stress stimuli. The most

rapid production involves enzymatic activation of TNF- α -converting enzyme (TACE/disintegrin and metalloproteinase 17 or ADAM17/CD156q), which sheds cell surface proTNF- α by proteolysis, an inactive latent precursor protein, and thereby releases bioactive TNF- α . This is a process known as protein ecto-domain shedding. The presence of TACE was first reported by Black *et al.* [19], who purified and cloned a metalloproteinase that specifically cleaves proTNF- α . Like most ADAMs, TACE is a type I transmembrane protein with multiple domains, including a zinc-dependent catalytic domain and disintegrin-cysteine rich sequence. It is of interest that TACE mRNA expression is found in most, if not all, tissues. The expression of TACE protein is largely constitutive and only the bioactive form becomes localized on the cell surface. In the absence of cell activators, the surface pool of the enzyme is long lived, with a half-life of more than 8 hours. However, prolonged exposure of cells to a phorbol ester, for example, causes internalization and degradation of TACE [20].

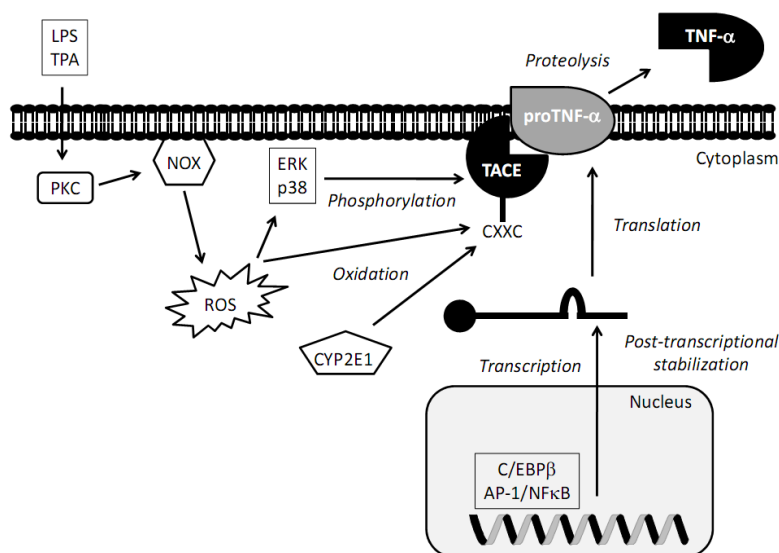


Figure 3. TNF- α protein production is regulated through multiple mechanisms. Both LPS and TPA are potent activators of PKC, which assembles and thereby activates NOX, a multi-component protein responsible for superoxide anion generation. The resultant ROS may oxidize the CXXC motif of TACE and/or activate p38 MAPK and ERK1/2, both of which potentiate TACE activity by phosphorylation. Alternatively, CYP2E1 may generate ROS to drive similar mechanisms. Activation of the p38 MAPK pathway leads to stabilization of TNF- α mRNA by post-transcriptional mechanisms (see Figure 3). TACE is a rate determining enzyme that sheds pro-TNF- α to form mature TNF- α , which is released from the cell. TLR, toll-like receptor; LPS, lipopolysaccharide; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PKC, protein kinase C; NOX, NADPH oxidase; ROS, reactive oxygen species; ERK, extracellular signal-regulated protein kinase; C/EBP, CCAAT-enhancer binding protein; AP-1, activator protein-1; NF κ B, nuclear factor κ B; TACE, TNF- α converting enzyme; TNF, tumor necrosis factor.

The mechanisms underlying TACE activation are not fully understood, though it is known to be tightly controlled by a component that does not affect notch processing [21]. However, redox regulation of TACE was recently suggested by Wang *et al.*, who found that oxidative stress induces thiol-disulfide conversion in two highly conserved vicinal cysteine sulfhydryl motifs (cysteine-X-X-cysteine) of the extracellular region of TACE, thereby

activating this enzyme [22]. This mechanism of action may be supported by numerous previous findings that treatments of cells with many, if not all, TNF- α -inducing stimuli induce redox-dependent responses in both enzymatic and non-enzymatic manners. For example, exposure of immune cells such as macrophages and Kupffer cells to the endotoxin LPS generates ROS via NADPH oxidase- and cytochrome P4502E1-dependent pathways [23, 24]. In addition, some protein kinases have been implicated to play major roles in the enzymatic activation of TACE. Soond and colleagues considered that phosphorylation of TACE at Thr735 highlights a key step in inducible TACE protein trafficking and maturation [25]. In accordance with this notion, Bell and Gooz recently reported that pro-fibrotic stimulus induced extracellular signal-regulated kinase (ERK) activation and thereby phosphorylated TACE for inducing a concomitant increase of TACE activity in kidney mesangial cells [26]. In platelets, hydrogen peroxide was found to activate TACE and this effect was mediated by p38 MAPK signaling pathways, but not protein kinase C, PI3K, or caspases [27]. Similar findings were also reported by Deng *et al.*, who noted that LPS exposure led to p38 MAPK activation that involved TACE activation for liver pathogenesis [28].

Other regulation mechanisms essential for TNF- α production involve both transcriptional and post-transcriptional events, which function in parallel with those of COX-2, as described above. In an early study, Rhoades *et al.* explored phorbol ester-activated promoter regions of the TNF- α gene, and found single AP-2- and AP-1-like consensus sequences, though the latter was more critical for promoter activity [29]. Following this observation, Pepe *et al.* reported that the transcription factor C/EBP β , but not C/EBP α , activated the TNF- α gene promoter in myelomonocytic cells [30], while c-jun was identified as a partner protein of C/EBP β [31]. Additionally, Myokai *et al.* cloned a novel TNF- α transcription factor in LPS-exposed THP-1 human monocytes, which was termed LPS-induced TNF- α factor [32]. However, the signaling molecules localized upstream of those transcription factors remain to be fully identified.

It is also important to note that the ras/raf-1/MEK/MAPK pathway is chiefly responsible for transduction of LPS signals to the level of TNF- α mRNA in macrophages [33]. Glucocorticoids including dexamethasone are a potent suppressor of TNF- α , as documented with many cell types. Steer *et al.* reported a significant observation that dexamethasone blocked LPS-stimulated TNF- α release from human monocytic cells by antagonizing both c-Jun/ATF-2 and NF- κ B transactivation via binding at the -106 to -88 bp region of the TNF- α promoter [34]. Along a similar line, recent studies have revealed adiponectin, an adipokinin with anti-adipogenetic and anti-inflammatory properties, as a novel endogenous negative regulator for TNF- α , as it decreased LPS-stimulated ERK1/2 phosphorylation and I κ B degradation, and thereby attenuated NF κ B [35]. Similar to COX-2, TNF- α mRNA is regulated by post-transcriptional mechanisms and adiponectin was found to disrupt the stability of TNF- α mRNA, indicating that it targets TNF- α at both transcriptional and post-transcriptional levels [36]. One of the earliest findings regarding TNF- α mRNA post-transcription regulation was reported by Lieberman *et al.*, who noted that the half-life of TNF mRNA was significantly decreased in the presence of protein kinase inhibitors [36]. The ARE-BP of TNF- α mRNA has been suggested to be a 35-kDa protein in primary rat astrocytes, as shown by electrophoretic mobility-shift assay results [37]. Later, a 55-kDa protein was suggested as an ARE-BP of TNF- α mRNA by another group [38] and then

identified as TIAR [39]. However, one of the critical ARE-BPs for TNF- α mRNA may be HuR [40], as Nabors *et al.* found that strong HuR protein expression was limited to high-grade malignancies in a study of clinical samples of central nervous system tumors, though RNA binding affinity was verified [41].

TTP is a *trans*-acting protein that accelerates degradation of ARE-containing mRNAs, including TNF- α . A recent study by Deleault *et al.* also demonstrated that proteasome inhibition led to blockade of TTP-mediated TNF- α mRNA decay, which was inhibited by the combined activation of ERK and p38 MAPK, but not by p38 MAPK activation alone, suggesting that both ERK and p38 MAPK are required to inhibit TTP function and stabilize TNF- α mRNA [42]. Furthermore, inhibition of protein phosphatase 2A resulted in a significant increase in the stability of TNF- α mRNA, which was associated with increased activation of both p38 MAPK and its substrate, MK-2 [43]. Meanwhile, glucocorticoids were shown to induce TTP expression in human bronchial epithelial cells [44], indicating that they are capable of regulating production of a number of inflammatory proteins in both transcriptional and post-transcriptional manners. In addition to the ARE-dependent regulation of TNF- α mRNA decay, Stoecklin *et al.* proposed an alternative mechanism that is dependent upon the constitutive decay element, which was mapped to an 80-nucleotide segment downstream of the ARE of TNF- α mRNA [45]. Interestingly, activation of p38 MAPK and PI3K does not inhibit constitutive decay element-mediated mRNA decay. A comparative study using wild-type and TIA-1(-/-) macrophages revealed that TIA-1 deficiency significantly increased the proportion of TNF- α transcripts associated with polysomes [46], suggesting that TIA-1 normally functions as a translational silencer.

2.3. Rankl

RANKL, a ligand of the receptor activator of NF- κ B (RANK), is a membrane-bound protein known to promote survival of dendritic cells and differentiation of osteoclasts, which are multinucleated giant cells that originate from monocyte-macrophage lineage cells. On the other hand, osteoblasts, or bone marrow stromal cells, have been suggested to be involved in osteoclastic bone resorption. Crosstalk between TNF- α and RANKL was well described by Zhang *et al.*, who observed that TNF- α and RANKL synergistically up-regulate RANK expression in wild-type precursors, whereas basal and stimulated levels of RANK were significantly lower in TNF receptor (TNFR)1 knockout cells [47]. The involvement of RANKL in the development of many inflammatory diseases, including osteoporosis [48], rheumatoid arthritis [49], and bone metastasis and multiple myelomas [50], has also been reported.

Many calciotropic hormones and cytokines, including 1,25(OH) $_2$ D $_3$, parathyroid hormone, PGE $_2$, IL-6, and IL-11, may have dual activities to inhibit production of osteoprotegerin (OPG), which is a decoy receptor for RANKL, and stimulate production of RANKL. The signaling pathways for RANKL expression by those ligands are complex, such as those of TNF- α , and not fully elucidated. They also may vary, as they are dependent on differences in cell types and stimuli that are capable of inducing RANKL. However, Liu *et al.* found that PGE $_2$ and IL-6 signaling enhance osteoclast differentiation by effects on the OPG/RANKL/RANK system in bone cells [51]. In addition, both Jak2 and Stat5a are

essential proteins for prolactin-induced RANKL expression in mammary gland [52]. Importantly, Bai *et al.* showed that ROS stimulate RANKL expression via ERKs and the PKA-CREB pathway in mouse osteoblasts, as well as via ERK and HSF2 in human MG63 cells [53]. Furthermore, E2F1 plays an important role in regulating RANKL transcription through binding to the E2F consensus binding site [54]. In addition, a recent study by Yuvaraj *et al.* indicated that the CXCL13/CXCR5 axis stimulates c-Jun and thereby promotes RANKL expression in oral squamous cell carcinomas [55]. The proteolytic cleavage of RANKL releases its soluble form (sRANKL), and this conversion requires ADAM or MMP family members. For example, TACE and ADAM19 have been shown to cleave RANKL *in vitro* [56]. It is also worth noting that sRANKL has more potent activity than its membrane-bound form [57] and important roles in tumor-induced osteolysis [58].

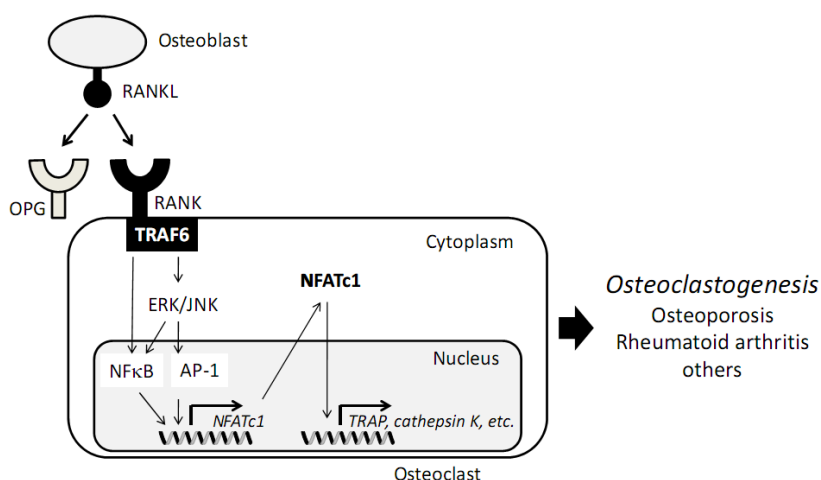


Figure 4. The binding of RANKL to RANK, but not OPG, induces osteoclastogenesis. RANKL activates both the TRAF6/AP-1 and TRAF6/NFκB pathways for inducing NFATc1, which is a master transcription factor of osteoclastogenesis. The presence of OPG disrupts these events, since it has a RANKL binding domain, though it lacks a signal transducing domain. RANK, receptor activator of NF-κB; RANKL, ligand of RANK; TRAF, TNF-α-associated factor; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; NFATc1, nuclear factor of activated T-cells-1.

2.4. Trail

TNF-α-related apoptosis-inducing ligand (TRAIL), also known as Apo 2 ligand (Apo2L), is another member of the TNF-α family of ligands and capable of initiating apoptosis through corresponding death receptors (DRs). TRAIL is a type-II membrane protein that has an intracellular terminal domain and a carboxyl terminus in the extracellular compartment. Like RANKL, TRAIL is generally expressed on the cell surface, and must lose its intramembrane and cytoplasmic domains before cleaving. Although TRAIL expression has been reported in a variety of cells and tissues, studies of mice and humans show that it is not expressed on the surface of freshly isolated T-cell, B-cells, monocytes, dendritic cells, or others. Thus, it is of great importance to note that TRAIL-induced cell apoptosis is selective for tumorigenic and transformed cells, but not for normal cells [59]. Even though mechanistic evidence to account for this selectivity remains to be provided, White-Gilbertson *et al.*

proposed an attractive hypothesis that links TRAIL sensitivity to protein translational arrest following various stresses that target eukaryotic elongation factor 2 [60]. In addition, Di Pietro and Zauli highlighted non-apoptotic functions of TRAIL, which may exhibit different responses to normal cells and tissues [61].

Gong and Almasan have demonstrated that TRAIL mRNA was induced by interferon (IFN)- α and - β , but not - γ , in Jurkat cells [62]. The promoter region of the TRAIL gene comprises several transcription regulatory factor binding motifs, including C/EBP, SP-1, Oct-1, and AP-1 [63]. On the other hand, NF κ B may be designated as master regulator for transcriptional activation of TRAIL expression in T lymphocytes [64]. Additionally, transcriptional induction of TRAIL was mediated via IFN regulatory factor 3 (IRF-3) for inducing apoptosis in Paramyxovirus-infected cells [65]. Microarray analysis by Fu *et al.* also revealed that EGR-1, a key transcriptional factor to mediate gene expression after vascular injury, represses TRAIL expression in human endothelial cells [66]. Along a similar line, activated protein C, an antithrombotic and anti-inflammatory serine protease, which plays an important role in vascular function, has been shown to inhibit TRAIL expression by activating the ERK pathway and thereby up-regulates EGR-1 in human umbilical vein endothelial cells [67].

2.5. DRs-Mediated Signaling

Each receptor for a TNF- α superfamily member plays an essential role in executing numerous events related to inflammation and apoptosis. Thus, regulation of DR family expression and/or their downstream signaling molecules is also recognized to be effective for anti-inflammation, as well as cancer therapy and prevention [68]. TNFR1 has been the focus of many studies as a prototype for members of the TNF superfamily. This receptor is constitutively expressed in most cell types, and a great amount of evidence showing its critical roles in mediating the physiological and pathophysiological activities of TNF- α has been accumulated. The binding of TNF- α to TNFR1 leads to recruitment of a key adaptor protein, TNFR1-associated death domain protein (TRADD), to the receptor complex [68]. Thereafter, a protein complex consisting of TNFR1, TRADD, the receptor interacting protein (RIP), and TNF- α -associated factor (TRAF)-2 is involved in transduction of signals for versatile cellular responses via ERK1/2 and NF κ B, which in turn suppresses apoptosis. Conversely, the association of FAS-associated death domain protein (FADD) and caspase-8 activates the JNK1/2 pathway to induce apoptosis [68].

The RANKL/RANK/OPG triad is the critical determinant of bone homeostasis as well as several diseases, such as osteoporosis, rheumatoid arthritis, and tumor-induced bone disease. Membrane-bound RANKL or sRANKL activates the receptor RANK, which is associated with TRAF proteins for inducing the PI3K, MAPK, and NF- κ B pathways to promote osteoclastogenesis. NF- κ B is activated via the canonical TAK1-IKK β pathway or non-canonical NIK-IKK α pathway, leading to transcriptional regulation of the target genes. On the other hand, MAPK family members control AP-1 family members, such as Jun, Fos, and Fra, which also regulate expression of important genes that play crucial roles in osteoclastogenesis. Concomitant activation of NF κ B and AP-1 induces the expression of nuclear factor of activated T-cells (NFAT)c1, a key determinant for osteoclastogenesis [69].

This particular transcriptional factor is also responsible for induction of several genes important for osteoclast functions, including tartrate-resistant acid phosphatase (TRAP), calcitonin receptor, cathepsin K, and $\beta 3$ integrin [69].

OPG, which is produced by osteoblasts and marrow stromal cells, lacks a transmembrane domain and acts as a secreted decoy receptor that has no direct signaling capacity, thus implicating it as an effective therapeutic target for diseases associated with excessive osteoclast activity. In accordance with this notion, Kostenuik and Shalhoub documented that overexpression of OPG in transgenic mice leads to profound osteopetrosis secondary to a near total lack of osteoclasts. Conversely, ablation of the OPG gene causes severe osteoporosis in mice [70]. Both DR4 and DR5 are the major receptors for TRAIL for executing apoptotic cell death in many malignant tissues and cells, while exogenous and endogenous stresses at excess levels to the endoplasmic reticulum (ER) are known to trigger apoptosis. Yamaguchi and Wang showed that the PKC inhibitor thapsigargin induced ER stress and thereby increased CHOP expression for apoptosis in HCT116 human colon cancer cells, which were accompanied with up-regulation of DR5 [71]. Importantly, Chen *et al.* found that a proteasome inhibitor induces NF- κ B activation and concomitantly ROS-mediated p53 activation, both of which are dominantly involved in DR5 transcription. This, in turn, leads to the subsequent enhancement of TRAIL-induced apoptosis in human lung cancer cells [72]. In addition, the transcription factor HIF-2 α was demonstrated to regulate DR5 in renal cancer cell lines [73], while treatment with glucocorticoids and IFN- γ can increase DR5 expression in cells with mutated p53, which is partially mediated by the transcription factor STAT1 [74].

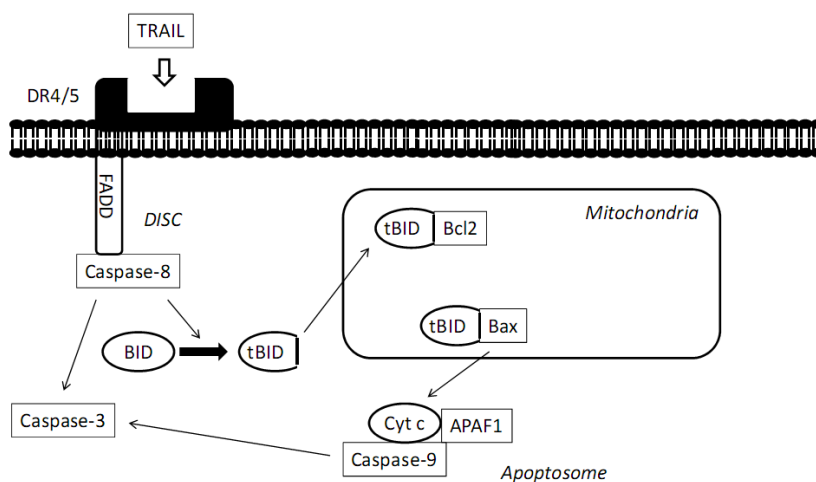


Figure 5. TRAIL triggers apoptosis inducing pathways via DR4/5 and the DISC complex. The binding of TRAIL to DR4 and DR5 induces activation of the caspase-8 and -3 pathways. Concomitantly, that binding converts BID into tBID, whose association with Bax, but not Bcl2, leads to the release of cytochrome c, which plays an essential role in caspase-3 activation and thus apoptosis induction. TRAIL, TNF-related apoptosis-inducing ligand; FADD, Fas-associated death domain; DISC, death inducing signal complex; APAF, apoptotic protease-activating factor; Cyt, cytochrome.

Meanwhile, a number of studies have indicated that most tumor cells and tissues resistant to TRAIL express higher levels of DR4 and DR5. One can argue that they are contradictory results because, as noted above, the TRAIL/DRs system executes apoptotic cell death. Increased TRAIL receptor expression in tumors is related to poor prognosis, indicating that

such tumors gain an advantage and progress more aggressively because they can escape TRAIL-mediated immune surveillance, for example, by inhibiting immune response against the tumor or resisting TRAIL-induced apoptosis due to the presence of intracellular inhibitory/anti-apoptotic proteins. Moreover, genetic mutations of those DRs increase their dysfunction, which may result in generation of TRAIL/DR-resistant tumor cells. In fact, Bin *et al.* described 5 types of somatic point mutations of DR5 identified in human tumors leading to its loss of function, which results in formation of death-inducing signaling complex (DISC) and induces apoptosis [75] (Figure 5). In support of those findings, homozygous genetic alterations within the DR4 gene may be implicated in the formation of osteosarcomas [76]. The major mechanism by which TRAIL induces apoptosis is associated with the formation of DISC and activation of caspase-8, both of which are associated with DRs. Therefore, the apoptotic process follows two signaling pathways, mitochondrial-independent activation of caspase-3 and mitochondrial-dependent apoptosis due to cleavage of BID by caspase-8, and the formation of apoptosomes, and activation of caspase-9 and downstream caspases [77].

3. Phytochemicals that Modulate Pro-Inflammatory Mediators

3.1. COX Suppressive Agents

Numerous dietary factors, micronutrients in particular, have been reported to suppress or inhibit COX-1 and -2. For example, resveratrol (3,4',5-trihydroxy-trans-stilbene, Figure 6), previously reported as a phytoalexin (an inducible phytochemical with anti-fungal activity) and found in grapes, is able to attenuate COX-2 transcription in several cell types [78-80]. Although its effect on mouse skin following topical application was ambiguous [81], oral administration attenuated trinitrobenzenesulphonic acid-induced COX-2 expression in rat colons, which was accompanied with colitis mitigation and oxidative stress reduction [82]. In addition, *N*-nitrosomethylbenzylamine-induced up-regulation of COX-2 in rat esophagus specimens was suppressed by resveratrol [83].

Curcumin is a major yellow pigment found in the rhizomes of turmeric (*Curcuma longa*) and is a curry spice used throughout the world. Along with its high potential for cancer prevention [84], the suppressive efficacy of this compound for COX-2 expression has attracted the attention of a great number of researchers. One of the first research groups to report the marked ability of curcumin to suppress COX-2 expression is Dannenberg and colleagues, who reported that it decreased chenodeoxycholate- and TPA-mediated induction of COX-2 in several gastrointestinal cell lines [85]. Their findings were later supported by other similar studies using colon cancer cells [86], leukocytes from healthy volunteers [87], Kupffer cells [88], non-small cell lung carcinoma cells [89], microglial cells [90], and macrophages [91]. Topical application of curcumin attenuated phorbol ester-induced COX-2 protein expression in mouse skin [92], while a *Curcuma* extract containing curcumin reduced blood PGE₂ concentration in 15 patients with advanced colorectal cancer, presumably by COX-2 repression [93].

Fish oil, which is rich in omega-3 fatty acids such as docosahexaenoic acid, is another reliable and safe food item that regulates COX-2 expression. Feeding rats with high-fat fish

oil, but not high-fat corn oil, resulted in a significant decrease in COX-2 expression in both colonic mucosa and tumors [94]. In cellular studies, fish oil and omega-3 fatty acids were shown to attenuate COX-2 expression in macrophages [95, 96] and smooth muscle cells [97]. On the other hand, a controversial report showed that LPS-induced COX-2 protein levels were increased without a change in COX-2 mRNA levels with omega-3 fatty acids pretreatment, suggesting that they may cause post-transcriptional stabilization of existing COX-2 mRNA [98]. Furthermore, Boudreau *et al.* reported a surprising finding that a fish oil-supplemented diet suppressed the growth of colon cancer cells in nude mice through a COX-2-independent pathway [99], implying that omega-3 fatty acids target other molecules to exhibit anti-cell proliferating activities.

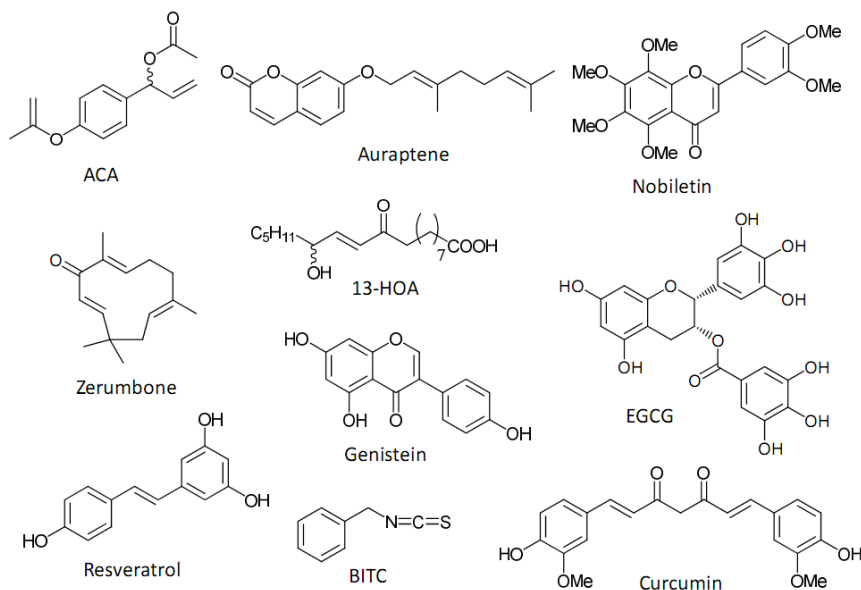


Figure 6. Chemical structures of phytochemicals referred to in this manuscript.

A green tea polyphenol, (-)-epigallocatechin-3-gallate (EGCG), has also emerged as a promising cancer preventive and anti-inflammatory agent. This polyphenol suppressed 2,2'-azobis (2-amidinopropane) dihydrochloride-induced expression of COX-2 in HaCaT human keratinocytes [100]. In addition, Kundu *et al.* presented interesting data showing that EGCG suppressed TPA-induced COX-2 expression in mouse skin as well as cultured human mammary epithelial cells [101], while it also attenuated COX-2 expression and tumor development in rat esophagus tissues [102]. Furthermore, IL-1 β -induced COX-2 upregulation in chondrocytes derived from osteoarthritis cartilage was reduced by EGCG [103]. In contrast, EGCG increased COX-2 expression via ERK1/2 and tyrosine phosphatase-related pathways in macrophages [104], and promoted the production of PGE₂ and TNF- α [105]. The above contrasting results may be associated with the experimental conditions used, including cell types and the presence or absence of stimuli.

Isothiocyanate (ITC)-related compounds occur widely in plants of the *Brassica* genus. Sulphoraphane, an aliphatic ITC, was reported to suppress LPS-induced COX-2 expression in macrophages [106], which appears to be important to better understand the anti-inflammatory actions of this compound. We also reported findings in support of those results using benzyl

ITC, which markedly blocked degradation of I κ B and thereby abrogated COX-2 expression [107]. However, a conflicting report showed that oral feeding of 6-phenylhexyl ITC promoted colon carcinogenesis with increased PGE₂ levels in colonic mucosa, suggesting enhancement of COX-2 expression by this ITC [107]. The soybean isoflavonoid genistein is another promising phytochemical for COX-2 suppression, whose ability was highlighted by its characteristic as a tyrosine kinase inhibitor [108]. Thereafter, a number of reports demonstrated COX-2 suppression by genistein in mesangial cells [109], islet cells [110], macrophages [111], pulmonary epithelial [112], colon cancer [113], and lung epithelial cells [114], though its *in vivo* activities remain to be demonstrated.

We have been searching for functionally novel phytochemicals that have potential to regulate the onset of carcinogenesis and other life style-related diseases. As a part of this search, we have screened hundreds of extracts from the plants of Southeast Asian countries for their suppressive effects on tumor promoter-induced EB virus activation in B-lymphoblastoid Raji cells over the past two decades and found that they are attractive sources of cancer preventive agents [115]. Activity-guided separation of active extracts identified 1'-acetoxychavicol acetate (ACA, from *Alpinia galanga*, Zingiberaceae), zerumbone (from *Zingiber zerumbet*, Zingiberaceae), and auraptene and nobiletin (from citrus fruits), which are readily available from natural sources and/or chemically synthesized. In addition, we recently isolated a new class of lipoxygenase metabolites in corn, (+/-)-13-hydroxy-10-oxo-*trans*-11-octadecenoic acid (13-HOA), which showed marked inhibition of LPS-induced mRNA expression of proinflammatory mediators in RAW264.7 macrophages [116]. Importantly, these five phytochemicals showed pronounced chemopreventive activities in multiple rodent models of chemical carcinogenesis. For example, ACA at a very low dose suppressed TPA-induced tumor promotion in mouse skin [117]. Also, 4-nitroquinoline 1-oxide-induced oral carcinogenesis was dramatically suppressed by this compound in other experiments [118]. Thereafter, ACA was shown to prevent azoxymethane-induced aberrant crypt foci formation [119] and colon carcinogenesis [120], as well as the formation of a putative preneoplastic marker of placental form-positive focal lesions, glutathione *S*-transferase (GST), in rat livers [121], and development of *N*-nitrosomethylbenzylamine-induced rat esophageal tumorigenesis [122] and *N*-nitrosobis(2-oxopropyl)-amine-induced initiation of cholangiocarcinogenesis in Syrian hamsters [123]. In parallel with those findings, auraptene has also exhibited a wide range of promising cancer preventive activities in chemical carcinogenesis experiments in mouse skin [124], rat colon [125-127], rat oral cavity [128], rat esophagus [129], and rat liver [130] specimens. Another citrus compound, nobiletin, also showed cancer preventive activity in rat colons [131, 132]. More recently, we found that oral feeding of nobiletin modulated serum leptin levels in mice with chemically [133] and genetically [134] induced colon tumors, together with significant suppression of tumor development. Also, pretreatment with 13-HOA abolished ear edema formation in an inflammation test, and reduced tumor incidence and the number of tumors per mouse in a two-stage skin carcinogenesis model [135], while zerumbone suppressed mouse skin carcinogenesis [136] and chemically induced colon carcinogenesis in both short- [137] and long-term [138] experiments, together with a noted reduction of COX-2 protein expression and PGE₂ formation in colonic mucosa [137]. In addition, our recent collaboration with Aggarwal and colleagues revealed that zerumbone decreased osteolysis in a dose-dependent manner in

MDA-MB-231 breast cancer tumor-bearing athymic nude mice, and was an effective blocker of RANKL-induced NF- κ B activation and osteoclastogenesis [139].

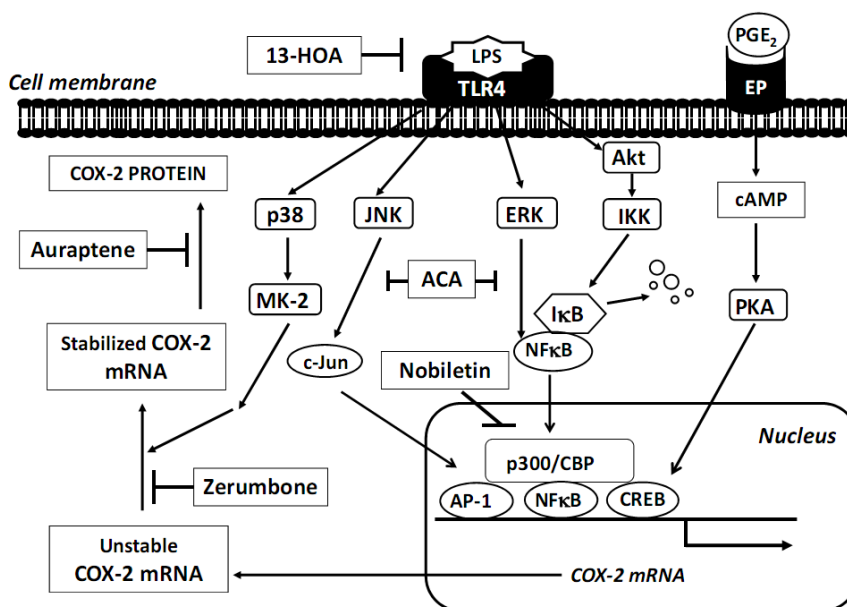


Figure 7. 13-HOA, ACA, nobiletin, zerumbone, and auraptene attenuate COX-2 protein expression via different mechanisms at various stages. Both ACA and 13-HOA suppressed JNK/ERK activation, whereas p38 MAPK and Akt activation were suppressed only by 13-HOA. Nobiletin did not have effects on the transcriptional activities of either AP-1 or NF- κ B, whereas it suppressed the expression of COX-2 mRNA. Zerumbone accelerated the degradation of COX-2 mRNA, which contains ARE at the 3'-UTR, while auraptene did not attenuate that mRNA expression, though it inhibited its protein synthesis, suggesting that it targets the translational step. ACA, 1'-acetoxychavicol acetate; 13-HOA, ($\pm$)-13-hydroxy-10-oxo-*trans*-11-octadecenoic acid; COX, cyclooxygenase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; NF- κ B, nuclear factor κ B; AP-1, activator protein-1; MAPK, mitogen-activated protein kinase; ARE, AU-rich element; UTR, untranslated region.

The modes of actions by which those phytochemicals exert chemopreventive activities have not been fully elucidated. However, it is notable that ACA, auraptene, and zerumbone are potent chemo-protectors that can induce the expression of self-defensive proteins, such as xenobiotic metabolizing enzymes (e.g., GST) and anti-oxidative enzymes (e.g., superoxide dismutase) [119, 125, 136]. On the other hand, 13-HOA has been characterized as a unique inducer of programmed cell death 4 (Pcd4), a novel tumor suppressor protein [135]. More strikingly, these 5 phytochemicals were found to possess anti-inflammatory activities derived from suppression of inflammatory cell activation. Previously, we uncovered strong COX-2 suppressive activities in RAW264.7 macrophages by these phytochemicals and addressed the underlying molecular mechanisms [115, 139] (Figure 7). It is interesting that LPS-induced mRNA expression of COX-2 was suppressed by 13-HOA, ACA, nobiletin, and zerumbone, but not by auraptene. As for the ability to suppress COX-2 protein expression, auraptene targeted the translational stage of COX-2 protein production, which was supported by our recent data showing that auraptene targets the translational step of pro-MMP-7 synthesis by inactivating ERK1/2 in HT-29 human adenocarcinoma cells [141]. Among these phytochemicals, the molecular target(s) of 13-HOA may be localized most upstream of the

LPS signaling pathways, since it abolished activation of MAPKs and Akt, all of which were up-regulated by LPS and shown to be related to COX-2 expression [116]. On the other hand, activation of p38 MAPK was not attenuated by zerumbone, ACA, or nobiletin. However, ACA substantially blocked both JNK1/2 and ERK1/2 activation, whereas both zerumbone and nobiletin allowed LPS-induced activation of those protein kinases. Although the phosphorylation of Akt was enhanced by LPS in a time-dependent manner, zerumbone, ACA, and nobiletin demonstrated no inhibition. In addition, ACA did not allow LPS-induced I κ B degradation, whereas both zerumbone and nobiletin were virtually inactive. LPS treatment also markedly elevated the activities of the transcription factors NF κ B and AP-1, while both ACA and nobiletin significantly suppressed those transcription factors, though zerumbone had no effect. The above results led us to propose a hypothesis that nobiletin allows nuclear translocation of NF κ B and AP-1, and disturbs transcription (e.g., by inhibiting DNA binding or coactivators such as CREB-binding protein/p300, etc.), while zerumbone disrupts the COX-2 mRNA stabilization step. In support of that latter portion of our hypothesis, zerumbone significantly promoted mRNA decay in LPS-exposed and actinomycin D-treated RAW264.7 cells [116]. As noted above, zerumbone does not suppress p38 MAPK. However, it slightly attenuates the activation of MK-2, a predominant substrate for p38 MAPK, and findings from an *in vitro* kinase assay showed that zerumbone does not have effects on the kinase activity of p38 MAPK [115]. Collectively, this compound may bind to MK-2 for declining downstream signaling pathways. Zerumbone has an α,β -unsaturated carbonyl group and thus demonstrates a highly electrophilic property, which is susceptible to nucleophilic addition by the cysteine residues of proteins via Michael reactions. Our recent data from a pull-down assay with RAW264.7 cell lysates and zerumbone-bound sepharose gel revealed the *in vitro* covalent binding of zerumbone to functional proteins, including HuR, which plays major roles in stabilization of COX-2 mRNA [142]. In addition, that binding specificity was shown in results of a competition assay using α -humulene, a zerumbone analog with a lower level of bioactivity [142].

Taken together, 13-HOA may act most upstream in suppressing the COX-2 and ACA targets of JNK1/2 and ERK1/2, while nobiletin may interfere with coactivators that suppress the transactivation of NF- κ B and AP-1, and/or inhibit DNA binding. Zerumbone allows LPS-induced MAPK/Akt activation and the transcription step, while it abrogates COX-2 mRNA induction. That activity may be associated with its binding to MK-2, HuR, or other functional proteins that are components of the post-transcription machinery, whereas the target molecule of auraptene may be associated with translation. In any case, COX-2 regulation of immune cells with phytotherapeutics may be a promising avenue for the prevention of numerous inflammatory disorders, including cancer [143].

3.2. TNF- α Suppressive Agents

As noted above, TACE regulation is necessary for maintaining proper local and systemic concentrations of bioactive TNF- α . Since tissue inhibitor of matrix metalloproteinase (TIMP)-3 is an endogenous inhibitor of TACE [144], TACE inhibitors and/or TIMP-3 activators are thought to be potential anti-inflammatory agents with reasonable modes of actions. In fact, TACE inhibitors have a wide range of potentials for treating rheumatoid

arthritis and various types of cancer [145]. Levin *et al.* documented the structure-activity relationships among acyclic sulfonamide hydroxamates in regard to development of acetylenic TACE inhibitors [146]. In an early study by Chang, curcumin suppressed LPS-induced production of TNF and IL-1 by Mono Mac 6 human monocytic macrophage cells by targeting NF κ B [147]. Also, supplementation with curcumin (100 mg/kg BW by gavage daily for 7 weeks) led to significant reductions of high glucose-elevated lipid peroxidation and TNF- α secretion in cultured monocytes from diabetic rats [148]. It is also worth noting that curcumin markedly inhibited NF- κ B and AP-1 activation, and thereby attenuated the induction of mRNAs for the cytokines IL-6 and TNF- α in pancreases of experimental pancreatitis model rats [149]. Moreover, it inhibited endotoxin-induced increases of TNF- α expression and markedly up-regulated peroxisome proliferator activating receptor (PPAR)- γ , which is known to act as a potent negative regulator of a variety of proinflammatory genes including TNF- α [150]. Furthermore, Wang *et al.* found that curcumin suppressed the transcription and secretion of TNF- α and IL-6 induced by palmitate in 3T3-L1 adipocytes through the NF- κ B and JNK pathways, suggesting its efficacy for regulating palmitate-induced insulin resistance state [151].

As noted above, targeting the production of certain, if not all, pro-inflammatory proteins including TNF- α may not be beneficial for health promotion or disease regulation. On the other hand, some phytochemicals occasionally exhibit contrasting profiles and modulate the production of those proteins, though the results presented have been dependent on the experimental conditions such as cell lines utilized and the presence or absence of stimuli. This was shown in part by our previous finding that zerumbone concentration-dependently suppressed the LPS-induced release of TNF- α from RAW264.7 murine macrophages [152], whereas it markedly induced the mRNA expressions of IL-1 α , IL-1 β , IL-6, and TNF- α in several different human colon adenocarcinoma cell lines, probably via upregulation of ERK1/2 and JNK1/2, but not p38 MAPK [153]. These conflicting results highlight the importance of the methods used to apply phytochemicals to anti-inflammatory strategies based on molecular targeting.

3.3. RANKL Suppressive Agents

RAW264.7 macrophages are frequently utilized for studies that focus on LPS-induced production of pro-inflammatory mediators. In addition, they are useful as a model of osteoclastogenesis when exposed to RANKL. In an intriguing study, Zou and Bar-Shavit investigated how LPS affects RANKL expression and its signaling when RAW264.7 macrophages were treated with LPS. Interestingly, LPS was found to inhibit RANKL activity by reducing the expressions of RANK and the M-CSF receptor, and stimulated osteoclastogenesis in RANKL-pretreated cells via TNF- α [154]. These observations showed the presence of both positive and negative interactions among endogenous and exogenous pro-inflammatory stimuli.

Meanwhile, the research group of Aggarwal *et al.* published interesting results showing that curcumin inhibits the RANKL-activated pathway, which involves activation of IKK and I κ B α degradation, and thereby inhibited osteoclastogenesis of RAW264.7 mouse macrophage [155]. In addition, exposure of IL-1 α -stimulated bone marrow stromal cells to

curcumin led to dose-dependent suppression of osteoclastogenesis and reduced expression of RANKL in co-cultures with bone marrow cells [156]. Taken together, curcumin can be designated as a dual inhibitor that suppresses RANKL expression and its signaling. Furthermore, a recent study by Hie *et al.* showed that curcumin suppresses increased bone resorption activity through attenuation of osteoclastogenesis, which may be associated with inhibition of the expression of c-fos and c-jun in diabetic rats [157].

We recently documented that phenethyl ITC (PEITC), a phytochemical present in various cruciferous plants, exhibited pronounced suppression of RANKL-induced osteoclastogenesis in RAW264.7 macrophages, which may have been associated with downregulation of both the ERK1/2 and p38 MAPK pathways, which attenuates the level of NF κ B-dependent mRNA expression of NFATc1, a master regulator of osteoclastogenesis [158]. Similar results have been reported by other groups that focused on caffeic acid phenethyl ester [159]. Following our *in vitro* study, we examined the *in vivo* efficacy of PEITC, zerumbone, auraptene, ACA, and nobiletin for suppressing osteoporosis in ovariectomized female ddY mice, among which the most active phytochemical was determined to be nobiletin, which markedly decreased type II collagen-induced arthritis following *i.p.* administration of 20 mg/kg BW in male DBA/1J mice every 2 days for 12 days [160]. In addition, nobiletin attenuated RANKL-induced osteoclastogenesis of RAW264.7 cells by targeting ERK1/2, JNK1/2, and p38 MAPK, and thereby regulated activation of the promoters of NF κ B and AP-1 [160].

Recently Hwang *et al.* demonstrated that momordin I, an oleanolic acid glycoside from *Ampelopsis radix*, inhibited the activation of NF κ B and AP-1 in RANKL-induced RAW264.7 cells, presumably by targeting I κ B degradation and c-Fos expression, but not MAPK signaling pathways, for suppressing osteoclast formation [161]. Garc'a *et al.* also studied the effects of genistein and estradiol on osteoclastic differentiation of RAW264.7 macrophages, and suggested that these estrogens protect against RANKL-induced bone loss by reducing osteoclast production, in addition to their effects on osteoblasts [162]. Also, the mRNA expression of OPG was increased in osteoblastic MC3T3-E1 cells treated with genistein [163]. In a human study conducted by Crisafulli *et al.*, supplementation with genistein (54 mg/day) for 1 year significantly decreased the sRANKL/OPG ratio in postmenopausal healthy women, implicating the beneficial effects of genistein on bone remodeling in postmenopausal women [164].

3.4. TRAIL-Potentiating Agents

TRAIL treatment in combination with chemo- or radiotherapy enhances TRAIL sensitivity or reverses TRAIL resistance by regulating the downstream effectors. Efforts to identify the agents that activate DRs and block specific effectors may improve therapeutic efficiency. A marked cancer chemotherapy potential of the combination of TRAIL with proteasome inhibitors was demonstrated by Sayers and Murphy [165]. In addition, Ishibashi and Ohtsuki recently investigated natural products targeting the TRAIL signaling pathway and identified the dimeric sesquiterpenoid from *Curcuma parviflora* (Zingiberaceae) as a potent inducer of the TRAIL receptor and isolated new isoflavones, termed brandisianins, from *Milletia brandisiana* (Leguminosae). In addition, a dihydroflavanol from *Blumea balsamifera* (Compositae) and fuligocandin B, a new anthranilylproline-indole alkaloid

isolated from myxomycete, were found to have a reversal effect on TRAIL resistance activity [166]. Sakai and colleagues have also published many excellent studies on the combination effects of TRAIL with natural compounds for potentiating their apoptosis inducing ability in cancer cells. For example, luteolin, a flavone present in numerous plants, synergistically functioned with TRAIL to induce apoptosis in HeLa cells, but not in normal human peripheral blood mononuclear cells [167], with similar results reported for structurally related flavonoids [168-171]. In addition, sulforaphane, a naturally occurring ITC, was demonstrated to enhance TRAIL-induced apoptosis in human osteosarcoma cells by promotion of Bid cleavage and activation of caspases-8, -10, -9 and -3, as well as upregulation of DR5 [172]. Combined treatment with halocynthiaxanthin, a carotenoid found in oysters and sea squirts, and TRAIL dramatically induced apoptosis in DLD-1 colon cancer cells, whereas individual treatments were marginally active [173]. In addition to halocynthiaxanthin, peridinin, but not alloxanthin, diadinoxanthin, or pyrrhoxanthin, induced DR5 expression and sensitized DLD-1 cells to TRAIL-induced apoptosis, suggesting that there are structural requirements for natural carotenoids to exhibit combinatorial effects with TRAIL [173]. This research group also showed that lactobacilli promote cell surface TRAIL expression, thereby facilitating NK activity for killing prostate cancer cell lines [174].

Resveratrol has drawn the attention of numerous investigators for its marked ability to regulate the onset of life-style related diseases such as cancer as well as lifespan (for review, see [175]). Treatment of prostate cancer cells with resveratrol resulted in generation of ROS, translocation of Bax to mitochondria and a subsequent drop in mitochondrial membrane potential, release of mitochondrial proteins (cytochrome c, Smac/DIABLO, and AIF) to cytosol, activation of the effectors caspase-3 and -9, and induction of apoptosis in prostate cancer cells, while the combination of resveratrol and TRAIL enhanced mitochondrial dysfunctions during apoptosis [176]. Although SIRT-1 has been implicated as the major target for exerting its anti-aging function, the recent elegant study by Mader *et al.* demonstrated that resveratrol enhances TRAIL-induced apoptosis of human pre-adipocytes in a highly synergistic manner that is independent of SIRT-1 [177]. On the other hand, Jung *et al.* presented interesting findings showing that curcumin enhanced TRAIL-induced apoptosis by CHOP-independent upregulation of DR5 in several cancer cell lines, including Caki human renal cancer cells [178].

Targeting the TRAIL/DRS system as a cancer therapeutic strategy is currently recognized as reasonable and promising, though it may occasionally lead to apoptosis resistance. For example, resistance to TRAIL-induced apoptosis by some cell types has emerged when TRAIL-signaling molecules (e.g., FADD and caspase-8) are down-regulated and/or TRAIL-suppressing molecules (e.g., Bcl-2) are up-regulated [179]. Thus, it is important to give attention to the phenotypes of target malignant cells to optimize the efficacy of TRAIL-potentiating agents.

Conclusion

Synthetic anti-inflammatory drugs are designed on the basis of their interactions with known molecular targets that are involved in inflammatory processes. Studies of structure-activity relationships presented thus far have provided great insight into how their efficacy

can be optimized and toxicity minimized. On the other hand, there is compelling evidence that a large portion of phytochemicals are effective and useful for mitigating inflammatory events. This notion is supported by the fact that the plants from which they are isolated have long been used as folk medicines for analgesic and anti-inflammatory purposes in local areas, and display no detectable toxicity. However, knowledge of the molecular mechanisms underlying their anti-inflammatory effects remains limited, thus additional studies to accumulate and expand important findings are needed. In addition, precise understanding of the molecular mechanisms involved in the multistep production of pro-inflammatory proteins is essential. Although the binding proteins and molecular targets of phytochemicals were largely unknown until recently, new studies have begun to identify them by using modern technologies [180, 181]. Progress in such advanced research will provide a solid rationale for using phytochemicals as powerful and reliable therapeutic agents.

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Chapter V

Dietary Polyphenols Exert Neuroprotective Effects by Attenuating Neuronal and Astrocytic Damage in Cerebral Ischemia

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Abstract

Polyphenols are natural substances with variable phenolic structures and are rich in vegetables, fruits, grains, bark, roots, tea, and wine. There are over 8000 polyphenolic structures identified in plants, but edible plants contain only several hundred polyphenolic structures. Recent interest in polyphenols has increased greatly due to their potential health benefits. In addition to their anti-oxidant effects, some polyphenols also have insulin-potentiating, anti-inflammatory, anti-carcinogenic, anti-viral, anti-ulcer, and anti-apoptotic properties. Beneficial effects of polyphenols in attenuating neuronal and astrocytic damage in ischemia/stroke have also been demonstrated. Stroke is a neurological injury in which the blood supply to a part of the brain is interrupted. It involves the sudden loss of neuronal function due to a decline in cerebral perfusion. The part of the brain with decreased blood flow no longer receives adequate oxygen. Ischemic stroke can lead to vascular leakage, inflammation, tissue injury, and necrosis. Given that oxidative stress and inflammation are hypothesized to contribute to increased neural damage in ischemia, polyphenols appear to have tremendous potential in attenuating such injuries. Neuronal death and brain edema are important consequences of ischemia and oxidative stress and inflammation have been implicated in their pathogenesis. The purpose of this article is to review the current literature on the potential of various dietary

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polyphenols in attenuating ischemic injury with the goal to identify newer approaches to attenuate neural damage. A review of currently known mechanisms underlying cell death and/or damage will be undertaken and the potential of dietary polyphenols to reduce such damage will be critically reviewed.

Abbreviations

AIF	apoptosis inducing factor;
AP1	5,7,3',4',5'-pentahydroxy dihydroflavanol-3-O-(2"-O-galloyl)-beta-d-glucopyranoside;
AQP4	Aquaporin 4;
ATP	adenosine triphosphate;
BBB	Blood-brain barrier;
cGMP	cyclic guanosine monophosphate;
CPE	Cinnamon polyphenol extract;
CsA	Cyclosporin A;
CSF	Cerebrospinal fluid;
EGCG	epigallocatechin-3-gallate;
ESAM	Endothelial cell-selective adhesion molecule;
GTE	green tea extract;
IAP	Inhibitor of apoptosis;
ICAM-1	Inter-cellular adhesion molecule 1;
IL-1 β	Interleukin-1 β ;
JAM	Junctional adhesion molecule;
L-NAME	N (G)-nitro-L- arginine methyl ester;
LPS	Lipopolysaccharide;
MCAO	middle cerebral artery occlusion;
MMP	Matrix metalloproteinase;
mPT	mitochondrial permeability transition;
NCX	Na ⁺ /Ca ²⁺ exchanger;
NHE	Na ⁺ /H ⁺ exchanger;
NKCC	Na ⁺ /K ⁺ /2Cl ⁻ co-transporter;
NO	Nitric oxide;
OGD	Oxygen-glucose deprivation;
RVI	Regulatory volume increase;
SIN-1	3-morpholinylsydnoneimine chloride;
SNAP	S-nitroso-N-acetyl penicillamine;
TBI	Traumatic brain injury;
TJP	Tight junction protein;
TNF- α	Tumor necrosis factor α ;
ZO-1	Zonula occludens-1;

1. Polyphenols

Polyphenols are natural substances with variable phenolic structures and are rich in vegetables, fruits, grains, bark, roots, tea, and wine (Bravo et al., 1998; Manach et al., 2004). There are over 8000 polyphenolic structures identified in plants but edible plants contain only several hundred polyphenolic structures (Ross et al., 2002). All polyphenols contain one or more aromatic rings with more than one hydroxyl groups. Most also have at least one sugar residue (glycoside) attached to the hydroxyl groups. They are classified into different groups depending on the number of phenol rings and chemical groups bound to the rings (Bravo et al., 1998). Flavonoids make up most of the polyphenols and they form the largest group of polyphenols. Categories of flavonoids include flavones (e.g. apigenin, luteolin,), flavonones (e.g. hesperetin,), catechins (e.g. epicatechin, epigallocatechin-3-gallate (EGCG)) and anthocyanins (e.g. cyanidin) (Hendrich, 2006). Another polyphenol that has received much attention is resveratrol, a stilbene polyphenol, present in grapes and red wine with demonstrated anti-oxidant properties

Diets rich in fruits and vegetables contain an abundance of various classes of polyphenols. Herbs and spices, as well as wine, are also important sources of polyphenols. For instance, flavonoids including anthocyanidins are rich in blackberries, blueberries, cherries, plums, and red wine. Flavonols including myricetin, quercetin and fisetin are found in celery, onions, spinach, apples, apricots, cranberries, dill weed, red wine, and green tea. Flavones, including apigenin and luteolin, are found in olives, fresh parsley, oregano, thyme, and rosemary. For a more detailed source of dietary polyphenols see Han et al. (2007).

2. Polyphenols in Cerebral Ischemic Injury

2.A. Ischemia and Apoptotic Signaling Pathways in the Brain

Stroke is a neurological injury in which the blood supply to a part of the brain is interrupted. It involves the sudden loss of neuronal function due to a decline in cerebral perfusion. The part of the brain with decreased blood flow no longer receives adequate oxygen. Ischemic stroke can lead to vascular leakage, inflammation, tissue injury, and necrosis. Associated changes include impairment of metabolism, energy failure, free radical production, excitotoxicity, altered calcium homeostasis, and activation of proteases (see Panickar and Norenberg, 2005 for review).

Cell death following cerebral ischemia is mediated by both necrosis and apoptosis (Snider et al., 1999). Necrosis is characterized by cell swelling and eventual loss of cell membrane integrity followed by cell lysis. Resultant inflammation is a significant part of necrotic death. In contrast, apoptosis (also known as programmed cell death) is an energy-dependent process and is characterized by a shrinking of the cytoplasm, condensation of the nucleus and eventual fragmentation of the cell body into smaller bodies. Activation of several intracellular enzymes during the process of apoptosis as well as non-random DNA fragmentation is also part of the apoptotic process. Generally, no inflammatory response is observed with apoptotic cell death.

Diverse groups of proteins are involved in the apoptosis pathway following ischemia (see Broughton et al., 2009 for review) and, as discussed below, several polyphenols appear to attenuate the levels of apoptotic proteins. One set of proteins that have been implicated in an overwhelming number of studies are the caspases. Caspases are a family of cysteine proteases which play an important role in apoptosis, necrosis, as well as in inflammation. Sequential activation of caspases plays a critical role in the execution of the apoptotic process. Caspase-3 exists as inactive proenzyme that undergoes proteolytic processing at conserved aspartic residues to produce active subunits. This protein also cleaves and activates other caspases including caspase 6, 7, and 9 and may also itself be a target for other caspases including caspase 8, 9, and 10. Another pro-apoptotic protein that is activated in ischemic injury is cytochrome C which is released from the mitochondria upon apoptotic stimuli. Release of cytochrome C can in turn activate caspase 9 which can then activate caspase 3. Another family of proteins that have been widely studied in ischemia is the Bcl-2 family of proteins. This family of proteins consists of both pro-apoptotic (Bad, Bax, Bim) and anti-apoptotic (Bcl-2, Bcl-xl, Bcl-w) members and it is hypothesized that they exert their effects by interacting with or controlling the inner mitochondrial membrane permeability transition pore opening. Overexpression of Bcl-xl protects rat septal neurons from ischemic injury in cultures (Panickar et al., 2005). Apoptosis-inducing factor (AIF) is another mitochondrial-associated protein that is normally located in the intermembrane space of mitochondria and upon a pro-apoptotic signal AIF is released from the mitochondria. AIF subsequently migrates to the nucleus and triggers DNA damage. In general mitochondrial dysfunction appears to mediate some of the critical pathways that are pro-apoptotic and preventing such dysfunction may mediate the anti-apoptotic effects. Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that belong to a larger family of proteases known as the metzincin superfamily. While MMPs are capable of degrading extracellular proteins, they are also known to be involved in the cleavage of cell surface receptors and the release of apoptotic ligands including the FAS ligand as well as chemokine/cytokine activation (Van Lint and Libert, 2007). Increased oxidative stress is a key feature of ischemic injury and some activators of the apoptotic signaling pathway are triggered by oxidative damage and hence anti-oxidants have often been characterized as having an anti-apoptotic effect.

2.B. Protective Role of Polyphenols on Apoptotic Signaling Pathways in Ischemia

Considerable interest has focused on the protective effects of polyphenols because of their antioxidant properties (Urquiaga and Leighton, 2000; Dajas et al., 2003). Phenolic antioxidants have been shown to inhibit the oxidation of lipids and other molecules and protect against free radicals (Nijveldt et al., 2001). Oxidative stress is a key event in the pathogenesis of cerebral ischemia. Overproduction of reactive oxygen species during ischemia and/or ischemia/reperfusion can damage lipids, proteins, and nucleic acids, thereby inducing apoptosis or necrosis.

Increasing evidence supports the hypothesis that plant polyphenols provide protection against neurodegenerative changes associated with cerebral ischemia (see Simonyi et al., 2005 for review). Inanami et al. (1998) observed a dose-dependent protection against

hippocampal neuronal death in ischemia in gerbils after *ad libitum* oral administration of catechin in the drinking water for 2 wk. Epigallocatechin-3-gallate (EGCG) injection immediately after ischemia in a rat model of transient focal cerebral ischemia was also shown to reduce neuronal damage in the hippocampal region (Sutherland et al., 2006). EGCG (50 mg/kg intraperitoneally) was effective even when it was administered 3 hr after the ischemic insult in gerbils (Lee et al., 2004). Hong et al. (2001) used green tea extract in the drinking water *ad libitum* for 3 wk before ischemia in gerbils. This treatment reduced the infarct volume, the number of apoptotic cells, and lipid peroxidation and inhibited the ischemia-induced hyperactivity. In another focal ischemia model using middle cerebral artery occlusion (MCAO) in rats, the protective effects of resveratrol were shown with pretreatment for 21 days (20 mg/kg intraperitoneally per day). The treatment reduced the infarct volume, prevented motor impairment, and inhibited lipid peroxidation (Sinha et al., 2002). A single dose of resveratrol (20 mg/kg) given orally 1 hr before permanent middle cerebral artery ligation in mice did not protect against ischemic damage. However, when given daily for 3 days before ischemia, resveratrol significantly reduced the infarct size. In another study, effects of resveratrol on transient global cerebral ischemic injury were examined in gerbils (Wang et al., 2002). Resveratrol (30 mg/kg intraperitoneally) was injected either during or shortly after common carotid artery ligation and 24 hr later. Resveratrol significantly decreased neuronal death in the hippocampus and also inhibited glial cell activation. Nanocapsule encapsulated quercetin treatment resulted in significant protection to endogenous antioxidant enzymes against ischemia induced oxidative damage in neuronal cells of young and old rats (Das et al., 2008). These studies indicate strong neuroprotective effects of polyphenols in ischemic injury possibly due to their antioxidant properties.

There is also evidence to indicate that some of the neuroprotective effects of polyphenols are mediated by a decline in apoptotic markers. Pomegranate polyphenols and resveratrol protect neonatal mouse brain from ischemic injury by reducing caspase-3 and calpain activation (West et al., 2007). In neonatal rats, amentoflavone blocked the activation of caspase 3 and the proteolytic cleavage of its substrates following hypoxic-ischemic injury (Shin et al., 2006). Pomegranate juice also diminished caspase-3 activation in the hippocampus and cortex of the neonatal brain against a hypoxic-ischemic insult through supplementation of the maternal diet with pomegranate juice (Loren et al., 2005). Mangiferin and morin, two antioxidant polyphenols, are neuroprotective in both *in vitro* and *in vivo* models of ischemia possibly by reducing Ca^{2+} influx as well as decreasing caspase-3 (Gottlieb et al., 2006). EGCG, a green tea polyphenol, reduced up-regulation of MMP-9 activity and neuronal damage following transient focal cerebral ischemia in C57BL/6 mice (Park et al., 2009). MMP-9 downregulation by resveratrol was also observed in an *in vitro* model of neuronal ischemic injury (Cheng et al., 2009). 5,7,3',4',5'-pentahydroxy dihydroflavanol-3-O-(2"-O-galloyl)-beta-d-glucopyranoside (AP1), a polyphenolic compound, isolated from *Anogeissus pendula* Edgew, was effective in reducing apoptotic cells in rat brain following transient focal cerebral ischemia (ArunaDevi et al., 2010). 2,3,5,4'-tetrahydroxystilbene-2-O-beta-D-glucoside (TSG), an active component of the rhizome extract from *Polygonum multiflorum*, protected rat brain from MCAO by increasing the anti-apoptotic Bcl-2 proteins (Wang et al., 2009). Curcumin, a potent polyphenol antioxidant enriched in turmeric, reduced cytochrome c release and subsequent caspase-3 activation following global cerebral ischemia in Mongolian gerbils (Wang et al., 2005). While the aforementioned studies have demonstrated a decrease in caspase-3 levels in the presence of

polyphenols, it is unclear whether polyphenols act directly on caspase-3 or whether they act on upstream caspases that are precursors to caspase-3. Alternatively, such polyphenols could also be activating inhibitor of apoptosis (IAP) which would then inhibit caspase-3 activation. In addition, effects of polyphenols may also involve protecting mitochondrial dysfunction in ischemic injury as seen in cell culture studies (Panickar et al., 2009a, b). Preventing the decline in mitochondrial membrane potential following ischemic injury may subsequently confer protection against apoptotic cell death. In addition, resveratrol can induce neuroprotection by increasing mitochondrial ATP synthesis efficiency in rat brain following ischemia (Della-Morte et al., 2009). In summary, polyphenols appear to have either direct and indirect effects in reducing apoptotic indices and thus exert neuroprotective effects in ischemic injury (see Figure 1).

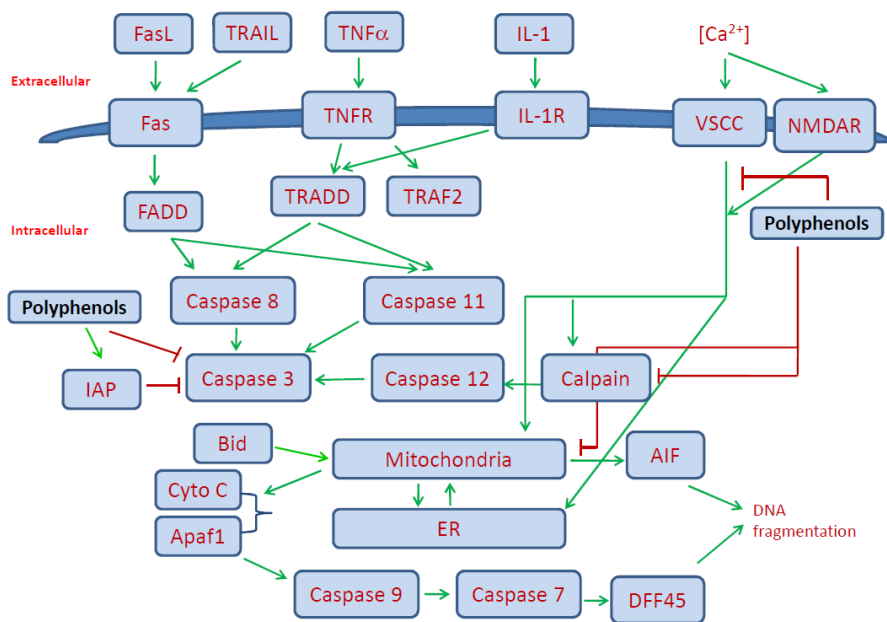


Figure 1. Schematic diagram of the apoptotic signaling pathway in neurons and the potential neuroprotective role of polyphenols. Polyphenols reduce caspase-3 protein levels following ischemic injury. This effect of polyphenols may be a direct action on caspase-3 or it may be indirect. For instance, a reduction in caspase-3 could be due to the action of polyphenols on upstream caspases or due to its action on calcium-activated calpain, another protease, or by acting to increase IAP, which would serve to inhibit caspase-3. Other actions of polyphenols include their effects on attenuating mitochondrial dysfunction including preventing the release of cytochrome C or attenuating mitochondrial calcium overload. Preserving mitochondrial function, or even attenuating mitochondrial dysfunction during or post-ischemia can diminish the severity of injury by reducing activation of downstream apoptotic events that result in DNA fragmentation, a key feature of cell death in neurons (see text for details). (Abbreviations: Apoptosis-inducing factor (AIF); apoptotic protease activating factor-1 (Apaf-1); Bcl-2 interacting protein (Bid); Cytochrome C (Cyto C); DNA fragmentation factor 45 (DFF45); Endoplasmic reticulum (ER); Fas-associated protein with death domain (FADD); Fas ligand (FasL); Inhibitors of apoptosis (IAP); Interleukin-1 (IL-1); Interleukin-1 receptor (IL-1R); NMDA receptor (NMDAR); Tumor necrosis factor α (TNFα); Tumor necrosis factor receptor (TNFR) Tumor necrosis factor receptor type 1-associated death domain (TRADD); TNF receptor-associated factor 2 (TRAF2); TNF-related apoptosis-inducing ligand (TRAIL); Voltage-sensitive calcium channel (VSCC)).

3. Brain Edema and Cell Swelling in Ischemic Injury

Brain edema is a key feature of ischemic injury (Klatzo, 1967). The early development of cerebral edema is an important clinical consequence following ischemia. Brain edema is characterized by an abnormal accumulation of fluid in the brain parenchyma resulting in a volumetric enlargement of cells or tissue. Edema can lead to increased intracranial pressure, brain herniation, irreversible brain damage, and ultimately, death. Brain edema is generally classified into cytotoxic or vasogenic edema (Klatzo, 1967; Katzman et al., 1977). Cytotoxic edema is defined as a cellular swelling with fluid accumulating within the cell. Astrocyte (glial) swelling is a major component of cytotoxic edema (Kimelberg, 1995). Swollen astrocytes following ischemic injury have been reported in cats (Garcia et al., 1977) and rats (Petito et al., 1982). Vasogenic edema is characterized by a breakdown of the blood-brain barrier (BBB) resulting in increased fluid accumulation; originating from blood vessels that amass around cells. Entry of serum proteins into brain parenchyma, normally limited by the tight endothelial cells of the BBB, constitutes an important feature of vasogenic edema. Cytotoxic edema is a relatively early event when compared to vasogenic edema (Ito et al., 1976; Klatzo, 1987) and secondary damage to cells surviving the primary ischemic insult may be reduced by suppressing brain edema (Kuriowa et al., 1994). In addition, the ability of swollen and dysfunctional astrocytes to effectively clear glutamate and K⁺ from the extracellular regions may be hampered and may further contribute to excitotoxicity (Tan et al., 1996; Panickar and Norenberg, 2005 for review). A reduction in edema may also improve neurological outcome (Albers, 1998) and cerebral microcirculation (Siesjo, 1984; Vexler et al., 1997). Interventions that reduce edema and associated secondary damage may be of considerable importance in diminishing the severity of ischemic injury (Mellergard et al., 1989). Both vasogenic and cytotoxic mechanisms contribute to overall ischemic brain edema (Klatzo et al., 1967; Ito et al., 1979) and consequently impair cerebral perfusion and oxygenation, and contribute to additional ischemic injuries. Another form of edema that has been described is the interstitial edema and such edema has been observed in animal models of ischemia (DeLaPaz et al., 1991; Dias et al., 2000; Melgar et al., 2005). Interstitial edema is due to rupture of the barrier of cerebrospinal fluid (CSF) and brain which results in transpendymal flow of CSF thereby allowing CSF to penetrate the brain and occupy the extracellular space of white matter. Interstitial edema differs from vasogenic edema in that unlike vasogenic edema such fluid does not contain any significant amount of protein. In general, a major consequence of brain edema is the development of increased intracranial pressure leading to brain herniation, brainstem compression and, ultimately, death. Currently there is a paucity of agents to attenuate brain edema effectively and surgical intervention is one of the choices (Brott and Bogousslavsky, 2000).

Dysfunction of astrocytes due to swelling is a major component of cytotoxic edema and can be deleterious. Astrocytes are known to carry out critical functions such as maintenance of ionic homeostasis, prevention of excitotoxicity, scavenging free radicals, provision of nutrients and growth factors, promotion of neovascularization, and support of synaptogenesis and neurogenesis that potentially may influence the outcome of ischemic injury. While astrocyte swelling in and of itself can be deleterious, loss of key astrocytic functions may contribute to neuronal injury/death (Panickar and Norenberg, 2005 for review). In addition to

astrocytes, neurons (Garcia et al., 1995; Zhang et al., 2000) as well as oligodendrocytes (Dewar et al., 2003) also swell in the brain in the acute phase of ischemia but such swelling may be followed by cell shrinkage and/or cell death. The relative contribution of each of these cell types to brain edema is not clear since neurons and oligodendrocytes are more susceptible to cell death than astrocytes. Given that astrocytes outnumber neurons, swelling of astrocytes likely plays a predominant role in cytotoxic brain edema. Reports on the beneficial effects of polyphenols on brain edema in ischemia are scarce. Lee et al. (2004) reported a protective effect of green tea polyphenol EGCG against neuronal damage and brain edema after unilateral cerebral ischemia in gerbils. AP1, a polyphenolic compound, also reduced brain edema in rats after transient focal ischemia (ArunaDevi et al., 2010). We recently reported protective effects of polyphenols from green tea (Panickar et al., 2009a) as well as cinnamon (Panickar et al., 2009b) on glial swelling following ischemia-like injury (Panickar and Anderson, 2009 for review). Myricetin and quercetin also attenuate cell swelling following oxygen-glucose deprivation in C6 cultures (Panickar et al., 2009 d).

Disruption of the BBB is a key feature of vasogenic edema and reperfusion may further increase damage to the BBB (Kuroiwa et al., 1989; Nakagawa et al., 1990). At the interface between blood and brain, endothelial cells and associated foot processes of astrocytes form "tight junctions." The tight junction is composed of smaller subunits that are transmembrane proteins including occludin, claudin, junctional adhesion molecule (JAM), and endothelial cell-selective adhesion molecule (ESAM). Each of these transmembrane proteins is anchored into the endothelial cells by the scaffolding protein complex that includes zonula occludens-1 (ZO-1), a membrane associated guanylate kinase homologue protein, and related proteins ZO-2 and ZO-3. Around the endothelial cells is a basal lamina composed of extracellular matrix proteins. Taken together the whole unit is also referred to as the "neurovascular unit" (del Zoppo, 2009). As discussed below, disruption to the BBB can occur as a result of damage to any number of elements that are involved in the composition of the neurovascular unit.

3.A. Intracellular Signaling Mechanisms Involved in Cell Swelling and Brain Edema

Mechanisms underlying cytotoxic brain edema are not clear (Ayata and Ropper, 2002; Liang et al., 2007; Panickar and Anderson, 2009) although a number of factors have been implicated in astrocyte swelling. These factors include increased free radical production, increased levels of Ca^{2+} , (Olson et al., 1990), elevated K^+ (Kimelberg, 1995), acidosis, free radicals, release of excitatory neurotransmitters, especially glutamate (Rutledge and Kimelberg, 1996; see Kimelberg, 2005 for review), and mitochondrial dysfunction, in particular the opening of the mitochondrial permeability transition (mPT) pore (McGregor et al., 2003; Rama Rao et al., 2003; Panickar et al., 2006; Jayakumar et al., 2008). The mPT is characterized by a sudden increase in permeability of the inner mitochondrial membrane to small solutes (ions and molecules, reducing equivalents; Zoratti and Szabo 1995; Bernardi et al., 2006). The mPT results in the depolarization of the inner membrane potential ($\Delta\Psi_m$) and may lead to osmotic swelling of the mitochondrial matrix (Gunter and Pfeiffer, 1990), mitochondrial dysfunction, defective oxidative phosphorylation, impaired ATP synthesis, and the generation of free radicals (Zamzami et al., 1997). Cyclosporin A (CsA), an immunosuppressant, is a relatively specific blocker of the mPT, but it also inhibits

calcineurin. Sensitivity to CsA, but not to FK506, another immunosuppressant that does not block the mPT but inhibits calcineurin, is generally indicative of the involvement of mPT (Schweizer, et al., 1993; Connern and Halestrap, 1994). Using such a combination of CsA and FK506 to implicate the mPT, it has been suggested that blockade of the mPT attenuates glial swelling in ammonia neurotoxicity (Rama Rao et al., 2003), in vitro trauma (Panickar et al., 2006; Jayakumar et al., 2008), and ischemic injury (McGregor et al., 2003; Panickar et al., 2009a,b).

Ion transporters or exchangers play an important role in the development of cytotoxic edema. One important co-transporter involved in cell volume regulation in glial cells is $\text{Na}^+/\text{K}^+2\text{Cl}^-$ (NKCC). NKCC is an electroneutral co-transporter and NKCC1 (CNS form) belongs to the cation-dependent Cl^- transporter family and transports 1 Na^+ , 1 K^+ , and 2 Cl^- into cells under physiological conditions in an electroneutral manner (Haas, 1994; Chen and Sun, 2005). In states of high K^+ , such as after ischemia, activation of the co-transporter leads to astrocyte swelling/brain edema, and such edema is significantly attenuated by bumetanide, an inhibitor of NKCC (Yan et al., 2003). Likewise, astrocytes from NKCC-deficient mice show less K^+ -induced swelling (Chen and Sun, 2005). Another ion exchanger that is involved in cell volume regulation is the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX). NCX extrudes Ca^{2+} , 3 Na^+ in, to 1 Ca^{2+} out. This exchanger may act in the "forward" mode resulting in Ca^{2+} efflux, or in the "reverse" mode causing Ca^{2+} influx depending on the electrochemical gradient of Na^+ across the plasma membranes and membrane potential (see DiPolo and Beauge, 2006 for review). Increased activity of NCX in forward mode is likely to increase intracellular Na^+ which in conjunction with Cl^- entry (via $\text{Cl}^-/\text{HCO}_3^-$; since HCO_3^- may be released to adjust the intracellular pH) may increase intracellular NaCl concentration thereby triggering the entry of water and causing cells to swell. In the reverse mode of operation, NCX will extrude Na^+ and consequently intracellular Ca^{2+} will increase which may induce the opening of the mPT and thus may contribute to astrocyte swelling. Other ion transporters involved in cell volume regulation that may play important roles in cytotoxic edema include the Na^+/H^+ exchanger (NHE), a protein that is expressed in many mammalian cell types (Orlowski et al., 1992; Pizzonia et al., 1996). More recently, an ion channel has been demonstrated to mediate brain edema in ischemia called the SUR-1 regulated $\text{NC}_{\text{Ca-ATP}}$ channel (Chen et al., 2003; Simard et al., 2006). This channel conducts all inorganic monovalent cations, but is impermeable to Ca^{2+} and Mg^{2+} , although Ca^{2+} is required for the opening of this channel. Depletion of ATP triggers channel opening. Blockade of this channel using glibenclamide reduced cerebral edema *in vivo* in two models of ischemia (Simard et al., 2006). Another membrane protein that is critically involved in cytotoxic brain edema is aquaporin 4 (AQP4). Aquaporins are membrane proteins that regulate the flow of water in and out of the cells selectively while generally preventing the passage of other ions or solutes (Agre, 2006). AQP4 is a transmembrane water channel that transports water bidirectionally along osmotic gradients. Generally found in astrocytic end feet, it is also found in endothelial cells (Amiry Moghaddam and Ottersen, 2003). Whether it works in co-ordination with an inward rectifying K^+ channel, Kir4.1, to reduce swelling is not clear (Nagelhus et al., 1989; Saadoun et al., 2003). AQP4-deficient mice show reduced brain edema after acute water intoxication and ischemic stroke (Manley et al., 2000).

Factors affecting cytotoxic edema may also contribute to vasogenic edema although the underlying mechanisms by which vasogenic edema develops may be different. The functional integrity of the BBB is critical in maintaining normal brain volume and disruption of the BBB

contributes to vasogenic edema. As with cytotoxic edema, oxidative stress is an important factor in inducing vasogenic edema. Swelling of the astrocytic end feet, which are rich in AQP4 as well as other ion transporters including NHE, may create a leaky environment leading to compromised microvascular integrity. Matrix metalloproteinases (MMPs), which are increased after ischemic injury (Heo et al., 1999; 2005), disrupt the BBB. Tight junction proteins (TJPs), occludin and claudin-5, which form the endothelial barrier, are vulnerable to attack by MMPs. Basal lamina proteins of the extracellular matrix proteins, such as fibronectin, laminin, and heparan sulfate, are also degraded by MMPs. Another feature of ischemia is the migration of neutrophils from the periphery to the area of ischemic lesion in the brain (del Zoppo et al., 1991). While neutrophils themselves can cause edema by infiltrating into the tissue, they are also a source of free radicals, MMPs, and myeloperoxidases that can aggravate endothelial damage and thus contribute to vasogenic edema. Mediators of inflammation, including TNF- α , IL-1 β , ICAM-1, augment endothelial dysfunction. In addition, oxidative and nitrosative stress can also disrupt BBB integrity through mechanisms that are not clear, considering the abundance of proteins that are involved in the structure and function of the BBB.

3.B. Effect of Polyphenols on Signaling Molecules in Attenuating Brain Edema and Cell Swelling in Ischemia

Oxidative stress is a key feature of ischemia injury including cell swelling and brain edema and polyphenols appear to be effective in attenuating such injury. It should, however, be noted that polyphenols can also have pro-oxidant effects depending on the cell type or at very high concentrations (see Halliwell, 2008 for review). Nevertheless, at lower doses, the anti-oxidant effects of polyphenols have been well-established. We have isolated and characterized a polyphenol-rich water-soluble cinnamon extract (cinnamon polyphenol extract; CPE) (Anderson et al., 2004). The major active components of CPE appear to be doubly linked procyanidin type-A polymers (Anderson et al., 2004). CPE has been reported to have anti-oxidant effects in rats (Lee et al., 2003; Lin et al., 2003; Anderson et al., 2004) and humans (Roussel et al., 2009). We recently demonstrated that CPE (Panickar et al., 2009b), as well as polyphenols from green tea (Panickar et al., 2009a), attenuate glial swelling in cultures following ischemic injury. Interestingly, known anti-oxidants were not effective in reducing cell swelling following ischemic injury (Panickar et al., 2009a, b). For instance α -tocopherol, a lipid peroxidation inhibitor, prevents glial swelling in cultures when exposed to pathological concentrations of ammonia (Jayakumar et al., 2006) or *in vitro* traumatic brain injury (TBI) (Jayakumar et al., 2008), but not oxygen-glucose deprivation (OGD; Panickar et al., 2009b). Several possibilities exist for α -tocopherol not having a protective effect. One possibility is that different cellular mechanisms underlie cell swelling in OGD when compared with ammonia toxicity or TBI in cultures and that lipid peroxidation is not a major contributor to cell swelling in OGD. This is somewhat surprising due to similarities between ischemic injury and TBI (Unterberg et al., 2004; Marmarou, 2003). Noteworthy, a differential effect of inhibition of lipid peroxidation that protects against neuronal damage after transient but not permanent middle cerebral artery occlusion (MCAO) in rats has been reported (Xue et al., 1992). Another possibility is the species of free radicals that dominate cell swelling in OGD are different from those seen in ammonia neurotoxicity or TBI and needs to be

investigated in future studies. Regardless, resveratrol, another polyphenol which also has anti-oxidant effects (Cherubini et al., 2008) did not prevent cell swelling. Taken together these studies indicate that while oxidative stress may be a contributing factor in cell swelling or brain edema, they probably act in conjunction with other factors which together contribute to cell swelling/edema.

One consequence of increased free radical production is the activation of intracellular signaling kinases (Remacle et al., 1995; Sen and Packer, 1996). Several intracellular signaling kinases have been implicated in glial swelling or brain edema. Blockade of protein kinase C (PKC) by H-7 reduced the extent of cerebral edema formation evoked by ligation of common carotid artery in gerbils (Hu et al., 1996) and common carotid artery occlusion in rats (Joo et al., 1989) and by middle cerebral artery occlusion in rats (Hu et al., 1996). Interestingly, phorbol 12-myristate 13-acetate (PMA), an activator of PKC, reduced brain water content in rats after MCAO possibly mediated by a downregulation of AQP4 in the ischemic area (Kleindienst et al., 2006; Fazzina et al., 2010). Reduction in brain water content with both an inhibitor as well as an activator of PKC is intriguing. However, non-specificity of such drugs is one possibility for such contrasting effects. For instance, H-7 in addition to blocking PKC also blocks cAMP and cGMP-dependent kinases (Hidaka et al., 1984). Likewise, PMA in addition to activating PKC also activates other MAP kinases including JNK, ERK, p38, as well as Jak/Stat signaling pathways in rat brain astrocytes (Hwang et al., 2007). It is possible that reduction of brain edema is dependent not simply on activation or inhibition of any one kinase or signaling pathway but on the interaction of multiple signaling pathways. Whether activation or inactivation of more than one signaling pathway depends on the location or severity of ischemic injury is not known and needs to be investigated. Blockade of c-Jun N-terminal kinase (JNK), in combination with hyperbaric oxygen reduced brain edema in rats after MCAO (Liu et al. 2009) or with JNK inhibitor alone following subarachnoid hemorrhage in rats (Yatsushige et al., 2007). Inhibition of sphingosine kinase (SphK), with the SphK inhibitor dimethylsphingosine, reduced brain edema in hypoxic preconditioned mice subjected to MCAO (Wacker et al., 2009). A novel small molecule, KDR kinase inhibitor Compound-1 (an inhibitor of kinase insert domain receptor (KDR), was effective in reducing edema following focal cerebral ischemia in rats (Foster et al., 2009). Intraventricular administration of SB 203580, an inhibitor of p38^{MAPK}, significantly attenuated BBB extravasation and subsequent edema (vasogenic) after transient focal ischemia in rats (Nito et al., 2008). Increased phosphorylation of the tyrosine kinase p125 focal adhesion kinase (p125(FAK)) and p38^{MAPK} has also been reported in ischemic chicken retinal swelling (de La Paz et al., 2002) and blockade of Src kinases reduces retinal edema in mice (Doukas et al., 2008) indicating an important role of kinases in the development of edema. Activation of such kinases could regulate cell volume by their subsequent phosphorylation of ion channels/transporters and modifying their action (Boron et al., 1988; Bae and Verkman, 1990). Polyphenols, due to their anti-oxidant properties, could also potentially reduce free radical-induced activation of such kinases and thus reduce cell swelling.

The effects of polyphenols in regulating ion transporters or aquaporin levels, following cerebral ischemia, are not clear, and are a subject of current investigation. Nevertheless, studies conducted in non-neural tissues or cell lines give us a glimpse on the ability of polyphenols to modulate ion channels and/or co-transporters. Quercetin, catechin, and resveratrol inhibited cardiac voltage gated sodium channel in rat cultured myocytes but had no effect on the reverse mode NCX, the sodium/calcium exchanger (Wallace et al., 2006).

Studies on the direct effects of polyphenols on ion channels or co-transporters involved in regulating intracellular calcium and thereby affecting cell volume regulation are lacking. But effects of polyphenols in modulating intracellular calcium levels $[Ca^{2+}]_i$ have yielded varied results. Elevated $[Ca^{2+}]_i$ was observed after exposure to tannins in HL-60 cells (Sakagami H et al., 1995). Red wine polyphenols increase calcium in bovine aortic endothelial cells (Martin et al., 2002). EGCG increases $[Ca^{2+}]_i$ in U87 cells by influx of extracellular Ca^{2+} as well as by releasing calcium from intracellular stores. In contrast, some studies have reported a decline in $[Ca^{2+}]_i$ following exposure to polyphenols. Quercetin attenuated the H_2O_2 -induced calcium dysregulation in PC12 cells (Wang and Joseph, 1999). Apple condensed tannins inhibit the increase in intracellular free Ca^{2+} concentration in RBL-2H3 cells induced by antigen stimulation (Kanda et al., 1998). EGCG reduces the glutamate-induced $[Ca^{2+}]_i$ increase by attenuating ionotropic Ca^{2+} influx in PC12 cells (Lee et al., 2004). These studies indicate that polyphenols have the potential to modulate some ion channels that are involved in cell volume regulation, but their role in attenuating glial swelling/cytotoxic edema in ischemia needs to be elucidated.

Mitochondrial dysfunction is a key component of ischemia (Sims et al., 1986; Sims, 1991). The mitochondrial permeability transition (mPT) has been implicated as one mechanism, or at least part of the mechanistic pathway, for cell swelling in cultured astrocytes following ammonia toxicity (Rama Rao et al., 2003) or TBI (Jayakumar et al., 2008) as well as in brain sections in ischemia (Macgregor et al., 2003). We recently demonstrated that the attenuation of cell swelling and the prevention of the decline in mitochondrial inner membrane potential ($\Delta\Psi_m$) by immunosuppressants, cyclosporin A (CsA), but not FK506, are consistent with the role of the mPT mediating such events. Similar to CsA, CPE and green tea polyphenols, also significantly prevented OGD-induced cell swelling and the decline in $\Delta\Psi_m$ in C6 glioma indicating that one mechanism by which CPE and GTE exert their protective effects is possibly by blocking the mPT. Interestingly, quercetin attenuated cell swelling in C6 glial cells following OGD but did not block the dissipation of the $\Delta\Psi_m$ (Panickar et al., 2009d) indicating that other factors, besides mitochondrial dysfunction, mediate the development of cell swelling in ischemic injury.

An important characteristic of polyphenols is their anti-inflammatory effect (Musim et al., 1976; Agarwal et al., 1993) and such effects of polyphenols have been reported in cerebral ischemia (Gao et al., 2006; Shin et al., 2006; ArunaDevi et al., 2010). Polyphenols found in cinnamon also have anti-inflammatory effects in cell culture studies (Cao et al., 2008; see also Panickar et al., 2009c for review). A reduction in $TNF-\alpha$, an inflammatory cytokine, has been reported for green tea polyphenols (Fujiki et al., 1999) as well as dried plum polyphenols (Bu et al., 2008). As mentioned above, $TNF-\alpha$ is one agent that increases endothelial permeability in vasogenic edema. Also, increases in intercellular adhesion molecule (ICAM-1) and myeloperoxidases in rodent lung injury are attenuated by green tea polyphenols (Di Paola et al., 2005). In addition, anti-cyclooxygenase 2 effects of resveratrol (Subbaramaiah et al., 1998), as well as anti-MMP9 effects of resveratrol (Li et al., 2003) and other polyphenols have been demonstrated. Given the role of inflammation in vasogenic edema and the anti-inflammatory effects of polyphenols, it is reasonable to hypothesize that polyphenols may have a protective role in reducing vasogenic brain edema in ischemia.

Conclusion

Ischemia induces neuronal loss, which is mediated by both apoptosis and necrosis. Increased free radical production and excitotoxicity are key features that affect neuronal viability after ischemia. In addition, brain edema is a major consequence of ischemia and can be fatal if not resolved. Edema can further aggravate neuronal injury by affecting cerebral perfusion. Currently, there are few remedial agents to effectively reduce neuronal death or brain edema. There is tremendous potential for the use of polyphenols in the prevention of cell loss and edema in cerebral ischemic injury. Given the large proportion of the population affected by stroke, and that there are not many effective strategies to attenuate brain edema and associated neural damage, it is important to determine the effects of potential beneficial dietary agents in the prevention and alleviation of the damaging effects in ischemia. Considering the multifactorial properties of dietary polyphenols, their potential in alleviating the neural damage associated with ischemic injury needs to be elucidated.

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Chapter VI

Holistic Approach Versus Scientific Evidence, what Do We Know about Anti-Aging Herbal Medicine in Aging-Associated Neurodegenerative Diseases?

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Abstract

In Asian countries such as China, Japan and Korea, some herbs are considered to be anti-aging herbs. According to the traditional medicine descriptions, these herbs have beneficial effects on aging-associated diseases and they intervene disease progression through a holistic approach. For a long time, this “holistic approach” statement remains difficult to be interpreted and is considered to be lack of scientific-evidence support. *L. barbarum* is a commonly used herb in China for both disease treatment and as functional food. It is also famous for its anti-aging properties. In this review, we use *L. barbarum* as an example to explain the “holistic approach” concept of anti-aging herbs. We discussed its effects on aging-associated neurodegenerative diseases such as glaucoma and Alzheimer’s disease (AD). We proposed that the holistic properties of *L. barbarum* can be reflected in the following areas. Firstly, *L. barbarum* is a common food ingredient in

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Eastern countries. Carotenoid and polysaccharides components of *L. barbarum* are beneficial to the eye and therefore explained why it can be used as food therapy. Secondly, *L. barbarum* utilize multiple protective mechanisms to protect neurons against pathological damages. *L. barbarum* can directly modulate a number of intracellular signaling pathways and increase the expression of survival factors to enhance neuronal survival and attenuate apoptosis. On the other hand, *L. barbarum* indirectly regulate neuronal survival through modulating the activities of the surrounding microglia. Furthermore, *L. barbarum* provides beneficial effects on a number of aging-associated conditions, which can be risk factors of neurodegenerative diseases. These holistic actions for therapy and prevention may explain why *L. barbarum* is regarded as an anti-aging herb.

Introduction

Herbal medicine refers to the use of plant or plant extracts for medical purpose. There has been a long history for human to use medicinal plants based on traditional medical theories, beliefs, and experience indigenous to different cultures. Medicinal plants are not just widely used in primary health care in developing countries. On the other hand, there is a growing trend of using medicinal plants for treatment of chronic diseases and disease prevention in developed countries such as the United States, Germany and France in the last few decades [1, 2].

Despite of the increasing sales and marketing size of medicinal herbs as either food supplement or as alternative medicine, the concept or theories of traditional medicine that the product claims to base on are still confusing to many people. Herbal medicine is often considered to be lack of “scientific evidence” even though many lines of research have been conducted on them. Among various categories of medicinal herbs, the “anti-aging herbs” is one of the good examples. In traditional medicine, like Chinese medicine, anti-aging herbs are used as functional food, as preventive medicine and for disease treatment. The traditional theory also emphasizes a holistic approach for anti-aging purpose. While advertisement always uses the term “holistic approach” to promote their “anti-aging” herbal products, little is known or intended to explain this term in a scientific language. In general, the holistic approach in complementary medicine is interpreted as “restoring the balance in our body and facilitating body’s own healing response” [3]. However, can the statement be explained precisely with scientific evidence and orthodox medical theories? With the advance in phytotherapy research and more understanding in mechanisms of some neurodegenerative diseases, we think it is the time to summarize our knowledge and re-analyses the actions of anti-aging herbs particularly their holistic properties. In this review, we use *Lycium barbarum* as an example to illustrate how this anti-aging herb can be used in treatment or prevention of aging-associated neurodegenerative diseases such as glaucoma and Alzheimer’s disease. We will discuss some scientific findings of *L. barbarum* and try to explain the holistic concept with this anti-aging herb.

1. What is Anti-Aging Herbs? The Differences between Advertisement and the Traditional Theories

The term “anti-aging” has been used extensively in advertisements of natural products, which are usually herbs extract in their crude or processed form. In many cases, the promotion claims that the “anti-aging” natural products are able to “slow down the aging process”, “boosting the body to excrete waste/toxin” and “help the body and mind to resist stress”. While these promotion phases may list out some of the beneficial effects of the “anti-aging” herbal products, they help little in the understanding of anti-aging theories or precise usage of herbs for the purpose. We think that some basic concept of traditional anti-aging theories is essential for the understanding of herb usage. This is also the milestone for using the traditional theory for the inspiration of new scientific research directions. The term “anti-aging” may have different meanings according to different types of traditional medicine. In this review, we only focus on the traditional Chinese medicine and discuss the meaning of anti-aging according to its medical theories.

1.A. Aging is a Process of Decline in Vital Energy

The concept of vital energy plays a central role in traditional Chinese medicine. Vital energy is a collective term for describing both physical and mental energy. It is the essential element for maintaining all body functions, such as growth, reproduction, and body defense [4]. Aging is caused by the progressive decline of vital energy. As the levels of vital energy diminish, aging-associated diseases will occur. Among various organs and body systems, the “kidney” and “liver” play the most critical roles in the aging process. Decline of vital energy in these two organs is the foundation for all aging process. The traditional theory also believes that “kidney” is the origin of an essential substance called “marrow”, and brain is referred to the “sea of marrow”, suggesting that cognitive functions and degeneration of the central nervous system is primarily affected by the health states of these two organs [5]. Although these theories are very different from the modern medicine, we should be aware that the term “kidney” and “liver” used in traditional medicine is not completely equivalent to the meaning used in modern medicine. Modern science has tried to explain the concept of vital energy and the effects of some herbs with mitochondrial ATP production or modulation of immune responses [6, 7], yet none of these approach can fully explain the traditional theories.

1.B. A Holistic Approach for Anti-Aging

While decline of vital energy is the cause of aging, anti-aging can be achieved by either replenishing the deficient energy or preserving the present amount of energy. According to the traditional theory, preservation of vital energy can be achieved by multiple means including proper diet, physical exercise, maintaining emotion stability and reducing stress. Thus, the holistic approach of anti-aging strategy involves the mind, body and environment [8]. Since the consideration of mind and environmental interaction is beyond the scope of this

review, we would like to focus on the “holistic” actions of herbs and elaborate how anti-aging herbs can achieve their effects.

The holistic action of anti-aging herbs can be reflected in their multi-target and multi-disease stage intervention. Moreover, research shows that herbs provide their beneficial effects through multiple mechanisms. Herbs are able to provide direct protection against pathological factors through up-regulation of survival pathways or down-regulation of pro-apoptotic pathways [9-11]. They can also act as a biological response modifier to modulate host immune responses [12]. We think that these properties are particularly important in fighting against aging-associated neurodegenerative diseases since these diseases are usually multi-factorial and have a complicated pathogenesis. In the following sections, we will use *Lycium barbarum* as an example to illustrate the holistic approach or action of anti-aging herbs.

2. *Lycium Barbarum* – The Traditional Anti-Aging Herb and its Usages

L. barbarum belongs to the family Solanaceae and it can be found in many regions of the world. The fruit of *L. barbarum* is a common medicinal herb in traditional Chinese medicine. It is also used in other Asian countries such as Korea and Japan. In China, where it is also named as *Gouqizi*, *L. barbarum* are primarily grown in the north part of the country, and the most famous origin is the Ningxia Autonomous Region. In some Western countries, *L. barbarum* is also called Wolfberry and “Gogi berry” is often used in the health food market [13].

There has been a long history of using *L. barbaum* for medical purpose. In Chinese pharmacopoeia, *L. barbarum* has been classified as a “yin-tonifying herb”, which means it can help replenishing the level of “yin” (a kind of vital energy) in our body. It has also been documented that the major targeting organs of *L. barbarum* are the “liver” and “kidney”. As these organs play essential roles in the aging process, it is not surprising that people will use the herb for anti-aging purpose. In traditional medicine, *L. barbarum* are used to treat various aging-associated diseases including diabetes and glaucoma. It is also particularly famous for its eye-protective property [14].

In Western countries, *L. barbarum* is mainly sold in the form of juice. However, in China, *L. barbarum* is usually sold as dried fruit. The dried fruit can be used directly for dietary or medical purpose. In most cases, the fruit is boiled in hot water for hours with other herbs to achieve maximum therapeutic effects. Its bright red color and sweetness also make it a common ingredient in Chinese cooking recipes.

3. *L. Barbarum* as a Food Ingredient, the Food Therapy Approach for Health Maintenance

A common feature of anti-aging herb is that they are frequently used as ingredients in dietary cooking. The traditional Chinese medicine places an importance on food therapy,

which believes that the intake of certain food can raise the amount of energy flow in the body [15]. The use of *L. barbarum* as food is one of the holistic approaches in anti-aging as this reflects a disease preventive measure. Modern findings on the nutritional value of *L. barbarum* may help us to explain its “tonifying” property for preservation of vital energy.

Carotenoid Components of *L. Barbarum* are Beneficial to the Eye

In Chinese *Materia Medica*, the function of *L. barbarum* is described as “brightening the eye” and it is used for aging-associated eye dysfunctions. Studies have shown that *L. barbarum* contains high amounts of two essential eye-beneficial carotenoids, namely beta-carotene and zeaxanthin, which may partly explain the traditional description.

The high beta-carotene contents in *L. barbarum* also account for its eye-protective property. The content of beta-carotene in *L. barbarum* (19.6 mg/100 g) has been found to be even higher than that of carrot (5.1 mg/100g) [16, 17]. Beta-carotene is the most common form of carotene. It is a precursor of vitamin A and can provide strong anti-oxidative effects. Many studies have shown that beta-carotene is an important nutrient for maintaining the healthy eye. Long-term intake of beta-carotene is associated with reduced risk of age-associated cataract and slow down the progression of age-associated macular degeneration [18, 19].

Zeaxanthin is the predominant carotenoid of the human retinal macular pigments. It has been reported that zeaxanthin and lutein nourishing the retina [20, 21]. They can improve visual acuity [20]; reduce the risk of age-associated macular degeneration [22, 23] and directly reduce apoptosis of photoreceptor triggered by oxidative stress [24, 25]. These unique characteristics of *L. barbarum* regarding to its zeaxanthin content can explain why the herb can “brighten our eye”.

Studies have shown that *L. barbarum* is one of the richest plant sources of zeaxanthin. The content of zeaxanthin in *L. barbarum* can reach 300 µg/g of fruit, which is comparable to that in egg yolk (210 µg/yolk) [26]. Most zeaxanthin in *L. barbarum* presents as an esterified form, zeaxanthin dipalmitate [27, 28]. Zeaxanthin dipalmitate comprises 31 to 56% of the total carotenoids [27, 29]. Although zeaxanthin can be found in many dark-green vegetables and yellow-to-orange fruits, its bioavailability varies from different foods. Studies have shown that intake of spinach and corn, both contain high levels of zeaxanthin, cannot lead to an increase in plasma zeaxanthin level [30, 31]. However, intake of *L. barbarum* can markedly increase the levels of plasma zeaxanthin, indicating the high bioavailability of the carotenoid in this herb [26, 32]. Furthermore, unlike egg yolk which also contains high levels of bioavailable zeaxanthin, *L. babrarum* is cholesterol-free. Using the herb for daily supplement of zeaxanthin does not have the risk of increasing blood LDL-cholesterol levels.

However, the total carotenoid concentration is only about 0.03% to 0.05% of the *L. barbarum* [29], indicating a large amounts of *L. barbarum* are required for preventive effects. It has been shown that at 0.5 mg zeaxanthin /kg/day (7.3 g of dry *L. barbarum*) feeding to rhesus monkeys can significantly increase zeaxanthin levels in the macula [32].

The Polysaccharides that Have Diverse Biological Activities

L. barbarum is made up of a group of components including polysaccharides, betaine, zeaxanthin, beta-carotene, beta-sitosterol, flavonoids, groups of vitamin, 19 kinds of amino acid, fatty acids and trace minerals. Among the components, *L. barbarum* polysaccharide (LBP) accounts for the major constitutions. *L. barbarum* contains about 40% polysaccharides. These polysaccharides are found to be active components responsible for the diverse biological activities of the herb [33]. Different laboratories have attempted to purify LBP into various fractions, in which the chemical and physical properties of the isolated glycoconjugates are identified [34, 35]. In general, the carbohydrate content of *L. barbarum* is found to contain arabinose, rhamnose, xylose, galactose, glucose, glucuronic acid, galacturonic acid and mannose [34, 36, 37]. LBP has been shown to have various beneficial effects on the aging-associated diseases. The anti-cancer, hypoglycemic, hypolipidemic and anti-oxidative effects of LBP in different cell or animal models have been documented [38-42]; and thus providing scientific bases for using *L. barbarum* in food therapy for health maintenance.

4. The Holistic Approach of *L. Barbarum* in the Intervention of Aging –Associated Neurodegenerative Diseases

Since *L. barbarum* has a strong reputation as an “anti-aging” herb, its significance in the anti-aging process is obviously not limited to its role in nutritional supplement for health maintenance. More importantly, it should be able to provide multiple paths for intervention on aging-associated diseases. The traditional “holistic concept” can be interpreted as the multiple actions of herbs, which means they can provide direct protection on the target organ, or provide protection through an indirect approach to modulate the body immune responses (i.e. biological response modifier). In the following sections, we will use *L. barbarum* as an example of anti-aging herb and discuss its holistic protective actions on two common aging-associated neurodegenerative diseases, glaucoma and Alzheimer’s disease. Increasing lines of evidence have shown that *L. barbarum* can modulate some cell survival and apoptotic signaling cascades; and attenuating pathological damages involved in neurodegenerative diseases [37, 43-46]. Furthermore, the immune modulation properties of *L. barbarum* serve as a good example to demonstrate how anti-aging herbs can protect our body in a macroscopic way [47]. Modulation of immune response by medicinal herbs serves as an important therapeutic strategy for neurodegenerative diseases.

4.A. The Polysaccharide from *L. Barbarum* is Neuroprotective to Retinal Ganglion Cells in Glaucoma

Overview of Glaucoma

Glaucoma is an aging-associated neurodegenerative disease, characterized by progressive degeneration of retinal ganglion cells (RGCs) and their axons, resulting in irreversible loss of

vision [48]. In 2002, statistics gathered by WHO shows that glaucoma is the second leading cause of blindness worldwide after cataract [49]. Glaucoma accounts for 12.3% of 37 million people affected by blindness, and 82% of which were 50 years or older. Age is an important factor for both in the incidence and prevalence of primary open angle glaucoma (POAG) and angle-close glaucoma (ACG). For example, the prevalence of POAG for white people 70-79 years old compared to those 40 to 49 years old is 3.5 times higher; and in African-Americans it is 7.4 [50]. The dramatic increase of life expectancy implies that the prevalence of glaucoma will significantly increase. It has been estimated that among people aged 40 or above, there will be 60.5 million with glaucoma, and over 8.4 million will be bilaterally blind from primary glaucoma by 2010. The number of glaucoma patients will increase to 79.6 million and the resulting blindness will increase to 11.1 million by 2020 [51].

Increase in intraocular pressure (IOP) is often considered to be the major risk factor in glaucoma. The prevalence of glaucoma rises proportionately with increasing IOP [52]. The medical therapy or surgical procedures to treat glaucoma are designed to reduce the IOP by either increasing the rate of aqueous outflow or decreasing aqueous production. General guidelines state that initial reduction should be around 20 – 30% from the baseline IOP, and this reduction decreases progressive worsening in POAG by 50 – 60% [53].

Effectively lowering IOP could not completely stop glaucomatous vision loss; efforts have been made for the treatment of glaucoma without modulating IOP. Neuroprotection was first introduced in the late 1970s. It is based on the concept that surrounding neurons are vulnerable to secondary neuronal degeneration adjacent to the area of ischemic stroke [54]. In 1990s, the concept of neuroprotection was applied to glaucoma [54, 55]. Investigation on potential neuroprotective agents is critical to prevent progressive vision loss in glaucoma.

Immune Modulation and Glaucoma

Increasing lines of evidence obtained from clinical and experimental studies strongly suggest an aberrant activity of the immune system in glaucoma [56;57]. The roles of the immune system in glaucoma are unclear and have been a controversial issue describing it as neuroprotective or neurodestructive. The fate of RGCs in glaucomatous eye is determined by the balance between beneficial immunity and harmful autoimmune neurodegeneration.

The most important sites for immune modulation in glaucoma are retina and the optic nerve, which have long been considered to be ‘immune privileged’ in the CNS. Despite immune privilege, T lymphocytes are found to patrol the healthy CNS as part of the constitutive immune surveillance [58, 59]. Following optic nerve crush injury, T cells have been found to accumulate at the injured site [60]. Passive transfer of anti-myelin basic protein T cells to treat the injured optic nerve exhibit protective effect on the damaged optic nerve. This has been confirmed using both morphological and functional assessments [61-63]. Further studies using glatiramer acetate (GA, also known as Cop-1) as an antigen to evoke active immunization also show neuroprotection against CNS injury. GA is a synthetic oligopeptide of 4 naturally occurring amino acids and is an FDA-approved drug for multiple sclerosis. Vaccination of GA significantly reduces loss of RGCs in rodent models of optic nerve crush injury, intraocular glutamate toxicity and chronically high IOP [64].

It is still not clear how autoimmune T cells elicit neuroprotection. It has been reported that active T cells produce various kinds of neurotrophins: nerve growth factor (NGF), NT-3, NT-4/5 and BDNF [62]. In addition, they communicate with resident innate immune cells - microglia and control their functions [65]. In optic nerve crush model, systemic injection of

these T cells increases infiltration of B cells and microglia/macrophages to the injured site and increases their expression of different kinds of neurotrophins [66].

Microglial cells are the major immunocompetent cells in the CNS. It has been reported that microglia have diverse phenotypes, which secret beneficial or destructive factors [67]. In human glaucomatous ocular specimens, microglia in the ONH and the parapapillary chorioretinal region of the ONH are activated and redistributed [68]. In argon laser-induced rat glaucoma model, there was no significant increase or change in either number or phenotype of microglia for up to three months in the ocular hypertensive eyes [69]. Since ocular hypertension (OH) is not an acute injury causing massive neuronal death, microglia in the glaucomatous retina were not activated as they were in the optic nerve axotomy model. There are increasing lines of evidence from *in vitro* studies showing that it is possible to manipulate the activation state of microglia, so that their activation can be beneficial - i.e. protecting rather than destroying neurons [70]. Whether microglia exhibit neuroprotective or neurodestructive effects depends on the disease state or the type of stimulus. In the OH model, intravitreal injection of 100 ng of monocyte chemoattractant protein-1 (MCP-1)/CCL2 activated retinal microglial cells to a moderately activated status and provided neuroprotection on RGC in OH experimental model. By raising the MCP-1 dose to 1000 ng or injecting lipopolysaccharide (LPS) intravitreally, loss of RGC in this model was exacerbated. In response to injury, activated microglia have been shown to induce neuronal death by releasing excess cytotoxic factors such as superoxide [71, 72], nitric oxide and tumor necrosis factor- α (TNF- α) [73-75].

Glial cell activation including macroglial and microglial activation is a hallmark of CNS injury. In human glaucomatous ocular specimens, macroglial activation in the peripapillary region was also detected by increased immunoreactivity of glial fibrillary acidic protein (GFAP) [56, 76], which is an intermediate filament protein and can be used as an index for glial activation and gliosis in response to various types of neural injury [77]. Increased expression levels of GFAP were detected in the vicinity of the RGCs where astrocytes and endfeet of the Müller cells are located [78]. It is believed that glial cells in the retina are integral components of and intimately involved in maintaining normal retinal neuronal activity through bidirectional communication [56, 79].

Immune Modulation Effect of LBP

The modulation of body immunity is often the first line index to access how a Chinese medicine improves our overall body health [80-82]. LBP as food supplement enhances body defense system by restoring atrophied thymus in aged subjects and regulates the proliferation and the immunoactivity of splenocytes and T cells [83-87]. It has been shown that LBP can increase phagocytic activity of macrophages, immunoactivity of cytotoxic T cells and natural killer (NK) cells in cyclophosphamide-treated mice and S180-bearing mice [39, 88, 89]. LBP modulates T cell proliferation by regulating the level of Con-A, enhancing cAMP/cGMP system and PKC activity [90]. LBP increased IL-2 receptors on isolated human peripheral lymphocytes [91]. LBP can be purified into different fractions, glycoconjugate LbGp4, LbGp5B and LBP_{3P}. LbGp4, LbGp5B promotes proliferation of splenocyte in mice, which is probably associated with activating the expression of nuclear factor kappa B (NF- κ B) and activator protein 1 (AP-1) [29, 92]. LBP_{3P} can increase expression of mRNA and protein levels of IL-2 and TNF- α in human peripheral blood mononuclear cells [83] and increase

macrophage phagocytosis, antibodies secreted by spleen cells, proliferation of spleen lymphocyte and cytotoxic T cells activity in S180-bearing mice [39]. Recently, LBP has been demonstrated to induce maturation of murine bone marrow - derived dendritic cells to secrete IL-12 p40 and increase the expression of membrane molecules I-A/I-E and CD11c [36]. Immune modulation effects of LBP are related to its anti-tumor property. In fact, LBPs have been extensively used in improving the immune activity of cancer patients after chemical or radiation therapy [93]. With increasing lines of evidence demonstrating the beneficial effects from immune modulation to neurodegenerative diseases [94-96], it is important to understand and elucidate how LBP works in glaucoma as an example of CNS neurodegenerative diseases.

Oral feeding the rats with LBP from 1 to 100 mg/kg can protect RGC from OH [47]. Concomitant with neuroprotective effect of LBP, microglia in the retina are moderately activated. Confocal image of the moderately activated microglia in the inner retina exhibits ramified morphology with thicker and focally enlarged processes. The important role of moderately activated microglia was further strengthened by using monocyte-inhibiting factor (MIF). Both *in vitro* and *in vivo* experiments show that MIF directly inhibits the activation of microglia/macrophages [97-99] and has no direct effect on neuron viability [100]. MIF can attenuate neuroprotective effects of LBP. The results suggest that activation of microglia at least partially contributes to the neuroprotective effect of LBP [101]. Furthermore, LBP also suppresses up-regulated GFAP immunoreactivity in the OH retinas.

Neuromodulation of LBP

In view of the direct cytoprotective and anti-aging effects of LBP, our work on Alzheimer's disease research has initiated a comprehensive investigation of LBP in counteracting β -amyloid peptide toxicity in primary neuronal cell culture (See 5b in following content). Intraperitoneal injection of LBP (60 g / 0.5 ml) has been shown to reduce stroke index and neurological score in a mouse cerebral ischemia and reperfusion model [102]. LBP can improve cognitive functions by enhancing the spontaneous electrical activity of the hippocampus [103]. To explore *in vivo* cellular mechanisms of LBP neuroprotection in experimental model of OH, proteomics has been adopted to evaluate pharmacological effects of LBP on the neuronal survival pathways. Among the changes of different proteins in the LBP-fed rat retinas compared with PBS-fed rat, the crystallin family has been investigated further using the Western-blot analysis of the retinas and immunohistochemical staining of the retinal sections. Crystallins are the dominant structural proteins in the lens and augment the refractive power of the transparent lens tissue. Crystallin gene families are divided into major ones: α -crystallins, and $\beta\gamma$ -crystallins [104]. Since Bhat and Nagineni (1989) first found the extralenticular expression of the α B-crystallin in the retina, heart, brain and other tissues [105], it is now known that crystallins are prominent proteins both in normal retina [106-110] and in retinal pathologies [111, 112]. Alpha B crystallin was later proved to be functional chaperones that protect other proteins against thermal insults [113, 114]. Increased expressions of crystallins has been considered to be a cellular response mechanism against stress [115-119]. Up-regulated expressions of crystallins in various retinal degenerations (genetic, age, light-mediated degeneration) demonstrate their important role in maintaining survival of retinal cell [111, 112]. Members of the $\beta\gamma$ -crystallins have also been detected outside of lens [107-110]. It has been shown that axonal regeneration is related to crystalline

β B2 movement [120]. Unlike other β -crystallins, β B2-crystallin has been shown to be heat-stable, similar to α -crystallin [121]. The thermal stability and Ca^{2+} binding capacity of β -crystallins [122] are both useful properties under stress conditions. We have demonstrated that neuroprotective effect of LBP on the survival of RGCs may be mediated via direct up-regulation of neuronal survival signal β B2-crystallin [69]. In addition, there are up-regulation of the expression of IGF-1 and IL-10R on the survival RGCs in LBP fed rats compared with the PBS-fed group.

Taken together, neuroprotective LBP is multi-targets. All these effects are summarized in Figure 1. Up to now, our studies have proved that LBP neuroprotective effect on the survival of RGC may include direct up-regulation of survival factors in the RGCs such as crystallins [69], IGF-1 and IL-10R, moderate activation of retinal microglia [101], deactivation of retinal macroglia (astrocytes and Müller cells) by attenuating their activation and preventing gliosis, the possible adverse effects.

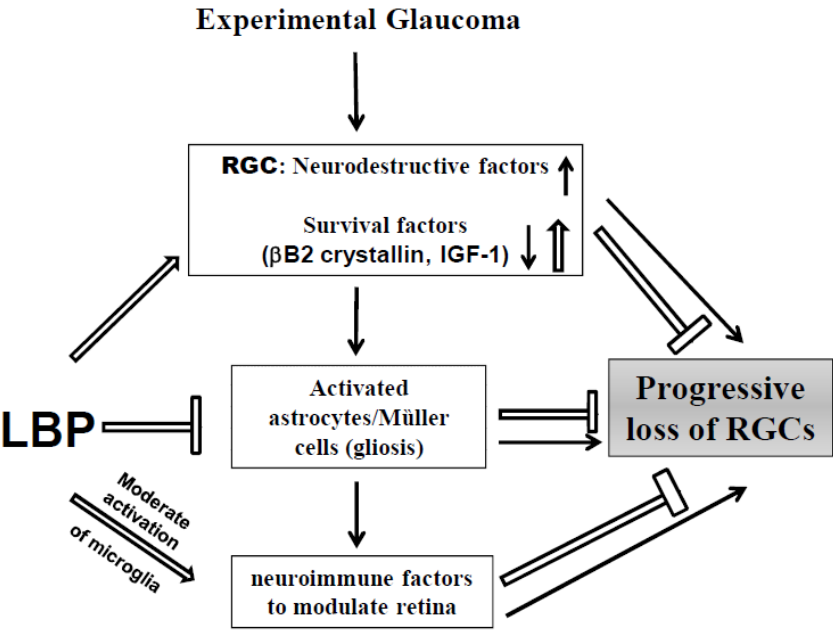


Figure 1. Schematic diagram to depict the intercellular effects of *L. barbarum* among different cells in the central nervous system. Glaucoma can increase neurodestructive facts and reduce survival factors, which will subsequently over-activate glial cells (as shown in solid arrow). LBP, however, can reduce neurodestructive factors and increase survival factors to modulate glial responses so that glial cells produce more trophic factors (as shown in open arrow).

4.B. *L. Barbarum* As A Potential Neuroprotective Herb For Alzheimer’s Disease

Alzheimer’s Disease – Some Basic Information

Alzheimer’s disease (AD) is an aging-associated progressive neurodegenerative disease. It accounts for 60 to 80% of dementia cases. Although the cause of AD is not clear, occurrence of AD is definitely strongly associated with age. It has been estimated that the

prevalence of AD increases gradually from 10.3% in people aged 65 to 47.2% in those aged 85 or above [123]. The global aging problem has made AD a major health concern as this disease can bring huge social and economical impacts to the society. According to the report conducted by the Alzheimer's Association, the total expenses on AD (include direct and indirect expenses) in United States will be around \$172 billion US dollar in 2010 [124].

The most noticeable clinical symptom of AD is decline in memory. The manifestation usually begins as a very mild impairment and presents as increasing difficulty in remembering new information. As the disease progresses, more cognitive functions will be affected. The impairment in spatial memory can create problems during driving and patients may get lost even in a familiar environment. Deterioration in language function can be presented as shrinking vocabulary and decreasing word fluency [125]. Some patients will developed neuropsychiatric manifestations such as agitation, irritability and anxiety [126]. All these neuropsychiatric disturbances create more burden and pressure for their family members and the caregivers.

Current pharmacological interventions approved by the U.S. Food and Drug Administration (FDA) for AD treatment can be divided into to two types. The first type is the acetylcholinesterase inhibitors (AChEIs) which include tacrine (no longer use due to its hepatotoxicity), donepezil, rivastigmine and galantamine. The principal of using symptom-relieving drugs are based on the "cholinergic-deficit hypothesis" which suggests that the degeneration of cholinergic neurons and the associated loss in cholinergic neurotransmission is responsible for the deterioration of cognitive functions observed in AD patient [127]. AChEIs inhibit the degradation of acetylcholine by acetylcholinesterase, thus increasing the availability of the neurotransmitter [128]. Memantine, which is approved by the FDA in 2003, is the only candidate for the second type of drug for AD treatment. Memantine acts as a specific, non-competitive, moderate-affinity NMDA antagonist to modulate NMDA receptor activity and reduce abnormal excitatory glutamate signal (glutamate excitotoxicity) [129]. Although both AChEIs and memantine can provide improvement on cognitive and global measures relevant to dementia in clinical trials [130-136], their effects have lots of limitations. It is known that AChEIs are purely symptomatic therapies that have no or little effects on the underlying disease pathology. While memantine is approved for the treatment of moderate to severe AD only and also has modest effects on slowing down disease progression, it has recently been shown that it can be potential neuroprotective agent. More importantly, it has been shown that neuronal dysfunction and brain atrophy precede the onset of clinical symptoms for years or even decades [137-139]. The development of disease-modifying therapy to stop or even reverse disease progression is therefore emphasized. It is expected that this type of drug can precisely target the pathological changes in AD development and therefore would be efficient for preventing or slowing the onset of disease [140]. Since AD pathogenesis is complicated and multi-factorial, research effort has been made to develop multifunctional or multipotential neuroprotective drug [141]. Green tea extracts is one of the examples to show that natural products are good source for the development of multifunctional drug [142]. Other anti-aging herbs such as *L. barbarum* have also been shown to be potential in neuroprotective drugs development.

L. Barbarum Attenuates Beta-Amyloid Nerurotoxicity

Among various pathological changes in AD, the deposition of senile plaques has long been a major concern. Senile plaques are the extracellular deposits of beta-amyloid (A β)

peptides surrounded by dystrophic neurites and activated glial cells [143, 144]. Two variants of A β peptides, A β ₁₋₄₀ and A β ₁₋₄₂, are found to be the predominant protein in plaque. A β ₁₋₄₂ peptide is more hydrophobic and readily to aggregate [144]. A β is produced from the enzymatic cleavage of the amyloid precursor protein (APP) [145, 146]. According to the “Amyloid Hypothesis”, A β peptide can trigger neurotoxic cascades leading to neurodegeneration in AD [147]. Many studies have shown that A β peptide can induce oxidative stress and neuronal apoptosis [148]. Activation of pro-apoptotic signals such as c-Jun-N-terminal kinase (JNKs), p38 mitogen activated protein kinase (MAPK) and double stranded RNA-dependent protein kinase (PKR) have been shown to be responsible for A β -induced apoptosis in *in vitro* models [149, 150]. Other proteins such as Akt, p53 and Bcl-2 are also involved in A β neurotoxicity [151]. These signaling pathways may serve as therapeutic targets for attenuating A β toxicity.

Several studies have actually demonstrated that *L. barbarum* is able to modulate some A β -mediated pro-apoptotic and survival signals, hence antagonizing A β neurotoxicity [37, 43, 152]. Interestingly, these studies show that different polysaccharide fractions extracted from *L. barbarum* employ differential protective mechanisms against A β toxicity. In a study conducted by Yu and colleagues [37], it has been shown that *L. barbarum* polysaccharides extracted by an aqueous extraction method (named as LBA for easier understanding) are able to attenuate A β peptide-induced neuronal cell death. LBA can attenuate the caspase-3-like activity as well as the LDH cytotoxicity triggered by A β peptide. Pretreatment of cortical neurons with LBA markedly reduces the number of apoptotic neurons upon challenge by A β peptide. The effective and safety dosages of LBA have also been found to be wider than that of lithium chloride (LiCl), a well-known Western neuroprotective agent. The study also suggests that LBA can suppress A β -induced JNK activation. Phosphorylation of JNK causes its activation, which will in turn activate its substrate c-Jun. This activation allows c-Jun to subsequently activate the transcription of apoptotic related genes [153]. Neurons incubated with LBA show marked reduction in the level of phospho-JNK and phospho-c-Jun upon A β challenge, suggesting that the polysaccharides can protect neurons through suppression of the pro-apoptotic pathway [37].

Although inhibition of JNK by LBA plays a significant role for its neuroprotection, the authors proposed that *L. barbarum* might have effects on other pro-apoptotic kinases. This speculation is actually proved to be correct. One should know that *L. barbarum* contains not just one particular type of polysaccharides but groups of these sugars. In a crude extract, mixture of different types of polysaccharides is often found. The chemical composition hence biological activities can be diverse. In another word, even all of these polysaccharides can protect neurons against A β toxicity, they may mediate different protective mechanisms. As shown in another study, a different aqueous fraction from *L. barbarum*, namely LBP-III, also protects cortical neurons from A β -induced damage [152]. LBP-III has a relatively low molecular weight (<30 kDa) when analyzed by HPGPC. Chemical analysis indicates that it contains an especially high content of galacturonic acid. In an *in vitro* model of primary cortical neurons, LBP-III can significantly reduce the phosphorylation of PRK in A β toxicity. Phosphorylation of PKR induces its activation, which can then phosphorylate eIF2 α to inhibit the initiation of protein translation and causes neuronal apoptosis [154, 155]. A number of studies have demonstrated the involvement and importance of PKR in the pathogenesis of

AD. Elevating the levels of phospho-PKR can be found in the nuclei of autopsied postmortem AD brain tissues [156]. Alteration of the kinase can also be observed in lymphocytes of AD patients. The levels of phospho-PKR and phospho-eIF2 α have found to be significantly higher in lymphocytes of AD patients, and these changes are correlated with cognitive and memory test scores [157]. The suppression of A β -induced PKR activation by LBP-III can partly explained its neuroprotective function [152]. In the same time, these data of *L. barbarum* serve as good examples to show that anti-aging herbs are able to modulate multiple pro-apoptotic signaling pathways to achieve its function.

A very important theory for many traditional medicines including traditional Chinese medicine is the balance concept. Chinese medicine believes that balancing different body components is essential for health keeping, disease prevention and intervention [158]. Based on this concept, it is also reasonable to speculate that there are additional actions provided by the anti-aging *L. barbarum* to counteract the devastating neurodegenerative processes and “restore body balance”, which is a general explanation for the meaning of holistic approach. We think the effects of *L. barbarum* on the survival Akt pathway can be a scientific evidence for the “balance” concept. By using an alkaline extraction method to extract polysaccharides from *L. barbarum*, a novel fraction namely LBB has been extracted by our laboratory. This novel alkaline fraction of *L. barbarum* can also protect neurons against A β toxicity. Instead of suppressing the activation of pro-apoptotic stress kinase, LBB can enhance the phosphorylation of Akt in A β -treated neurons [43]. It is well-known that activation of Akt plays an important role in cell survival and may safeguard cells from harmful conditions. Activation of Akt is in response with neurotrophic factors such as IGF-1 [159] and nerve growth factor [160]. Studies have shown that Akt can have anti-apoptotic effects and it serves as one of the neuroprotection pathways [161-163]. As documented in many publications, A β peptide can down-regulate Akt expression [164, 165]. The effects of LBB from *L. barbarum* on Akt may be one of the possible explanations of its protective actions. In fact, other anti-aging herbs such as ginseng [166] are also able to regulate the survival pathways hence protect neurons from harmful insults. The data of *L. barbarum* together with other anti-aging herbs suggested that the bi-directional modulation of body response, including pro-apoptotic and survival signaling pathways, can help us to “restore the balance” hence providing beneficial effects in aging-associated neurodegenerative diseases.

ER-Stress Can Be Attenuated by L. Barbarum

The endoplasmic reticulum (ER) plays an important role in protein synthesis. Protein are synthesized, modified, secreted and delivered to target sites. In the ER lumen, proteins are folded into correct configurations with the aid of ER-resident molecular chaperones, foldases, and lectins. Any incomplete or miss-folded proteins are retained in the ER to wait for completing the folding process or are subjected to the degradation pathways. Disruption of normal ER functions cause ER stress, which leads to an ER response called unfolded protein response (UPR) [167]. This response can be reflected by the alterations of ER stress transducers such as inositol-requiring protein-1 (IRE1), activating transcription factor-6 (ATF6) or protein kinase RNA (PKR)-like ER kinase (PERK). Increasing lines of evidence have shown that ER stress is involved in neurodegenerative diseases including AD [168]. It has been reported that the protein levels of BiP and phospho-PERK increase in the neurons in AD patients. These elevations of ER stress-related protein indicate the occurrence of UPR in

AD patients [169, 170]. Moreover, it has been suggested that the occurrence of ER stress is involved in early pathogenesis of AD and may contribute to the formation of neurofibrillary tangles, which is another pathological hallmark in AD [169]. Attenuation of ER stress-induced neuropathology may therefore be a possible therapeutic strategy for AD.

L. barbaum has also shown protection against ER stress-induced neuronal damage. In an *in vitro* model, dithiothreitol (DTT) was used as ER stress inducer to trigger reducing stress in cortical neurons. It has been found that extract from *L. barbarum* could significantly reduce DTT-induced neuronal cell death and apoptosis. *L. barbarum* also attenuate DTT-induced phosphorylation of PERK, suggesting that neuroprotective effects of *L. barbarum* are related to ER stress signaling pathways [46]. Although this study did not further investigate the downstream signaling pathway modulated by *L. barbarum*, it is worth to notice that DTT can induce the PERK-dependent activation of JNK [171]. Since studies have shown that *L. barbarum* can affect the phosphorylation of JNK in A β -treated neurons [37], it is possible that *L. barbarum* can also suppress phospho-PERK-induced JNK activation to provide its protection against ER stress.

L. Barbarum Reduces Glutamate Excitotoxicity

Glutamate is an excitatory amino acid that plays important roles in neurotransmission. After the release from pre-synaptic vesicles, glutamate can bind to post-synaptic glutamate receptors (e.g. N-Methyl-D-Aspartate (NMDA), α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), kainate and some metabotropic glutamate receptors) to trigger depolarization and action potential. Besides its physiological functions, high concentrations of glutamate in synapse can result in excitotoxicity leading to neuronal cell damage. Glutamate excitotoxicity can be found in brain injury, hypoxia, ischemia, AD, Parkinson's disease, and Huntington's disease [172-174]. It has been reported that A β peptide can impair the expression and functions of glutamate receptors [175, 176]. Excessive activation of NMDA receptors by A β peptide has been reported in *in vivo* study [177]. A β peptide can also affect the glutamate uptake by affecting glutamate transporter in glial cells [178]. Furthermore, when there is neuronal damage, glutamate release from damaged neurons would further increase its concentrations in synapses, forming a vicious cycle to cause neurodegeneration. Attenuation of glutamate excitotoxicity thus offers a good intervention to slow down the progression of AD.

The protective effects of *L. barbarum* against glutamate excitotoxicity have been investigated [45]. It has been shown that *L. barbarum* extract LBA can reduce glutamate-induced neuronal damage and its effects are comparable to memantine. It is also found that LBA reduces cellular damage-induced by NMDA, the agonist of NMDA glutamate receptor, suggesting that LBA may provide its protection through modulation NMDA receptor-mediated signaling pathways. The levels of glutamate-induced elevation of phospho-JNK have been found to be significantly lower in LBA treated groups. Although direct modulation of activities of glutamate receptors is a possible mode for reducing glutamate excitotoxicity, the various physiological functions that are also modulated by the receptors make it very difficult to synthesize a perfect drug that can only attenuate toxicity but not affecting the normal receptor functions. Therefore, it has been suggested that downstream signaling events rather than blockage of the receptors should be targeted [179]. The data in this study show that *L. barbarum* can modulate the downstream JNK signaling pathway, which supports an

idea that it can be a potential therapeutic candidate. All these direct effects are summarized in Figure 2.

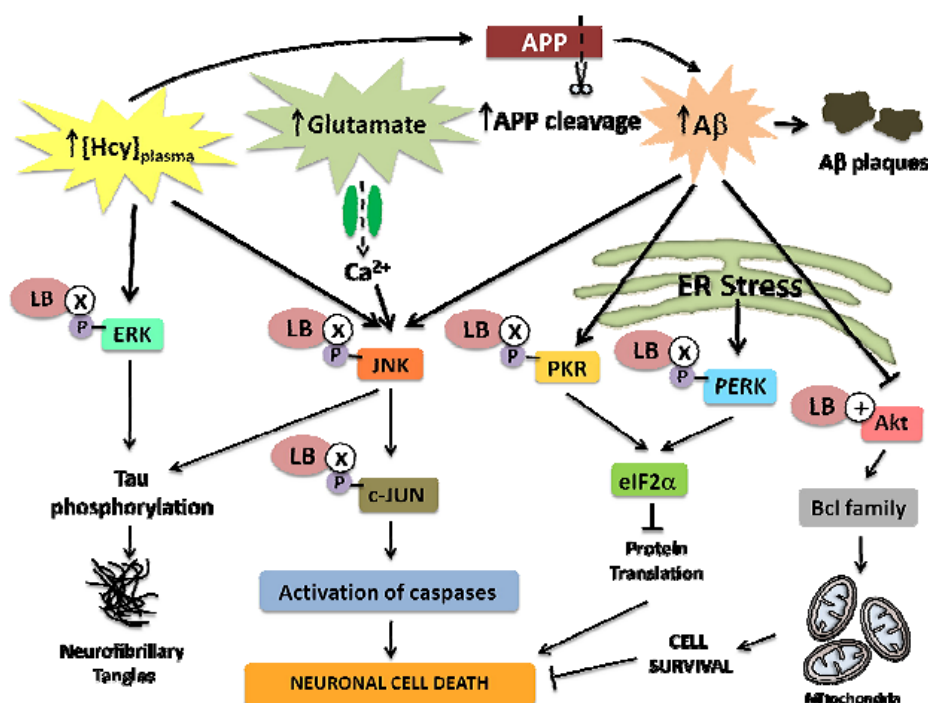


Figure 2. Schematic diagram showing the effects of *L. barbarum* on intracellular signaling pathways. *L. barbarum* (as indicated as LB in the diagram) can modulate the pro-apoptotic (JNK, ERK, PERK, PKR) and survival (Akt) pathways to protect neurons against different pathological damages.

Effects of *L. Barbarum* in Some AD Risk Factors

Besides the direct effects of *L. barbarum* on Aβ and glutamate neurotoxicity, which have been known for playing central roles in AD pathogenesis, it is also possible for the anti-aging herbs to preserve neurons indirectly on AD risk factors. It is well-known that medicinal herbs, which are unlike to orthodox medicine, have non-specific actions. In the past, these non-specific actions are also explained as its holistic properties. In another words, anti-aging herbs can modulate the functions or status of organs or systems other than the target pathological site to achieve therapeutic effects. With the advance in medicine especially in epidemiological studies, many diseases are actually proved to be multi-factorial and have a number of risk factors. Modulation of these risk factors can serve as possible disease-modifying approaches. Here we think that the effects of *L. barbarum* on homocysteine-induced neuropathology, diabetes and hypercholesterolaemia further support its multi-target hence holistic actions in AD intervention.

Elevation of plasma homocysteine levels, which is also called hyperhomocysteinaemia, has been shown to correlate directly to AD [180-182]. In The Hordaland Homocysteine Study, it is found that elderly who have memory deficit have higher plasma total Hcy levels in baseline and follow-up study [183]. Another study has shown that high Hcy and low folate may precede dementia onset, which may indicate a causal relation between Hcy and AD

[184]. The mechanisms that Hcy affects AD prevalence and memory decline have been explored. Homocysteine can induce direct cytotoxicity to neurons by inducing calcium influx through NMDA receptor activation [185]. Homocysteine can also sensitize neurons to A β toxicity [186] as well as increase APP cleavage and A β overproduction in animal hyperhomocysteinaemia models [187]. The effects of hyperhomocysteinaemia on tau pathology may also explain its relation with AD. Homocysteine can induce tau hyperphosphorylation, which can contribute to the formation of neurofibrillary tangles (another pathological hallmarks in AD) [188, 189]. Studies actually have shown that *L. barbarum* can attenuate homocysteine-induced neurotoxicity. *L. barbarum* can also significantly reduce the level of phospho-tau and cleavage of tau triggered by homocysteine. These studies show that *L. barbarum* has no effect on glycogen synthase kinase 3 beta (GSK-3 β) but it may provide its protection through modulating the activation of JNK and extracellular signal-regulated kinases (ERK). These kinases do not just take part the apoptotic pathways but they can also phosphorylate tau.

L. barbarum also has hypoglycemic effects which may allow it to help controlling of blood glucose level in diabetic patients. Diabetic mellitus has been identified as one of the risk factors in AD [190, 191]. A better control of blood glucose in AD patients who also has non-insulin dependent diabetes has proved to be beneficial to their cognitive performance as well as reducing the density of neuritic plaques in their brains [192, 193]. The exact pathological linkage between diabetes mellitus and AD has yet to be further explored. However, oxidative stress and the formation of advanced glycation end products (AGE) might be a common link between the two diseases. Receptor for AGE (RAGE), which mediate the effects of A β on neurons and microglia, is up-regulated in AD brain [194]. *L. barbarum* does not just simply provide hypoglycemic effects; it can also attenuate the oxidative damage in diabetes mellitus. The serum levels of malondialdehyde and nitric oxide in experimental diabetic rats fed with *L. barbaum* have been found to be significantly lower than the control group [41].

Apart from the effects on elevated blood glucose levels, *L. barbarum* also has hypolipidemic effects. Elevated plasma cholesterol levels are also considered as risk factor for AD. Cholesterol-rich diet can increase the levels of intracellular A β in animal models [195]. The effects of a cholesterol-lowering drug in reducing A β production in neurons [196] also support the hypothesis. Oral feeding of alloxan-induced diabetic or hyperlipidemic rabbits with *L. barbarum* extract can markedly reduce their serum total cholesterol and triglyceride level. The author suggested that both the vitamin and polysaccharides from *L. barbaum* might be the active components responsible for the effects [38]. It is also possible that the anti-oxidative properties of *L. barbarum*, as demonstrated in a number of studies [197-200], allow it to antagonize oxidative changes in diabetic and hyperlipidemic animals [41, 201].

Potential Use of *L. Barbarum* for the Management of Depression

Depression is one of the neuropsychiatric manifestations in AD. It is estimated that about 20 to 50% of AD patients also has depression. The pathological linkage between depression and AD is not very clear. One of the possible linkage of the two diseases might be hypothalamo-pituitary-adrenal (HPA)-axis hyperactivity, which can result in increased levels of cerebrospinal fluid (CSF) cortisol [202]. Although it is still unclear if this increased levels

of CSF cortisol has a direct effect on AD pathogenesis [202], it has been shown that elevation of plasma basal cortisol levels are correlated with reduction of hippocampal volume and cognitive decline in AD [203]. In animals, daily injection of corticosterone can elevate its plasma levels and induce depression-like behaviors. New findings from Zhang et al. actually show that orally feeding of LBP can attenuate the depression-like behavior in this experimental depression model. The corticosterone-treated rats with LBP feeding have shorter period of immobility in forced swimming test when compared with the control rats. Inhibition of neurogenesis by corticosterone is a possible explanation for the depression-like behavior. Data shows that orally feeding LBP reverses the inhibitory effect of corticosterone on the neurogenesis in the dentate gyrus [204]. Although it is too early to conclude that *L. barbarum* has a positive effect on depression, *in vivo* data demonstrate a great potential of it in depression therapy and this certainly deserve our further exploration.

5. A Short Summary on the “Holistic Approach” - The Intercellular and Intracellular Effects of Anti-Aging Herbs

We have used *L. barbarum* as an example to demonstrate the properties of anti-aging herbs. We propose that many of the scientific studies can, at least partly, explain the holistic concept of these anti-aging herbs. As illustrated in our previous sections, *L. barbarum* has multi-target and multi-disease stage intervention properties. These biological activities can be summarized into two aspects. The first one is the intercellular effects of *L. barbarum* on neurons. *L. barbarum* has immune modulation effects and it can activate microglia to an appropriate state (moderately activated) to protect RGCs against OH in glaucoma. It also suppresses the up-regulated GFAP immunoreactivity in the OH retinas [101]. This suggests *L. barbarum* may be able to safeguard neurons through indirect effects on the surrounding micro-environment. The second aspect is the intracellular effects of *L. barbarum* on neurons. *L. barbarum* is able to modulate a number of intracellular signaling pathways to reduce different pathological damages. It modulates both survival and pro-apoptotic pathways to maintain homeostasis of our body under stress conditions. It can up-regulate the expression of β B2-crystallin, IGF-1 and IL-10R on the RGC [69]; it can enhance the phosphorylation of Akt in cortical neurons treated with A β peptide [43]. It also attenuates neuronal apoptosis and damages through suppressing the phosphorylation of JNK, ERK, PKR and PERK [37;44-46;152]. All these lines of evidence suggest that *L. barbarum* utilizes multiple mechanisms for its protective effects. Interestingly, the activation of many of the survival and apoptotic signals are not unique in a particular pathological condition. They are in fact common pathways in various pathological insults. The ability of *L. barbarum* to interfere these pathways may explain why it can provide a broad-spectrum of protection, and this is the holistic approach of anti-aging herbs.

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Chapter VII

Beneficial Effect of Neem on Human Health

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Abstract

Background: The existing knowledge of ethno pharmacology using plant extracts or phytotherapy is a potential source to identify and characterize novel lead molecules for therapeutic applications and to overcome the limitations of existing drugs. Neem (*Azadirachta indica*), ‘The greatest Boon of the Century’, as mentioned by UN (1968), is an important medicinal plant ever since the ancient Indian culture even to this era of globalization. It made a journey from being a ‘native folklore’ to the status of being a real panacea’.

Material and Methods: Potential therapeutic qualities have been identified from the various pharmacological constituents obtained from different parts of the Neem as evident from preclinical studies and limited clinical trials. The Neem is traditionally used as antiviral, antifungal, antimicrobial, antibacterial, antipyretic, anti-inflammatory, anti-tumor, analgesic, immune stimulation, anthelmintic and anti-emetic.

Results: Neem leaf and bark extracts can scavenge hydroxyl radical (·OH) and offer gastroprotective effect by preventing oxidative stress-mediated gastric mucosal cell apoptosis.. The Neem limonoids showed potential chemopreventive activity against both human and animal cancer cell lines. Neem extract helps body to fight against infection by boosting both the cell mediated and lymphocytic immune system. Neem based tablets not only lowered blood glucose level significantly but also increased the effectiveness of the well known hypoglycemic drugs when taken together. The Neem leaf extract showed antiplaque activity and reduced average bacterial count in saliva. Emphasis is given on the Neem derived triterpenes targeting lipid rafts/caveolae of the plasma membrane to prevent HIV, as it is widely accepted that HIV-1 enters into and buds out from these lipid

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rafts. Neem extract prevented AIDS virus from infecting cells although the detail mechanism is still unknown. The oxidative stress leads to various pathological conditions including cardiovascular dysfunction, neurodegenerative diseases, gastroduodenal pathogenesis, cancer and premature aging. Neem has the inherent capability of preventing oxidative stress by scavenging $\cdot\text{OH}$.

Conclusion: Therefore, identification of compound(s) responsible for $\cdot\text{OH}$ scavenging activity, a new challenge, may reveal lead molecule for treating oxidative stress-mediated diseases like Alzheimer, Parkinson and Huntington. In spite of various medicinal values, Neem has not been considered as a major drug due to the lack of clinical studies and perfect characterization of different active principles. Here we will try to report the beneficial role of Neem on human health and the underlying mechanism of antioxidant action and signal transduction pathway explored hitherto.

Abbreviations

ROS	Reactive oxygen species;
NF- κ B	Nuclear factor- κ B;
ETC	Electron transport chain;
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced);
PCNA	Prolifrating cell nuclear antigen;
NBE	Neem bark extract;
$\cdot\text{OH}$	Hydroxyl radical;

1. Introduction

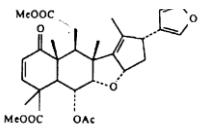
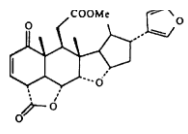
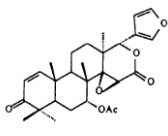
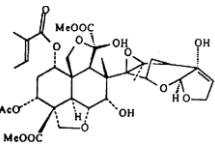
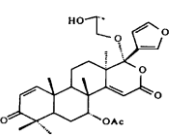
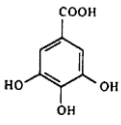
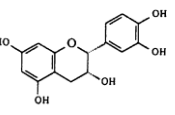
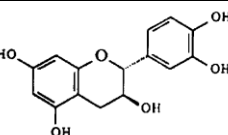
Neem (*Azadirachta indica* A. Jussieu, Meliaceae) is an evergreen tree in the Indian subcontinent and Southeast Asian countries. Neem survives primarily in tropical climates with annual rainfall of 400-800 mm [1]. Every part of this versatile plant deserves importance for its beneficial effects on human health. The existence of Neem in the Indian subcontinent is evident since the era of the proto-australoid and proto-dravidian culture of Mohandjodaro and Harappa (2000 BC). The Neem has a genetic chromosome number of $n=14$ and somatic $2n=28$. $2n=30$ has been reported from the root tip mitosis. Beside the *Azadirachta indica* other species include *A. excelsa* (Philippine Neem) and *A. siamensis* (Thai Neem) [2]. *A. indica* A.Juss and *M. azadarach* are very closely related species of Meliaceae. The former is the Indian Neem or Indian lilac and the latter is the Persian lilac. Since the early report of isolation of nimbin by Siddiqui [3] in 1942 about 300 bioactive phytochemicals have been isolated from Neem [4]. A large number of herbal products of Neem are already commercially available which includes Neem based toothpaste, skin lotion, Neem face wash, mouthwash, Neem cake etc. Neem has a wide range of medicinal property as it is used against multiple health problems as antiviral, antifungal, antimicrobial, antibacterial, antipyretic, anti-inflammatory, anti-tumor, analgesic anthelmintic and anti-emetic agent [5-7]. The leaves are considered beneficial in the treatment of skin diseases and anorexia and the fruits are used as emollients and purgatives, and are effective in the treatment of intestinal urinary diseases, worms and piles [8]. Various parts of Neem have also been reported to have *in vivo* anti-inflammatory and antipyretic effects [9]. *In vitro* studies show that the bark extract possesses

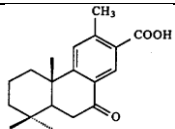
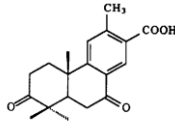
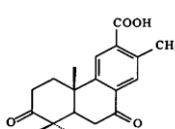
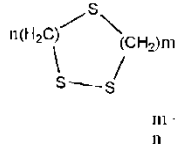
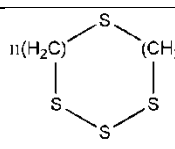
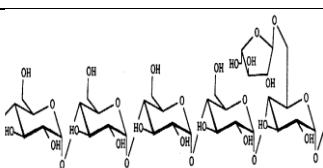
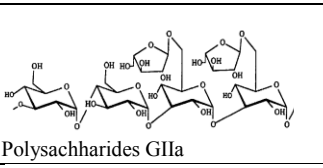
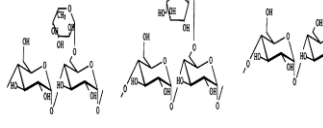
antiinflammatory [10] and immunomodulatory [11] activities. Neem leaf shows insecticidal, insect repellent, antituberculous and antiseptic activities [12]. Studies also reveal that the leaf extract has anticancer [13], antiviral [14] and immunomodulating [9, 15] activities *in vitro* and antimalarial [16], hepatoprotective and hypoglycaemic [17] activities *in vivo*. Both Neem bark and leaf contains several compounds, some of which (such as, nimbin, sitosterol, and nimbinene) are present in both the bark and leaf and some are exclusively present in the bark or the leaf [18]. The US National Academy of Sciences also recognized the importance of Neem and published a report in 1992 entitled 'Neem-a tree for solving global problems'. Here we will try to emphasize the effects of different Neem compounds and crude extracts on human health with special attention to their mode of action and signaling pathways.

2. Neem: Some Compounds, Structures and Medicinal Properties in General

The leaves, bark, fruit, oil and root of Neem tree have been used extensively in traditional medicine to treat various human health problems [19-21]. Although various compounds have been isolated from Neem [18, 21, 22], the pharmacological actions of most of these compounds are still unknown. The biological activities of some of these compounds together with their structure are shown in Table 1. The compounds can be divided into two major classes: isoprenoids and others. The isoprenoids include diterpenoids and triterpenoids containing limoids, protomeliacins, azadirone and its derivatives, genudin and its derivatives, valsinin type compounds and C-secomeliacins such as salanin, nimbin, and azadirachtin. The nonisoprenoids include carbohydrates (polysaccharides), proteins (amino acids) and sulphurous compounds, polyphenolics such as flavonoids and their glycosides, coumarin and tannins, dihydrochalcone, aliphatic compounds, etc [5]. Few new compounds from Neem seeds have been identified namely azadirone-type (15-hydroxyazadiradione, 7-benzoyl-17-hydroxynimbocinol, 23-deoxyazadiranolide, limocin E, 23-epilimocin E) and one diterpenoid-type (7a-acetoxy-3-oxoisocopal-1,13-dien-15-oic acid) [23]. Compounds isolated from different parts of Neem have been attributed for wide biological and pharmacological activities [5-7, 24]. Nimbidin and sodium nimbidate possess significant dose dependent anti-inflammatory activity against carrageenin induced acute paw oedema in rats and formalin-induced arthritis [3, 25]. Antipyretic and hypoglycaemic activity has also been reported and confirmed in nimbidin [26, 27]. Nimbidin significantly showed antiulcer effect as it prevented acetyl salicylic acid, indomethacin, stress or serotonin-induced gastric lesions as well as histamine or cysteamine-induced duodenal ulcers [28, 29]. Nimbidin acts as an antihistamine by blocking H₂ receptors, thereby acting as an antiulcer agent [30]. Nimbidin exhibited antifungal activity by inhibiting the growth of *Tinea rubrum* [31]. *In vitro*, it can inhibit *Mycobacterium tuberculosis* growth and was found to be bactericidal [31]. The spermicidal activity of sodium nimbidinate, nimbidin and nimbin was reported in human and rats [5, 32]. Sodium nimbidinate showed diuretic activity in dogs [33]. Nimbolide prevents the growth of *Plasmodium falciparum* to show antimalarial activity [34, 35].

Table 1. Some bioactive compounds from Neem

No.	Name and Structure	Source	Biological activities
1	 Nimbin	Seed oil	Sprmicidal
2	 Nimbolide	Seed oil	Anti-bacterial Anti-cancer Anti-malarial
3	 Gedunin	Seed oil	Anti-fungal Anti-malarial
4	 Azadirachtin	Seed	Anti-cancer Anti-malarial
5	 Mahmoodin	Seed oil	Anti-bacterial
6	 Gallic acid	Bark	Anti-inflammatory Immunomodulatory
7	 (-)-Epicatechin	Bark	Anti-inflammatory Immunomodulatory
8	 Catechin	Bark	Anti-inflammatory Immunomodulatory

No.	Name and Structure	Source	Biological activities
9	 Margolone	Bark	Anti-bacterial
10	 Margolonone	Bark	Anti-bacterial
11	 Isomargolone	Bark	Anti-bacterial
12	 Cyclic trisulphide	Leaf	Anti-fungal
13	 Cyclic tetrasulphide	Leaf	Anti-fungal
14	 Polysaccharides GIa	Bark	Anti-tumor
15	 Polysaccharides GIb	Bark	Anti-inflammatory
16	 Polysaccharides GIII	Bark	Anti-inflammatory

Nimbolide also shows antibacterial activity against *S. aureus* and *S. coagulase* [36]. Gedunin, isolated from Neem seed oil possesses both antimalarial and antifungal activities [37]. Azadirachtin, isolated from Neem seed, have strong antifeedant and antimalarial property as well [38-40]. Mahmoodin, isolated from seed oil, a deoxygedunin exhibits moderate antibacterial action against some strains of human pathogenic bacteria [41]. Gallic acid, (+) gallo catechin, (–) epicatechin, (+) catechin and epigallocatechin, of which gallic acid, (–) epicatechin and catechin, derivatives of tannins from bark, inhibited oxidative burst of polymorphonuclear neutrophil (PMN) during inflammation [11]. Three tricyclic, diterpenoids, isomargolonone, margolone and margolonone isolated from Neem stem bark are found to be active against, *Serratia* sp., *Klebsiella* sp. and *Staphylococcus* sp. [42]. The polysaccharides from Neem exhibit various biological effects. A polysaccharide extracted from bark inhibits carrageenin-induced inflammation in mice [5]. Two water-soluble polysaccharides GIa and GIb when administered in mice for four days at a daily dose of 50 mg/kg from 24 h after subcutaneous inoculation of Sarcoma-180 cells showed strong antitumour effect [43]. Two more polysaccharides GIIa and GIIIa also showed significant anti-inflammatory effect on carrageenin-induced edema in mice [44]. Two polymers were also isolated from an aqueous extract of Neem bark. They possess anticomplement activity. Out of the two polymers a peptidoglycan of lower molecular weight, NB-II, was found to be more potent [45, 46]. Even in stress induced gastric lesions few active ingredients (phytosterol fraction) obtained from the lipid part of Neem fruits, exhibited antiulcer activity [47].

3. The Mechanism of Biological and Pharmacological Actions of Neem

Several preclinical studies are going on around the world with different Neem crude extracts and active principles. Majority of these studies are non-clinical using different animal models (*in vivo*) as well as human and non human cell lines (*in vitro*) indicating future prospects for human trials. Effective clinical trials are although not sufficient yet. Here, we will concentrate mainly on the anti-oxidant and anti-apoptotic property, gastroprotective activity, anti-inflammatory and anti-carcinogenic effect of Neem with mechanistic details and signaling pathways involved.

3.A. Antioxidant and Antiapoptotic Property of Neem

Generation of ROS in Cells

The electron transfer mechanism in the electron transport chain (ETC) of mitochondria is coupled with the energy deriving process for living organisms. During this procedure some amount (about 5% of the inhaled oxygen) of reactive oxygen species (ROS) is always produced which are taken care of by the inherent antioxidant system of the body [48]. ROS include superoxide anion radical ($O_2^{\cdot-}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), free radical form of nitric oxide ($\cdot NO$), peroxynitrite ($ONOO^-$) and the highly reactive hydroxyl radical ($\cdot OH$). $O_2^{\cdot-}$, a relatively stable intermediate, is produced due to one-electron reduction

of molecular oxygen. $O_2^{\bullet -}$ is considered as the precursor of most of the ROS [49, 50]. Electron leakage from the mitochondrial ETC to molecular oxygen leads to the generation of superoxide anion ($O_2^{\bullet -}$) (Figure 1). Complex I (NADH-ubiquinone oxidoreductase, C-I) is an integral inner membrane multi-protein complex exposed to both matrix and intermembrane space. It oxidizes NADH using coenzyme Q as electron acceptor in a reversible reaction coupled with proton pump generating transmembrane potential [51]. Reports regarding the number and site(s) of ROS production in Complex I vary. It may be located between flavin and the rotenone binding site. Flavin [52, 53] or a complex of half-reduced NAD[•] radical bound to the flavin [54] may also be the ROS producing site in Complex I. Studies by Herrero et al. indicated that the complex I oxygen radical generator may be located between the ferricyanide and the ubiquinone reduction site, making iron-sulfur centers possible candidates, although the unstable semiquinones may also play the same role [55]. Superoxide is produced from Complex I and mainly appears in the mitochondrial matrix. Complex II and IV do not contribute much in the production of $O_2^{\bullet -}$. Complex III (ubiquinol:cytochrome *c* oxidoreductase) can also generate considerable amount of $O_2^{\bullet -}$, especially when mitochondrial respiration is suppressed. $O_2^{\bullet -}$ produced at Complex III is found on both sides of the inner mitochondrial membrane (IMM) [56]. Subsequent dismutation of superoxide anion by Mn-dependent superoxide dismutase (MnSOD) generates H_2O_2 , which in turn can lead to the production of $\bullet OH$ (Figure 1 A). These oxygen radicals are normally inactivated by endogenous scavenger mechanisms present within the cell, such as antioxidant enzymes and lipid-soluble antioxidants, including vitamin E and reduced CoQ [57]. In addition to these toxic electron transport chain reactions of the inner mitochondrial membrane, the mitochondrial outer membrane enzyme monoamine oxidase (MAO) catalyzes the oxidative deamination of biogenic amines and is a quantitatively large source of H_2O_2 that contributes to an increase in the steady state concentrations of reactive species within both the mitochondrial matrix and cytosol [58]. $O_2^{\bullet -}$ may further interact with Fe^{2+} via the Fenton reaction and form the highly cytotoxic and reactive $\bullet OH$ [48]. The aconitase is very susceptible to $O_2^{\bullet -}$. $O_2^{\bullet -}$ attacks the aconitase which in turn releases iron from its Fe-S cluster forming the source of Fe^{2+} inside the cell [59] (Figure 1 A). Another valid source of iron may be the cytochrome C which releases its iron from its heme moiety in presence of H_2O_2 [60].

Besides ETC there are several other ways of generation of ROS in cells (Figure 1 B). ROS is deliberately generated in phagocytes like neutrophils and macrophages by an enzyme complex called the phagocyte NADPH oxidase (Phox). The oxidase consists of the catalytic subunit gp91phox (also called Nox2), together with the regulatory subunits p22phox, p47phox, p40phox, p67phox and the small GTPase RAC [61]. The enzyme activity of gp91phox is regulated by the assembly of these regulatory subunits with gp91phox to form an active complex [61]. The non-mitochondrial source of ROS in non-phagocytes is attributed to some flavoproteins similar to Nox2. Homology searches in human genome databases resulted in the discovery of six novel NADPH oxidase enzymes: Nox1, Nox3, Nox4, Nox5, Duox1 and Duox2 [62-64]. This enzyme complex uses electrons derived from intracellular NADPH to generate superoxide anion, which is further processed to form H_2O_2 and other ROS [65]. Another source of ROS is xanthine oxidase [66], which catalyses the oxidation of hypoxanthine to xanthine and then to uric acid. Molecular oxygen is the oxidant, and products include superoxide and H_2O_2 . This enzyme can also produce $\bullet NO$, suggesting that it might have a dual role in cell signaling. Cytochrome P450, which is involved in the five-electron oxidation of nitrogen, is known to uncouple from substrate oxidation under certain conditions

and leads to ROS generation. Levels of cytochrome P450 2E1 (CYP2E1), a member of the cytochrome P450 mixed-function oxidase system, are elevated under a variety of physiological and pathophysiological conditions, and after acute and chronic alcohol treatment. CYP2E1 is also an effective generator of ROS such as $O_2^{\cdot-}$ and H_2O_2 and in the presence of iron catalysts, produces powerful oxidants such as $\cdot OH$ [67]. Monoamine oxidases (MAOs) are mitochondrial enzymes that catabolize prohypertrophic neurotransmitters, such as norepinephrine and serotonin, generating hydrogen peroxide. Because excess reactive oxygen species and catecholamines are major contributors to the pathophysiology of congestive heart failure, MAOs could play an important role in this process [68].

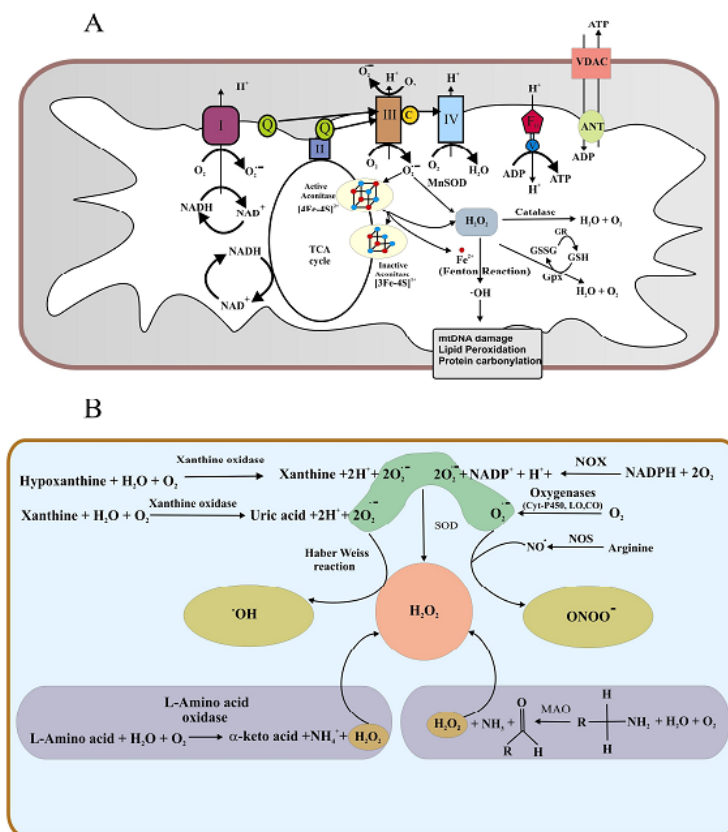


Figure 1. Generation of ROS by ETC- dependent and ETC- independent pathways. (A) In the mitochondria, superoxide is generated by the capture of electrons escaping from the electron transport chain by molecular oxygen. Superoxide is the precursor of most ROS and can be rapidly converted to H_2O_2 by superoxide dismutases (SOD). In the presence of transition metals (such as Fe^{2+}), formed from aconitase or cytochrome c, H_2O_2 can be converted to hydroxyl radicals ($HO\cdot$). H_2O_2 may be scavenged by glutathione peroxidase (GPx) in presence of GSH. The oxidised glutathione (GSSG) is reduced back to GSH by glutathione reductase (GR) in presence of NADPH. (B) ROS is also generated by other than ETC. $O_2^{\cdot-}$ is formed by NAD(P)H oxidases (NOX), xanthine oxidase and oxygenase like cytochrome P450, lipoxygenases (LO) and cyclooxygenases (CO). L-amino acid oxidase and monoamine oxidase (MAO) generates H_2O_2 by reducing O_2 while catalyzing their respective reactions.

Oxidative Stress and Antioxidants

Although ROS is essential for the organism's beneficial activities, including regulation of cell proliferation, intracellular signaling, phagocytosis, synthesis of biologically active compounds and energy but it develops oxidative stress by toxic insult when produced in excess [4]. Oxidative stress, in general, can be defined as a harmful condition in which the generation of ROS or pro-oxidants far exceeds its neutralization resulting in irreversible damage to the key components of the cell including DNA, proteins and lipids. This imbalance can either be due to a depletion of antioxidants, an overproduction of the ROS in the mitochondria or both. Oxidative stress has recently gained a lot of attention for playing a central role in the pathogenesis of various diseases which includes chronic inflammation, atherosclerosis, cancer, diabetes, gastropathy, cardiovascular diseases, neurodegenerative disorders, viral infection, and ischemia-reperfusion injury [49, 69, 70]. It is also involved in the process of aging by induction of a process named mitohormesis [71]. Understanding the signaling process leading to oxidative stress during disease process and targeting this mechanism or pathway will be realistic approach for therapeutic intervention. Thus the identification of competent and target oriented antioxidant is necessary for therapeutic purpose.

Antioxidants are mostly reducing agents which work by neutralizing the ROS by being oxidized themselves. On the basis of solubility antioxidants can be of two types, either soluble in water (hydrophilic) or in lipids (hydrophobic). In general, water-soluble antioxidants react with oxidants in the cell cytosol and the blood plasma, while lipid-soluble antioxidants protect cell membranes from lipid peroxidation. The term antioxidant is a misnomer because most antioxidants can turn into pro-oxidants under certain conditions like the concentration of the antioxidants, presence of some transition metals. This is a major problem with many antioxidants that are being synthesized artificially. Some of the well known natural antioxidants are glutathione (GSH), vitamins (C and E), melatonin, catalase, superoxide dismutase, thioredoxin and various peroxidases. The mitochondrial ROS generation is controlled by antioxidants present inside the mitochondria, such as MnSOD or SOD 2, thioredoxin 2 and GSH. The artificially synthesized spin trap α -phenyl-N-tert-butyl nitrone and $\cdot\text{OH}$ scavengers, dimethylsulfoxide and mannitol are well known antioxidants. The other major drawbacks are toxicity to the host and inability of many antioxidants to move inside the cell and mitochondria. Since mitochondria are the major site of ROS production, mitochondrially targeted antioxidants can be an effective tool for ROS induced diseases. The mitochondria-targeted antioxidant S(-) enantiomer of pramipexole is a potent D_3/D_2 receptor agonist and used for therapy against Parkinson's disease [72]. The major limitations with some of the artificially synthesized antioxidants are their solubility in physiological conditions, and cell membrane permeability.

Neem and its Antioxidant and Antiapoptotic Properties

The leaf and bark fraction extracts of Neem were evaluated for their antioxidant activity. Antioxidant activity was determined by measuring $\cdot\text{OH}$ scavenging activity, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging property, DNA protection test, metal chelating and the inhibition of peroxidation [73]. $\cdot\text{OH}$ when generated by Fenton's reaction can break the intact supercoiled DNA into an open circular form compared with the untreated DNA. DNA treated with Neem (bark and leaf) fractions showed a protective effect on hydroxyl

radical induced DNA damage [73, 74]. Iron and copper (transition elements) with one or more unpaired electrons participate in electron transfer reactions and are powerful catalysts of oxidation reactions [75]. In the Fenton's reaction iron and copper can take part in the conversion of H_2O_2 to $\cdot OH$ and also in the decomposition of alkyl peroxides to the highly reactive alkoxy and hydroxyl radicals [76]. The Neem bark extract (NBE) showed a comparatively higher metal chelating ability than that of the leaf. The data indicate that the phenolics are responsible for the variation in the antioxidant activity of the plant [77]. Neem has been found to inactivate lipid free radicals and prevent hydroperoxides' decomposition into free radicals [78, 79] or chelate metal ions and protect against pathogens and predators [80]. An *in vitro* study demonstrated that the water fraction of the bark with higher phenolic compounds exhibited highest radical scavenging activity. In contrast to bark, leaf represented a poor scavenging activity. Even the bark and the leaf extract showed a good inhibition of lipid peroxidation proving Neem as a potent antioxidant [73, 81]. The antioxidant effect of Neem has been used as a treatment against various clinical conditions. Cerebral reperfusion injury enhances lipid peroxidation, SOD activity and fall in total sulphydryl content. Neem pretreatment (500 mg/kg/day x 7 days) attenuated the reperfusion induced effects and thus showed neuroprotective response. [82]. Neem effectively attenuated reactive changes in brain histology like perivascular lymphocytic infiltration, gliosis, cellular edema and recruitment of macrophages following extended hypoperfusion [82]. In the rat colon Neem also showed antioxidative effects on azoxymethane-induced preneoplastic lesions [83]. Hepato-protective efficacy of Neem was observed in 7,12-dimethylbenz(a)anthracene (DMBA) induced skin tumor bearing male Balb/c mice. Skin tumor is characterized by the liver injury as evident from a decreased hepatosomatic index and increased activities of the hepatic tissue injury marker enzymes, namely alkaline phosphatase, alanine aminotransferase and aspartate aminotransferase. Treatment with neem can reverse the above mentioned alterations in marker enzymes significantly in skin tumor bearing mouse showing a protective effect [84]. In preventing cancer development at extrahepatic sites Neem leaf has modulatory effects on hepatic and blood oxidant-antioxidant status [85]. Pretreatment of ethanolic Neem leaf extract at a dose (200 mg kg^{-1} body weight) for five consecutive days showed very promising protective effects against N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-induced oxidative stress in male Wistar rats [86]. Antioxidant properties of Neem leaf fractions may be responsible for modulating cell proliferation, angiogenesis and apoptosis in the hamster buccal pouch carcinogenesis model [87]. Ethanolic Neem leaf extract significantly prevented MNNG-induced genotoxicity and oxidative stress in male Swiss albino mice by augmenting host antioxidant defense mechanisms [88].

ROS can induce both mitochondrial and death receptor pathway of apoptosis. Mitochondria are the source and target of ROS. Mitochondrial oxidative stress causes the release of cytochrome C in the cytosol that activates caspases and leads to apoptosis. The mitochondrial pathway of apoptosis further involves the fall of mitochondrial membrane potential associated with the formation of permeability transition pores by the oligomerization of bax. The upregulation of the bax protein is connected with the downregulation of bcl-2 protein. In this situation antioxidants provided from outside can help body to cope with the oxidative stress and the subsequent apoptosis and restore its normal cellular functions. As Neem has been found to prevent oxidative stress, it is assumed that Neem may also prevent the oxidative stress induced cell apoptosis. Nonsteroidal anti-inflammatory drug (NSAID)-induced gastropathy involves oxidative stress mediated apoptosis. Omeprazole is a known drug

that prevents it by inhibiting both ROS mediated damage and apoptosis [89]. It is found that the antioxidant effect of bark and leaf extracts of Neem to block oxidative damage and gastric lesions are comparable with that of omeprazole. NBE scavenged the endogenous hydroxyl radical to block restraint-cold stress and indomethacin induced gastric ulcer [74]. NBE is found to be highly effective in preventing this apoptosis by blocking the decrease of mitochondrial membrane potential, and by inhibiting cytochrome-c release and caspase-3 activation [74, 90]. It is intriguing why crude Neem bark and leaf extracts are so effective in offering protection against oxidative stress and this stress mediated apoptosis. Antioxidant-antiapoptotic effect of Neem is probably due to the presence of a number of polyphenols in Neem. Polyphenols in Neem includes flavones, catechins, epi-gallocatechins, kaempferol-3-o-rutinoside, quercitin, quercitin rhamnoside, quercitrin, quercitrin iso, rhamnetin iso, rutin etc [24]. These polyphenol compounds are already reported to have antioxidant activities in various systems [91, 92]. Flavonoids are known to offer gastroprotection with increased prostaglandin synthesis [93], decreased histamine secretion from the mast cell [94] and by scavenging free radicals [95]. Quercitin has been found to prevent ethanol-induced gastric lesions involving its antioxidant and antihistaminic properties [96]. Critical analysis of the structure of the polyphenols unfolded many facts supporting their antioxidant function. Polyphenols are a group of highly hydroxylated phenolic compounds present in the extractive fraction of several plant materials. Polyphenols are generally good antioxidants and their antioxidant property is dependent on the structural conformation. It is known that greater the number of hydroxyl group greater is the antioxidant property [97]. The position of the hydroxyl group is another important factor for its antioxidant activity. An antioxidant is defined as something which significantly delays or prevents oxidation i.e. having the reducing and scavenging (of ROS) property. The reducing property depends on the position of the hydroxyl group in the flavonoid molecules (poly phenol). Literature shows the importance of the 3-hydroxy group in scavenging hypochlorite [98], peroxy radical [99], peroxy nitrite [100] and superoxide radical [101]. The o-dihydroxy substitution in the B ring of flavonoids also offers almost equal antioxidant potential as offered by the flavonoids containing 3-hydroxy group [100, 102]. Due to the presence of 3- hydroxyl group and 2,3 unsaturation in conjugation with a 4-oxo-functional group in the C ring of flavonoids it is capable of binding transition metal ions, such as iron and copper [103] to stop the Fenton's reaction and generation of hydroxyl radical. This structural configuration of flavonoids is also responsible for reducing capability. Due to the presence of 3-OH group and the 2,3 unsaturation in conjugation it can easily be oxidized into quinoloid structure and prevents the oxidation of the substrate. One of the important advantages of the presence of 3-OH group and 4-oxo moiety is the possibility of hydrogen bonding interactions between hydroxy group as donor and 4-oxo moiety as acceptor [104], which may influence the antioxidant activity.

3.B. Neem Protects Oxidative Stress-Mediated Gastroduodenal Damage

Oxidative damage is the cause of several diseases including the gastropathy. Gastric ulceration is a multifactorial process where inflammation, ROS-mediated oxidative damage, apoptosis and decreased gastroprotective factors including loss of cell proliferation are common. Although it is the disease of multifactorial origin, development of oxidative stress and augmentation of apoptotic cell death in the mucosal region are considered to be the major

etiological factors for the development of gastropathy (Figure 2) [105-107]. The potential of ROS in inducing apoptosis is also well established [108]. Although, it seems that the mechanism behind all the ulcerogen-induced gastropathy is varied but all are associated with the overproduction of free radicals and apoptosis as indicated by lipid peroxidation, depletion of antioxidants, mitochondrial dysfunction, DNA damage [89, 96, 109-115]. Migration of neutrophil to the site of gastropathy is already documented. Respiratory burst of neutrophil can generate ROS leading to gastropathy (Figure 2) [116, 117]. In both stress and indomethacin induced gastropathy the inactivation of gastric and glutathione peroxidases by the $\cdot\text{OH}$ have been demonstrated. Ischemia helps in the release of some redox-active metal ions (iron and copper) from their storage proteins, which may bind to a specific site of lipid, protein and DNA to catalyze generation of $\cdot\text{OH}$ from H_2O_2 causing oxidative damage of the molecules along with thiol depletion [118, 119]. In mitochondria, $\text{O}_2\cdot^-$ attacks the iron-sulfur center of aconitase and release iron, thus helps in generating $\cdot\text{OH}$ to cause oxidative damage of mitochondrial lipid and macromolecules [120-122]. In cultured gastric cells H_2O_2 causes injury through the involvement of iron and $\text{O}_2\cdot^-$, suggesting the role of $\cdot\text{OH}$ in the cell damage [123]. H_2O_2 can directly activate the parietal cell for acid secretion [124] thereby accelerating the mucosal damage by acid-peptic juice. H_2O_2 decreases prostaglandin level by inactivating prostaglandin synthetase [125], thereby affecting almost all aspects of gastroprotection [125-127]. H_2O_2 , through the involvement of transcription factors NF κ B and activator protein-1 (AP-1) regulate gene expression of various proteins such as TNF- α interleukins, collagenase, matrix metalloproteases etc and thus acting as a signaling messenger [128]. These proteins are involved in neutrophil accumulation and activation, inflammation and gastric mucosal damage [129-134]. Besides, H_2O_2 produced by the activated neutrophil also diffuses to the cell and adds up to increase the intracellular level of ROS to augment the gastric damage. Cells mainly undergo apoptosis through two main pathways, viz., mitochondrial (intrinsic pathway) and death receptor (extrinsic pathway) [135]. The mitochondrial pathway of apoptosis is initiated by the stimuli induced up-regulation of Bcl-2 family of pro-apoptotic proteins such as, Bax, Bak and/or down-regulation of antiapoptotic Bcl-2, BclxL [136, 137]. The death receptor pathway is initiated due to binding of death ligand to its receptor, leading to activation of caspase-8 [138, 139]. Activated caspase-8 in turn activates caspase-3 directly or can converge with the mitochondrial pathway through cleaving Bid. Truncated Bid translocates to mitochondria and participates in forming pores through which cytochrome c is released (Figure 2) [140]. Therefore, a portion of the death receptor activity is channeled through mitochondrial pathway [140]. Previous studies showed that NBE possesses significant antioxidant activity and inhibited development of oxidative stress in the gastric mucosa during pathogenesis of gastric ulcer [105].

The role of Neem in controlling gastroduodenal ulcer was first evident when nimbidine, a bitter compound from Neem seed oil was shown to block gastroduodenal lesions in several experimental animal models [141]. Both the bark and leaf extracts of Neem show antiulcer and gastroprotective activities against stress, NSAIDs and ethanol induced ulcer [74, 142-145]. Aqueous extracts of Neem leaves prevent mast cell degranulation and increase the amount of adherent gastric mucus [145]. Aqueous and ethanolic extracts, both showed antiulcer activity in different ulcer models. [146].

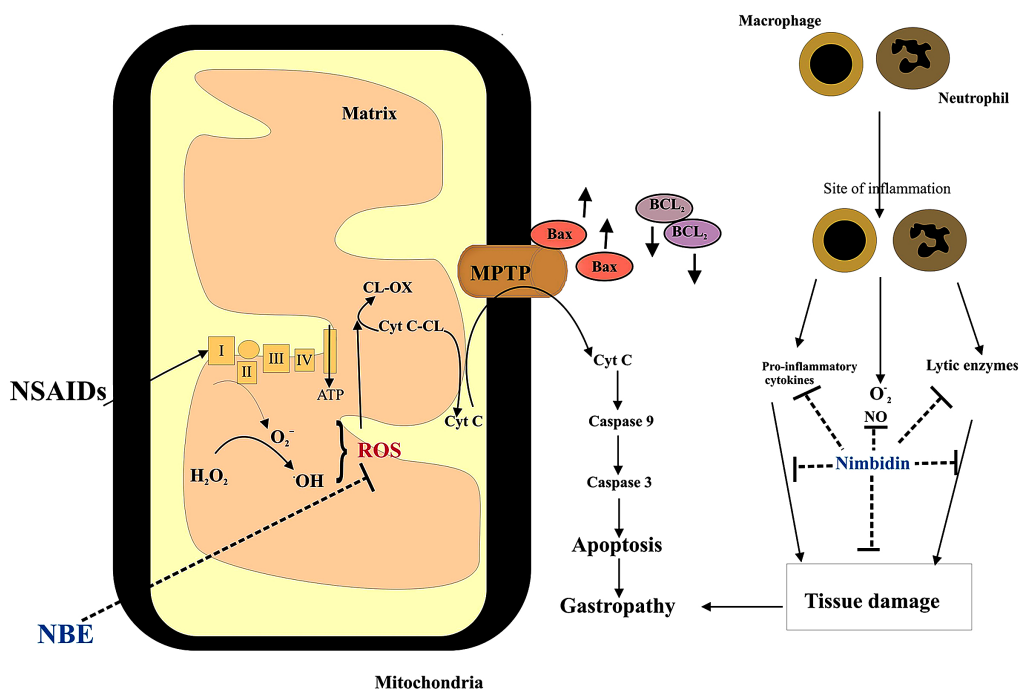


Figure 2. Scheme for NSAID-induced oxidative stress-mediated gastropathy and the effect of Neem on gastropathy. Neem compounds prevent oxidative stress, mitochondrial pathway of apoptosis and subsequent gastropathy. Infiltration of neutrophil to the site of inflammation also causes tissue damage and gastropathy. Nimbodin is supposed to prevent this pathway. NSAIDs = non steroidal anti-inflammatory drugs, ETC= electron transport chain, MPTP = Mitochondrial permeability transition pore, cyt C = cytochrome C, CL= cardiolipin, CL-ox = oxidized cardiolipin, ↑ indicates upregulation, ↓ indicates downregulation, - - - - indicates inhibition.

The bark extract was also effective in inhibiting pylorus-ligation induced gastric damage in rats (85% inhibition at 20 mg/kg) and histamine-induced gastric lesions in guinea-pigs (80% inhibition at 40 mg/kg) [74]. The ethanol extract of Neem bark was also shown to block indomethacin induced gastric damage (250 mg/kg i.p. or 800 mg/kg orally), which was accompanied by a significant decrease in gastric acid secretion [147]. From both ED₈₀ and ED₅₀ values, the bioefficacy of bark extract was found to be more effective and more potent than leaf extract in blocking stress and indomethacin-induced gastric lesions. When compared with currently used antiulcer drugs bark extract was found to be more potent than ranitidine but as potent as omeprazole in blocking both stress and indomethacin-induced gastric ulcer. Limited clinical studies have been reported on the therapeutic potential of Neem bark extract in patients suffering from gastric acid-related problems and gastroduodenal ulcer [148]. Neem bark extract also protects the gastric mucosa against oxidative damage induced by stress, indomethacin and ethanol [105, 149]. Both lipid peroxidation and thiol depletion are prevented by the bark extract. Leaf extract is also effective in preventing lipid peroxidation in stress ulcer model [144] and is almost as effective as bark extract to prevent oxidative damage at the dose of 20 mg/kg. The $OH^{\cdot}$ -mediated oxidative damage of DNA isolated from rat or human gastric mucosa is also significantly protected by both bark and leaf extracts *in vitro* [105, 144]. Neem bark extract has anti-inflammatory effect and also inhibits activation of neutrophils [11]. These effects might contribute in preventing inflammation and activated neutrophil-mediated damage observed during ulceration. Moreover, induction of TNF- α and

release of cytochrome c to activate caspase-3-like proteases are involved in apoptotic cell death in indomethacin-induced gastric ulcer [113, 130]. By holding back the TNF- α induced biological response Neem may contribute in preventing gastric mucosal damage.

Both Neem bark and leaf extracts are rich in flavonoids, flavonoglycosides, polyphenolics, tannins etc [22] and it is known that flavonoids offer gastroprotection via increased prostaglandin synthesis [93], decreased histamine secretion from mast cells [94] and by scavenging free radicals [24, 150]. Increased prostaglandin formation and decreased histamine level could prevent the development of ischemia and ROS associated oxidative damage. A phenolic glycoside having antisecretory and antiulcer property, isolated from NBE has been shown to be a potent inhibitor of H⁺-K⁺-ATPase [142]. It is highly effective in inhibiting both pylorus-ligation and mercaptomethylimidazole-induced acid secretion, and also offers gastroprotection against stress-induced ulcer. Other antiulcer compounds have also been isolated from Neem, and these include nimbidine [3, 28] and salanin [151] isolated from Neem seed oil; there is also an antiulcer phytosterol isolated from Neem fruit lipid fraction [47].

3.C. Anti-Inflammatory and Immunomodulatory Activities of Neem

Inflammation is the defensive mechanism of body against foreign substances and injury. The five well known cardinal signs of inflammation include redness (rubor), heat (calor), swelling (tumor), pain (pain) and loss of function (function laesa). Inflammation can be broadly classified as chronic and acute. Inflammation related health problems include arthritis, heart disease, irritable bowel syndrome, Alzheimer's disease, Parkinson's disease, and many others. The proinflammatory molecules are responsible for inducing the inflammatory response of the body (Figure 3). The proinflammatory cytokines include tumor necrosis factor alpha (TNF- α) and interleukins. Neutrophils and macrophages are the two major inflammatory cells. An inflammatory response involves the migration of these cells to the site of inflammation where they destroy the harmful foreign particles. Sustained activation of macrophages and neutrophils release large amount of bioactive substances such as prostaglandins, pro-inflammatory cytokines such as interleukin-1 (IL-1), oxidants such as nitric oxide (NO) and superoxide radical (O₂^{•-}) and the lytic enzymes. These factors are mostly attributed for the pathophysiology and associated tissue damage (Figure 3) [152-154]. Neem has been used traditionally in treating inflammation and inflammatory disorders. Studies revealed that nimbidin dose dependently inhibited chemotaxis, phagocytosis and stimulated respiratory burst, release of inflammatory mediators namely O₂^{•-}, NO, IL-1 and lytic enzymes. Nimbidin has been shown to inhibit O₂^{•-} production by interfering with components of NADPH oxidase. It further inhibits chemotaxis by inhibiting PGE₂. Release of lytic enzymes namely β -glucuronidase, myeloperoxidase and lysozyme by activated neutrophils is inhibited by nimbidin [155].

TNF α , a proinflammatory cytokine, plays a pivotal role in the signaling pathways of inflammation, apoptosis, and tumorigenesis [156]. TNF α exerts its effect via NF- κ B activation which enters the nucleus and up-regulates a number of proinflammatory cytokines, adhesion molecules and chemokines that augments the inflammation (Figure 3). TNF α binds to its transmembrane receptor TNFR which further transduces the signal through a number of pathways involving TNF receptor associated factor (TRAF) and NF- κ B for inflammation as

well as the FADD and caspase-8 of the death receptor pathway [157]. Azadirachtin have been reported to inhibit $\text{TNF}\alpha$ -induced biological responses.

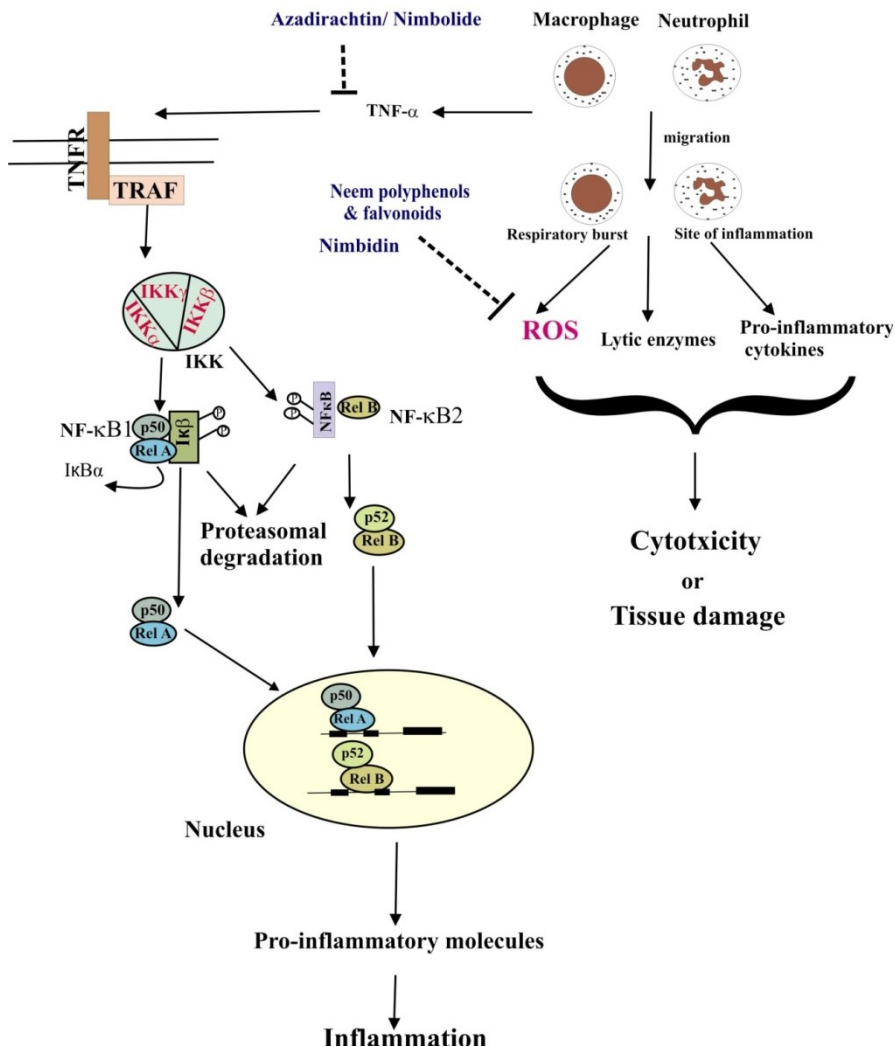


Figure 3. The role of neem compounds in exhibiting anti-inflammatory activity and preventing tissue damage. TNFR = receptor of TNF α , - - - | indicates inhibition.

The anti-inflammatory role of Neem is well known in traditional medicines. Carageenin or kaolin-induced paw edema has been found to be prevented by Neem [158]. A recent study reported that azadirachtin pretreatment inhibited TNF-mediated NF- κ B DNA binding and down-regulated the expression of adhesion molecules indicating its anti-inflammatory action. In mammals the NF- κ B transcription factor family consists of five proteins, p105/p50 (NF- κ B1), RelA (p65), and p100/52 (NF- κ B2) RelB, c-Rel, that associate with each other to form distinct transcriptionally active homo- and heterodimeric complexes [159]. NF- κ B activation needs sequential phosphorylation, ubiquitination, and degradation of I κ B α . Azadirachtin blocks activation of IKK which is followed by the degradation I κ B α . Further investigation reported that azadirachtin interacts with TNFRs and prevents TNF binding through

downstream signaling like ($\text{I}\kappa\text{B}\alpha$ kinase) IKK activation, $\text{I}\kappa\text{B}\alpha$ degradation, p53 nuclear translocation, and NF- κB -dependent gene transcription. Therefore, by blocking TNF receptor azadirachtin prevents TNF induced NF- κB activation and thus control inflammation (Figure 3) [160].

Several works have established the immunomodulatory effects of Neem oil [161, 162]. Immunomodulatory effects of Neem have been reported in a study that indicates that NIM-76 (a volatile fraction from Neem oil) activates macrophages and lymphocytes through a cell-mediated mechanism. This action of NIM-76 is concentration dependent. Enhanced macrophage activity and lymphocyte proliferation response were found at a dose of 120 mg/kg body weight. At higher dose (300 mg/ kg) macrophage activity remained unaltered while up-regulation of mitogen-induced lymphocyte proliferation was observed [162]. Neem leaf glycoprotein (NLGP) is found to maintain normal immune homeostasis effectively by up-regulating type 1 response in immunosuppressed hosts. This significantly adds to its host protective antitumor functions [163]. NLP enhanced the cytotoxic ability of natural killer (NK) cells against tumor cells and stimulate the release of IL-12 from macrophages from healthy individuals and head and neck squamous cell carcinoma patients [164]. Neem leaf preparation enhances Th1 type immune response and antitumor immunity against breast tumor associated antigen [165]. B16 melanoma cell surface antigen (B16MelSAg) is poorly immunogenic. But combined application of B16MelSAg with NLP in C57BL/6 mice enhanced the immunogenicity [166]. Neem leaf glycoprotein (NLGP) restricts the growth of mice tumors showing its immunomodulatory activity. Chemotaxis of cytotoxic cells to the site of tumor formation is necessary. This chemoattraction process is controlled by both CXCR3 (receptor) and the ligand CXCL10. The interaction between these receptor and ligand gets dysregulated in head and neck squamous cell carcinoma. NLGP is found to restore this dysregulation [167]. NLGP is associated with the activated cytotoxic T lymphocytes (CTL) and the natural NK cells-mediated killing of oral cancer cell (KB) and K562 (erythroleukemia) respectively. This is achieved by upregulation of perforin/ granzyme B expression in killer cells and by the activation of IFN γ [168].

3.D. Anti Carcinogenic and Chemopreventive Action of Neem

Cancer accounts for 7 million deaths annually worldwide. Increased cell proliferation and decreased apoptosis are associated with cancer. Inducing apoptosis in cancer cells will be a therapeutic strategy against cancer. The ability of medicinal plants in inducing apoptosis in the cancer cells as a chemopreventive strategy against carcinogenesis is gaining interest. Deregulation of the cell cycle, the occurrence of several harmful mutations and aberrant substratum attachment can make a cell unwanted for the body. Programmed cell death is the natural process of removing cancer cells. When the delicate balance between the cell proliferation and cell death gets disrupted cancer may be an inevitable event [169]. Proliferating cell nuclear antigen (PCNA), nicknamed ‘the ring master’, actively participates in a number of signaling pathways essential for the life and death of mammalian cell, is a good marker for the cell proliferation indicating cancer [170, 171]. p53 is the first tumor suppressor gene to be identified, preventing neoplastic development [172]. Mutations in p53 are responsible for carcinoma development [172]. p53 normally activates its down stream target p21 which consequently arrests cell cycle via downregulation of the cell cycle

regulators cyclin B cyclin D1. Azadirachtin and nimbolide induce apoptosis by both the mitochondrial and the death receptor pathway [173]. Increase in the ratio of Bcl-2 and bax is a characteristic of the cancer cells [174]. Therefore, activation of several proapoptotic factors and inhibition of the antiapoptotic factors are considered to be favorable targets for controlling cancer. Moreover, NF- κ B has been considered as a major culprit in cancer [175, 176]. A number of signaling pathways activate the NF- κ B modulated gene expression and malignant phenotype of cancer [177]. NF- κ B is activated by two pathways: in the first, the canonical pathway, the Rel A/p52 subunits enter the nucleus whereas in the second pathway Rel B/p52 gets the chance to enter the nucleus. Two catalytic subunits (IKK α and IKK β), and one regulatory subunit, (IKK γ) together comprises the inhibitor of κ B (I κ B) kinase (IKK) complex. The IKK β subunit is activated in response to stimuli such as tumour-necrosis factor- α (TNF- α), CD40 ligand (CD40L), interleukin-1 (IL-1) or lipopolysaccharide (LPS), and phosphorylates the I κ B proteins (bound to the NF- κ B heterodimers).

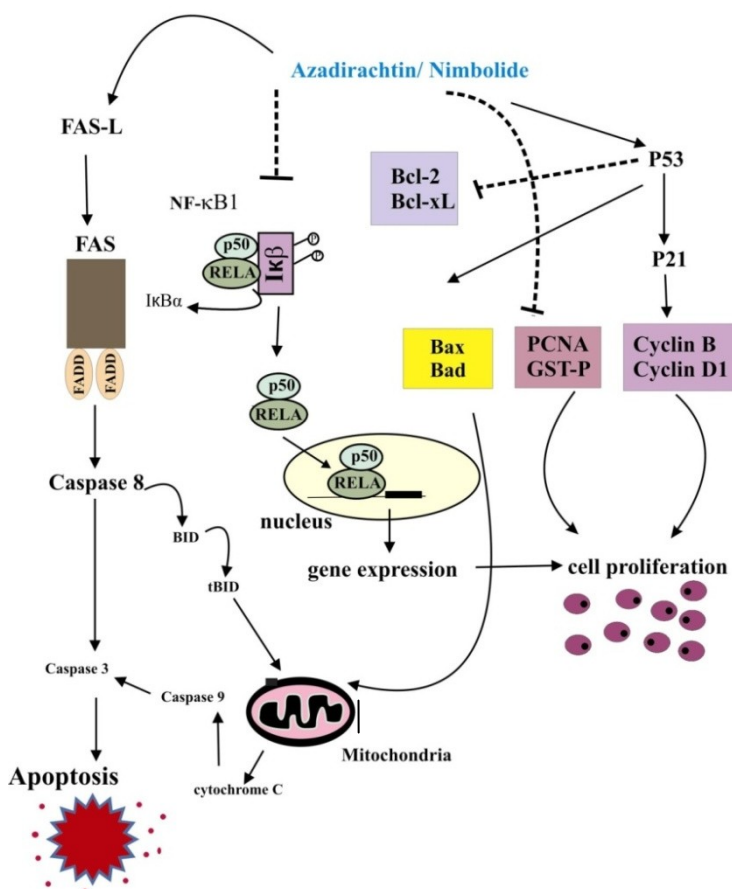


Figure 4. Anti carcinogenic and chemopreventive action of Neem compounds (modified from G. Harish Kumar et.al, 2009). PCNA = proliferating cell nuclear antigen, GST-P= glutathione S-transferase pi.

The consequence of this phosphorylation event triggers the 26S proteasome mediated degradation of I κ B followed by the nuclear translocation of RELA-p50 (or c-REL-p50) heterodimers and transcriptional activation of target genes. In response to other stimuli, such

as the TNF family members lymphotoxin B (LT β) and BAFF, IKK α is activated to induce the phosphorylation of p100 (bound to RELB). p100 undergoes ubiquitin-dependent degradation after this phosphorylation event. The released amino-terminal half, the p52 polypeptide, together with its heterodimer partner, RELB, translocates to the nucleus to activate transcription [175] (Figure 4). Considerable evidences are now available reporting the prevalence of sustained or constitutive activation of NF- κ B in cell lines and tumor tissue specimens. Thus NF- κ B involved pathway is an attractive target of research for cancer therapy.

Secondary metabolites of many plants have the potential to treat tumors as an alternative therapy. Neem acts as a double-edged sword. Along with its antioxidant and antiapoptotic role it has the potential of killing cancer cells by inducing apoptosis. Different chemical constituents of the different parts of Neem may be attributed for its variation in functions. Further in each case specific signaling pathways are involved. The cytotoxic effects of different Neem extracts have been already established in several reports. Neem limonoids have attracted ample research attention for their anticancer activity [173, 178]. To test the efficacy of putative chemopreventive agents the hamster buccal pouch (HBP) carcinogenesis model is the most popular [179]. Ethanolic Neem leaf extract (ENLE) protects N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-induced oxidative stress, genotoxicity and gastric carcinogenesis [88]. Crude Neem leaf extract reduces the incidence of 7, 12-dimethylbenz[a]anthracene (DMBA)-induced HBP carcinomas at a concentration lower than crude extract. HPLC analysis of the extract revealed the presence of nimbolide, nimbin, 2'3'-dehydrosalannol and quercetin in the CEE while EAF showed high concentration of nimbolide and quercetin. These phytochemicals may be attributed for the antioxidant and anticarcinogenic activities of Neem leaf. Down regulation of PCNA, glutathione S-transferase pi (GST-P), and NF- κ B and upregulation of caspase 3 represents the anti-proliferative properties of Neem preparations [87]. Neem limonoids including azadirachtin and nimbolide are found to be effective in inducing apoptosis and inhibiting cell proliferation in hamster buccal pouch carcinogenesis [173]. Analysing the expression and large number pro and anti cancer and apoptotic factors it is reported that azadirachtin and nimbolide mediate their anti-proliferative effects by down-regulating proteins involved in cell cycle progression and transducer apoptosis by both the intrinsic and extrinsic pathways. In limonoids treated DMBA induced HBP cancer cells downregulation of PCNA, GST-P, NF- κ B, Bcl₂, cyclin D₁, and enhanced expression of p21 and p53, bax and caspase 3 has been observed indicating the role of azadirachtin and nimbolide in cell cycle progression. Inhibition of PCNA and NF- κ B by the limonoids is considered to be the key factors for restraining the proliferation of HBP carcinomas and inducing apoptosis. The Neem nimbolide has higher efficacy than azadirachtin in chemoprevention which is achieved even at a very low dose of 10 μ g/kg body weight. This may be due to the presence of α,β -unsaturated ketone element in nimbolide. The PCNA is considered as a marker for cell proliferation [173]. The Neem bioflavonoid quercetin has been reported to inhibit tumor cell growth by down-regulating the expression of PCNA, NF- κ B and Bcl-2 and enhancing caspase-3 expression [180, 181]. Neem oil has been efficiently used to induce apoptosis in murine 3T6 fibroblast cells by counteracting and impairing PCNA [182]. Neem has efficiently controlled the prostate cancer, the most common cancer of men [183]. The ethanolic extract of Neem dose dependently reduced the Bcl-2 expression and increased the expression of Bax in the PC-3 (prostate cancer cell line) cells. Neem induced apoptosis in the PC-3 cells are evident by the DNA laddering [183].

Growth inhibitory role of nimbolide is established in a study on human colon carcinoma HT-29 cells. Cell cycle arrest and p21 are good targets for controlling cancer. Nimbolide, by blocking the cell cycle at the G2/M and G0/G1 phases in a time-dependent manner inhibited the growth of colon cancer cell line (HT-29), that is associated with the up-regulation of p21, cyclin D2 and Chk2 and down-regulation of cyclin A, cyclin E, Cdk2 [173]. Beside nimbolide, the other limonoid the azadirachtin, has also been found to arrest cell cycle together with actin depolarization and caspase independent apoptosis in *Drosophila melanogaster* [184]. For therapeutic use the toxicity of a compound is a major issue. Nimbolide was found to be not toxic to some experimental animals when given through the intragastric route [185]. Thus from the above discussion it is clear that different compounds are responsible for the cytotoxic and chemopreventive action of Neem in its therapeutic application against cancer, targeting multiple molecules responsible for cell proliferation and apoptosis. However, extensive pre clinical and clinical trials are necessary before introducing Neem compounds as drugs in the market.

3.E. Effect of Neem on Several Other Human Health Hazards/Diseases/ Pathology

The viral diseases are also a potential threat for human health. Several pre clinical studies depicted the Neem as a potential medicine against a number of viral diseases. A fractionated Neem-leaf extract having activities against malaria, HIV/AIDS and cancer has been developed into a drug and marketed in Nigeria as (IRACAP) [186]. NBE is found to have an antiviral effect against herpes simplex virus (HSV) type I infection. The virus causes a broad range of diseases including labial herpes, ocular keratitis, genital disease and encephalitis [187]. The study showed that 1000 µg/ml did not alter any cell viability, using Vero cells (kidney epithelial cell line of African green monkey). Preincubation of the HSV-1 virus with NBE significantly inhibited viral attachment and entry. NBE blocked the HSV-1 entry in both glycoprotein D receptor expressing CHO-K1 cells and the naturally susceptible cells such as HeLa, Vero and retinal pigment epithelial (RPE) cells even at a lower concentration (100 µg/ml). Interestingly cells preincubated with NBE had no or minor effect in the entry of HSV-1 while virus preincubated with NBE showed no entry into the cells. Further the experiments established that NBE significantly affected viral entry at the attachment step. It also prevented the HSV-1 glycoprotein-mediated cell-cell fusion and polykaryocytes formation [187]. Neem is a source of polyphenols, terpenes, flavonoids, glycoproteins. These phenolics, polyphenols, terpenes (e.g., mono-, di-, tri-), flavonoids, sugar-containing compounds, have been already reported to be promising anti-herpetic agents [188]. Dengue is another viral disease which can potentially infect 100 million people annually in tropical and subtropical countries of the globe. The currently available drugs have limited usefulness due to high toxicity against target cells. Herbal medicine thus can be the right alternative. The inhibitory effect of aqueous extract of Neem leaf (NL) on Dengue virus type-2 by suppressing the virus replication in both in vitro as well as in vivo systems has been reported. Human immunodeficiency virus (HIV), the cause of acquired immunodeficiency syndrome (AIDS) is a great danger to human civilization with its high death rate. According to the Avert (Avert is an international AIDS charity) website (www.avert.org) about 25 million people have died since 1981 of AIDS. 2 million died in 2008. In 2008 about 33.2 million people were living

with AIDS on earth. Moreover the cost of the treatment is still beyond the reach of a large number of people. Therefore, search for traditional herbal medicine will meet the need of a cost effective treatment. An acetone-water Neem leaf extract showed antiretroviral activity with a mechanism of action that may involve inhibition of cytoadhesion [189]. The lipid rafts/caveolae of plasma membranes of infected cells are the main entry and budding out sites of the HIV. The lipids play a crucial role in raft associated signaling. Modulating the composition/structure of lipid rafts can have some influence on the life cycle of HIV-1 inhibiting its replication. The multi-drug treatment against retrovirus has severe side effects. Therefore, studies concerning the consequence of dietary and plant-derived compounds having lipid rafts as their sole target could be the future strategy to check the devastating AIDS, as the plant derived compounds are with no side effects and can be used in a cost effective manner. Thus, Neem derived triterpenes targeting lipid rafts and its major component cholesterol may be a good strategy [190].

Diabetes mellitus is another highly complicated disease of human being. There is an increased focus on the plants having hypoglycemic/ antihyperglycemic agents. Streptozotocin (STZ) causes diabetes mellitus. Diabetes mellitus can further induce oxidative stress mediated tissue damage. Neem has been found to have anti-hyperglycemic effect [191-193]. Petroleum ether extracts of kernel and husk of Neem found to block oxidative stress in STZ- induced diabetes mellitus. The aqueous extract of Neem also has a good therapeutic potential as hypoglycemic agent [193]. Neem is not involved in modulating effect of insulin on glycogen metabolism rather it inhibits the inhibitory function of serotonin on insulin release in pancreas of rats mediated by glucose, eventually controlling glucose [192, 194].

Conclusion

Thus, it is evident from various preclinical studies that Neem has the potential for the discovery of novel drugs from its constituents against various diseases.

1. Most important aspect for future is to identify different active principles from Neem. Research should be directed to structure-functional analysis of those chemically characterized active principles along with the identification of suitable targets to exhibit their biological effects.
2. In the field of drug discovery the systems biology is gaining attention. Systems biology involves an integrated approach to study and understand the function of biological systems, ultimately to develop predictive models of human disease and can have an impact on drug discovery. Human diseases are complex phenomenon. This system biology approach will be helpful to design Neem based medicines containing a combination of molecules. This will lead to the standardization of different compounds of Neem with different percentage to formulate medicines with multiple targets.
3. Preclinical studies are not sufficient enough to claim a molecule to be a potent drug until a proper clinical trial or toxicity evaluation is conducted. Therefore, proper toxicity studies of molecules/compound identified from Neem and their clinical correlation are essential before making a drug/formulation for therapy.

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Chapter VIII

Beneficial Effects of Propolis on Human Health: Pharmacological and Molecular Aspects

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Abstract

Propolis is a natural resinous substance that is collected by honeybees *Apis mellifera* from various plant sources. Honeybee workers mix their own waxes and β -glucosidase with resins collected from plants, and use it as a sealant and antimicrobial agent to construct and protect their hives. Propolis has been used as a folk medicine since ancient times. Currently, it is used as an alternative medicine due to its broad spectrum of biological activities, such as antimicrobial, antioxidant, anti-inflammatory, anti-proliferative, immunomodulatory, and others. At present more than 300 constituents of propolis, mainly polyphenols (flavonoids, phenolic acids, esters, aldehydes and ketones) have been identified. Flavonoids have been suggested as the key compounds, contributing to its biological activities. The chemical composition of propolis varies considerably depending on the geographical and botanical origins, greatly influencing species and contents of flavonoids, thereby affecting the biological properties of propolis. This chapter describes the chemical composition, biological activities, and a potential molecular mechanism underlying the beneficial effects of propolis on human health.

Keywords: *Apis mellifera*, flavonoids, immunomodulatory, anti-oxidative, anti-inflammatory, antimicrobial, antiproliferative

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1. Introduction

Propolis has been used as a natural remedy to treat dermatoses, burns and a great variety of human diseases since ancient times [1,2,3]. Propolis is derived from two Greek words: "pro" means before or in front, and "polis" means "city" or "community". Chemically propolis is a complex resinous bee product, which is collected by honeybees *Apis mellifera* from buds, barks, and exudates of certain trees (such as poplars and conifers) and plants (such as *Clusia* and *Dalechampia*), and processed in the beehive in combination with products of bees salivary glands, wax and pollen [4]. Honeybees use propolis to seal holes in the hive, smooth out the internal walls, and shorten the hive entrance against harmful intruders, which help in the maintenance of the hive inner temperature $\sim 35^{\circ}\text{C}$. Furthermore, inhabitants in bee hives are protected from bacterial, fungal, and viral infections due to antimicrobial activity of propolis, thus it contributes to the attainment of an internal aseptic environment [5]. Now there is a substantial evidence that propolis exerts antimicrobial, anti-inflammatory, antioxidant, and immunomodulatory activities [5-10].

The color, consistency, and chemical composition of propolis vary according to the geographical region, botanical origin, and the season in which it is collected by honeybees. The biological activities of propolis also vary from one geographical region to other due to differences in its composition, making it difficult to determine its quality as the available chemical methods for propolis quality control are unsatisfactory [11]. The pharmacologically active constituents of propolis are chemical compounds, such as polyphenols (flavonoids, phenolic acids and their esters), terpenoids, steroids, and so on [10,12,13]. Among these compounds, flavonoids are main active propolis constituents that are responsible for large part of propolis's biological activity, therefore, total flavonoid contents are often used as an index for evaluating the quality of propolis. Propolis with the flavonoid content of less than 11% is considered as low quality, whereas propolis containing 11-17% or greater is referred as good quality, and high quality, respectively [11]. Based on the chemical diversity of propolis due to their plant source, a chemical profile has been suggested for "poplar" propolis, typical for the temperate zone, using following parameters: total flavone and flavonol content, total flavanone and dihydroflavonol content, and total phenolics content [14], however, a lot more work has to be done to standardize other propolis types.

Although, it is well known that propolis contains flavonoids, phenolic acids, and their esters, but the molecular mechanism associated with beneficial effects of its components in terms of signal transduction pathway still remains obscure. This chapter discusses the chemical composition, biological activities, and possibility of using propolis as a potential therapeutic agent along with proposed molecular mechanism underlying the flavonoid-mediated protective effects of propolis.

2. Chemical Composition of Propolis

The chemical composition of propolis is extremely complex due to differences in time, vegetation, and the area of collection [15,16]. In addition, phenological differences in plant chemistry may also account for divergences in the chemical composition of propolis [17]. Propolis is composed of resin (flavonoids and related phenolic acids), beeswax, essential and

aromatic oils, pollen, and other substances. Honeybees themselves produce the bee-wax and use it when constructing their combs, while resin and volatiles are obtained from plants. Minor constituents of propolis are pollen, and other substances such as vitamins and minerals. Typical raw propolis contains ~50% resins and vegetable balsams (R & B), 30% wax, 10% oils, 5% pollen, and 5% other substances (Figure 1A) [8,18]. Other substances include vitamins (B1, B2, B6, C, E), minerals (aluminum, Al; chromium, Cr; barium, Ba; cobalt, Co; calcium, Ca; copper, Cu; manganese, Mn; magnesium, Mg; nickel, Ni; iron, Fe; lead, Pb; silicon, Si; strontium, Sr; titanium, Ti; vanadium, V, and zinc, Zn (Figure 1B)) as well as a number of fatty acids [8,18,19]. Fe and Zn are important for the synthesis of collagen [6,20]. Propolis also contains some enzymes, including, succinic dehydrogenase, glucose-6-phosphatase, adenosine triphosphatase, and acid phosphatase [21].

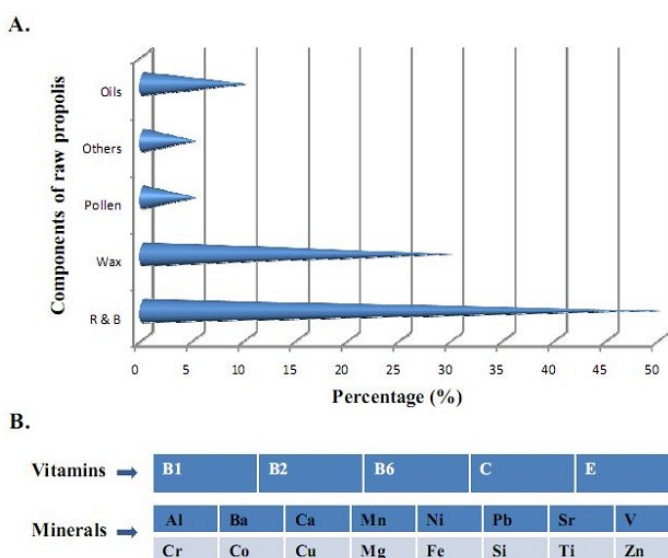


Figure 1. General composition of raw propolis. A. Propolis contains 50% resin and vegetable balsam (R & B), 30% bee-wax, and 10% essential and aromatic oils, 5% bee-pollen, and 5% other substances. Others substances include organic matter, vitamins and minerals. B. List of vitamins and minerals in propolis.

To date, more than 300 constituents of propolis have been identified and quantitated [6,8]. Major active constituents of the resinous fraction of propolis are phenolic compounds (flavonoids and phenolic acid and their esters, and phenyl propanoids (compounds derived from phenylalanine), constituting ~50% of its composition [22,23]. In addition, propolis also contains glucosidases (enzymes secreted by honeybees from their hypopharyngeal glands during the propolis collection), therefore flavonoids in propolis are aglycones, without sugar component. The main phenolic constituents of propolis are flavonoids, which contain a common phenylchromanone structure, containing at least one hydroxyl group substituent or a hydroxyl derivative, such as a methoxy group. Flavonoids are derived from 2-phenylchromen-4-one (bearing ring B at position 2 of the heterocyclic ring), whereas isoflavonoids and neoflavonoids are derived from 3-phenylchromen-4-one (bearing ring B at position 3) and 4-phenylcoumarine (bearing ring B at position 4), respectively (Figure 2).

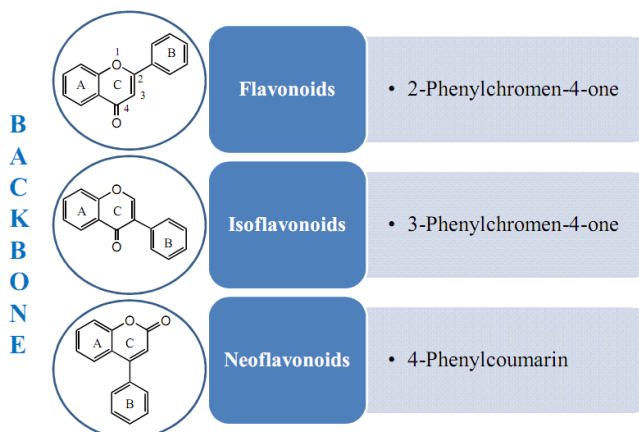


Figure 2. Structural backbone of lipophilic flavonoids, isoflavonoids, and neoflavonoids.

Flavonoids occur as glycosides, aglycones (in propolis), as well as in the form of methylated derivatives. As aglycone flavonoids, the benzene ring (A) is condensed with a six membered ring (C), carrying a phenyl ring (B) at position 2 [24,25]. Flavonoids are grouped into four major classes: (1) flavones (2-phenylchromen-4-one backbone; IUPAC name: 2-phenyl-1-benzopyran-4-one), (2) flavonols (3-hydroxyflavone backbone; IUPAC name: 3-hydroxy-2-phenylchromen-4-one), (3) flavanones (generally glycosylated at position 7 giving flavanone glycosides), and (4) flavanonols (3-hydroxyflavanone or 2,3-dihydroflavonol; IUPAC name: 3-hydroxy-2,3-dihydro-2-phenylchromen-4-one) (Figure 3). Flavonoids are often hydroxylated at various positions (3,5,7,2',3',4',5'), and glycosylated at positions 3 or 7, using carbohydrate such as L-rhamnose, D-glucose, glucorhamnose, galactose or arabinose [25,26]. The molecular structures of each class of flavonoids vary from the other by the presence or absence of hydroxyl group at position 3, replacement of the hydroxyl group at position 3 or 7 with sugar molecule, and reduction of C2-C3 carbon-carbon double bond (Figure3), and hydroxylation at various positions (as shown in Table 1).

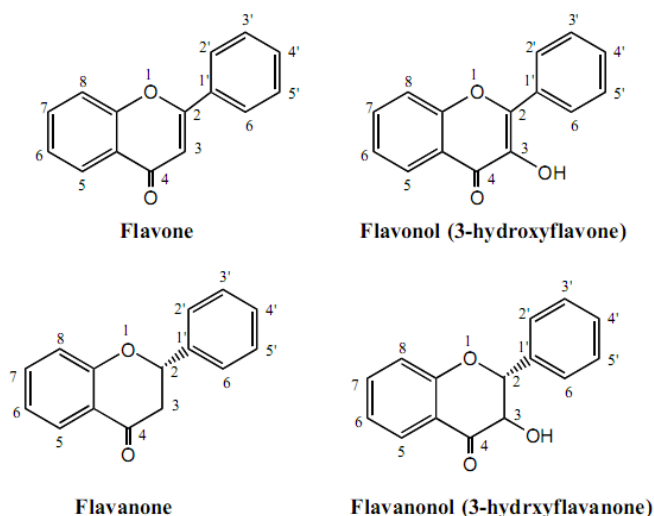


Figure 3. Structural backbone of flavone, flavonol, flavanone, and flavanonol.

Table1. Major classes of flavonoids

CLASSIFICATION	3	5	7	2'	3'	4'	5'
I. Flavones							
Chrysin	H	OH	OH	H	H	H	H
Apigenin	H	OH	OH	H	H	OH	H
Luteolin	H	OH	OH	H	OH	OH	H
Techtochrysin	H	OH	OCH3	H	H	H	H
II. Flavonols							
Kaempferol	OH	OH	OH	H	H	OH	H
Quercetin	OH	OH	OH	H	OH	OH	H
Galangin	OH	OH	OH	H	H	H	H
Myricetin	OH	OH	OH	H	OH	OH	OH
Fisetin	OH	H	OH	H	OH	OH	H
Morin	OH	OH	OH	OH	H	OH	H
Rutin	O-R	OH	OH	H	OH	OH	H
III. Flavanones							
Pinocembrin	H	OH	OH	H	H	H	H
Naringin	H	OH	O-R	H	H	OH	H
Naringenin	H	OH	OH	H	H	OH	H
Hesperitin	H	OH	OH	H	OH	OCH3	H
IV. Flavanonols							
Pinobanksin	OH	OH	OH	H	H	H	H
Taxifolin	OH	OH	OH	H	OH	OH	H
Aromadedrin	OH	OH	OH	H	H	OH	H

Taxifolin (dihydroquercetin), Aromadedrin (dihydrokaempferol). O-R represents flavonoid conjugated with a sugar molecule.

Major phenolic compounds of propolis include caffeic acid phenethyl ester (CAPE), propolins, galangin, kaempferol, quercetin, pinocembrin, pinostrobin, and pinobanksin. Other phenolics identified include quinic acid, ferulic acid, isoferulic acid, caffeic acid, cinnamic alcohol, cinnamic acid and vanillin [27]. Plants often synthesize O-linked or C-linked glycosides of these compounds, but in propolis, flavonoids occur as aglycones. Recently, for the first time, triterpenoids (belonging to oleanane, lupane, ursane, and lanostane skeletons) and polymethoxylated flavones or flavanones have been found in yellow Cuban propolis [28]. This group has classified yellow Cuban propolis samples into two type: (1) rich in triterpenic alcohols and with the presence of polymethoxylated flavonoids as minor constituents, and (2) containing acetyl triterpenes as the main constituents [28], not covered in this chapter. Although, so many factors affecting propolis chemical composition make its quality variable but the interest in propolis is mainly due to phenolic constituents (flavonoids) responsible for their valuable physiological activities, which can be responsible for the beneficial effects of propolis.

3. Biological/Pharmacological Properties of Propolis

Emerging evidence suggests that propolis is a natural medicine with multiple activities such as antimicrobial activity against a wide range of microorganisms (including bacteria, fungi and viruses), anti-oxidative, anti-inflammatory, immunomodulatory, anti-proliferative, anti-ulcer, hepatoprotective, cardioprotective, local anaesthetic, and other beneficial properties (Figure 4), but only few of them have been supported by clinical and experimental evidence [4,8,9]. These properties may markedly vary in each sample of propolis depending upon its polyphenolic constituents, which vary widely due to climate, season of collection, location and plant source [29-31].

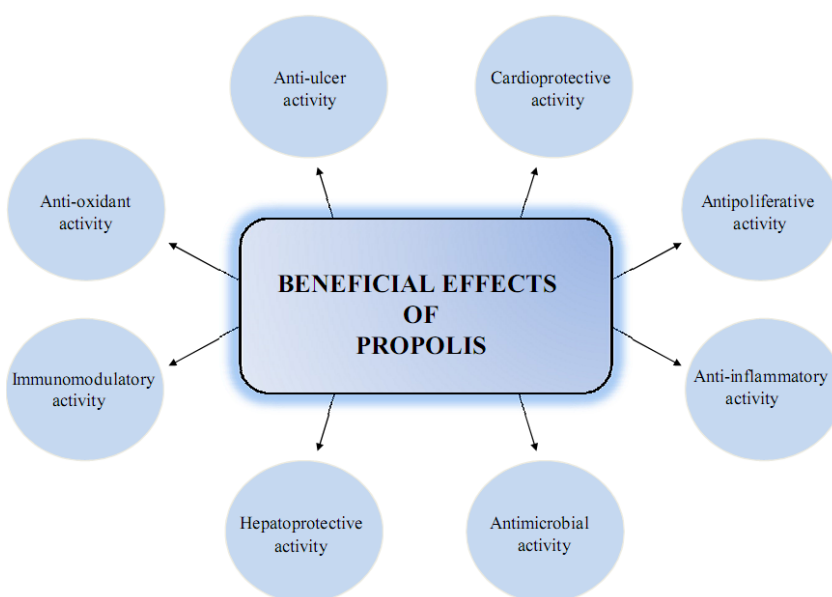


Figure 4. Beneficial effects of propolis.

3.1. Anti-Microbial Activity

Propolis exerts antimicrobial activity against a wide range of microorganisms (bacteria, fungi and viruses). Its strongest antibacterial activity against Gram-positive and limited action against Gram-negative bacteria may be due to richness in flavonoids [32-35]. Pinocembrin and galangin possess the antibacterial activity of propolis; pinocembrin also has fungicidal activity. The cytotoxic and anti-herpetic effects of propolis extract against anti-herpes simplex virus (HSV-1 and HSV-2) as well as anti-influenza virus further support its antimicrobial potential [36-39]. The antimicrobial properties of propolis, focusing on respiratory pathogens make propolis a valid therapeutic for treating the upper respiratory tract infections [4]. Flavonoid constituents (such as galangin, pinocembrin, and quercetin) and phenolic acids (e.g. caffeic acid, benzoic acid, cinnamic acid) of propolis probably act on the microbial membrane or cell wall site and cause functional and structural damages [6,40].

Propolis exerts mucoprotective properties for oral and gastric mucosa [41]. The administration of standardized propolis preparations of mouth rinse has been suggested to be more effective on oral microorganisms and non-cytotoxic to gingival fibroblasts [42]. Collective evidence suggests that antimicrobial effect of propolis [43-45] may not be based on individual constituent of propolis but rather a synergistic effect of flavonoids and other phenolic compounds.

3.2. Immunomodulatory Activity

Immunomodulatory action of propolis has been well documented, but the exact mechanism of propolis on immune pathway is still unclear. *In vitro* and *in vivo* assays demonstrate increased stimulatory action of propolis not only on the lytic activity of natural killer cells against tumor cells and increased antibody production, but also on inhibitory action on lymphoproliferation associated to its anti-inflammatory property [31]. Thus, propolis treatment activates macrophages [46,47] and stimulates antibody production in sheep red blood cells-immunized mice [48]. *In vitro* model of peripheral blood mononuclear cells-treated with mitogen phytohaemagglutinin (T cell mitogen) shows increased neopterin production, increased tryptophan degradation, and markedly enhanced cytokine release, whereas propolis treatment of T cell mitogen-treated cells suppresses these effects [49], supporting the immunomodulatory actions of propolis against stress-induced negative effects in various inflammatory diseases. Caffeic acid phenethyl ester (CAPE) mediates immunostimulatory effect by strengthening phagocyte activity, and thus increases the ratio of CD4/CD8T cells in mice [46,49]. CAPE exerts strong anti-inflammatory effects, cell suppression of T lymphocytes, and inhibition of store-operated Ca^{2+} entry channels and K^{+} channels, which may be another intriguing way of signals activating immune responses [50]. More immunostimulatory than antiplasmodial activity (antimalarial activity) in hydroalcoholic solution of propolis proved by the increase of IgG and the macrophage phagocytosis activity and capacity supports cell-mediated and humoral antibody mediated immune responses [51].

3.3. Anti-inflammatory Activity

Propolis exerts anti-inflammatory activity [52-54]. Chrysin and quercetin, two biologically active components of propolis, have been shown to significantly suppress the lipopolysaccharide (LPS)-induced cyclooxygenase-2 (COX-2) protein and its mRNA expression [55], and inhibit inducible nitric oxide synthase (iNOS) expression by decreasing nitric oxide production in endotoxin/cytokine-stimulated microglia [56], respectively. These results indicate that flavones (chrysin and quercetin) display similar anti-inflammatory activity via different mechanisms. Anti-inflammatory effect of CAPE is through its inhibition of LPS-induced up-regulation TNF- α and interleukin (IL)-8 production, resulting in the suppression of I- κ B- α degradation [57]. Low concentrations of CAPE (<1 μM) have been reported to suppress osteoclastogenesis and bone resorption through the inhibition of NF- κ B activation and nuclear factor of activated T cell activity [58], implying CAPE can be used as a potential treatment for osteolytic bone diseases. Propolis not only suppresses prostaglandin

and leukotriene generation, and lipoxygenase pathway of arachidonic acid metabolism [52] by inhibiting the expression and activities of cyclooxygenases and lipoxygenases activities, but also retards the gene expression of nitric oxide synthase [59,60]. Propolis also blocks tumor necrosis factor- α (TNF- α)-mediated NF- κ B activation, and reduces immune response in T cells [61,62], suggesting the synergistic effects of compounds present in the propolis.

3.4. Anti-oxidative Activity

Propolis extracts are rich in anti-oxidant compounds, including pinobanksin 3-acetate, naringenin, pinocembrin, chrysin, daidzein, quercetin 3',7-dimethyl ether, apigenin, and kaempferid, caffeic acid, coumaric acid, ferulic acid, cinnamic acid, aromadendrin-4'-methyl ether, isosakuranetin, drupanin, artepillin C, baccharin, and 2,2-dimethyl-6-carboxyethenyl-2H-1-benzopyran acid [63,64]. The position of the geranyl or prenyl groups on the flavonoid skeleton plays an important role in exhibiting anti-oxidant activity [65]. In addition, caffeoylquinic acid derivatives, artepillin C, and p-coumaric acid, and CAPE exert many neuroprotective effects, including anti-oxidative activity [66,67]. CAPE alone exhibits a higher anti-oxidant activity than galangin, confirming that the anti-oxidant activity of propolis depends on the presence of CAPE [67]. Brazilian green propolis and its main constituents attenuate the cell damage in rat retinal ganglion, and pheochromocytoma neuronal cells. Following oxygen-glucose deprivation/ reoxygenation-induced cell damage, the expression of anti-oxidant- and apoptosis-related genes (casein kinase 2 and Bcl-2-related ovarian killer protein) is modulated, but these damaging effects are normalized by propolis [66]. CAPE significantly inhibits the formation of reactive oxygen species (ROS) in the experimental autoimmune encephalomyelitis model system in rats, suggesting that CAPE exerts its anti-oxidant effect by inhibiting ROS production at the transcriptional level through the suppression of NF- κ B activation, and/or by directly inhibiting the catalytic activity of iNOS [67,68]. CAPE found in Chinese red propolis exhibits potent 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, stronger than that of ascorbic acid and trolox. Caffeic acid, a metabolite of caffeoylquinic acid, also exhibits powerful DPPH radical scavenging activity while quinic acid (another metabolite of caffeoylquinic acid) has no such activity. Therefore, it appears that CAPE and other derivatives of caffeoylquinic acid in propolis may have powerful scavenging radical activity that may be partly dependent on the nature of their caffeoyl groups [69]. However, flavones (e.g. luteolin and chrysin) and flavonols (e.g. quercetin) possess anti-oxidant activities, but may act by different mechanisms [30,70].

4. Molecular Mechanism of Propolis

Propolis exhibits many biological activities, which have been proven beneficial in bacterial infection, allergies, asthma, diabetes, hypertension, and many model systems of several human diseases. It is speculated that many therapeutic effects of propolis may mainly be associated with anti-oxidant, anti-inflammatory, and immunomodulatory properties. Mitochondrial dysfunction is the major source of ROS (O_2^- , H_2O_2 , and $\cdot OH$) production. Other mechanisms of ROS production include arachidonic acid cascade and activation of

NADPH oxidase [71,72,73] (Figure 5). In brain, ROS have been implicated in the pathophysiology of many chronic human diseases, including Alzheimer disease (AD), Parkinson disease (PD), and amyotrophic lateral sclerosis (ALS) [74]. In visceral tissues, ROS are closely associated with the pathogenesis of rheumatoid arthritis [75], systemic lupus erythematosus [76,77], sickle cell anemia [78], cardiovascular disease [79], and various forms of cancer and tumor formation [80-82]. Overproduction of ROS breaks DNA strands, induces oxidation of DNA bases, and facilitates the oxidation of lipids and proteins. These processes have been implicated in the mutagenicity and carcinogenicity.

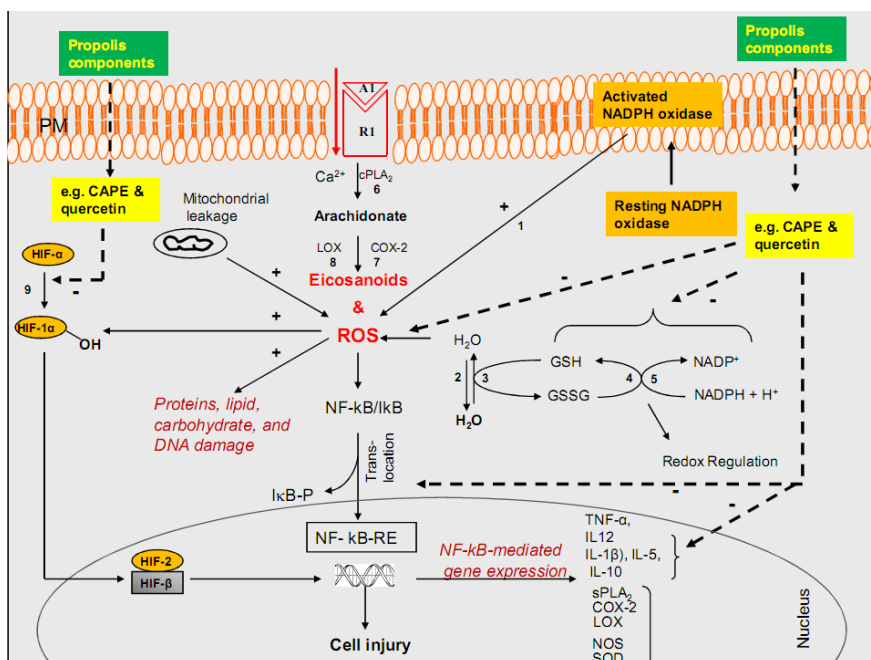


Figure 5. Hypothetical molecular mechanism underlying beneficial effects of propolis. Cellular injury involves the generation of ROS by multiple mechanisms, activation of NF-κB, alterations in redox status, and gene expression during oxidative stress. NADPH oxidase (1); superoxide dismutase (2) catalase (3); glutathione peroxidase (4); glutathione reductase (5); cytosolic phospholipase A₂, cPLA₂ (6); secretory phospholipase A₂ (sPLA₂); cyclooxygenase-2, COX-2 (7), lipoxygenase, LOX (8); superoxide dismutase (SOD); nitric oxide synthase (NOS); nitric oxide (NO); peroxy nitrite (OONO⁻); cytokines, TNF-α and IL-1β; reduced glutathione (GSH); oxidized glutathione (GSSG) and hydrogen peroxide (H₂O₂). Nuclear factor-κB (NF-κB); Nuclear factor-κB response element (NF-κB-RE). Stimulation of NF-κB by ROS results in its translocation in the nucleus, where it not only facilitates the transcription of sPLA₂, COX-2, NOS, and SOD, but also cytokines (TNF-α and IL-1β) in the nucleus. The expression of TNF-α and IL-1β up regulates activities of cPLA₂ and sPLA₂ through a positive loop type of mechanism in cytoplasm and neural membranes. Propolis inhibits the translocation of HIF-α to the nucleus. + indicates stimulation, - represents inhibition.

In addition to oxidative stress, the pathogenesis of chronic diseases also involves inflammation, a protective mechanism, which not only isolates the injured cells from uninjured cells, but also destroys damaged cells, and repairs the extracellular matrix. The main mediators of inflammatory reaction are macrophages in visceral organs and microglia in brain. Prostaglandins and platelet activating factor are major lipid mediators of inflammation [71,83,84]. They not only initiate the induction of inflammatory reaction, but also facilitate

the maintenance of inflammation. Lipoxin, another arachidonic acid-derived lipid mediator induces resolution of inflammation [83]. Although, the activation of macrophages in visceral organs and microglia in brain are hallmarks of pathology of visceral and neurodegenerative diseases, but it remains controversial whether macrophages or microglial cells have beneficial or detrimental functions in various pathological conditions. The chronic activation of microglia may cause neuronal damage through the release of cytotoxic molecules (Figure 5), including proinflammatory cytokines, eicosanoids, tumor necrosis factor- α (TNF- α), leukotrienes (IL-1 β , IL-5, IL-10), and complement proteins [83,84]. Very little is known about molecular mechanisms and internal and external factors that control and modulate the dynamics of acute and chronic inflammation in the body. In addition, receptors, like toll like receptors (TLR), and transcription factors such as peroxisome proliferator-activated receptor (PPAR), and NF- κ B also play an important role in modulation of inflammatory processes [84]. Components of propolis, such as caffeic, ferulic, *p*-coumaric and cinnamic acids, CAPE, quercetin, and chrysin block the generation of inflammatory mediators (eicosanoids and platelet activating factor) by inhibiting the expression of phospholipase A₂, cyclooxygenases, lipoxygenases, and nitric oxidase synthase [52-54]. Some components of propolis also inhibit stimulation of NF- κ B by blocking its translocation to the nucleus (Figure 5).

Age-related chronic diseases, such as rheumatoid arthritis, atherosclerosis, and cancer, are characterized by the presence of pockets of hypoxic cells growing behind aerobic cells and away from blood vessels. Hypoxic cell injury is caused by an uncontrolled growth and insufficient vascularization. Cells respond to hypoxic injury by stimulating the expression of hypoxia inducible factor (HIF), which is critical for survival under hypoxic conditions. HIF is a heterodimer consisting of the O₂-regulated subunit, HIF-1 α , and the constitutively expressed aryl hydrocarbon receptor nuclear translocator, HIF-1 β . Under hypoxic conditions, oxidative stress promotes the translocation of HIF-1 α to the nucleus where it interacts with HIF-1 β to form the complex (HIF-1 α + HIF-1 β) (Figure 5). The initiation of transcription is facilitated by the binding of the complex (HIF-1 α + HIF-1 β) to hypoxia responsive elements (HREs). This complex [(HIF-1 α + HIF-1 β) + HREs] stimulates the expression of genes involved in angiogenesis, vascular permeability, and inflammation [85,86,87]. CAPE, a major component of propolis, inhibits the translocation of HIF-1 α to the nucleus, therefore prevents the expression of genes responsible for the induction of inflammation.

Toll-like receptors (TLRs) are key receptors of the innate immune system. Microglial cells, the immune cells of brain, are activated in chronic diseases like AD and its mouse models. Microglia express the Toll-like receptor 2 (TLR2) [88]. A robust increase in the transcriptional expression of TLR2 may be related to inflammation during infection, brain injuries, and chronic brain diseases [89]. Although, propolis treatment does not antagonize the inhibitory effects on proinflammatory cytokine (IL-1 β and IL-6) production, however it partially restores the TLR2 mRNA expression and counteracts the inhibition on TLR-4 expression in infected or injured animals, suggesting that propolis modulates the mechanisms of innate immunity [90-93].

In conclusion, we propose that inhibition of ROS formation along with modulation of HIF-1 α , and TLR2 receptor by propolis and its components may provide a potential molecular basis for the protective actions of propolis in chronic diseases with inflammation and oxidative stress as major components.

5. Medicinal Dose of Propolis

A number of clinical trials have tested the use of propolis for treating various health problems, including common cold, dental health (dental caries, periodontal disease), rheumatic diseases, genital herpes, bacterial or parasitic infections, cold symptoms, burns, Legg-Calvé-Perthes disease and endometriosis (women infertility) [94-113]. However a wide range of doses has been used to treat these conditions (Table 2), indicating no proven effective dose of propolis has yet been established. Propolis is available in a wide variety of forms, which include tablets, capsules, powders and liquid extracts. It is also available as topical ointment, cream, lotion, and present in cosmetics, but whether the propolis concentration and constituents in these preparations closely resemble topical propolis products used in scientific research studies is not guaranteed. In one study, investigators have administered the natural propolis extract NIVCRISOL to a group of preschool children during the whole cold season to treat acute and chronic rhinopharyngitis, and reported the favorable effects of this local treatment [95]. In a randomized single-blind study (masked investigator) using 90 men/women with recurrent genital HSV type 2, the healing ability of a group in which subjects were treated by 3% Canadian propolis skin cream was compared with two other groups in which subjects used ointment containing acyclovir, and vehicle (placebo), respectively [104]. These investigators observed a higher % of individuals healed from the genital herpes in the propolis-treated group than the control or acyclovir-treated groups, suggesting a higher efficacy and more healing ability in the ointment containing natural flavonoids than both acyclovir and placebo ointments [104]. In another pilot randomized controlled trial, investigators compared the effect of propolis with placebo in the treatment of infertility associated with minimal or mild endometriosis, but this information is based only on published abstract [111]. These investigators showed a pregnancy rate of 60% by using 500 mg of propolis twice daily as compared to a pregnancy rate of 20% in the placebo group [111], without discussing the mechanism involved with this effect. Propolis has been shown to be considerably effective against microorganisms associated with dental cavities, gingivitis and periodontal disease in test tube studies [98,99]. However, Özcan et al. (2007) compared the effects of four different mouthrinses containing 10%, 5%, 2.5% and 1% propolis solutions with the mouthrinse containing 0.2% chlorhexidine (bactericidal) on oral microorganisms and human gingival fibroblasts, and reported that mouthrinses with these concentrations of propolis were not as effective in cytotoxicity as chlorhexidine [112]. Thus, standardized preparations of propolis at appropriate concentrations are needed to treat dental cavities, gingivitis and periodontal diseases. Furthermore, in an investigation involving 12 patients (35-62 years old) with chronic fungal sinusitis and its treatment with an alcohol-oil emulsion of propolis (2-4 ml) by introducing this preparation into the sinuses after irrigation with isotonic saline resulted in recovery of 9 and improvement in 3 patients [113]. Although, *in vitro* tests performed by this group reported that the fungus was sensitive to propolis in 8 cases, weakly sensitive in 2 and resistant in 2 [113]. The variability in the recovery of these patients may be due to dose-response of propolis among patients, which may vary depending on age, gender, race, or health of a patient. Although, collective evidence [94-113] shows reliable and relatively consistent scientific data regarding a substantial health benefit of propolis, but effectiveness and safety of its doses (Table 2) have to be further established. Therefore, additional research and clinical trials with larger group of subjects, proper dose

response, and different frequency of propolis administration are needed before a clear recommendation can be made about its role as a potential therapeutic agent.

Table 2. A wide range of propolis doses used in studies to treat various conditions

Dose used	Treatment	Reference
Aqueous propolis extract solution (NIVCRISOL)	Cutaneous herpes and herpes zoster	[94]
NIVCRISOL (0.5 ml propolis nasal spray)	Acute and chronic rhinopharyngitis in children under 18 years of age	[95]
500 mg Propolis XNP (500 mg for 13 days)	Prophylactic effectiveness of propolis for immunostimulation	[96]
5% Propolis in aqueous alcohol solution (mouth rinse)	Surgical wounds in mouth	[97]
1%-10% propolis mouth rinse	Dental cavities, gingivitis, periodontal disease	[98-100]
Aqueous propolis extract solution (NIVCRISOL)	Common cold	[101,102]
*10-30% Propolis	Intestinal parasitic infection	[103]
3% Propolis skin cream	Genital herpes (HSV)	[104]
5% Aqueous propolis solution as a dressing or douche for seven days	Vaginal parasites and acute cervicitis; chronic vaginitis	[105,106]
[▮] 30 % Ethanolic extract of propolis (27 mg propolis/well)	Against <i>Helicobacter pylori</i> strains <i>in vitro</i>	[107]
20 Drops of an alcoholic preparation of Brazilian green propolis (3 times a day/7 days)	<i>Helicobacter pylori</i> infection	[108]
500 mg Propolis capsule daily	Aphthous stomatitis (mouth ulcers)	[109]
Intra-articular injections of aqueous propolis extract (2 ml)	Legg-Calve-Perthes disease (avascular necrosis of the hip)	[110]
500 mg Propolis capsule (twice daily)	Mild endometriosis and infertility	[111]
Quapolis, a hot water extract of mulberry <i>Morus alba</i> leaves mixed with the ethanol extract of propolis (0.7 ml/meal = 0.705 mg of acarbose per day)	Type 2 diabetes	[112]
An alcohol-oil emulsion of propolis (2-4 ml)	Chronic fungal sinusitis	[113]

The doses listed in this table are based on scientific research publications. Effectiveness and safety of these doses shown in the table have not been established, and seems to depend on the preparation, use, and manufacturer. *Dose variation: in children (10% concentration) and in adults (20 and 30% concentration). [▮] Although the effect of propolis on *H. pylori* is promising *in vitro*, further clinical trials are required.

6. Toxic Effects of Propolis

Propolis is a potent sensitizer and is well recognized as a cause of occupational allergic eczematous contact dermatitis (such as perioral eczema and stomatitis) in apiarists [114]. Since than multiple cases of allergic reactions, including contact dermatitis, acute oral mucositis with ulceration, erythema, vesiculitis, and pruritis have also been reported with

propolis [115,116]. People who are allergic to pollen, honey, or conifer and poplar trees should not use propolis unless tested first by their physician. Effectiveness and safety of propolis have not been established during pregnancy and breast-feeding, therefore women should not use it during these times unless directed to do so by a physician. Due to high alcohol content in some propolis tinctures (15%-90%), they may result in vomiting when given in combination with drugs such as (disulfiram, metronidazole). A preventive effect of a propolis product (containing Echinacea (herb), propolis, and vitamin C) has been observed on the incidences of respiratory tract infections [117].

Since propolis is a potent sensitizer, therefore it should not be used in patients with an allergic predisposition, in particular an allergy to pollen. A significant association with balsam of Peru sensitivity has been reported in children reacting to propolis [118]. Therefore, propolis should not be used in topical products specifically for children because of its high sensitization rate at the paediatric age. Healthcare practitioners and public should be aware of the risk of allergic reactions to propolis. Although some allergic reactions with the use of propolis have been reported, but majority of studies indicate that it is relatively non-toxic. More clinical trials are required to investigate its toxicity and synergism between propolis and antimicrobial, as well as anti-inflammatory, antibiotic, anticancer, immunosuppressants, anticoagulants and anaesthetic agents.

Conclusion

Propolis, a resinous substance from various plant sources, is collected and manufactured by honeybees. Therefore it is a plant-derived substance as well as an animal product. Since the biological activity of propolis is assigned to plant-derived substances thus it is obviously a phytochemical. There are over 300 known substances in propolis, and it possesses a broad spectrum of biological activities, which are mainly due to few substances (such as flavonoids, terpenes, caffeic, ferulic, CAPE, and cumaric acids and esters). Although, propolis is becoming increasingly popular because of its biological properties, but in spite of its beneficial properties, its heterogeneous chemical composition creates a big problem in its use as a potential alternative medicine. The highly variable chemical composition, largely depending on factors like phytogeographical area of collection (time, origin, alternative resin plant sources, proper collection and handling techniques) and unsatisfactory quantification make it difficult to determine the quality control of propolis. It will also be difficult for practitioners to define the right dosage, and for distributors of propolis products to have any control on this challenging problem. New studies, using advanced techniques and more researches are needed to investigate and solve this problem. Some individuals show hypersensitivity to propolis therefore these individuals should be warned to avoid using propolis or its supplements. Furthermore, flavonoids in propolis may exert beneficial effects in the brain by protecting neurons against oxidative stress-induced injury, by reducing neuroinflammation, and improving cognitive function [119]. Water-soluble derivatives of propolis have been shown to inhibit scopolamine-induced learning and memory impairment in mice [120]. Therefore, there is a possibility that propolis extract may be used as a therapeutic agent in improving cognitive functions in neurodegenerative diseases, such as AD

and PD. Taken together, propolis indicates a relatively non-toxic profile and may prove to be a promising alternative medicine for the therapy of a wide spectrum of human diseases.

Conflict of Interest

Authors show that there is no conflict of interest.

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Chapter IX

Health Effects, Antioxidant Activity, and Sensory Properties of Virgin Olive Oil

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Abstract

Virgin olive oil (VOO) is a natural product obtained directly from olives without any further refining process. If extracted from fresh, healthy olives and properly processed and stored, it is characterized by unique sensory characteristics that are markedly different from those of other edible fats and oils. Many studies have been carried out to clarify the relationships between the sensory attributes in a VOO and its volatile and phenolic profiles, which are responsible for aroma and taste, respectively. Moreover, several lines of evidence suggest that VOO, a basic ingredient of the Mediterranean diet, can play an important role in the prevention of several immune and inflammatory and degenerative diseases. The health value of VOO has been attributed to its high levels of oleic acid and other minor components such as phytosterols, carotenoids, tocopherols, and in particular hydrophilic phenolic molecules. These natural antioxidants, both lipophilic (carotenoids and tocopherols) and hydrophilic (phenol and polyphenols) may exert an effective antiradical activity that is able to limit lipid oxidation. Its health value and pleasant flavour have contributed to an increase in VOO consumption, also outside the traditional olive-oil-producing regions of the Mediterranean basin. In this chapter, a survey on the main positive properties of the VOO will be made. Since hydrophilic

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phenolic molecules have been the subject of intense investigation in recent years, this review will focus on their specific health, antioxidant and sensory effects.

Abbreviations

ABTS	2,2'-azinobis(3-ethylbenzothiaziline-6-sulfonate)
AOM	Active Oxygen Method
COX	Cyclooxygenase
CUPRAC	Copper Reduction Assay
DPPH	2,2-diphenyl-1-picrylhydrazyl
FRAP	Ferric Reducing Antioxidant Power
HDL	High Density Lipoprotein
LDL	Low Density Lipoprotein
LO [•]	Alkoxyl Radical
LOO [•]	Peroxyl Radical
LOX	Lipoxygenase
MUFA	Monounsaturated Fat
OSI	Oxidative Stability Instrument
PAI-1	Plasminogen Activator Inhibitor Type I
PH	Phenolic Compounds
TBAR	Thiobarbituric Acid Reactive Substance
VOO	Virgin Olive Oil

1. Health Effects of Virgin Olive Oil

In 2004, an important international conference concerning the beneficial effects of virgin olive oil (VOO) was organized in Spain. The primary aim of this scientific meeting, which brought together the majority of researchers working on these aspects, was to summarize new findings in VOO, including fat and non-fat components. The results published one year later [1] support that a Mediterranean diet based in VOO is a good dietary model to achieve healthy aging and to prevent some of the most important causes of morbidity and mortality. Some of the most interesting points were as follows:

- A Mediterranean diet, rich in VOO, improves protection against major risk factors for cardiovascular disease, such as the lipoprotein profile, blood pressure, glucose metabolism and antithrombotic profile. Endothelial function, inflammation and oxidative stress are also positively modulated. Some of these effects are attributed to minor components of VOO. Therefore, the definition of the Mediterranean diet should include VOO.
- Several observational studies in humans have shown that the intake of monounsaturated fat may be protective against age-related cognitive decline and Alzheimer's disease.

- Microconstituents from VOO are bioavailable in humans, and have shown antioxidant properties and capacity to improve endothelial function. Furthermore, they are able to modify the haemostasis, showing antithrombotic properties.

1.A. Some Health Benefits Related to Monounsaturated Fatty Acids and Triterpenoid Compounds

Olive oil, which is particularly rich in monounsaturated fat (MUFA) due to a high percentage of oleic acid, improves postprandial lipoprotein metabolism inducing lower levels of postprandial triacylglycerols and higher HDL (high density lipoprotein) cholesterol. Low density lipoprotein (LDL) particles from individuals consuming a favourable MUFA pattern are protected from oxidative modification compared with individuals consuming diets rich in high polyunsaturated fatty acids (PUFA). A rich MUFA diet elicits a less prothrombotic environment by modifying different haemostatic components, such as platelet aggregation, fibrinogen, Von Willebrand factor, total plasma factor VII, tissue factor and PAI-1 (plasminogen activator inhibitor type I) plasma levels. The postprandial increase in activated factor VII is reduced by the intake of VOO in comparison with saturated fats [2].

Oleic acid is not exclusively accountable for the healthful properties of olive oil. In fact, it is also noteworthy that some seed oils obtained from genetic selection, such as high-oleic sunflower oil, can express a similar percentage of this monounsaturated fatty acid. Several investigations indicate that the minor components, either characteristic or particularly abundant in VOOs, such as phenols and squalene, may play a key role in atherosclerosis protection [3-4].

Squalene is a triterpenic hydrocarbon molecule that is an intermediate in sterol biosynthesis. Several studies have shown that squalene is able to contribute to the anti-tumoral activity of VOO, and also modulates cholesterol, triglyceride, glucose and leptin plasmatic levels [5-7]. In particular, Bellosta and co-workers have shown that squalene strongly inhibits the activity of beta-hydroxy-beta-methylglutaryl-CoA reductase, a key enzyme in cholesterol synthesis. Squalene has been also found to inhibit chemically-induced tumors in animal studies [8-9].

Farina and co-workers [10] focused on the interesting anti-ulcer properties of pentacyclic triterpenoids as the derivatives of oleanolic and ursolic acids, proposing the synthesis of new molecules practically devoid of mineralcorticoid activity and potentially non-toxic.

1.B. Some Health Benefits Related to Polar Phenolic Compounds

Pathologies such as cardiovascular disease and cancer have been linked to excessive free radical formation, and thus a higher intake of antioxidants appears to have beneficial effects on human health. Several scientific studies have suggested that phenol-rich olive oil could be particularly useful in contributing to the dietary intake of biologically active compounds [3]. The biological effects of VOO phenolics have been well detailed in a recent review [11].

It must be remembered that only VOO (according to the current regulations) contains a significant amount of polar phenolic compounds, as other oils have almost totally lost them as a consequence of the chemical-physical procedures employed during refining.

1.B.1. Phenolic Bioavailability

Important studies have been carried out to demonstrate the dose-dependent absorption of olive oil phenolics in humans and their urinary excretion as *O*-glucuronide conjugates [12-13]. Phenolic aglycones, such as secoiridoids, are subject to time-dependent hydrolysis in the acidic gastric environment, leading to an approximate 5-fold and 3-fold increase, respectively, in the amount of free hydroxytyrosol and tyrosol after only 30 min following assumption [14]. The major site for its absorption is the small intestine. Following ingestion of VOO, the levels of hydroxytyrosol and tyrosol increase rapidly achieving a peak concentration at approximately 1 h in plasma and 2 h in urine [15]. Studies have confirmed that hydroxytyrosol is absorbed by passive diffusion from the gut, through a pathway independent of chylomicron formation [16]; during the post-prandial phase this molecule is incorporated into human lipoproteins, thereby increasing plasma antioxidant activity and decreasing LDL oxidation, thus exerting a significant antioxidant effect *in vivo* [17]. Recent investigations have also demonstrated that secoiridoids, which do not appear to be absorbed in the small intestine, undergo bacterial catabolism in the large intestine with oleuropein undergoing rapid degradation by the colonic microflora producing hydroxytyrosol as the major end product [18].

It is noteworthy that hydroxytyrosol exists in the brain as an endogenous catabolite of catecholic neurotransmitters such as dopamine and norepinephrine that are formed via monoamino oxidase-catalyzed deamination and subsequent reduction. Therefore, it is plausible that hydroxytyrosol is recognized and metabolized by catecholamine enzymatic systems (catechol-*O*-methyltransferase). In this regard, the formation of homovanillyl alcohol in Caco-2 cells has been reported [13]. Trials conducted on human volunteers who ingested olive oil supplemented with increasing amounts of phenolics suggested that the catechol-*O*-methyltransferase derived metabolite of hydroxytyrosol (i.e. homovanillyl alcohol) enters into cellular compartments where it exerts its antioxidant activity [19].

1.B.2. Enzyme Activity Modulation

The protective effects of hydroxytyrosol and oleuropein (this latter molecule contains hydroxytyrosol in its structure) have been demonstrated through both *in-vitro* and *in-vivo* tests. The activities of olive oil phenolics on enzymes have been tested on macrophages, leukocytes and platelets: several studies [20-22] have demonstrated the capacity of hydroxytyrosol to inhibit chemically induced *in-vitro* platelet aggregation, accumulation of thromboxane in human serum, production of pro-inflammatory leukotrienes and inhibition of arachidonate lipooxygenase.

1.B.3. Antioxidant Activity

The mutagenic properties of oxidatively-damaged DNA suggest that antioxidants might have a protective activity toward cancer formation. The orthodihydroxy (catecholic) substitution, as for hydroxytyrosol and oleuropein, confers high antioxidant capacity as shown by several *in-vitro* tests. For example, both hydroxytyrosol and oleuropein were able to scavenge superoxide anion generated by the xanthine/xanthine oxidase system and by human polymorphonuclear cells, and to block potent oxidant species produced *in vivo* in the site of inflammation by activated neutrophils [23].

During inflammation events, macrophages react to the endotoxin by increasing nitric oxide production, which in turn inhibits platelet aggregation and exerts a protective role in preventing oxidative LDL modification that may occur at the site of inflammation. Visioli and co-authors [23] verified that oleuropein significantly increases the production of nitric oxide as a functional response of the immunocompetent cells.

A scavenging effect of hydroxytyrosol and oleuropein has also been demonstrated with respect to hypochlorous acid [24], a potent oxidant produced *in vivo* by neutrophil myeloperoxidase at the site of inflammation.

A potent and dose-dependent reduced formation of various markers of LDL oxidation has been observed, indicating protection of the apoprotein layer [25] and confirming their metal chelator and radical scavenger activities [24]. Data obtained on human oxidized LDL [26] demonstrated that phenols present in the hydroalcoholic extract of VOO inhibit the formation of oxysterols in a dose-dependent manner. Moreover, protection against the oxidative modifications of apoproteins may play an important role in preventing the accumulation of lipids in the arterial wall.

Recently, Visioli and co-workers [27] showed a significant increase in total plasma glutathione levels, both in the reduced and oxidized forms, in 98 healthy subjects after ingestion of 2 ml of a phenolic extract obtained from olive mill waste waters that were particularly rich in hydroxytyrosol. It was suggested that the observed effects might be governed by the antioxidant response element mediated increase in phase II enzyme expression, including that of γ -glutamylcysteine ligase and glutathione synthetase.

1.B.4. Anti-Inflammatory Activity

One VOO phenolic molecule of particular interest due to its putative health-benefiting properties is decarboxymethyl ligstroside aglycone (also named oleocanthal), the main component responsible for the throat irritation associated with VOO consumption (codified as pungent positive taste). Beauchamp and colleagues [28] demonstrated that oleocanthal possesses anti-inflammatory activity due to its dose-dependent ability to inhibit the same cyclooxygenase (COX) enzymes that are involved in the same prostaglandin biosynthesis (inflammatory) pathway as ibuprofen. It has also been demonstrated that the dose-dependent anti-inflammatory properties exhibited *in vitro* are mimicked by its dose-dependent irritation in the oral cavity.

Interestingly, in 2009 Cicerale and co-authors [29] demonstrated a minimal decrease in oleocanthal upon heat treatment, but a significant decrease in the biological activity of this compound with extended heating (up to 31%). The authors hypothesized that the reduction in biological activity after heating may be due to the formation of an oleocanthal antagonist, which could mask the anti-inflammatory activity.

1.B.5. Anti-Tumoral Activity

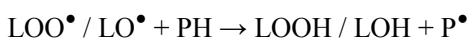
The effects of VOO phenols on colorectal cancer have gained particular attention, since it is the second most common cause of cancer mortality in Western countries. Their ability to inhibit colon cancer development has been demonstrated in cancer cell models [30], animals [31] and humans [32]. In spite of their direct antioxidant effects, VOO phenolics may exert chemopreventive effects via their interactions with cellular signalling pathways that are important in controlling the growth, differentiation and metastasis of cancer cells [33-36]. The anti-cancer properties in the gut seem to be mediated at least in part by the phenolics through

modulation of MAPK kinases and down-regulation of the expression of COX-2 and Bcl-2 proteins, which play a crucial role in colorectal carcinogenesis.

2. Antioxidant Activity of Phenolics in Virgin Olive Oil

2.A. General Concepts Relating to Antioxidant Activity of Phenolic Molecules

Phenolic compounds can slow lipid oxidation, inhibiting initiation and propagation steps, by inactivating or scavenging free radicals. By acting as free radical scavengers or chain-breaking antioxidants, phenolic compounds (PH) in VOO are able to accept a radical from oxidizing lipids species such as peroxy ($\text{LOO}^\bullet$) and alkoxy ($\text{LO}^\bullet$) radicals through the following reaction:



Hydrogen donation generally occurs through the hydroxyl group of phenols, and the subsequently formed radical is stabilized by resonance delocalization throughout the phenolic ring structure.

A second hydroxyl group, especially at the *ortho* position, stabilizes the phenoxyl radical through an intramolecular hydrogen bond; thus phenols with a catechol structure, known as *o*-diphenols, such as hydroxytyrosol, decarboxymethyl oleuropein aglycon and oleuropein aglycon, are the most potent antioxidants in VOO [37].

The antioxidant efficiency of phenols in VOO is dependent on the ability of the antioxidant molecule to donate hydrogen to the free radical, such that the transfer of the hydrogen to the free radical is more rapid and energetically favourable as the hydrogen bond energy of the phenols decreases. A phenol (e.g. catechol $E^\circ=530$ mV) having a reduction potential lower than the reduction potential of a radical (e.g. peroxy radical $E^\circ=1000$ mV) is capable to react, by donating a hydrogen to it, with a feasible reaction from a kinetic point of view [38].

A phenolic molecule can lose antioxidant activity if present in a lipid substrate at concentrations that are too high, and also behave as pro-oxidants by involvement in initiation reactions [39].

Another general concept to keep in mind, which has important implications for antioxidant activity, is related to the distribution in lipidic substrates of different molecules. In fact, natural antioxidants exhibit complex properties between air-oil and oil-water interfaces that significantly affect their relative activities in different lipidic systems. The presence of hydrophilic phenolic compounds in VOO, and their high antioxidant activity, can be explained by the so-called “polar paradox” [40-41]. According to this, “polar antioxidants are more effective in non-polar lipids, whereas non-polar antioxidants are more active in polar lipid emulsions”. The polar paradox is based on the fact that in a bulk oil system hydrophilic antioxidants, such as polar phenols, are oriented in the air-oil interface (a low quantity of air

is always trapped in the oil) and become more protective against oxidation than the lipophilic antioxidants, such as tocopherols, which remain in solution in the oil.

2.B. General Concepts On Direct and Indirect Methods to Determine Antioxidant Activity of Phenols in Virgin Olive Oil

There are several approaches to determine the antioxidant activity of the phenols present in VOO. For simplicity, these can be divided into two groups: indirect and direct methods [42]. Whereas the former measure the ability of phenols to block free radicals that are not linked to oxidative degradation, the latter are closely associated with the study of the effect of phenols with respect of lipid peroxidation. Generally, indirect methods are used more frequently than direct methods, and each type of method has its advantages and disadvantages. Direct methods are more satisfactory in principle, and are generally more sensitive; on the other hand, they are more time-consuming and more difficult experimentally.

2.B.1. Indirect Methods

Indirect methods commonly provide information on the ability of phenols to scavenge stable free radicals as 2,2-diphenyl-1-picrylhydrazyl (DPPH test) and 2,2'-azinobis(3-ethylbenzothiaziline-6-sulfonate) (ABTS assay) or to reduce a metal, such as FRAP (ferric reducing antioxidant power) and CUPRAC (copper reduction assay) tests [43]. One widely applied indirect method is the Folin–Ciocalteu test, which allows estimation of the reductive capacity of phenolic molecules (previously extracted by VOO) [44]. The Folin–Ciocalteu test is a well-standardized indirect method that is useful for routine estimation of antioxidant activity of phenols in VOO. It is also possible to use methods based on chemiluminescence to assess the antioxidant capacity of phenols [45].

2.B.2. Direct Methods

The first important choice concerns the lipidic substrate in which the antioxidant activity of phenolic compounds is tested. Preference should be given to individual lipids such as methyl linoleate, linoleic acid or trilinolein since their commercial accessibility provides reproducibility [46]. Moreover, these compounds are relatively inexpensive, and their oxidation is representative of the most essential features of lipid peroxidation. Another possibility is the use of a VOO in which its antioxidants and pro-oxidant components were eliminated through washing with appropriate solvent mixtures.

The initiation of lipid peroxidation can be start by using an initiator, such as a thermolabile azo-compound (i.e. water soluble AAPH or lipid-soluble AMVN), or a transition metal (i.e. Fe^{3+} or Cu^{2+}) [47].

Monitoring of lipid peroxidation is possible through two means: discontinuous, via collection of several aliquots, or continuous. This latter is preferable as it allows both a higher throughput and more detailed observation of the process.

The most popular determinations are based on the quantification of the primary products of oxidation (i.e. through the measure of peroxides and of conjugated dienes), of secondary products (i.e. through the TBARS assay or of specific volatile aldehydes as hexanal or nonanal) or of oxygen consumption. Measurement via oxygen consumption seems to be more representative of the oxidation development and is also quite sensitive.

Studies on lipid matrices enriched in phenolic compounds or VOO can be performed under normal storage conditions or under accelerated oxidation such as active oxygen method (AOM), Schaal oven test, oxygen uptake/absorption, or by using a fully automated oxidative stability instrument (Oxidative Stability Instrument called OSI or Rancimat apparatus) [48-49]. Automatic assays of lipid oxidation in bulk using enzymatic sensors to record the consumption of phenolic components have been demonstrated [50-51].

2.C. Evaluation of Antioxidant Activity in Virgin Olive Oil

Various investigations have studied the importance of the total or individual phenol contents with regards to VOO stability. The active phenols in VOO are mainly *o*-diphenols such as hydroxytyrosol and its oleosidic forms. However, monophenol tyrosol and its oleosidic and derivative forms show less antioxidant activity. In 1999, Aparicio *et al.* [49] used Rancimat (set to 100°C) to study the relative contribution of several chemical compounds (phenols, carotenoids, tocopherols, chlorophylls) to the oxidative stability of 79 VOOs. It was observed that the main components protecting VOO against oxidation are *o*-diphenols, and that other purported antioxidants do not contribute significantly to VOO stability in the presence of phenols.

In 2003, Mateos and co-authors [52] spiked a purified olive oil with several single phenols and tocopherols (with concentration ranges similar to those found in VOO) to evaluate their antioxidant or pro-oxidant activities under accelerated oxidation in a Rancimat apparatus at 100°C. The data were in agreement with the fact that antioxidant activity is correlated with the number of phenolic hydroxyls and the *o*-disubstitution in the molecule. In fact, hydroxytyrosol, hydroxytyrosyl acetate and oleuropein aglycon showed similar antioxidant activities per mmol of substance, whereas the activity of α -tocopherol was significantly lower than that of similar concentrations of these phenols. There was only a slight contribution of tyrosol to oil stability. The flavone luteolin showed an antioxidant activity similar to that of hydroxytyrosol, whereas apigenin did not show any effects.

In investigations carried out by Carrasco-Pancorbo and co-authors [46], the antioxidant activity of several single phenolic compounds of VOO (hydroxytyrosol, tyrosol, elenolic acid, decarboxymethyl oleuropein aglycon, (+)-pinoresinol, (+)-1-acetoxypinoresinol, oleuropein aglycon and ligstroside aglycon) was evaluated by three different chemical approaches: radical assay (DPPH), accelerated oxidation in a lipid model system by OSI and an electrochemical method (flow injection analysis FIA amperometry and cyclic voltammetry). These authors verified that, as is generally assumed, the presence of a single hydroxyl group on a benzene ring conferred only limited antioxidant activity. On the other hand, the presence of a catechol structure enhances the ability of phenolic compounds to act as antioxidants. In fact, the results obtained with all three different approaches showed that *o*-diphenols of VOO (hydroxytyrosol, decarboxymethyl oleuropein aglycon and oleuropein aglycon) were the strongest in terms of antioxidant power. Moreover, by OSI the authors demonstrated a pro-oxidant effect of elenolic acid, (+)-pinoresinol, tyrosol, ligstroside aglycon and (+)-1-acetoxypinoresinol.

In 1999, Mannino *et al.* [53] proposed a procedure to evaluate the antioxidant capacity of VOO based on electrochemical properties by direct injection of the samples in an FIA system with an electrochemical detector operating at a potential of +500 mV (vs. Ag/AgCl). The

results from this procedure were compared with those by Rancimat and the ABTS^{•+} assay (after dilution of samples with *n*-hexane). In a previous work by the same authors [54], it was shown that molecules with an oxidation potential lower than +600 mV (vs. Ag/AgCl) possess good antioxidant activity, whereas molecules with higher values show no or moderate antioxidant activity. Through the study of the hydrodynamic voltammetric profiles of VOO compounds, it was demonstrated that only a few compounds in VOO are oxidizable at potentials lower than +600 mV (i.e. 3,4-dihydroxyphenylacetic acid, caffeic acid, gallic acid, α -tocopherol, oleuropein, protocatechuic acid), and no compounds can be detected at potentials lower than +400 mV. There was good agreement between the results of the proposed electrochemical method in several VOO and those of the ABTS^{•+} decolouration assay ($r=0.988$), whereas a lower linear correlation was observed with data by Rancimat ($r=0.893$).

In 2004, Del Carlo and co-workers [55] applied the hydrodynamic voltammetry in flow injection analysis (FIA) for the antioxidant power evaluation of 22 samples of VOO. They also analysed the total phenol content and the antioxidant activity by Folin-Ciocalteu and DPPH, respectively. The antioxidant activity of phenolic extracts determined by DPPH and hydrodynamic voltammetry in flow injection analysis was positively correlated with the oxidative stability measured with Rancimat ($r=0.810$ and $r=0.808$, respectively).

3. Sensory Properties of Virgin Olive Oil

VOO is a natural oil obtained directly from olives without any further refining process. Its flavour (combination of odors and tastes) is characteristic and highly appreciated by consumers and is markedly different from those of other edible fats and oils. Many studies have been performed to clarify the relationships between the sensory attributes in a VOO as perceived by assessors and its volatile and phenol profiles, which are responsible for aroma and taste, respectively [37, 56].

Some phenols mainly elicit the tasting perception of bitterness; however, other phenolic molecules can stimulate the free endings of the trigeminal nerve located in the palate and in the gustative buds giving rise to the chemesthetic perceptions of pungency, astringency and metallic attributes.

Some researchers have suggested that the secoiridoid derivatives of hydroxytyrosol are the main contributors to olive oil bitterness: Andrewes et al. [57] assessed the relationship between polyphenols and olive oil pungency. The decarboxymethyl ligstroside aglycone or oleocanthal (an ester between tyrosol and elenolic acid) was the key source of the burning sensation found in many VOO. In contrast, the decarboxymethyl oleuropein aglycone (an ester between hydroxytyrosol and elenolic acid), tasted at an equivalent concentration, produced very little burning sensation. This is a clear example of the different sensory properties of a secoiridoid derivative of tyrosol and hydroxytyrosol. In 2003, Gutierrez-Rosales and co-authors [58] isolated the major peaks found in the phenolic profile of VOO using preparative HPLC; after dissolving in water, these molecules purified were then tasted to evaluate the intensity of bitterness. It was concluded that the peaks corresponding to the decarboxymethyl oleuropein aglycone, oleuropein aglycone and decarboxymethyl ligstroside aglycone were those mainly responsible for the bitter taste of VOO. As previously reported,

Mateos et al. [59] verified the better correlation between the aldehydic form of oleuropein aglycon and bitterness.

In 2005, Beauchamp and co-authors [28] measured the pungent intensity of oleocanthal isolated from different VOO, confirming that this molecule is the principal agent responsible for throat irritation.

As above mentioned, volatile compounds are responsible for the aroma of VOO. The C6 and C5 compounds are enzymatically produced from polyunsaturated fatty acids through the so-called lipoxygenase (LOX) pathway. Quantitatively, linear C6 unsaturated and saturated aldehydes are the most important fraction of volatile compounds in high quality VOO [60], but several other compounds belonging to different classes (e.g. alcohols, esters, ketones, acids, hydrocarbures, terpenes) are also present. The quali-quantitative profiles of these volatile compounds depend on the level and activity of each enzyme involved in the LOX pathway [61]. It should be noted that although the *E*-2-hexenal content, which gives the typical “green note” to VOO, is by far the major C6 aldehyde in all fresh oils, hexanal seems to contribute more to the green odour than *E*-2-hexenal because of its lower odour threshold [62-65].

In addition to fruity, the “green” sensation reminiscent of freshly-cut grass, leaf, tomato, artichoke, walnut husk, apple or other fruits generally contributes to the aroma of high quality oil. “Green” notes include *Z*-3-hexenal, and *Z*-3-hexenyl acetate, whereas alcohols such as *E*-2-hexen-1-ol, *Z*-3-hexen-1-ol and hexan-1-ol have less sensory significance than aldehydes due to their higher odour threshold values. Their sensory descriptions are associated with ripe fruity, soft green and aromatic sensory notes [66].

Esters are compounds associated with fruity nuances [64]. Hexyl acetate and *Z*-3-hexenyl acetate are present in the aroma of all fresh VOOs, but are minor components compared with aldehydes or alcohols. Different authors [62, 64] indicate that *Z*-3-hexenyl acetate is linked to the pleasant green and banana notes. Among the C5 compounds, 1-penten-3-one has been mostly associated with fruity, sweet and pleasant attributes such as tomato and strawberry [64]. It has a very low odour threshold (0.7-50 µg kg⁻¹) and so its contribution to the total aroma can be considered important.

When a low quality of olives or incorrect agricultural and technological practices is used, VOO can be characterized by unpleasant odours coded as defects. The molecules responsible for these negative attributes originate from sugar fermentation (e.g. ethanol, ethyl acetate and acetic acid), degradation reactions of amino acids (e.g. 3-methyl-1-butanol), mould enzyme action (e.g. octen-1-ol) or fatty acid auto-oxidation (i.g. *E*-2-heptenal) [67].

Conclusion

In spite of their low amount, phenolic molecules are very important compounds that are characteristic of VOO. Several experimental lines of evidence have shown that the amount of phenols, particularly those with a catecholic structure, is related to several healthy attributes that include reduction of risk factors for coronary heart disease, prevention of several types of cancer and modification of immune and inflammatory responses. It is also of interest to highlight that phenolic molecules are responsible for bitterness, pungency, astringency taste sensations and for the antioxidant activities of the polar fraction.

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Beneficial Effects of Mulberry on Human Health

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Abstract

Background: Mulberry fruit juice is rich in carbohydrates, proteins, dietary fibers, vitamins, amino acids, minerals, alkaloids, polyphenols, flavonoids and anthocyanins. This review summarizes diverse biological activity of Mulberry extracts and components. Most of previous studies have focused on lower molecular weight of polyphenols. We present new two biological activities of Mulberry fruit: that is anti-stress activity of the non-fibrous materials, and the anti-HIV activity of fibrous fractions.

Materials and Methods: Anti-stress activity was evaluated by measuring the plasma level of lipid peroxide in mice loaded with water immersion restraint stress. Anti-HIV activity was evaluated by the selectivity index. Lignin carbohydrate complex was isolated by alkaline extraction and acid precipitation.

Results: Oral administration of supernatant of mulberry juice (that contains most of the lower molecular weight polyphenols) significantly showed anti-stress activity. Precipitating fibrous materials, inhibited the cytopathic effect of HIV-infection *in vitro* more efficiently than blueberry juice that does not contain fibrous materials. Almost of the anti-HIV activity was concentrated in the lignin carbohydrate complex fractions (Fr. IV and VI) that occupy about 2% (w/w) of mulberry juice. Frs. IV and IV showed higher superoxide anion scavenging activity than Fr. I, in a synergistic fashion with sodium ascorbate.

Conclusion: The presence of various attractive substances in the mulberry juice suggests its possible functionality as an alternative medicine.

Abbreviations

MCH-1	melanin-concentrating hormone-1
GABA	gamma-aminobutyric acid
LPS	Lipopolysaccharide
NF- κ B	Nuclear factor- κ B
SI	selectivity index
DPPH	1,1-diphenyl-2-picrylhydrazyl
CC ₅₀	50% cytotoxic concentration
OSCC	Oral squamous cell carcinoma

1. Introduction

Mulberry (*Morus alba* L.) is a plant distributed into the tropics and subtropics zones, and has been cultivated as a nutrition for silkworms. With the downhill of sericulture, this plant has not been spread so well as before. However, the anti-diabetic activity of deoxynojirimycin in the leaf fraction, antioxidative activity of anthocyanins in the fruit body, and the abundance of minerals has drawn our attention. We first review previous reports of component analysis and diverse biological activity of mulberry including ours, and then discuss the possible mechanism of two new biological activity of mulberry juice: anti-stress activity and anti-HIV activity.

2. Component Analysis

Mulberry fruit juice is rich in major nutritional ingredients, alkaloids, polyphenols, flavonoids, anthocyanins, minerals and amino acids [1-3] (Tables 1, 2). Different mulberry species have shown different content of 1-deoxynojirimycin, resveratrol, oxyresveratrol, anthocyanins, and flavonoids [2]. Mulberry juice also contains nonanthocyanin phenolics such as rutin, quercetin, procatechuic acid, chlorogenic acid, 4-caffeoylquinic acid, 3,5-dicaffeoylquinic acid, taxifolin, taxifolin-hexoside and kaempferol-hexoside [4]. Total polyphenols are present in Mulberry juice at 152.06 mg/100ml, approximately 80 and 40 mg of which may be ascribed to anthocyanins and chlorogenic acid, respectively (Table 2). Major vitamins are vitamin K₁ (phyloquinone) [5] and β -carotene (Table 2). Vitamin K₂ (menaquinone) was not detected. Potassium content was high compared with other minerals (calcium, magnesium, sodium, phosphorus, sulfur) [3] (Table 1). Relatively higher amount of iron (Table 1) suggests its possible application to anemia. Asparagine, alanine, γ -butyric acid (GABA), serine and aspartic acid are major amino acids in Mulberry juice (Table 2). Mulberry juice contains higher amounts of dietary fiber (especially insoluble fiber that contains lignin carbohydrate complex) than other fruit juices (Satsuma mandarine, valencia orange, grapefruit juice, pine apple, grape, apple) [6] (Table 1). These enriched ingredients of Mulberry juice have supported the survival of periodontal ligament cells [7]. Diverse biological activity of Mulberry leaf, root bark and fruit is summarized in Table 3.

Table 1. Components of straight fruit juices

	Straight fruit juice						
	Mulberry	Satsuma	Valencia	Grapefruit	Pineapple	Grape	Apple
		mandarine	orange				
Energy (kcal/100 g)	33	41	42	40	41	55	44
Moisture (g/100 g)	91,5	88,5	87,8	88,7	88,2	84,8	87,7
Protein (g/100 g)	0,9	0,5	0,8	0,6	0,3	0,3	0,2
Lipid (g/100 g)	trace	0,1	trace	0,1	0,1	0,2	0,1
Carbohydrate (g/100 g)	7,3	10,6	11,0	10,3	11,0	14,5	11,8
Ash (g/100 g)	0,3	0,3	0,4	0,3	0,4	0,2	0,2
Dietary fiber (g/100g)	1,1	0,0	0,3	0,1	0,0	0,1	trace
soluble	0,3	0,0	0,2	0,1	0,0	0,1	trace
insoluble	0,8	0,0	0,1	0,0	0,0	trace	trace
α -Carotene (μ g/100 g)	0	2	7	0	0	0	0
β -Carotene (μ g/100 g)	11	53	12	0	9	0	0
Retinol equivalent (μ g/100 g)	1	35	3	0	1	0	0
Vitamin D (μ g/100 g)	0	0	0	0	0	0	0
α -Tocopherol (mg/100 g)	0	0,2	0,2	0,2	trace	0	0,1
Vitamin K (μ g/100 g)	12	0	0	trace	0	trace	0
Vitamin B ₁ (mg/100 g)	0,02	0,06	0,07	0,04	0,04	0,02	0,01
Vitamin B ₂ (mg/100 g)	0,02	0,01	0,01	0,01	0,01	0,01	0,01
Niacin (mg/100 g)	0,6	0,2	0,2	0,2	0,2	0,1	0,1
Vitamin B ₆ (mg/100 g)	0,03	0,03	0,06	0,03	0,07	0,05	0,03
Vitamin B ₁₂ (μ g/100 g)	0	0	0	0	0	0	0
Folic acid (μ g/100 g)	4	15	25	11	9	1	3
Pantothenic acid (mg/100 g)	0,26	0,14	0,14	0,23	0,19	0,06	0,21
Vitamin C (mg/100 g)	0	29	22	38	6	trace	3
Na (mg/100 g)	4	1	1	1	1	1	3
K (mg/100 g)	143	130	180	180	210	30	77
Ca (mg/100 g)	13	8	9	9	22	3	2
Mg (mg/100 g)	11	8	10	9	10	14	3
P (mg/100 g)	17	11	20	12	13	7	6
Fe (mg/100 g)	2,0	0,2	0,1	0,1	0,4	0,1	0,4
Zn (mg/100 g)	0,1	Tr	Tr	Tr	0,1	0,1	Tr
Cu (mg/100 g)	0,02	0,02	0,04	0,03	0,03	0,02	0,03
Mn (mg/100 g)	0,08	0,03	0,02	0,01	0,87	0,13	0,03

Components except for dietary fiber in mulberry juice were analyzed by Japanese Food Research Lab. Data of other juices were derived from the standard tables of food composition in Japan, Fifth Revised and Enlarged Edition [6].

Table 2. Amino acid and other components in mulberry juice

Amino acids	nmol/mL	mg/100 mL
Asp	1060±17	14,11
Thr	317±4	3,78
Ser	1600±18	16,81
Asn	7499±89	99,08
Glu	536±6	7,89
Gly	118±1	0,89
Ala	2343±24	2,09
Cit	16±12	0,28
α-ABA	115±6	1,19
Val	442±10	5,18
Met	174±2	2,60
cysta	8±0	0,18
Ile	253±9	3,32
Leu	574±8	7,53
Tyr	884±9	16,02
β-Ala	112±7	1,00
Phe	471±12	7,78
GABA	1613±22	16,63
MEA	93±10	0,57
Orn	8±0	0,11
Lys	28±1	0,41
Arg	404±4	7,04
Polyphenols		150,06
3-Caffeoylquinic acid	151	5,34
5-Caffeoylquinic acid(chlorogenic acid)	703	24,87
4-Caffeoylquinic acid	334	11,82
Cyanidine 3-glucoside	1495	67,19

Each juice was mixed with an equal volume of 10%trichloroacetic acid, and the supernant was determined for amino acid. Polyphenols were determined by Folin-Denis method. Caffeoylquinic acids and cyanidine were determined by HPLC and LC/MS.

Table 3. Biological activities of mulberry extracts

Activities	Materials	System, possible mechanism etc	Ref.
Anti-aging effect			
Anti-diabetic effect	<i>Morus alba</i> L leaf extract	Streptozotocin-induced chronic diabetic rats	[8]
	<i>Morus alba</i> root bark 20 K glycoprotein	Blood glucose↓ glucose transport↑	[9]
	<i>Morus alba</i> leaf extract	AMPK activation glucose transporter	[10]
	<i>Morus alba</i> extract	No insulin secretagogue activity	[11]
	<i>Morus alba</i> root extract flavonoids, triterpenes	Glucose↓ insulin↑ lipid peroxidation↓	[12]
Anti-atherosclerosis effect	<i>Morus alba</i> L leaf extract	MMPs↓GTPase↓ Akt/NF-κB↓	[13]
Anti-obesity effect	<i>Morus alba</i> L leaf extract	MCH-1 receptor binding↓	[14]
	(Hydrocy)pheophorbide a methyl esters	Inhibition of MCH-1 to its receptor	[15]
	<i>Morus alba</i> leaf extract	Glucose transport↓ α-glucosidase↓	[16]
Anti-Alzheimer's diseases	Pheophorbide a, kaempherol glucosides	Aβ (1-42)↓	[17]
Anti-Parkinson	Oxyresveratrol	Protection from 6-	[18]

Activities	Materials	System, possible mechanism etc	Ref.
disease		hydroxydopamine neurotoxicity	
Neuroprotection	Cyanidine- <i>O</i> - β -d-glucopyranoside	Protection of neuronal cells from oxidative stress	[19]
Hepatoprotection	2,5-dihydroxy-4,3'-di(β -D-glucopyranosyloxy)- <i>trans</i> -stilbene	Protection from CCl ₄ -induced hepatotoxicity	[20]
Antinociceptive effect	<i>Morus nigra</i> L. leaf extract	Formalin, hot plate, tail immersion test, writhing mice	[21]
Anti-melanogenesis activity	<i>Morus alba</i> L. extract	Inhibition of tyrosinase and DOPA auto-oxidation	[22]
	Flavanones, flavone, phenylbenzofuranes, moracin	Inhibition of mono- and di-phenolases	[23]
Anti-stress activity	Mulberry juice	Inhibition of plasma lipid peroxidation	[24]
	Mulberry juice of <i>Morus nigra</i> fruit	Protective action against peroxidative damage	[26]
	Mulberry juice, <i>Moruba alba</i> L, <i>Morus nigra</i> L leaf extract	O ₂ ⁻ , OH, NO, DPPH radical scavenging	[24, 25, 46, 47]
	Nonanthocyanin phenolics from <i>Morus alba</i> L. cultivar	O ₂ ⁻ , DPPH radical scavenging	[4]
	Dietary fruits	Antioxidant activity against liposome and linoleic acid	[29]
Anti-tumor activity			
Tumor-specificity	Flavonoids	Low tumor-specificity	[31-34]
	Chlorogenic acid	Low tumor-specificity	[38]
	Procyanidins, hydrolyzable tannins	Tumor-specificity increased with oligomerization	[35, 36]
Anti-metastatic activity	Resveratrol	Inhibition of heparinase and pulmonary metastasis	[39]
Anti-MDR activity	<i>Morou mesozygia</i> Stapf extract	Inhibition of ABCG2 transporter	[40]
Anti-mutagenic activity	<i>Morus alba</i> L. fruit extract	Inhibition of chromosome aberrations	[41]
Anti-microbial activity			
	<i>Morus mesozygia</i> stem bark extract	Isolation of moracin T and moracin C	[42]
	Prenylated favonoids	Antimicrobial spectrum and cytotoxicity	[43]
Anti-viral activity			
	Lignin carbohydrate complex from Mulberry juice	Relatively higher anti-HIV activity	[25]
	Flavonoids	Relatively lower anti-HIV activity	[31]
	Morus alba L. root bark flavonoids	Anti-HSV-1 activity of flavonoids	[34]
Immuno-modulating activity			
Anti-inflammatory activity	<i>Morus alba</i> L cortex extract	NF- κ B↓ ICAM-1↓ lung cells	[45]
	Prenylflavonoids	Anti-nephritis activity	[46]
Pro-inflammatory activity	<i>Mori Fructus</i> extract	TLR4↑ NO↑ TNF ↑ RAW264.7 macrophage	[47]
Immunopotensiation	<i>Morus nigra</i> lectin	Interaction with cell membrane carbohydrate	[48]
Others			
Inhibition of CYP3A	Fruit juice	Inhibition of CYP3A-catalyzed hydroxylation	[49]
Serine protease	<i>Morus indica</i> cv. K2 latex	Purification and characterization	[50]

3. Anti-Aging Activity

3.A. Anti-Metabolic Syndrome Activity

Oral administration of *Morus alba* L. leaf extract significantly reduced the fasting blood glucose level and high blood pressure, restored the vascular reactivity possibly by alleviating the oxidative stress (reducing the malondialdehyde content) in diabetic rats [8]. 20K glycoprotein from the root bark of *Morus alba*. lowered blood glucose level in streptozotocin-induced hyperglycemic mice model and increased the glucose transport in cultured epididymist fat cells [9]. *Morus alba* leaf hot water extract stimulated 5'-AMP-activated protein kinase (AMPK) in isolated epitrochlearis skeletal muscle, causing the increase in the phosphorylation of Thr172 of the catalytic α subunit of AMPK and the phosphorylation of Ser79 of acetyl CoA carboxylase, an interacellular substrate of AMPK. This was accompanied by an increased rate of 3-*O*-methyl-D-glucose transport in the absence of insulin and with phosphorylation of AS160, a signaling intermediary leading to glucose transporter 4 translocation [10]. There is a negative report that *Morus alba* ethanol extract did not show the insulin secretagogue activity in INS-1 cells [11]. However, oral administration of 70% alcohol extract of the Egyptian *Morus alba* bark to streptozotocin-induced diabetic rat models resulted in the hypoglycemic effect, increased the insulin level and diminished the lipid peroxidation [12].

Morus alba L is rich in polyphenols including gallic acid, procatechuic acid, catechin, gallocatechin gallate, caffeic acid, epicatechin, rutin and quercetin and inhibited the migration of vascular smooth muscle cells by inhibiting matrix metalloproteinases (MMP-2, MMP-9) and blocking small GTPase and Akt/NF- κ B [13].

Oral administration of ethanol extract of *Morus alba* leaves reduced the body weight and hepatic lipid accumulation in diet-induced obese mice, possibly by inhibiting the binding melanin-concentrating hormone-1 (MCH-1) to its receptor [14]. Pheophorbide a methyl ester and 13(2)(S)-hydroxypheophorbide a methyl ester from *Morus alba* L. aqueous extract has been identified as an inhibitor of the binding of MCH-1 to its receptor and the phosphorylation of MCH-1-mediated extracellular signal-regulated kinase (ERK) in Chinese hamster ovary cell expressing human MCH-1 receptor [15]. Oral administration reduced fasting blood glucose, insulin, homeostasis model assessment-insulin resistance(HOMA-IR), C-reactive protein and triglyceride in a spontaneous non-obese diabetic rats, possibly by the inhibition of glucose transport and α -glucosidase in the small intestine [16].

3.B. Neuroprotective Effect

Pheophorbide a, kaempherol-3-*O*-glucoside, kaempherol-3-*O*-(6-malonyl) glucoside, from the methanol extract of mulberry leaves inhibited the formation of amyloid β -peptide (1-42)-fibrils, and protected hippocampal neurons from amyloid β -peptide (1-42)-induced cell death [17]. This indicates their possible application to Alzheimer's diseases.

Oxyresveratrol, a polyhydroxylated stilbene existing in mulberry, showed higher neuroprotective activity than resveratrol, inhibiting the cytotoxic effect of 6-hydroxydopamine on human neuroblasoma cell line SH-SY5Y via inhibition of JNK and c-

Jun phosphorylation [18]. Cyanidine-3-*O*- β -D-glucopyranoside isolated from mulberry fruit also showed neuroprotective effect on the cultured rat neutrol PC12 cells exposed to hydrogen peroxide and oxygen deprivation [19]. This suggests their possible application to Parkinson disease.

3.C. Others

2,5-Dihydroxy-4,3-di(β -D-glucopyranosyloxy)-*trans*-stilbene from *Morus bombycis* Koizumi roots showed a liver protective action against CCl₄-induced hepatotoxicity (cell ballooning) [20].

Dichloromethane extract from the leaves of *Morus nigra* L. showed more potent antinociceptive effect than indomethacine and morphine in mice [21].

Morus alba extract inhibited the mushroom tyrosinase activity and DOPA auto-oxidation activity [22]. Flavanones, flavone, phenylbenzofuranes in methanol extract of roots of *Morus lhou* also inhibited tyrosinase activity [23]. These components may be utilized as skin-whitening or antibrowning preparations.

4. Anti-Stress Activity

4.A. Inhibition of Plasma Lipid Peroxide Level

Oral administration of mulberry juice significantly reduced the elevated plasma level of lipid peroxide in mice loaded with water immersion restraint stress [24]. The anti-stress activity was concentrated in the supernatant fraction that contains most of the lower molecular weight polyphenols such as anthocyanins and inhibitory amino acids such as GABA [25] (Figure 1). GABA may be expected to exert the relaxing effects. We observed that anti-stress condition maintained during at least 4 hours after the cessation of mulberry juice intake, but abrogated after 8 hours. This suggests that intake of mulberry juice should be continued to maintain the anti-stress condition.

4.B. Radical Scavenging Activity

Mulberry juice scavenged superoxide anion (generated by hypoxanthin-xanthin oxidase reaction), hydroxyl radical (generated by Fenton reaction) and NO (generated from NOC-7) [24]. Lignin carbohydrate fraction of mulberry juice scavenged the superoxide anion and hydroxyl radical synergistically with vitamin C [25]. *Morus nigra* fruit inhibited the glucose-induced haemoglobin glycosylation, hydrogen peroxide-induced haemolysis of human erythrocytes, production of malondialdehyde produced *tert*-butyl hydroperoxide-induced lipid peroxidation of LDL, and Cu²⁺-induced lipid peroxidation [26].

Water extract and anthocyanin-rich extract of *Morus alba* L inhibited the relative electrophoretic mobility (REM), ApoB fragmentation, thiobarbituric acid reaction substances (TBARS) formation in Cu²⁺-mediated oxidation LDL, and scavenged 1,1-diphenyl-2-

picrylhydrazyl (DPPH) radical [27]. Aqueous extract of *Morus nigra* leaf moderated the DPPH radical scavenging activity [28]. The anthocyanin-rich fruits inhibited the copper-induced peroxidation of liposome, and the co-oxidation of linoleic acid and β -carotene [29]. We found that 20-fold diluted mulberry juice or green tea extract (original concentration of total polyphenol=186.4 mg/ml) inhibited the DPPH radical intensity by 20.4 and 68.4%, indicating that the DPPH radical scavenging activity of mulberry juice is approximately one-third that of green tea (Watanabe et al., submitted).

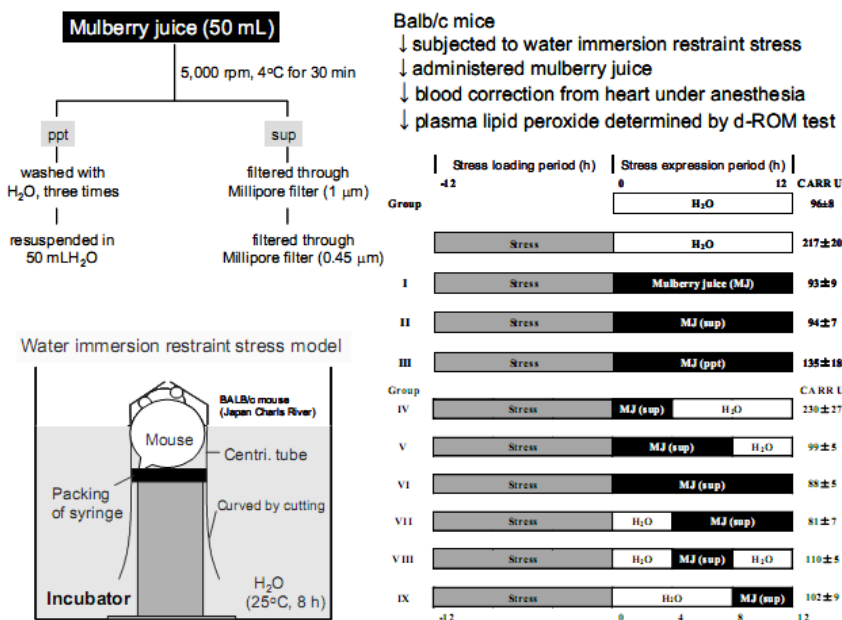


Figure 1. Anti-stress activity of Mulberry juice. Part of data cited from ref. 25.

5. Antitumor Activity

We have established the assay system to evaluate the tumor-specificity of test samples using human oral squamous cell carcinoma (OSCC) cell line (HSC-2, HSC-3, HSC-4) and human oral normal cells [human gingival fibroblast (HGF), pulp cells (HPC), periodontal ligament fibroblast (HPLF)]. The tumor-specificity index (TS value) was defined as the ratio of mean CC₅₀ value for normal cells to that for OSCC cell lines. Low molecular weight polyphenols (flavonoids, dibenzoylmethanes, dihydrostilbenes, dihydrophenanthrenes and 3-phenylchromen-4-ones) showed weak tumor-specificity (TS=1-4) [30-34]. Prenylation of isoflavone and 2-arylbenzofuran significantly enhanced the cytotoxic activity. Glabrol and 5'-(1,1-dimethyl-2-prenyl)glabridin failed to induce internucleosomal DNA fragmentation in HSC-2 cells [30]. We found that tumor-specificity of (-)-catachin was enhanced with oligomerization (TS=4.0 to 7.4) [35]. Similarly, the tumor-specificity of hydrolysable tannins was increased from monomers (TS=1.0-2.5) to oligomers (TS=1.2-8.2) [36], suggesting the importance of highly polymerized structure for the higher tumor-specificity. Chlorogenic acid showed slightly higher cytotoxicity against HSC-2 and human salivary gland tumor cell line (HSG) than HGF cells, yielding TS value of 1.7 [37]. Chlorogenic acid induced apoptotic

phenotypes (nuclear condensation and caspase activation) and produced large DNA fragmentation without induction of internucleosomal DNA fragmentation in HSC-2 and HSG cell lines [37]. Cytotoxic activity of chlorogenic acid was significantly reduced by CoCl_2 , possibly due to the conformational changes detected by absorption spectrum [38].

Reseveratrol, purified from the methanol extract of *Morus alba* L., inhibited the heparinase, and the pulmonary metastasis of murine B16 melanoma cells [39]. QSAR analysis of flavonoids in *Morus mesozygia* Stapf. demonstrated that shape parameters and hydrophobicity were major physicochemical descriptors responsible for the inhibition activity of flavonoid derivatives against the wild-type breast cancer resistance protein (BCRP/ABCG2) [40].

Fruits of *Morus alba* L. inhibited the frequency of chromosome aberrations induced by genotoxicants (X-rays, N-methylnitrosourea, cyclophosphamide, sodium fluoride) and aging in bone marrow cells from mice and rats [41].

6. Anti-Microbial Activity

Moracin T, moracin C in ethanolic extract of stem bark of *Morus mesozygia* showed antimicrobial activity against nine bacteria including *Escherichia coli*, *Shigella dysenteriae*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Bacillus cereus*, comparable with gentamycin used as reference antibiotic [42]. We found that supernatant of mulberry juice at 10 mg/mL inhibited the growth of *Porphyromonas gingivalis* and *Prevotella melaninogenica*, although it was ineffective against *Streptococcus mutans*, *Streptococcus gordonii*, *Actinomyces viscosus*, *Prevotella intermedia*, *Fusobacterium nucleatum*, *Capnocytophaga ochracea*, *Candida albicans*, *Escherichia coli* and *Staphylococcus aureus*. Anti-microbial activity of prenylated flavonoids isolated from *Morus Alba* L. and *Morus mongolica* Schneider, have been reported [43]. Further analysis of anti-bacterial activity against *Porphyromonas gingivalis* and *Prevotella melaninogenica* are necessary for the application of mulberry juice to the oral cavity.

7. Anti-Viral Activity

7.A. Anti-Hiv Activity

We have reported for the first time that mulberry juice showed much higher anti-HIV activity than blueberry juice, due to the higher content of water insoluble fiber [25]. The relative potency of the anti-HIV activity was evaluated by the selectivity index (SI), determined by the following equation: $\text{SI} = \text{CC}_{50} / \text{IC}_{50}$, where the CC_{50} is the concentration that reduced the viable cell number of mock-infected cells by 50%, and IC_{50} is the concentration that increased the viable cell number to 50% of mock-infected cells. Mulberry juice, that contains precipitating fibrous materials, inhibited the cytopathic effect of HIV-infection *in vitro* ($\text{SI}=3.7$) more efficiently than blueberry juice that does not contain fibrous materials ($\text{SI}<1.0$) [25]. Oolong tea ($\text{SI}<1.0$), gallic acid (component unit of tannin) ($\text{SI}<1.0$) and most of chemically defined polyphenols (flavanones, 3-hydroxyflavanones, isoflavans, isoflav-3-

enes, flavans, 2-arylbenzofurans, isoflavones, isoflavanones, 3-arylcoumarins, coumestans, pterocarpanes, chalcones, dibenzoylmethanes, dihydrostilbenes, dihydrophenanthrenes, diphenylpropanes, flavone, flavonols, 5-hydroxy-3-phenoxychromen-4-ones) [31] (SI=1.0) did not show anti-HIV activity, due to higher cytotoxicity against mock-infected cells (Figure 2). The anti-HIV activity was concentrated in the precipitating fibrous fraction. We further fractionated the precipitate with hot water and 1% NaOH. The hot water extract was fractionated by step-wise ethanol precipitations (that yields Frs. II and III). The 1%NaOH extract was fractionated by acid precipitation (that yields Fr. IV) and step-wise ethanol precipitations (that yields Frs. V and IV). The most of the anti-HIV activity was found to be concentrated in the lignin carbohydrate complex fractions (Frs. IV, VI) (SI=7.2 and 7.0, respectively) [25].

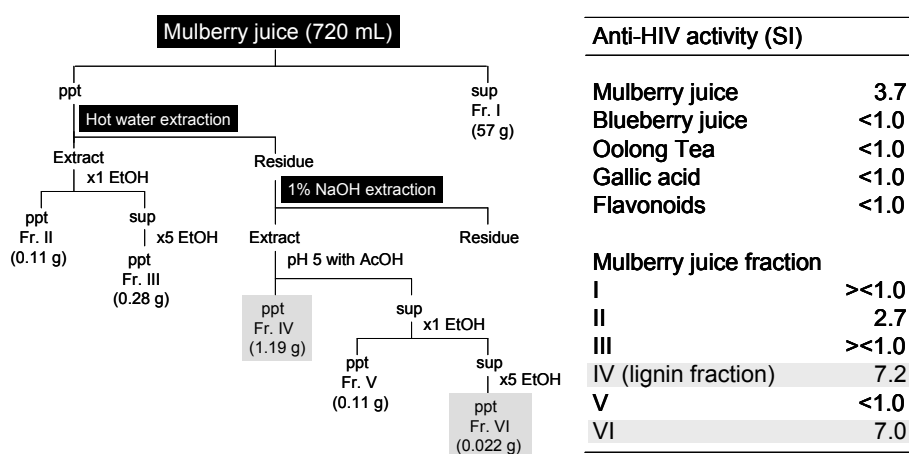


Figure 2. Anti-HIV activity of lignin carbohydrate complex fraction in the precipitate of Mulberry juice. Part of the data cited from ref. 25.

7.B. Anti-Hsv Activity

Flavonoids isolated from the root bark of *Morus alba* L. (moralbanone, kuwanon S, mulberroside C, cyclomorusin, eudraflavone B hydroperoxide, oxydihydromorusin, leachianone G, alpha-acetyl-amyrin) showed anti-herpes simplex type 1 virus (HSV-1) activity with IC_{50} of 1.6-75 $\mu\text{g/mL}$ [44].

8. Immuno-Modulating Activity

The decoction of *Morus alba* inhibited the LPS-stimulated NF- κ B activation and ICAM-1 up-regulation of human pulmonary epithelial cells A549, suggesting its possible anti-inflammatory activity [45]. Morusin, a prenylated flavonoid of mulberry tree (*Morus* spp.), showed anti-nephritis activity (urinary protein excretion, total cholesterol) and superoxide anion scavenging activity [46]. On the other hand, the aqueous extract of *Mori Fructus* stimulated the NO production and TNF- α secretion from mouse macrophage-like RAW264.7

cells by activating the Toll-like receptor 4-mediated pathways including the activation of NF- κ B [47].

Lectin from *Morus nigra* induced the proliferation and apoptotic cell death of lymphocytes by interacting with the cell surface carbohydrate moiety and incorporating intracellularly [48].

9. Inhibitory Effect on CYP3A Activity

Black mulberry inhibited the CYP3A activity (measured as midazolam 1'-hydroxylase activity) dose-dependently, but to much lesser than that achieved by white grape fruit juice [49]. However, the interaction between black mulberry juice and CYP3A substrates should be monitored to determine whether the effect of fruits juice is clinically relevant.

10. Purification of Serine Protease

Indicain, a serine protease, was purified from the latex of *Morus indica* cv. K2, and various enzymatic properties were characterized. The enzyme showed similarity with subtilisin in regards of the stability against pH, temperature and organic solvent, the substrate specificity and the *N*-terminal residues [50]. These unique properties suggest its possible application in food and pharma industry.

11. Future Direction

11.A.Anti-Stress Experiment

We found two new biological activity of mulberry juice: anti-stress activity and anti-HIV activity. The anti-stress activity of Mulberry juice [24] seems to be derived from its anti-oxidant activity, since there are numerous anti-oxidants and radical scavengers in the mulberry juice. We found that 4 hours administration of mulberry juice is enough to induce anti-stress activity in mice, but it is recommendable that the mulberry juice is administered at the most stressful time. On such condition, the anti-stress effect can be maintained even 4 hours after cessation of administration (Figure 1). Our preliminary study showed that one gulp of mulberry juice by two healthy male volunteers without stress did not significantly change the visual acuity, evaluated 4 hours later by distant visual acuity, near visual acuity, refraction, intraocular pressure, critical flicker fusion frequency and night vision [51]. Thus, the anti-stress activity of mulberry juice in human should be evaluated under more stressful or fatigue conditions, or continuous administration.

11.B. Possible Action Mechanism of Lignin Carbohydrate Complex

We unexpectedly found that mulberry juice that contains fiber showed anti-HIV activity, whereas blueberry juice that does not contain fiber was inactive [25]. The anti-HIV activity was concentrated in the lignin carbohydrate fractions [25]. Lignin carbohydrate complex is an amorphous substance of polymerized phenylpropanoids complexed with polysaccharides. The anti-viral activity of Mulberry lignin carbohydrate complex seems to be derived from the direct inactivation of the virus [52] by the phenylpropanoid portions [53], or the inhibition of HIV-1 penetration into the cells [54]. The binding site of lignin carbohydrate complex in the outer membrane of HIV remains to be investigated.

Our data suggest the positive relation between the molecular weight and tumor-specificity. However, lignin carbohydrate complex showed extremely low cytotoxicity, and even stimulated the growth of mouse splenocyte by yet unknown mechanism [55], making it difficult to accurately determine its tumor-specificity index.

Toll-like receptor4 (TLR4) is the central signaling receptor for LPS in mammals. TLR4 not only engages LPS but it recognizes an envelope glycoprotein encoded by mouse mammary tumor virus, ligands such as heat shock proteins and extracellular domain A in fibronectin [56]. Recently, aqueous extract of Mori Fructus activated the TLR4-mediated pathway including NF- κ B, ERK1/2, p38 and JNK [47]. Declin-1 is a receptor for β -1,3- and β -1,6 glucan, and consists a single C-type, lectin-like, carbohydrate recognition domain, a short stalk, and a cytoplasmic tail possessing an immunoreceptor tyrosine-based activation motif. Dectin-1 is expressed on cells of monocyte/macrophages lineage, and neutrophils [57]. On the contrary, the receptor for lignin carbohydrate complex has not been identified. DNA array analysis demonstrated that lignin carbohydrate complex from *Lentinus edodes* mycelia extract (L·E·M) activated TLR2-mediated pathways, and expression of chemokine gene expressions, without induction of autophagy related genes [58].

11.C. Factors Affecting the Biological Activity of Lignin Carbohydrate Complex

The biological activity of lignin carbohydrate complex strictly depends on the molecular weight, solubility and sterilization method. It remains to characterize the minimum size of lignin carbohydrate complex of mulberry and elucidate the action point.

We have previously reported synergistic action of vitamin C and lignin carbohydrate complex [59, 60] and vitamin K [61, 62]. Mulberry juice contains relatively higher amounts of lignin carbohydrate complex and vitamin K, but very low concentration of vitamin C (Table I). Supplementation of vitamin C may further enhance the biological activity of lignin carbohydrate complex and vitamin K present in mulberry juice.

The biological activity of antioxidants are affected by metal ions. We have previously reported that the cytotoxic activity of chlorogenic acid was significantly reduced by CoCl_2 [38]. The cytotoxicity of sodium ascorbate, gallic acid, EGCG, curcumin and dopamine was also reduced by CoCl_2 , possibly due to prooxidant action of cobalt ion: Co^{2+} may be easily oxidized to Co^{3+} by oxygen in the circulating air, and the resultant unstable Co^{3+} then oxidize the antioxidants [63]. The cytotoxic activity of sodium ascorbate was enhanced by CuCl and

CuCl₂, but neutralized by Fe²⁺ and Fe³⁺ [64]. These data suggest that the importance of appropriate amounts of metals in the nutrients. The presence of various attractive substances in the mulberry juice suggests its possible functionality as an alternative medicine.

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Chapter XI

Benefits of Grape Chemicals on Human Health

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Abstract

Benefits ascribed to healthy dietary patterns are mainly due to plant-derived foodstuffs rich in bioactive constituents. In particular, in the context of Mediterranean diet, grapevine (*Vitis vinifera* L.) products (grape, grape juice and wine) represent a valuable source of polyphenols, an extensive and heterogeneous group of secondary metabolites abundant in plant tissues. Typically, polyphenols include flavonoids, stilbenes and proanthocyanidins, compound with a broad spectrum biological activity, though grape chemistry is much more complex. According to the structure-activity relationship, antioxidant power has been extensively attributed to these phytochemicals, due to the hydroxyl groups and conjugated double bonds of the flavan nucleus, the basic skeleton of polyphenols. In this survey, we focus on the antitumoral, cardio- and neuroprotective activity of these phytotherapeutics, emphasizing on the molecular and biochemical mechanisms involved in these properties. Finally, we briefly introduce some aspects of polyphenol bioavailability, a critical process whose knowledge is essential to better understand the *in vivo* activities of these compounds.

Keywords: *grape, wine, Mediterranean diet, polyphenols, flavonoids, anthocyanins, resveratrol, proanthocyanidins, chemoprevention, cardioprotection, neuroprotection*

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1. Introduction

Though the impressive number of studies on the biological activities of specific food components, so far carried out mainly on *in vitro* and animal models, the contribute of diet to human health is far from being completely elucidated. Consequently, it still remains an open task to define the precise role of healthy dietary patterns on prevention of chronic degenerative diseases, taking into account that other environmental and genetic factors play a prominent role on the etiology of these disorders. First of all, healthy diet is crucial for reducing overweight and obesity, the leading causes of many chronic conditions, including hypertension, cardiovascular diseases, type II diabetes and some types of cancers (National Institute of Health, 1998). Therefore, the decrease of total energy (calorie) intake is fundamental in promoting overall health, in association with regular, moderate-intensity aerobic activity to increase the caloric expenditure (Haskell et al., 2007).

A diet rich in fruits and vegetables represents a typical hypocaloric (low-energy) regimen. Additionally, regularly consumed as part of a normal diet, plant foods may provide health benefits beyond basic nutritional functions, by virtue of their content of bioactive phytochemicals, contributing to improve life quality and expectancy. Results from several large longitudinal studies have shown that individuals consuming five or more servings per day of fruits and vegetables significantly reduce their risk of cardiovascular diseases (Hung et al., 2004; Dauchet et al., 2006; He et al., 2006, 2007). Clinical trials have also reported that diets rich in plant foods significantly decrease blood pressure (Appel et al., 1997). With respect to cancer risk, the evidence is less consistent, though both case-control and cohort studies have shown an inverse association between plant food intake and several cancers. A cohort study has suggested a protective effect of fruit consumption on bladder cancer (Riboli and Norat, 2003), while, in a larger cohort study, consuming five or more servings of fruits and vegetables per day did not reduce the total cancer risk (Hung et al., 2004). A meta-analysis of cohort studies on gastric cancer has found that plant food intake is associated with lower incidence, but not with mortality (Lunet et al., 2005), and both case-control and cohort studies have shown that high consumption of fruits and vegetables is inversely associated with oral cancer (De Stefani et al., 2000; Boeing et al., 2006; Pavia et al., 2006).

Compelling evidences suggest that the health-promoting effects attributed to plant foods are due to some their bioactive constituents (phytochemicals). The latter are secondary metabolites (phytoalexins) produced by plants to defend themselves against pathogen and pest attack and to improve their adaptation to the adverse environmental conditions (Iriti and Faoro, 2004, 2009). Because of their antimicrobial and antioxidant activity, phytoalexins are able to arrest the pathogen spreading and to scavenge the reactive oxygen species (ROS) involved in the etio-pathogenesis of many plant (and animal) diseases (Iriti and Faoro, 2004, 2009).

In this survey, we provide a brief description of the complex chemistry of grapevine (*Vitis vinifera* L.) products, among the main components of Mediterranean diet. In particular, we focus on specific grape chemicals, emphasising on the molecular and biochemical mechanisms involved in their antitumoral, cardio- and neuroprotective activities, important properties ascribed to these phytotherapeutics.

2. Grape Chemistry and Products

Plant food matrices include a huge collection of secondary metabolites arising from different metabolic pathways. In particular, grape represents the paradigm of this complexity, with thousands of compounds present in different tissues, among which polyphenols are the most investigated metabolites for their biological activities (Jeandet et al., 2002; Pezzuto, 2008). The term ‘polyphenols’ refers to some groups of phenylpropanoids, a class of compounds arising from the aromatic amino acid phenylalanine (Phe) (Figure 1). *Sensu stricto*, polyphenols are metabolites with more than one aromatic (phenol) ring, one deriving from Phe and the other from the malonate pathway, a biosynthetic route which starts from acetate, the precursor of lipidic compounds (Iriti and Faoro 2004, 2009).

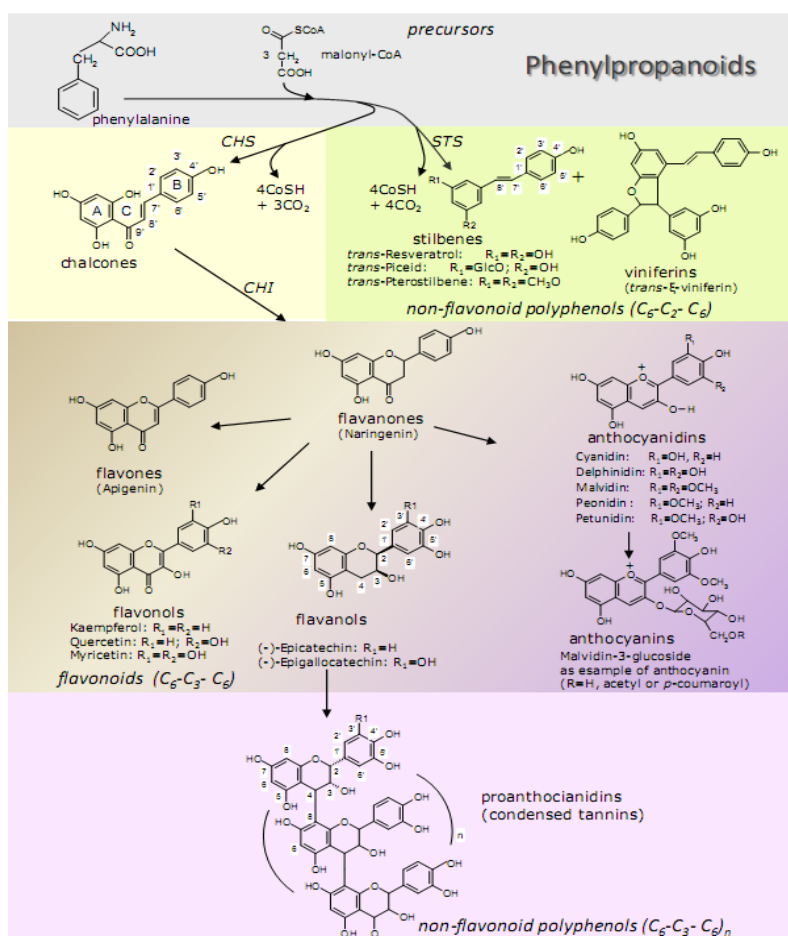


Figure 1. Phenylpropanoid pathway leading to flavonoids and non-flavonoid polyphenols (stilbenes and proanthocyanidins). Phenylalanine derivative is condensed with 3 molecules of malonyl-coenzyme A (CoA) by chalcone synthase (CHS) to form chalcone, then converted to naringenin, the precursor of flavonoids, by chalcone isomerase (CHI). Alternatively, the enzyme stilbene synthase (STS) produces stilbenes from the same precursors. The compounds belonging to the stilbene family possess the skeleton based on the trans-resveratrol structure. Proanthocyanidins are oligomeric or polymeric flavanol derivatives arising from catechin monomeric units. See the text for more details.

Polyphenols are subdivided into flavonoids ($C_6-C_3-C_6$), stilbenes ($C_6-C_2-C_6$) and proanthocyanidins [$(C_6-C_3-C_6)_n$]. The basic flavonoid chemical structure is the flavan nucleus, consisting of 15 carbon atoms arranged in three rings: two benzene rings (A and B) combined by an oxygen-containing pyran ring (C) (Pietta, 2000). The diverse classes of flavonoids in grape (flavanones, flavones, flavonols, flavanols and anthocyanidins) differ among them in the level of oxidation and saturation of the C-ring, while individual compounds within a class vary in the substitution pattern of the A- and B-ring (Figure 1). Flavonoids may be hydroxylated in different positions, 3, 5, 7, 3', 4' and/or 5', and one or more of these hydroxyl groups can be in turn methylated, acylated, prenylated or sulphated (Viskupiřová et al., 2008). Among flavonoids, anthocyanidins are the most abundant pigments in grape berry skin. Their conjugated derivatives (glycones), anthocyanins, generally bound to sugars, are water soluble pigments conferring blue, dark blue, red and purple hue to flowers, fruits and other plant organs. Anthocyanins of *Vitis* are structurally based on five aglycones/anthocyanidins, malvidin, cyanidin, delphinidin, peonidin and petunidin, which differentiate on the basis of number and position of their hydroxyl groups and their methylation degree (Figure 1) (Iriti et al., 2004; Horbowicz et al., 2008; de Pascual-Teresa and Sanchez-Ballesta, 2008). Flavonols include mainly kaempferol, quercetin and myricetin aglycones, whereas apigenin is the main flavone in grape. Flavanols provide catechins, the monomeric units for the proanthocyanidin biosynthesis (see ahead this section) (Iwashina, 2000). Molecules belonging to the stilbene family are also abundant in berry skin cells, and possess the basic chemical structure of the *trans*-resveratrol skeleton (Figure 1). Stilbenes comprise piceids, pterostilbenes and viniferins that are glucosides, dimethylated derivatives and oligomers of resveratrol, respectively (Jeandet et al., 2002). Proanthocyanidins, also known as condensed tannins or simply tannins, are both oligomeric and polymeric compounds arising from flavanol condensation (Figure 1). In grape, the most common monomers include catechin epimers [(+)-catechin and (-)-epicatechin], whose polymerization degree (PD) ranges mainly between 3 and 11. In *Vitis*, proanthocyanidins are principally present in seeds, where they represent the major fraction of the total polyphenols (Gavetta et al., 2000).



Figure 2. Main components of the Mediterranean diet and their suggested timely intake.

Among grape products, wine represents a main component of Mediterranean diets, the different traditional dietary patterns developed in geographical subareas of the Mediterranean basin, (southern Italy and France, Spain, Greece, Turkey, northern Africa and Middle East). Traditionally, these dietary habits originated in areas where olive (*Olea europaea* L.) and grapevine were cultivated, and their products, olive oil and wine, regularly consumed. Besides these foodstuffs, other main constituents of Mediterranean diets include whole grains, fruits, vegetables, legumes, nuts, yogurt and ricotta as dairy product, fish and white meat as protein source (Figure 2) (Simopoulos 2001).

3. Grape and Cancer Chemoprevention

Cancer is a multistage and multifactorial disease, the second leading cause of death worldwide after heart disease, whose risk and incidence augments with age. In addition to genetic factors, environmental and nutritional factors play a main role in cancer aetiology. In industrialized developed countries, breast, prostate and colorectal cancers predominate, because of a diet rich in animal foods, refined carbohydrates and deficient in plant foods. Conversely, in developing countries, where diet is largely based on cereal/starchy foods, esophageal, stomach and liver cancers have a higher incidence (Young and Le Leu, 2002; La Vecchia and Bosetti, 2007).

Prevention has become as important as therapy to control cancer, in order to reduce both cancer morbidity and mortality. Chemoprevention of cancers is the strategy of preventing, arresting or reversing carcinogenesis with chemopreventive agents, dietary therapeutics pharmacologically safer than chemotherapeutic agents used in cancer patients. Chemoprevention can interfere with each step of neoplastic progression, consisting of three mechanistically distinct, temporally ordered and closely linked stages: malignancy initiation, promotion and progression. Tumor initiation is a rapid, mutagenic and irreversible process transforming a normal cell into a initiated cell with a benign tumor phenotype. It includes the exposure to a procarcinogen, its transport and distribution to organs and tissues where metabolic activation and detoxification can occur, and the genotoxic damage due to the covalent interaction of its ultimate electrophilic species with target-cell DNA. The second stage, promotion, is a slow and reversible clonal expansion (proliferation) of preneoplastic cells producing a benign tumor. In the final step, progression, a neoplastic transformation occurs irreversibly, that is the conversion of the benign tumor to a malignant phenotype, with invasive and metastatic potential (Wattenberg, 1985; Greenwald, 2002; Suhr, 2003; Aggarwal et al., 2004; Fresco et al., 2006; Athar et al., 2007; Hail et al., 2008; Khan et al. 2008a; Saiko et al., 2008; Bishayee, 2009).

Chemopreventive agents can be divided into two categories. Blocking agents arrest the tumour initiation stage, inhibiting the metabolic activation of procarcinogens and preventing their ultimate reactive species to reach the cellular target sites. Blocking agents can also promote the detoxification of procarcinogens, thus stimulating their excretion from the body. Differently, suppressing agents act on the tumor promotion and progression, inhibiting the malignant transformation of initiated cells (Wattenberg, 1985; Greenwald, 2002; Suhr, 2003; Aggarwal et al., 2004; Fresco et al., 2006; Athar et al., 2007; Hail et al., 2008; Khan et al. 2008a; Saiko et al., 2008; Bishayee, 2009). Again, chemopreventive agents can avoid the

time-dependent tumour resistance (chemoresistance) to chemotherapeutic agents and their non-specific toxicity towards non-target cells, thus resulting pharmacologically safer (Garg et al., 2005).

An intricate network of signalling pathways is involved in cancer pathogenesis, regulating the (im)balance between cell growth-promoting and growth-inhibiting mechanisms (Gatz and Wiesmuller, 2008). At molecular level, the interactions with both transcription factors and receptors have been proposed as putative mechanisms for the reported anticarcinogenic activity of (grape) polyphenols (Table 1, Figure 3) (Shankar et al., 2007; Kundu and Suhr, 2009). Furthermore, chemopreventive dietary agents can promote apoptosis in premalignant and malignant cells by modifying different stages of the apoptotic process, including the cell redox status (Khan et al., 2008b).

Table 1. Targets and biological activities of grape chemicals relevant to cancer prevention

Class of target	Molecular and biochemical targets	Biological activity	References
Inflammation	COX-2	Anti-inflammatory	Richard et al., 2005
Oxidative stress	ROS	Antioxidant	Seyoum et al., 2006
Transcription	NF- κ B, AP-1	Antitumoral	Hou et al., 2004 Kundu et al., 2006
Cell cycle	ODC, DNA polymerase, ribonucleotide reductase	Antiproliferative	Fontecave et al., 1998 Schneider et al., 2000 Tsan et al., 2002
Apoptosis	P53, BAX, Bcl2, survivin	Proapoptotic	Huang et al., 1999 Tinhofer et al., 2001 Tang et al., 2007
Steroid hormones	ERs, ARs	Modulation of steroid receptors	Bhat et al., 2001 Xing et al., 2001
Extracellular matrix	MMPs	Inhibition of metastasis	Tang et al., 2008
Tissue oxygen metabolism	HIF-1 α	Inhibition of angiogenesis	Cao et al., 2004

Chemopreventive properties of grape products are more likely attributable to the combined effect of their bioactive components, rather than to one or a few specific molecules, although resveratrol represents the most studied example of grape biologically active compound (Iriti and Faoro, 2006; Pezzuto, 2008; Wang and Stoner, 2008; Nandakumar et al., 2008; Thomasset et al., 2009). In his seminal and pioneering study, Pezzuto and his group reported, for the first time, the chemopreventive potential of resveratrol in assays representing the three carcinogenesis stages. They also showed that resveratrol inhibits the development of preneoplastic lesions in a mouse mammary gland culture treated with the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA), as well as tumourigenesis in a mouse skin cancer model (Jang et al., 1997). Nevertheless, it must be underlined that, when consuming a food or beverage, we ingest its potpourri of (phyto)chemicals.

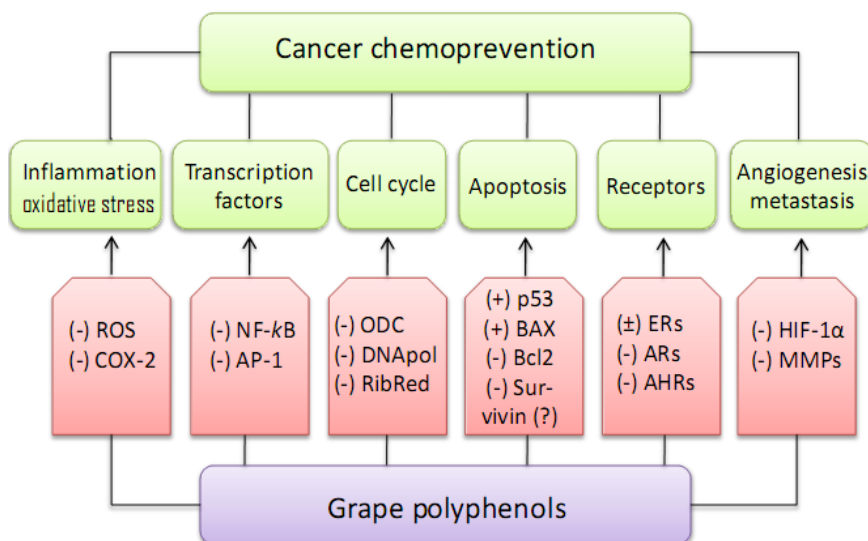


Figure 3. Mechanisms of cancer prevention of polyphenols by inhibiting (-) or enhancing (+) reactive oxygen species (ROS) and cyclooxygenases (COX-2), important transcription factors (NFκB, AP-1), ornithine decarboxylase (ODC), DNA polymerase (DNAPol), ribonucleotide reductase (RibRed), P53 and BAX proapoptotic proteins, apoptosis inhibitory proteins Bcl-2 and survivins, androgen and estrogen receptors (ARs, ERs), aryl hydrocarbon receptors (AHR), hypoxia inducible factors-1α (HIF-1α), matrix metalloproteinases (MMPs) (see text for details).

Cancer and chronic inflammation are causally linked, as demonstrated by the increased gene expression and/or activity of enzymes synthesizing arachidonate-derived proinflammatory mediators, and by the augmented production of these mediators (prostanoids) in various cancers (Tabel 1, Figure 3) (Kundu and Surh, 2005; Aggarwal et al., 2006). Hence, inhibition of cyclooxygenases (COXs), particularly of inducible COX-2 isozyme, and blockage of prostaglandin cascade are relevant and effective mechanisms to counteract multistage carcinogenesis (Subbaramaiah and Donnenberg, 2003). Among polyphenols, resveratrol capability of blocking various components of the proinflammatory cascade has been known for a long time, as reported in phorbol ester-treated human mammary epithelial cells, where resveratrol inhibited both COX-2 gene transcription and enzymatic activity (Subbaramaiah et al., 1998). Antiinflammatory properties of resveratrol were then reported in a variety of models and at different biochemical levels. This compound significantly inhibited the expression of COX-2 in: *i*) mouse peritoneal macrophages treated with lipopolysaccharide (LPS), 12-*O*-tetradecanoylphorbol-13-acetate (TPA) or H₂O₂ (Martinez and Moreno, 2000); *ii*) RAW 264.7 macrophages stimulated with LPS plus interferon γ (INF- γ) (Murakami et al., 2003); and *iii*) mouse skin treated with TPA (Kundu et al., 2006). *In vivo* (F344 rat), resveratrol at concentrations between 1 and 2 mg/kg body weight suppressed both the number and size of *N*-nitrosomethylbenzylamine (NMBA)-induced esophageal tumors per rat, by targeting COXs and prostaglandin E₂ (PGE₂) (Li et al., 2002). Resveratrol and other stilbenes also decreased COX-2 activity and reduced the production of PGE₂ in peripheral blood leukocytes treated with LPS plus INF- γ (Richard et al., 2005).

Diverse pathways have been proposed as putative mechanisms for the reported anticarcinogenic activity of grape chemicals, above all the interaction with transcription

factors. NF- κ B (nuclear factor κ B) is a pleiotropic transcription factor involved in different signalling pathways, ranging from inflammation, oncogenesis and apoptosis (Table 1, Figure 3). Normally, it resides in the cytosol, forming a complex with the inhibitory subunit I κ B, whose phosphorylation, ubiquitylation and subsequent degradation, by 26S proteasome, allows NF κ B to move into the nucleus, where it activates gene transcription by binding to specific gene promoter regions. Therefore, aberrant activation of NF- κ B, associated with proliferation of malignant cells and prevention of apoptosis, is causally linked to the neoplastic transformation (Bharti and Aggarwal, 2002). The mechanism of resveratrol-induced inhibition of the NF- κ B activity includes the suppression of the pathway regulated by the inflammatory mediator tumor necrosis factor α (TNF α) (Manna et al., 2000; Pervaiz, 2003; Suhr, 2003). Additionally, resveratrol inhibits the I κ B kinase (IKK) activity and the subsequent I κ B phosphorylation, thus blocking its degradation, NF- κ B translocation and DNA binding (Holmes-McNary and Baldwin, 2000).

Activator protein 1 (AP-1) is another transcription factor that regulates the expression of genes involved in cell adaptation, differentiation, proliferation and apoptosis, whose activation is inhibited by resveratrol and anthocyanidins (Table 1, Figure 3) (Manna et al., 2000; Dong, 2003; Hou et al., 2004). The mammalian AP-1 proteins are homodimers and heterodimers composed of basic region-leucine zipper (bZIP) proteins that belong to the Jun (c-Jun, JunB and JunD), Fos (c-Fos, FosB, Fra-1 and Fra-2), Jun dimerization partners (JDP1 and JDP2) and the closely related activating transcription factors (ATF2, LRF1/ATF3 and B-ATF) subfamilies. They are activated in response to oxidative and proinflammatory stimuli via the upstream MAP kinase signalling (Shaulian and Karin, 2002).

The interaction with both androgen (ARs) and estrogen receptors (ERs), belonging to the nuclear steroid hormone receptor family, represents another molecular mechanism involved in resveratrol-mediated chemoprevention (Table 1, Figure 3). A decrease in cell proliferation was reported in the androgen-responsive prostate cancer cell lines LNCaP treated with resveratrol and the flavonoid quercetin, due to the inhibition of both expression and function of ARs (Mitchell et al., 1999; Xing et al., 2001). ARs represent essential mediators of the androgen activity, controlling the transcription of androgen-inducible genes, such as the prostate specific antigen (PSA). Therefore, they are involved in the development of the hormone-responsive prostate cancer. Moreover, the growth of androgen-unresponsive prostate cancer cells was also inhibited by resveratrol, though to a lesser extent than that of the androgen-responsive cell lines (Hsieh and Wu, 1999).

Estrogens regulate the transcription of target genes by binding to different intracellular estrogen receptors (ER α and ER β) with tissue and ligand specificity, influencing the growth, differentiation and function of target tissues and playing a pivotal role in breast cancer. As a phytoestrogen, resveratrol was shown to possess either estrogen agonist and antagonist activity, thus rising some controversy regarding its therapeutical application against the estrogen-responsive breast cancers (Lu and Serrero, 1999; Basly et al., 2000; Bhat et al., 2001). Phytoestrogens are diphenolic plant metabolites that exert estrogen agonist/antagonist activity because of their structural similarities to natural and synthetic estrogen steroids. They are either hormone-like compounds, with inherent estrogenic activity, or can be converted by intestinal flora to weakly estrogenic compounds. Other phytoestrogens include lignans and isoflavones, present in whole cereals and legumes respectively, classified as selective estrogen receptor modulators (SERMs) (Ososki and Kennelly, 2003).

Inhibition of both aromatase (estrogen synthetase) expression and activity by grape seed extracts and red wine phytochemicals represents another mechanism of breast cancer suppression, as demonstrated in an aromatase-transfected MCF-7 breast cancer xenograft model (Eng et al., 2001; Kijima et al., 2006). Aromatase is a cytochrome P-450 enzyme which converts C₁₉ androgens to aromatic C₁₈ estrogens, expressed at higher levels in breast cancer than in normal tissues. Therefore, its overexpression in breast cancerous cells can influence the tumoral progression itself, because of the major role of estrogens on the breast cancer development (Sun et al., 1997).

Aryl hydrocarbon receptor (AHR) is a cytosolic protein that translocates to the nucleus upon ligand binding. Metabolic activation of aryl hydrocarbons (AH) results from their binding to AHR, that, after migration, activates the transcription of the *CYP1A1* gene, encoding for the cytochrome P450 (CYP450) isozyme CYP1A1. CYP450 enzymes are involved in the metabolism of a variety of xenobiotics, including carcinogens such as AH, and are overexpressed in a variety of tumors. The metabolized active forms of carcinogens can subsequently interact with DNA, thus causing mutations. Resveratrol was shown to exert a strong inhibitory effect on the AH-induced *CYP1A1* expression, both at the mRNA and protein level, as well as on other CYP450 isozymes, such as CYP1A2 and CYP3A4 (Ciolino et al. 1998; Ciolino and Yeh, 1999; Chan and Delucchi, 2000; Chang et al., 2001).

Besides the above mentioned signalling networks regulated by polyphenols, the induction of apoptosis is another molecular mechanism involved in their anti-proliferative effects (Table 1, Figure 3) (Khan et al., 2008b). In a variety of tumor cell lines, among them leukaemia cells, it was reported that resveratrol activates the mitochondrial-dependent apoptotic pathway, by the up-regulation of pro-apoptotic p53 and Bax proteins and the down-regulation of the death inhibitory protein Bcl-2 (Clement et al., 1998; Huang et al., 1999; Suhr et al., 1999; Dorrie et al., 2001; Tinhofer et al., 2001; Roman et al., 2002). The gene *p53* is an important oncosuppressor, whose mutations, as well as the loss of p53 protein function, are related to more than half of human cancer (Khan et al., 2008b). However, in different cell lines, polyphenols induce apoptosis by mechanisms other than *p53* gene modulation (Soleas et al., 2001; Schneider et al., 2004). Suppression of antiapoptotic protein survivin may be another proapoptotic mechanism promoted by grapevine polyphenols, as reported for green tea polyphenols, which decreased both mRNA and protein expression of survivin (Tang et al., 2007). Survivin is a member of the inhibitor of apoptosis protein (IAP) family, overexpressed in several human neoplasms (Johnson and Howerth, 2004).

The anti-proliferative effects of polyphenols can also be due to the inhibition of ribonucleotide reductase, the enzyme that catalyzes the reduction of ribonucleotides into deoxyribonucleotides, as well as of the DNA polymerase and ornithine decarboxylase (ODC), the latter a key enzyme of the polyamine synthesis deeply involved in cancer growth (Fontecave et al., 1998; Schneider et al., 2000; Tsan et al., 2002). Moreover, a number of studies have established that resveratrol blocks cell proliferation by inducing cell cycle arrest at the G1/S and S/G2 phase (Ragione et al., 1998; Hsieh et al., 1999; Agarwal et al., 2000; Bernhard et al., 2000; Joe et al., 2000).

Probably, the most investigated biological activity of polyphenols is their antioxidant power, another mechanism involved in aberrant growth inhibition, as a prooxidant intracellular milieu represents a strong stimulus for cell proliferation (Pietta, 2000; Vinson et al., 2000; Amic et al., 2003; Heim et al., 2002; Soobrattee et al., 2005; Seyoum et al., 2006). The antioxidant capacity of red grape was evaluated in HepG2 (human hepatocellular liver

carcinoma) cell line and positively correlated to the total phenolic content and to the oxygen radical absorbance capacity (ORAC) values of grape extracts. The authors concluded that increasing fruit consumption may represent a suitable strategy to counteract oxidative stress and to reduce the risk of cancer (Wolfe et al., 2008). Oxidative DNA damage, leading to modifications of DNA bases, is related to mutagenesis and carcinogenesis too. Daily grape juice supplementation (480 mL), for 8 weeks, reduced DNA strand breaks in peripheral lymphocytes, as detected by the single cell gel electrophoresis (comet assay, a powerful tool in mutagenesis studies), beside decreasing the amount of released reactive oxygen species (ROS) (Park et al., 2003). Similarly, treatment of human lymphocytes with a grape seed extract decreased the frequency of micronuclei (an assay for the detection of DNA damage) by 40 % and the production of malonyldialdehyde (a biomarker of lipid peroxidation) by 30 %, while increasing the activities of the antioxidant enzymes catalase and glutathione S-transferase by 10 and 15 %, respectively (Stankovic et al., 2008).

Grape seed extracts showed promising efficacy also against two important processes involved in cancer progression, angiogenesis and metastasis, inhibited in prostate and breast carcinoma, respectively (Singh et al., 2004; Mantena et al., 2006). Because of increased metabolic activity and oxygen consumption of rapidly proliferating cells, solid tumors are likely to maintain an intratumoral hypoxic environment, which, in turn, induces a set of hypoxia-responsive genes in order to allow tumor cell adaptation. The expression of these genes is regulated by hypoxia inducible factor (HIF), a major regulator of cellular oxygen homeostasis (Liao and Johnson, 2007). Resveratrol was reported to inhibit the expression of HIF-1 α in human ovarian cancer cells OVCAR-3, as well as of the vascular endothelial growth factor (VEGF), an HIF-regulated angiogenic factor (Cao et al., 2004). Angiogenic factors promote neovascularization of interstitial stroma, the tissue surrounding the primary tumor site, a process which supplies nutritional requirements to proliferating neoplastic cells and facilitates their access to vascular system (intravasation). Before penetrating blood vessel endothelium, from the primary tumor site, and gaining access to the blood stream, cancer cells must invade local tissues by degrading extracellular matrix (ECM) components and, ultimately, transverse the basement membrane. Once in circulation, these cells can form metastatic colonies at secondary locations. Resveratrol was reported to inhibit the invasiveness of diverse cancer cells, by reducing the expression and activity of matrix metalloproteinase (MMP)-2 and MMP-9, involved in ECM degradation (Sun et al., 2006; Yu et al., 2007; Tang et al., 2008).

Apart from the beneficial effects exerted by polyphenols mainly on prostate, breast and blood cancers, as discussed above, other types of tumors can benefit from regular, moderate consumption of grape products (Figure 4). Carcinomas of the digestive tract are common and their risk increases with age. In gastric cancer cells, resveratrol was shown to inhibit cell proliferation and to induce apoptosis (Holian et al., 2002; Atten et al., 2005; Riles et al., 2006). Resveratrol and red wine also inhibited the growth of 15 clinical isolates of *Helicobacter pylori*, the primary etiological determinant of gastric cancer (Mahady and Pendland, 2000). Grape polyphenols, mainly quercetin, were shown to suppress the formation of aberrant crypt foci, in animal models of carcinogenesis, by modulating both cell proliferation and apoptosis (Johnson, 2002). The crypt is the fundamental unit of epithelial proliferation in the colonic mucosa, where genetically damaged stem cells are removed from the epithelium by apoptosis, before they undergo clonal expansion. Hence, increased apoptosis in the proliferating zone of the colonic crypt provides a protective mechanism

against the crypt cell hyperproliferation and neoplasia (Johnson, 2002). In both cancerous and noncancerous human colon tissues, black grape extracts modified the activity of enzymes involved in DNA-turnover, adenosine deaminase, 5' nucleotidase and xanthine oxidase, thus depriving cancer cells of nucleotides for proliferation (Durak et al., 2005). Similar results were reported on cancerous and noncancerous human urinary bladder tissues (Durak et al., 2007). Moreover, the growth of human colorectal carcinoma cells was inhibited by a grape seed extract rich in proanthocyanidins (Kaur et al., 2006), and a red grape dietary fiber (obtained from seeds) induced epithelial hypoplasia with a decrease in the depth of crypts, in both rat cecal and distal colonic mucosa, as well as a reduction of crypt density and mucosal thickness (López-Oliva et al., 2006).

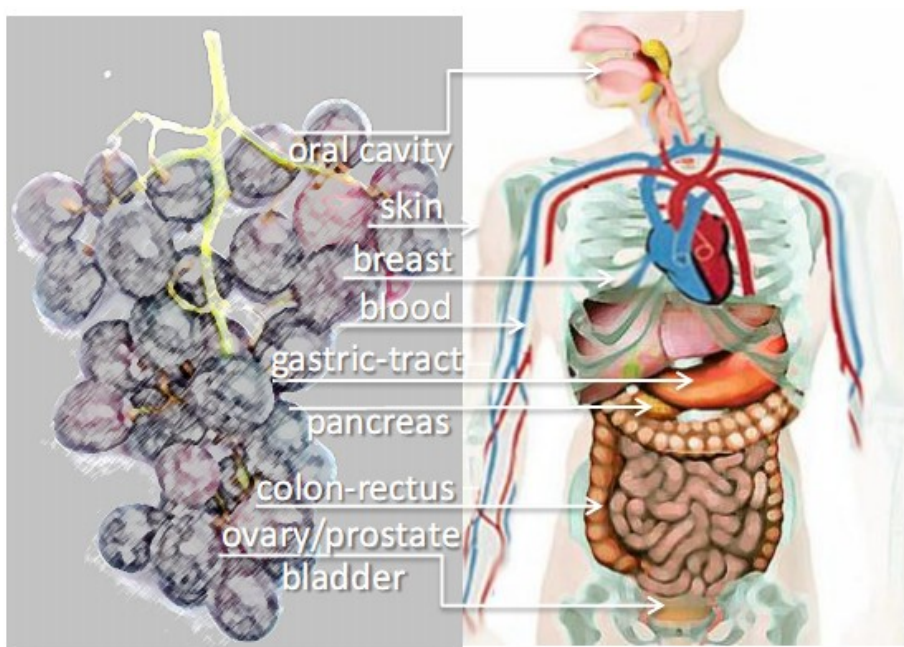


Figure 4. Grape polyphenols exert beneficial effects in the prevention of different cancers.

Photochemoprevention by botanical agents may prevent skin cancer at various stage of carcinogenesis, as shown in different models. Topical application of resveratrol and apigenin to SKH-1 hairless mice, prior to UV exposure, effectively prevented the radiation-induced carcinogenesis (Birt et al., 1997). Mechanisms involved in photochemoprevention by resveratrol include the inhibition of NF κ B signalling, as demonstrated in UV-exposed normal human epidermal keratinocytes (Adhami et al., 2003). In SKH-1 mice, grape seed proanthocyanidins also prevented the UV-induced oxidative stress, decreased lipid peroxidation, inhibited the activation of NF- κ B signalling and, finally, photocarcinogenesis (Mittal et al., 2003; Sharma et al., 2007). As for other cell lines, resveratrol induced apoptosis in two human melanoma cell lines, by activating a MAP kinase pathway (Niles et al 2003).

In human pancreatic cancer cell line Panc02 inoculated into C57BL/6 mice, both resveratrol and quercetin suppressed pancreatic cancer via different mechanisms, namely apoptosis induction (by caspase 3 and 8), cell cycle arrest at G1 phase and inhibition of tumor cell migration (invasiveness) through the ECM barrier (Cui et al., 2008). Pancreatic cancer is

a highly aggressive malignancy, currently treated with limited success by conventional therapeutics and with an extremely poor prognosis (Jemal et al., 2008). A mixture of isoflavone 10 nM + curcumin 500 nM + epigallocatechin-3-gallate (EGCG) 125 nM + resveratrol 125 nM inhibited by 40%, up to 72h, the cell growth of BxPC-3 cells, a human pancreatic cancer cell line, via a mechanism partly due to the inactivation of NF- κ B. Isoflavones, curcumin and EGCG are polyphenolic compounds occurring in legumes, *Curcuma longa* (a plant used in Ayurvedic medicine) and tea, respectively. The authors concluded that a combined treatment with phytochemicals induces a greater inhibition of cell growth than that obtained after treatment with single compounds (Wang et al., 2008).

Among the tumors of head and neck, carcinomas of the oral cavity, mainly of squamous-cell type, comprise an important group of malignancies, whose incidence is increasing all over the world. Cancer of the upper aerodigestive tract, including cancers of lip, tongue, oral cavity and pharynx, ranks as the seventh most common cause of cancer worldwide and represents 2-4 % of all diagnosed cancers, associated with a poor prognosis and survival rate if not early diagnosed (Jane-Salas et. al., 2003; Davies and Welch; 2006; Pavia et al., 2006). Administration of proanthocyanidins showed to suppress the proliferation of human oral squamous-cell carcinoma in a dose-dependent manner (King et al., 2007). More interestingly, proanthocyanidins inhibited the proliferation of cell carcinoma also after transfection with human papillomavirus (HPV), another putative risk factor for oral cancer (King et al., 2007). It has also been reported that, in the two well-characterized oral squamous-cell carcinoma cell lines CAL-27 and SCC-25, the antiproliferative effect of a grape seed extract was correlated to the dramatic upregulation of mRNA expression of caspases 2 and 8, proteases considered the executioners of apoptosis (Chatelain et al., 2008). In human tongue squamous-cell carcinoma cells SCC-9, resveratrol inhibited angiogenesis by reducing HIF-1 α protein accumulation and VEGF expression, promoting the proteasomal degradation of HIF-1 α without affecting HIF-1 α mRNA expression (Zhang et al., 2005).

4. Grape and Cardiovascular Health

Endothelial dysfunction, oxidative modification of LDL, inflammation and platelet aggregation are key factors in atherogenesis and hypertension (Figure 5). Endothelial cells exert multiple physiological functions, maintaining the integrity of the vascular wall and representing a permeable barrier through which diffusion and active transport of several substances occur. Furthermore, endothelial cells constitute a non-thrombotic and non-adherent surface for platelets and leukocytes, and they regulate the vascular tone by producing nitric oxide (NO), eicosanoids, endothelins and cytokines (da Luz and Coimbra, 2004).

In cardiovascular diseases, an important inflammatory process takes place after leukocyte mobilization. Phospholipase A2 leads to the hydrolysis of plasma membrane phospholipids, mainly phosphatidylcholine and phosphatidylethanolamine, and to the subsequent release of arachidonic acid. This fatty acid represents the substrate of COX and lipoxygenase (LOX), the enzymes involved in the synthesis of prostanoids (prostaglandins and thromboxanes) and leukotrienes, respectively, the inflammation mediators collectively grouped in eicosanoids. Among COX-derived platelet modulators, thromboxanes, mainly thromboxane A2 (TXA2),

potentiate platelet reactivity, whereas prostacyclins help to maintain platelets in a quiescent state (Smith, 1989). Polyphenols, mainly quercetin and resveratrol, were shown to inhibit COX and LOX activities and eicosanoid synthesis (Figure 5) (Kimura et al., 1985; Ferrandiz and Alcaraz, 1991; Laughton et al., 1991; Pace-Asciak et al., 1995; Kim et al., 1998).

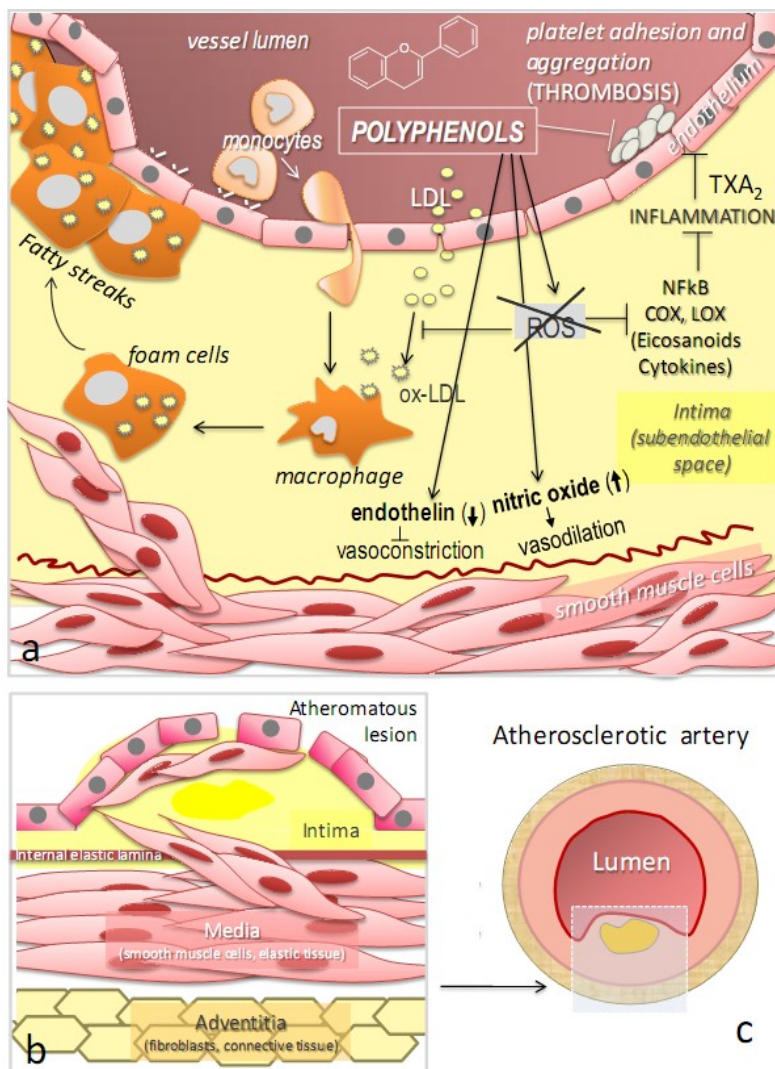


Figure 5. Cardioprotective effects of polyphenols. Molecular mechanisms by which polyphenolic compounds may counteract (a) endothelial dysfunction and (b,c) atherosclerosis include: prevention of low density lipoprotein (LDL) oxidation and atheromatous plaque deposition; suppression of nuclear factor κB (NFκB), cyclooxygenase (COX), lipoxigenase (LOX) and thromboxane A₂ (TXA₂); inhibition of platelet aggregation and thrombus formation; decrease of endothelin-mediated vasoconstriction; release of nitric oxide and vasodilation.

Atherosclerosis is a chronic, inflammatory, fibro-proliferative process of large and medium-sized arteries. It results in the progressive formation of fibrous plaques that impair the blood flow inside the vessels. In the affected artery, atherosclerotic lesions, resulting from an eccentric thickening of the intima, can either promote an occlusive thrombosis or produce

a gradual stenosis of the arterial lumen (Figure 5). In the first case, thrombus formation due to the disruption of the lesion surface can lead to infarction of the organ supplied by the afflicted vessel, such as in a heart attack, when a coronary artery is suddenly blocked, or in a thrombotic stroke, when a cerebral artery is damaged. In the second case, the stenosis of the vessel limits the blood supply to local tissues, leading to a progressive and gradual injury of the affected organ (Berliner et al., 1995).

A great deal of evidences suggests an inverse relationship between grape product consumption and cardiovascular diseases. Clinical trials have demonstrated that grape and grape juice consumption improves endothelial function, reduces platelet aggregation and platelet-endothelial cell interactions, decreases blood pressure and positively influences biomarkers such as LDL (low density lipoproteins) and HDL (high density lipoproteins). Additionally, the prevention of LDL oxidation further contributes to the cardioprotective effect of grapevine products (Bertelli et al., 1995, 1996; Di Castelnuovo et al., 2002; Bradamante et al., 2004; Dell'Agli et al., 2004; Stoclet et al., 2004; Cordova et al., 2005; Perez-Vizcaino et al., 2006; Lindberg and Amsterdam, 2008; Dohadwala and Vita, 2009).

In patients with coronary hearth disease, ingestion of red grape juice (8 mL/kg body weight) daily for 2 weeks improved endothelial function, increasing the flow-mediated dilation of the brachial artery to 6.5 %, compared to baseline values measured by high-resolution brachial artery ultrasonography (a measure of the endothelium-dependent vasodilation). The authors also reported a reduction of LDL susceptibility to oxidation in the same subjects (Stein et al., 1999; Chou et al., 2001). Similar results were reported in hypercholesterolemic patients supplied with red grape juice (500 mL/day) for 2 weeks, with a 6.7 % increase of the brachial artery flow-mediated dilation, related to the baseline (Coimbra et al., 2005). The improvement of arterial endothelial dilation was also shown in subjects with coronary hearth disease, peaking 60 min after the acute intake of a red grape polyphenolic extract (600 mg in 20 mL of water) (Lekakis et al., 2005).

Inhibition of platelet activity after grape juice supplementation was extensively reported. Red grape juice reduced platelet-mediated thrombus formation in stenosed canine coronary arteries, abolishing the cyclic (thrombogenic) flow reduction in coronary blood flow (Demrow et al., 1995). *In vitro* incubation of platelets with diluted red grape juice reduced their aggregation, decreased the O_2^- production and enhanced the release of platelet-derived nitric oxide (NO). In turn, the release of NO by platelets contributes to further inhibit their own aggregation and their recruitment to a growing thrombus (Freedman et al., 1997). The same results were reported in plasma samples from healthy subjects daily supplemented with grape juice (7 mL/kg) for 2 weeks (Freedman et al., 2001). Intriguingly, orange and grapefruit juices failed to inhibit the platelet function in healthy patients, probably because of the lower amount of polyphenols in these products than in red grape juice, as asserted by the authors (Keevil et al. 2000). In fact, in that study, only the daily intake of grape juice (5-7.5 mL/kg) for 7-10 days reduced the baseline values of the platelet aggregation response to 1.0 mg/L of collagen by 77 % (Keevil et al. 2000). Moreover, the collagen-mediated platelet aggregation was greater when grape seed and grape skin extracts were supplied individually than when used in combination, both in human and dog blood samples (Shanmuganayagam et al., 2002).

In haemodialysis patients, daily supplementation with red grape juice (100 mL) for 2 weeks ameliorated the lipoprotein profile, by decreasing plasma concentration of LDL and increasing that of HDL, besides reducing the plasma level of oxidized LDL and monocyte chemoattractant protein 1 (MCP-1), an inflammatory biomarker associated with

cardiovascular disease risk. In the same study, a rapid absorption of quercetin was reported, with the maximum plasma concentration reaching 3 h after the grape juice ingestion (Castilla et al., 2006). Also the levels of TNF- α , a plasma proinflammatory cytokine released by both endothelial cells and leukocytes, decreased in pre- and postmenopausal women after the supplementation of a lyophilized grape powder (36 g daily) for 4 weeks. As previously introduced, vascular inflammation and endothelial activation induced by TNF- α play a critical role in atherosclerosis. Plasma triglyceride concentrations were also reduced in the same patients (Zern et al., 2005).

Blood pressure parameters and atherosclerosis were ameliorated by grape juice intake. Supplementing hypertensive men with red grape juice (5.5 mL/kg body weight), daily for 8 weeks, reduced on average by 7.2 and 6.2 mm Hg the systolic and diastolic blood pressure, respectively, compared to the baseline values, and by 3.5 and 3.2 mm Hg, respectively, compared to a calorie-matched placebo (Park et al., 2004). By using a hamster model of atherosclerosis, it was shown that grape juice intake reduced by 10 % the aorta area covered by foam cells, i.e. macrophages internalizing oxidized LDL, in animals supplied with a cholesterol/saturated fat diet for 10 weeks (Vinson et al., 2001).

Interestingly, another antithrombotic effect of red wine polyphenols is their capacity to prevent the rebound phenomenon of platelet hyperaggregability observed after an acute alcohol consumption and responsible for ischemic strokes or sudden deaths occurring after episodes of drunkenness. This rebound effect was not observed after acute wine intake, possibly because of the bioactive components present in this product with respect to other alcoholic beverages (Ruf et al., 1995).

Other molecular mechanisms by which polyphenols may counteract the endothelial dysfunction, thus playing a role in the etiopathogenesis and pathophysiology of atherosclerosis, include: *i*) the release of NO by the endothelial nitric oxide synthase (eNOS); *ii*) the decrease of endothelin 1 (ET-1) production; *iii*) the suppression of NF- κ B expression (Figure 5).

Polyphenols, mainly anthocyanins, trigger an endothelium-dependent, NO-mediated vasorelaxation, and delphinidin (an anthocyanin) inhibits the apoptosis of endothelial cells (Martin et al., 2003; Fumagalli et al., 2006). NO exerts vasodilating, antithrombotic and antiproliferative effects, besides inhibiting leukocyte adhesion to the vascular wall. Alcohol-free red wine polyphenol extract increased eNOS expression and subsequent NO release (Leikert et al., 2002). Additionally, incubation of endothelial cells with red wines upregulated eNOS mRNA and protein expression, with a production of bioactive NO up to three times higher than that reported in control cells (Wallerath et al., 2002). Among ROS, $\text{O}_2^{\cdot -}$ can react rapidly with the endothelium-derived NO, leading to the strong oxidant peroxynitrate (ONOO $^-$) and reducing the amount of bioactive NO available for vasodilation. Therefore, the antioxidant power represents another cardioprotective mechanism of polyphenols, by scavenging ROS and reducing NO breakdown (Huie and Padmaja, 1993).

In particular, procyanidins, a class of proanthocyanidins, block the production of ET-1 by suppressing the *ET-1* gene transcription in cultured bovine aortic endothelial cells. ET-1 is a highly potent vasoconstrictor, which also promotes leukocyte adhesion, monocyte chemotaxis and smooth muscle cells proliferation, in addition to facilitate the LDL uptake by the endothelial cells (Corder et al., 2001). ET-1 acts as the natural counterpart to endothelium-derived NO which, besides its arterial blood pressure-raising effect, induces vascular and

myocardial hypertrophies, an independent risk factor for cardiovascular morbidity and mortality (Kiely et al., 1997).

As explained in the previous section, NF- κ B is a pleiotropic transcription factor subjected to a redox regulation and involved in different signalling pathways and processes, including vascular inflammation. Therefore, it can be evoked by an oxidative stress and inhibited by antioxidants (Brand et al., 1997). In human coronary endothelial cells, resveratrol (10^{-6} mol/L) reduced by 50 % the activation of NF- κ B mediated by TNF- α , in addition to attenuate the monocyte adhesiveness to endothelium (Csiszar et al., 2006). Disruption of the cytokine-activated NF- κ B signalling pathway exerts a vasculoprotective action, by reducing vascular inflammation and preventing atherogenesis. Additionally, a spectrum of different genes expressed in atherosclerosis was shown to be upregulated by NF- κ B, including those encoding for TNF- α , MCP-1, interleukin 1 (IL-1), vascular cell adhesion molecules (VCAM) and intracellular adhesion molecules (ICAM) (Brand et al., 1997).

Enhancement of fibrinolysis and inhibition of vascular smooth muscle cell (VSMC) proliferation and migration represent two additional processes involved in cardiovascular protective effects of polyphenols. Clinical and epidemiological studies suggest that impairment of the fibrinolytic system contributes significantly to the atherothrombosis development (Hamsten and Eriksson, 1995). It has been demonstrated that grape polyphenols (catechin, epicatechin, quercetin and resveratrol) increase mRNA levels of plasminogen activators (PAs) in human umbilical vein endothelial cells, independent of ethanol (Abou-Agag et al., 2001). PAs convert plasminogen to plasmin, the latter able to degrade fibrin within a thrombus and, eventually, leading to clot dissolution. The abnormal proliferation of VSMC in the arterial intima plays an important role in the pathogenesis of atherosclerosis. In an endothelial denudation model, rabbits fed with a high-dose resveratrol diet developed less intimal hyperplasia than control rabbits, with a considerably reduced number of SMCs in resveratrol-treated animals (Zou et al., 2000). In agreement with these results, bovine aortic SMC proliferation was inhibited after treatment with grape polyphenols (dealcoholized red wine, red wine polyphenol extract or resveratrol), according to a dose-response relation (Araim et al., 2002). In stroke-prone, hypertensive rats, resveratrol inhibited proliferation and collagen synthesis in VSMCs at concentration attainable in plasma at therapeutic doses (from 0.01 to 1.0 μ M) (Mizutani et al., 2000).

5. Grape and Neuroprotection

Neuroprotection refers to the strategies and relative mechanisms able to defend the central nervous system (CNS) against neuronal injury due to both acute (e.g. stroke or trauma) and chronic neurodegenerative disorders (e.g. Alzheimer's disease, AD, and Parkinson's disease, PD).

According to the World Health Organization (WHO), neurodegenerative diseases, in 2020, will represent the eighth greatest cause of disease burden for developed countries, and, by the middle of the century, they will become the world's second leading cause of death, overtaking cancer (Menken et al., 2000). Many important neurodegenerative disorders include amyloidogenic diseases, characterized by conformational changes (misfolding) and aggregation of proteins and peptides inside or outside neuronal cells. Major amyloid-related

diseases include AD and PD (Agorogiannis et al., 2004). Familial forms of these diseases, due to a mutation of the gene coding for the abnormally aggregating protein, represent a minority of cases, most neurodegenerative disorders occurring sporadically and arising through interactions among genetic and environmental factors (Elbaz et al., 2007). AD is a progressive, degenerative disorder which accounts for 65 % of all age-related dementias (Ritchie and Lovestone, 2002). Histopathology reveals that one of the major hallmark of AD is the abundant protein deposits in neurons that trigger neuronal degeneration. These deposits result from the extra- and intracellular accumulation of amyloid β ($A\beta$) peptides and phosphorylated tau ($P\tau$) protein, respectively. $A\beta$ and $P\tau$ aggregation leads to the formation and deposition of senile plaques and neurofibrillary tangles, respectively, which promote inflammation and neuronal cell death (Selkoe, 2001). PD is a movement disorder considered the most frequent neurodegenerative disease after AD, caused by the degeneration of dopaminergic neurons in the substantia nigra. One of the main pathological hallmarks of PD is the aggregation of the intracellular protein α -synuclein to form intracytoplasmic inclusions (Lewy bodies) in these neurons (Shastry et al., 2003). Currently, oxidative and nitrosative stress is believed to be one of the leading cause of neuronal degeneration in both AD and PD (Hunot et al., 1996; Perry et al., 2002; Jenner, 2003). Acute cerebrovascular attack (stroke) is currently the second leading cause of death in industrialized countries, ranking after heart disease and before cancer, and a major cause of permanent disability (Dannon et al., 2008). It is an ischemia (a reduction or a blockage of oxygen and glucose supply) generally caused by thrombosis or haemorrhage which block the blood flow to a specific brain region. In particular, the occlusion of a cerebral vessel due to a blood clot (thrombus) discharged from the surface of an atherosclerotic lesion may cause an ischemic stroke, whereas an intracranial or intracerebral haemorrhage, commonly caused by hypertension, may lead to a haemorrhagic stroke (Ginsberg, 2003; Hachinski, 2007). Interestingly, about 25 % of patients develops dementia in 3 months after stroke (Tatamichi et al., 1994a, 1994b). In fact, both $A\beta$ and APP are deposited in cortical and subcortical brain areas of non-dementia patients following stroke (Jendroska et al., 1995).

As recently reviewed, many prospective, population-based cohort and case control studies have provided the substantial evidence that a regular (daily or possibly 3-4 times weekly) intake of moderate amounts (2 glasses/days) of red wine, at meals and in the context of a Mediterranean dietary style, is associated with a lower risk of developing neurodegenerative diseases (Figure 2) (Pinder, 2009). In particular, the largest meta-analysis on alcohol consumption and risk of dementia suggested that low to moderate wine drinking significantly reduced by 38 and 32 % the risk of dementia and AD, respectively (Peters et al., 2008). However, it has been suggested the hypothesis that the drinker and not the drink may produce benefits, because of the healthier life-style habits of wine drinkers in relation to their beer-, spirit-drinking and abstention counterparts. Wine drinkers are more often women, college graduates, non-smokers and perform a moderate physical activity (Klatsky 1999, 2003). In any case, immoderate or excessive consumption of wine and/or other alcoholic beverages is associated with an increased risk of dementia, possibly due to the neurotoxicity of ethanol or resulting from the concomitant malnutrition/nutrition deficiency (Charness, 1993).

Although association does not imply causality, mechanisms (putatively) involved in wine-induced neuroprotection have been deeply investigated. In general, protective effects of grape polyphenols against neurodegenerative diseases can be ascribed to their anti-

amyloidogenic, antioxidant and anti-inflammatory activity (Conte et al., 2003; Ono et al., 2003; Savaskan et al., 2003; Porat et al., 2006; Rivi re et al., 2008). The daily administration of resveratrol (50 or 100 mg/kg) for 1 or 2 weeks to adult male mice significantly prevented the nigrostriatal dopaminergic neuron depletion, after the acute treatment with the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) injected intraperitoneally (Blanchet et al., 2008). In different cell lines stably transfected with human A β precursor protein (APP), resveratrol was shown to promote the intracellular degradation (clearance) of A β peptides via a mechanism that involves the proteasome, without direct inhibition of the enzymes β - and γ -secretases implicated in the A β protein synthesis (Marambaud et al., 2005). Neuroprotective effects of three major grape polyphenolic constituents (resveratrol, quercetin and catechin) were assessed in cultured mixed (glial/neuronal) cells of rat hippocampus, a brain area severely affected in both AD and ischemia. Hippocampal cell treatment with polyphenols reduced the cytotoxicity induced by both the NO free radical donor sodium nitroprusside (SNP) and intracellular ROS accumulation (Bastianetto et al., 2000). In a mouse model of AD, the moderate consumption of Cabernet Sauvignon promoted the non-amyloidogenic processing of APP mediated by α -secretase, thereby preventing or delaying the generation of A β peptides (Wang et al., 2006). More recently, a grape seed polyphenolic extract significantly prevented A β protein oligomerization, by inhibiting the A β protein aggregation into high-molecular-weight oligomeric A β species, both *in vitro* and in Tg2576 mice. Besides, when orally administered to those animals, the extract attenuated the cognitive deterioration typical of AD (Wang et al., 2008).

In the Copenhagen City Hearth Study, wine intake on a monthly, weekly or daily basis was associated with a lower risk of stroke, compared with no wine intake. No association between beer or spirit consumption and risk of stroke was reported, thus suggesting that some wine components, in addition to ethanol, may be responsible for the beneficial effect of wine drinking on the risk of stroke (Truelsen et al., 1998). The Framingham Study evaluated the association between the type of alcoholic beverage and incidence of ischemic stroke, showing a protective effect of wine consumption among subject aged 60-69 years (Djouss  et al., 2002). Results from a case-control study on young women were consistent with the above reported data and, in general, beer and spirit drinking failed to exert neuroprotective effects against ischemic stroke (Malarcher et al., 2001).

From the above reported and other studies, the classical J-shaped relationship between alcohol consumption and all-cause mortality could also be extended to the relationship between wine intake and risk of neurodegenerative diseases, besides cardiovascular disorders and cancer Gr nbaek et al., 2000; Gr nbaek, 2006). In J-shaped curve, regular and moderate wine drinking, at the nadir of J, is beneficial, whereas abstinence and heavy intake, on the short and long arm, respectively, are both detrimental, though to a different extent (Pinder, 2009).

Other mechanisms by which polyphenols retard the ageing process and delay the onset of ageing-related diseases resemble those induced by caloric restriction (CR), suggesting that these compounds and CR share quite similar molecular pathways (Howitz et al., 2003; Wood et al., 2004). A moderate reduction in calorie intake of 20-40 % significantly extends lifespan in a wide spectrum of organisms, ranging from bacteria to primates, a process mediated by a family of nicotinamide adenine dinucleotide (NAD⁺)-dependent deacetylases, the sirtuins (from silent information regulator, SIR, proteins, Figure 6) (Bordone and Guarente, 2005; Chen and Guarente, 2006; Haigis and Guarente, 2006).

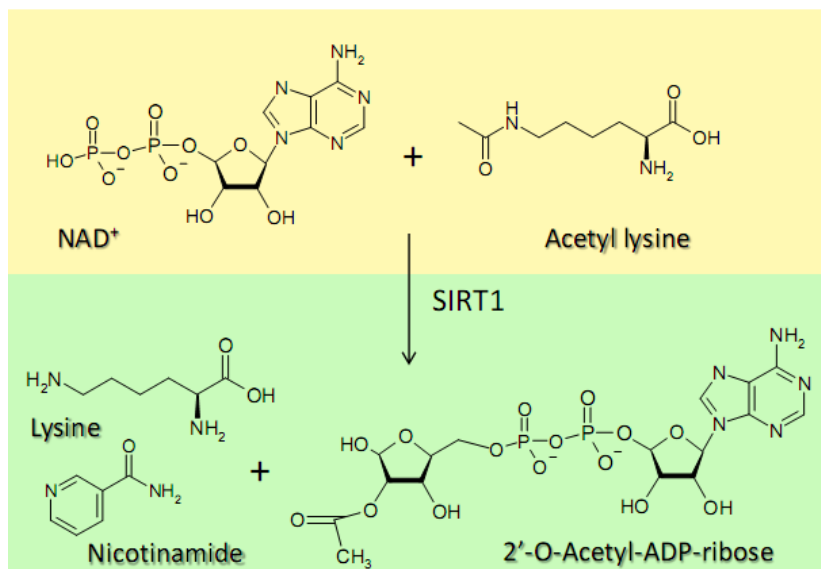


Figure 6. Sirtuins (SIRT1) are NAD-dependent enzymes that remove acetyl groups from specific lysine residues on various proteins (both histones and non-histone proteins); NAD⁺ hydrolysis yields 2'-O-acetyl-ADP-ribose and nicotinamide.

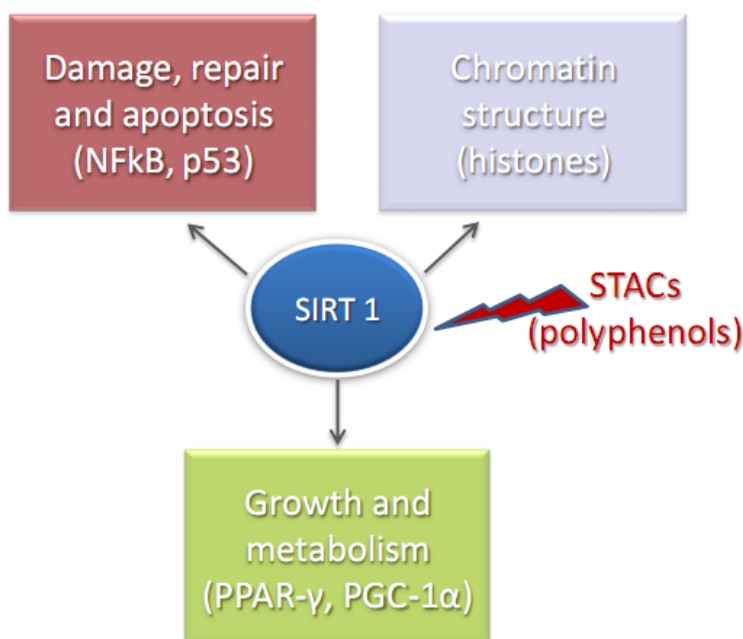


Figure 7. Proteins involved in different cellular processes are deacetylation targets of SIRT 1; STACs: sirtuin activating compounds; NF-kB: nuclear factor kB; p53: tumor suppressor protein p53; PPAR-γ: peroxisome proliferator-activated receptor γ; PGC-1α: PPAR-γ co-activator 1α.

In mammals, sirtuins represent novel therapeutic targets to treat age-associated and neurodegenerative diseases, being implicated in a variety of cellular functions, ranging from gene silencing, over the control of cell cycle and apoptosis, to mitochondrial function and

energy metabolism (Figure 7). Additionally, these proteins play a role in protecting neurons against damage and in prolonging their survival in AD (Outeiro et al., 2008; Michan and Sinclair, 2007; Yamamoto et al., 2007). Therefore, sirtuin activating compounds (STACs) represent a promising class of therapeutics, including resveratrol, quercetin and other polyphenols, able to activate SIRT1 (Figure 8) (Anekonda 2006; Anekonda and Reddy 2006). The latter deacetylates both histones and non-histone substrates, such as the tumor suppressor protein p53 and the transcription factor NF- κ B, thus regulating some pathophysiological processes affected during ageing and modified by CR (Porcu and Chiarugi, 2005; Lavu et al., 2008). Peroxisome proliferator-activated receptor γ (PPAR- γ) and PPAR- γ co-activator 1 α (PGC-1 α), master regulators of adipogenesis, fat storage in white adipose tissue, liver metabolism and differentiation of muscle cells are also relevant deacetylation targets of SIRT1 (Bordone and Guarente, 2005).

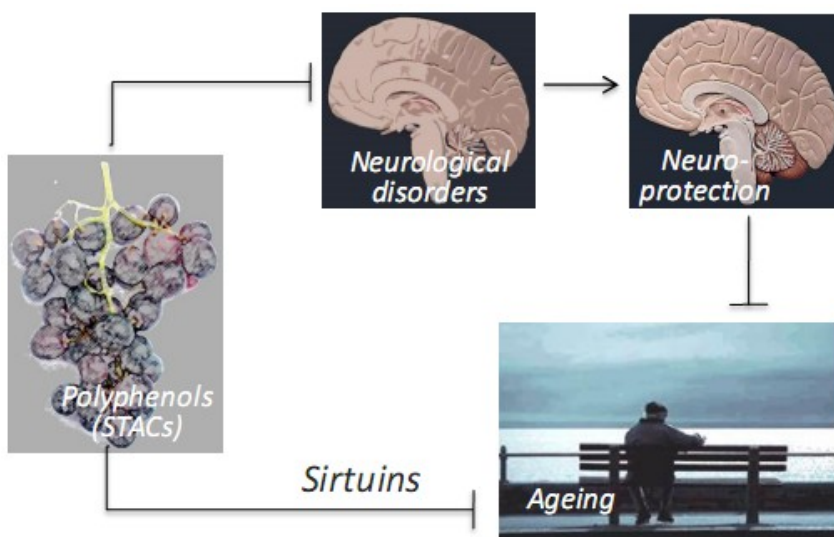


Figure 8. Resveratrol, quercetin and other polyphenols are sirtuin activating compounds (STACs), able to activate SIRT1, which in turn regulates some physiological processes affected during ageing, preventing or delaying neurological disorders.

6. Polyphenol Bioavailability

The bioavailability of polyphenols is greatly influenced by their chemical structure. As previously introduced, the structural variety of polyphenols is surprising. In plants (and consequently in human diet), they occur mainly as *O*-glycoside, with a saccharide substituent bounds to a hydroxyl group of the aglycone, usually in position 3 or 7. The most common saccharides are glucose, rhamnose, galactose and arabinose, and diglycosides are also frequently found in plant tissues. Furthermore, the saccharides can be often substituted by acyl residues, such as malonate or acetate (Clifford 2004; Karakaya, 2004; Viskupičová et al., 2008).

In general, dietary non-anthocyanic flavonoids are better absorbed than anthocyanins and proanthocyanidins, though absorption may be influenced by the (food) matrix in which these

compounds are distributed and consumed and by the heterogeneity of saccharides and other functional groups on the flavan nucleus. Moreover, gender and genetic differences, dietary habits, dosage and intestinal microflora may affect the absorption kinetics (Heim et al., 2002).

After ingestion, flavonoid glycosides need to undergo deglycosylation prior to be absorbed (Figure 9). In humans, hydrolysis of the saccharide moiety is carried out by three different β -glucosidases found in cytoplasm of small intestinal epithelial cells: a broad-specificity β -glucosidase, a lactase phloridzin hydrolase and a glucocerebrosidase (Day et al., 1998; Mackey et al., 2002; Németh et al., 2003). Next step after deglycosylation involves passive diffusion of the resulting aglycone through the epithelial cells, supported by increased hydrophobicity, though certain glycosilated flavonoids, such as quercetin-4'-*O*-glucoside, can be actively transported into epithelial cells via the active sodium-dependent glucose transporter (Gee et al., 2000).

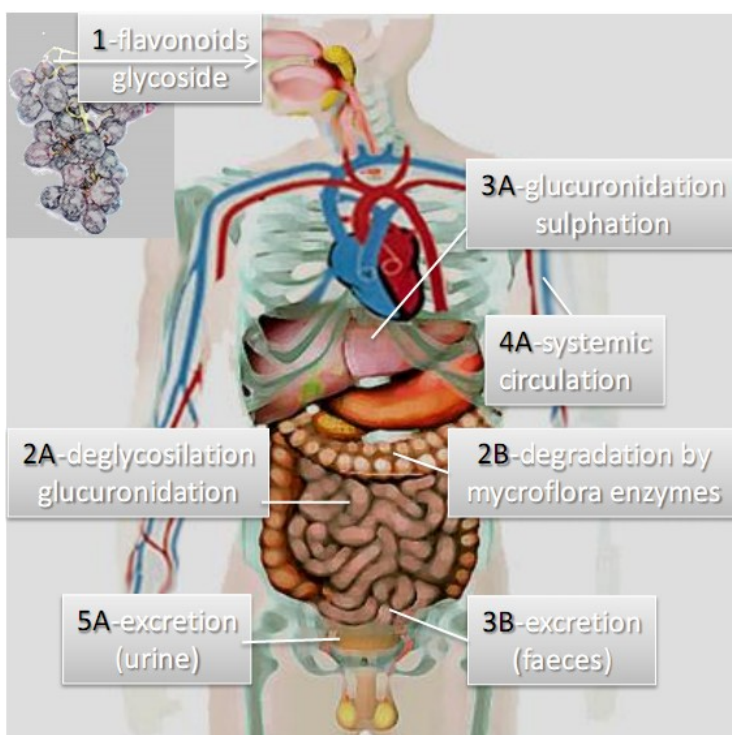


Figure 9. The fate of flavonoids in the human body.

At this point, active intestinal efflux of flavonoids may further limit their absorption into systemic circulation. Members of the adenosine triphosphate-binding cassette (ABC) superfamily of transporters are involved in this process. ABC transporters include multidrug-resistance protein, P-glycoprotein and breast cancer resistance protein, whose activities are relevant for the pharmacokinetic and pharmacodynamic of flavonoids (Walgren et al., 2000; Vaidyanathan and Walle, 2003; Brand et al., 2006). Interestingly, since ABC transporters are located in cell membranes all over the organism, their interaction with flavonoids also affect the distribution of these metabolites to the target sites of action (Morris and Zhang, 2006).

Absorbed flavonoids are bound to albumin and transported to the liver via the portal vein, where they undergo extensive Phase II metabolism prior to entry into systemic circulation

(Manach et al., 1996). Conjugation reactions with glucuronic acid or sulphate are the most common types of biotransformation for flavonoids and stilbenes, though other metabolic pathways involve oxidation, reduction, hydrolysis and *O*-methylation (Figures 9,10,11). Glucuronidation may occur both in small intestine and liver by uridine-5'-diphosphate glucuronosyltransferases (UGTs). The UGT supefamily of enzymes shows a wide diversity in substrate recognition, catalysing the glucuronidation of many functional groups, including -OH, -COOH, -NH₂ and -SH (Meech and Mackenzie, 1997; Otake et al., 2002). Sulphation occurs in hepatocytes by sulphotransferases (Vaidyanathan and Walle, 2002; O'Leary et al., 2003).

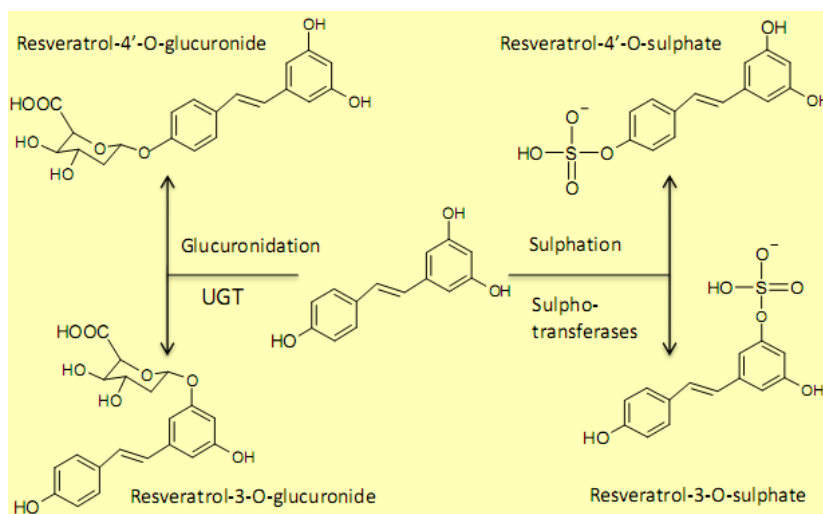


Figure 10. Biotransformation of resveratrol includes glucuronidation by UGT (Uridine-5'-diphosphate glucuronosyl transferase) and sulphation by sulphotransferases.

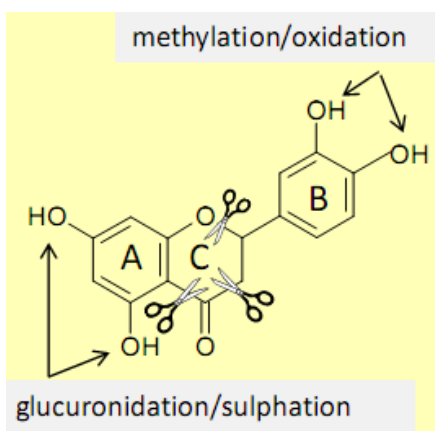


Figure 11. Potential molecular sites of biotransformation and C ring cleavage of flavonoids (adapted from Rice-Evans, 2001).

Unabsorbed flavonoids reaching colon can be extensively degraded by microflora enzymes, with the scission of the C ring and the further demethylation and dehydroxylation of the resulting phenolic acids (Figure 11) (Rice-Evans, 2001).

Studies in humans have shown that resveratrol is highly absorbed (~ 70 %) and rapidly transformed (~ 30 min) by hepatic Phase II drug-metabolizing enzymes to hydrophilic glucuronide and sulphate conjugates, the main metabolites detected in serum and urine (Walle et al., 2004). Conversely, anthocyanins are rapidly absorbed as intact glycosides. Their major metabolites isolated from human urine and serum are glucuronidated and methylated conjugates, whereas aglycones and sulphate conjugates have been identified as minor metabolites in the same body fluids (Felgines et al., 2003, 2005; Kay et al., 2004, 2005). Unabsorbed anthocyanins are degraded to protocatechuic acid (a phenolic acid) by the human colon microflora (Vitaglione et al., 2007). Proanthocyanidins occurring in foods cover a wide range of polymerization degree (PD), with the concentration of monomers (flavanols), dimers and trimers lower than that of the polymeric forms (Figure 1). Therefore, only the low-molecular-weight oligomers ($PD \leq 3$) are absorbed intact in the gastrointestinal tract, differently from polymers (Depréz et al., 2001). The latter are degraded into small phenolic acids by the colonic microflora, such as phenylacetic, phenylpropionic and phenylvaleric acids (Depréz et al., 2000).

Conclusion

Food plants constitute an unlimited source of molecules available for improving human health. Nonetheless, a single plant contains hundreds or thousands of secondary, bioactive metabolites, a chemical diversity that determined the evolutionary success of plants, favouring their adaptation to a changing environment (Iriti and Faoro, 2009). In this view, to ascribe the health-promoting effects of a plant food only to a molecule, or a single class of compounds (i. e. polyphenols), represents an inappropriate and inopportune task. It is likely that different phytochemicals contained in diverse foods produce *in vivo* additive and/or synergistic effects, thus amplifying (or reducing/inhibiting) their activities. In addition, compounds with multiple targets (i. e. polyphenols) appear as a potential and promising class of phytotherapeutics for the treatment of diseases with a multifactorial etiology, such as cancer, cardiovascular and neurodegenerative disorders.

Finally, a better comprehension of the bioavailability of dietary phytochemicals is critical in order to correctly evaluate their *in vivo* bioactivity, to interpret the experimental results and to design new approaches. In fact, data supporting their absorption, distribution, metabolism and excretion in human body are still fragmentary, despite the enormous amount of indications on their bioactivities.

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Chapter XII

Beneficial Effects of Indian Herbs for the Treatment of Diabetes

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Abstract

Diabetes is a major health problem, more so in the Asian region. In India, diabetes is emerging as a major health concern, especially in the urban areas. Indian medicinal plants and their formulations provide an alternate strategy in reducing the ill-effect of diabetes. They have lesser side effects and cost lesser than other means of treatment. Recent evidences suggest a casual role for oxidative stress resulting from high levels of reactive oxygen species (ROS) in diabetes and its secondary complications like macrovascular disease, nephropathy, retinopathy and neuropathy. On the other hand, low levels of ROS such as H₂O₂ act as metabolic signaling molecules for glucose stimulated insulin secretion in pancreatic beta cells. Redox signaling also has a co-regulatory function in insulin receptor activation. Hence ROS have dual roles to play at low and high concentrations. Antioxidant property, as a consequence, may be modulatory on antidiabetic effects in a biphasic manner. Indian traditional systems of medicine such as Ayurveda, have identified many plants with antidiabetic and antioxidant effects in different model systems. These include *Aegle marmelos*, *Allium sativum*, *Azadirachta indica*, *Butea monosperma*, *Citrus sinensis*, *Emelia ribes*, *Eugenia jambolana*, *Helicteres isora*, *Momoardica charantia*, *Murraya koenigii*, *Ocimum sanctum*, *Phyllanthus amarus*, *Pterocarpus marsupium*, *Punica granatum*, *Tinospora cordifoila*, *Trigonella foenum-graceum* and *Withania somnifera*. A number of polyherbal preparations containing such plants also have shown potential to help in diabetes and its complications. Some compounds isolated

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from these plants have been examined for their beneficial effects besides the possible mechanisms involved. These include charantin and polypeptide P from *Momordica charantia*, trigonellin, nicotinic acid, coumarin and naringenin present in *Trigonella foenum-graceum*. Naringin exhibit insulin like effect by activating MAPK/ERK pathway. Another compound from medicinal plant epicatechin, regenerate functional beta cells. Saponins and sapogenins from *Helicteres isora* act through PI3K/Akt pathway. Similar effects also have been observed by other compounds.

Conclusion; This chapter examines antidiabetic property and related beneficial effects of Indian medicinal plants, isolated compounds and formulations, besides the possible mechanisms including effects on redox signaling in various model systems and clinical situations.

Abbreviations

ACAT	acyl-CoA:cholesterol acyltransferase
AGEs	advanced glycation end products
APS	adaptor with pleckstrin homology
cAMP	cyclic adenosine monophosphate
CAP	cbl associated protein
DOK4	downstream of kinases 4
EGCG	epigallocatechin-gallate
ER	endoplasmic reticulum
Fe-NTA	ferric nitrilotriacetate
FFA	free fatty acid
FOXO1	forkhead boxO1
G6Pase	glucose-6-phosphatase
GAB	Grb2-associated binding protein
GLUT 4	glucose transporter 4
GS	glycogen synthase
GSH	glutathione
GSK-3	glycogen synthase kinase-3
GSPE	grape-seed procyanidin extract
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
IDDM	insulin dependent diabetes mellitus
IP3	inositol 1,4,5-triphosphate
IRS	insulin receptor substrates
MAPK	mitogen activated protein kinase
NA	nicotinic acid
NIDDM	non-insulin dependent diabetes mellitus
PDK1	phosphoinositide dependent kinase 1
PEPCK	phosphoenolpyruvate carboxylase
PGC-1 α	Peroxisome proliferator-activated receptor gamma coactivator-1 α
PI3K	phosphatidylinositol-3-kinase
PIP2	phosphatidyl inositol 1,4,5-triphosphate
PKB	protein kinase B

PLC	phospholipase C
PP1	protein phosphatase 1
PPAR γ	Peroxisome proliferator-activated receptor γ
ROS	reactive oxygen species
SoHo	sorbin homology domain
SOS	son-of sevenless
SREBPs	steroid regulatory element-binding proteins
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

1. Introduction

In recent years there is an increased interest in the use of alternate medicine especially for use against chronic diseases. Diabetes is emerging as a major crippling disease in the world leading to huge economic losses. Diabetes mellitus, long considered a disease of minor significance to world health, is now taking its place as one of the main threats to human health in the 21st century [1]. Past three decades have seen an explosive increase in the number of people diagnosed with diabetes worldwide. Pronounced changes in the human environment, human behavior and lifestyle, have accompanied globalization, and these have resulted in escalating rates of both obesity and diabetes [2-3]. The diabetes epidemic, although apparent right around the world, has been most pronounced in non-European populations, as evidenced by studies from Native American and Canadian communities, Pacific and Indian Ocean island populations, groups in India and Australian Aboriginal communities [4]. The potential for increase in the number of cases of diabetes is greatest in Asia [2]. Studies conducted in developing countries like India have also indicated that it is increasing rapidly in urban populations due to change in life styles. This scenario of diabetes has been successful in developing interest of clinicians and researchers world-wide in studying the pathophysiology and treatment of diabetes. Though there are conventional therapies involving life-long treatment, alternative therapies such as use of medicinal plants promise to be a better and safe option in the coming decades. In the last few years there has been an exponential growth in the herbal medicine and these drugs are gaining popularity due to their natural origin and less side effects.

The present chapter first deals with the molecular mechanisms of diabetes development followed by secondary complications and modulation by natural compounds derived from medicinal plants

2. Diabetes: A Metabolic Syndrome and its Molecular Mechanisms

Diabetes is a chronic disorder of carbohydrate, fat and protein metabolism characterized by increased fasting and post prandial blood sugar levels. Diabetes mellitus is a complex metabolic disorder resulting from either insulin insufficiency or insulin dysfunction. Type I diabetes is caused due to insulin insufficiency because of lack of functional beta cells. *Type I*

or insulin dependent diabetes mellitus (IDDM) is due to autoimmune mediated destruction of the pancreatic beta islets resulting in insulin deficiency, Patients suffering from this are therefore totally dependent on exogenous source of insulin while patients suffering from Type II diabetes (insulin independent) are unable to respond to insulin and can be treated with dietary changes, exercise and medication. *Type II* diabetes or non-insulin dependent diabetes mellitus (NIDDM) is associated with defects in both insulin action and secretion. A defect in insulin action is characterized by an inability of the cells of the body to respond to the presence of insulin and is often referred to as insulin resistance [5]. Type II insulin-resistant diabetes mellitus accounts for 90-95% of all diabetic cases. This disease is largely associated with obesity, which in turn contributes substantially to insulin resistance. Importantly, insulin resistance is also implicated in the pathogenesis of other major diseases including atherosclerotic cardiovascular disease, dyslipidemia and polycystic ovarian syndrome [6]. Diabetes is accompanied by alterations in cellular physiology, metabolism and gene expression.

2.1. Normal Metabolism of Glucose

Carbohydrates from various dietary sources are the primary exogenous source of glucose which is the main fuel for energy requirement of the body. Therefore, a continuous supply of glucose is necessary for proper function and survival of all organs. Mammals have evolved complex systems to maintain blood glucose in a tight range (of around 5 mM) during fasting and feeding conditions. This level of glucose is maintained chiefly through the hormonal modulation of production of glucose by the liver and the peripheral uptake of glucose by skeletal muscle, liver and adipose tissue.

Metabolism of carbohydrates starts from the gut, wherein carbohydrates are broken down by digestive enzymes into monosaccharides, especially glucose which is the primary stimulus for the beta cells of the pancreatic islets. This glucose induced stimulation of pancreatic islet cells initiates a cascade of events resulting in insulin secretion and is dependent on increase in intracellular Ca^{2+} . Glucose enters beta cells via glucose transporter 2 (GLUT2) and is metabolized generating ATP. With increase in concentration of ATP, the ATP dependent potassium channels close leading to membrane depolarization. On depolarization, voltage controlled Ca^{2+} channels open and calcium flows into cells [7]. An increased Ca^{2+} level leads to activation of phospholipase C (PLC), which cleaves the membrane phospholipid, phosphatidyl inositol 1,4,5-triphosphate (PIP2) into diacylglycerol and inositol 1,4,5-triphosphate (IP3). IP3 binds to receptor proteins in the membrane of endoplasmic reticulum (ER) allowing the release of Ca^{2+} from ER via IP3 gated channels and further raises the cellular concentration of calcium. Significantly increased amounts of calcium in the cells causes release of previously synthesized insulin, which has been stored in secretory vesicles [8].

2.2. Insulin Signaling

Release of insulin regulates metabolism and maintenance of normoglycemia and normolipidemia. Action of insulin begins by binding to its cell surface receptor. Insulin

receptor consists of two alpha subunits and two beta subunits that are disulfide linked into an $\alpha_2\beta_2$ heterotetrameric complex. Binding of insulin to the α subunit leads to activation of intracellular tyrosine kinase domain of the β subunit resulting in receptor autophosphorylation. Phosphorylation of receptor tyrosine creates recognition motifs for several insulin receptor substrates (IRS) such as insulin receptor substrate-1 (IRS-1), Grb-2 associated binding protein (GAB) and the IRS5/DOK4 (Downstream of kinases 4) and IRS/DOK6 [9]. Three major signal transduction pathways induced by the activated IRS are the Phosphatidylinositol-3-kinase (PI3K), the (c-cbl associated protein) CAP/Cbl TC10 (Rho family member GTPase) and the MAPK (mitogen activated protein kinase) pathway [10].

2.2.1. PI3K Pathway

PI3K has a pivotal role in metabolic and mitogenic action of insulin. PI3K is a heterodimeric lipid kinase with a broad range of cellular functions, including growth and differentiation, synthesis and degradation of carbohydrates, proteins, lipids and membrane trafficking. It catalyzes the phosphorylation of phosphoinositides to produce phosphatidylinositol-3-phosphate (mainly $\text{PI}(3,4,5)\text{P}_3$), which binds to the pleckstrin homology domain (PH domains) of variety of signaling molecules, thereby altering their activity or subcellular localization. Moreover PI3K also possess serine kinase activity and both the regulatory and catalytic subunits of the enzyme can interact with other signaling proteins [11].

Phosphatidylinositol-3-phosphate regulates three main classes of signaling molecules: the AGC family of serine/ threonine protein kinases, guanine nucleotide exchange protein of the rho family of GTPases, and the TEC family of tyrosine kinases [12]. These pathways act in concerted fashion to co-ordinate the regulation of vesicle trafficking, protein synthesis, enzyme activation - inactivation and gene expression that results in regulation of glucose, lipid and protein metabolism. One of the best characterized AGC kinase, is a serine kinase phosphoinositide dependent kinase 1 (PDK1), that phosphorylates and activates serine/threonine kinase Akt/PKB (Protein kinase B) [13]. Akt possesses PH domain that also interacts directly with $\text{PI}(3,4,5)\text{P}_3$, promoting membrane targeting of the protein and catalytic activation. Akt has been suggested to be important in transmission of the insulin signal as it can activate glucose transporter, phosphorylate enzyme glycogen synthase kinase-3 (GSK-3) and activate fork headed transcription factors. This then leads to either activation of glucose transport or glycogen synthesis or inhibition of gluconeogenesis [14-15].

2.2.2. CAP/Cbl Pathway and Lipid Rafts

A new pathway has suggested the role of CAP (cbl associated protein)/cbl in glucose uptake, in addition to that catalyzed by activated PI3K. This pathway involves tyrosine phosphorylation of *cbl* proto-oncogene by the activated insulin receptor. The insulin receptor recruits and phosphorylates Cbl by means of intermediary adaptor proteins APS (adaptor with pleckstrin homology and Src homology-2 domains) [16]. Binding of the APS SH2 domain to the phosphorylated insulin receptor facilitates phosphorylation of APS which then serves as a docking site for Cbl. Another adaptor protein termed Cbl associated protein (CAP) is involved in targeting Cbl to the lipid raft microdomains. CAP contains three Src3 homology

(SH3) domains at its C terminus that bind to Cbl and a Sorbin homology domain (SoHo) that interacts with the caveolae resident protein flotilin. Upon phosphorylation of Cbl, the CAP/Cbl complex provides docking sites for the recruitment of the adaptor protein CrkII and the guanyl nucleotide exchange factor C3G. Recruitment of C3G results in the activation of the small GTP binding protein TC10. Activation of TC10 results in cytoskeletal rearrangements that are needed to facilitate Glut4 translocation [17]. Thus, this signaling pathway induces a second signal to the GLUT4 protein in addition to PI3K pathway.

2.2.3. MAPK Pathway

Insulin stimulated MAPK cascade involves series of phosphorylation events. Autophosphorylation of the insulin receptor leads to tyrosine phosphorylation of IRS proteins/Shc, which in turn interacts with the adapter protein Grb2 recruiting the Son-of Sevenless (SOS) exchange protein to the plasma membrane for activation of Ras [18]. Ras is also activated via tyrosine phosphatase SHP-2, through its interaction with receptor substrates such as Gab-1 or IRS1/2. Activation of Ras leads to stimulation of serine phosphorylation cascade through the stepwise activation of Raf, MEK and ERK. Activated ERK can translocate to the nucleus and catalyse the phosphorylation of transcription factors such as p62 TCF, leading to cellular differentiation. A parallel MAPK signaling pathway is the mixed lineage kinase/apoptosis signaling kinase, MAPK kinase 3/6, p38 kinase cascade. The effect of insulin on the p38 pathway is inconsistent. In fetal neurons, insulin can inhibit the p38 pathway as well as apoptosis, whereas in myoblast insulin brings about cytoskeletal rearrangement and myoblast differentiation via p38 pathway. Although activation of MAP kinases by insulin appears to be less important in the control of glucose metabolism than components downstream of PI3K, positive and negative crosstalk can occur between these pathways [19].

2.3. Glycogen Synthesis

Apart from increasing the intake of glucose in the insulin sensitive organs, insulin also channels the excess glucose by activating enzymes necessary for glycogen synthesis. Insulin stimulates glycogen accumulation through a co-ordinated increase in glucose transport and glycogen synthesis. Glycogen synthase (GS) catalyzes the formation of glycogen from glucose. This enzyme is maintained in its inactive phosphorylated form by Glycogen Synthase Kinase-3. Activation of insulin receptor leads to activation of PI3K which in turn activates Akt. Akt on one hand phosphorylates GSK-3, thus inhibiting it from phosphorylating GS and on the other hand Akt activates (GS) which dephosphorylates GS converting it into its active form. This activation of glycogen synthase leads to glycogen synthesis, thereby storing excess glucose [20].

2.4. Modulation of Gluconeogenesis

Hepatic gluconeogenesis is absolutely essential for the survival during prolonged fasting or starvation. Glucocorticoid and glucagons have strong gluconeogenic action on liver. In contrast, insulin suppresses hepatic gluconeogenesis. Insulin inhibits the transcription of gene encoding phosphoenolpyruvate carboxylase, fructose 1-6-bisphosphatase and glucose-6-phosphatase and increases transcription of glycolytic enzymes such as glucokinase and pyruvate kinase and lipogenic enzymes such as fatty acid synthase and acetyl-CoA carboxylase. A finer tuning is observed when, two transcription factors Peroxisome proliferator-activated receptor γ (PPAR γ) coactivator-1 α (PGC-1 α) and forkhead boxO1 (FOXO1) interact to execute signals from insulin [21].

2.5. Lipid Regulation

Insulin promotes the uptake of fatty acids and the synthesis of lipids, whilst inhibiting lipolysis. Insulin regulated lipid homeostasis is modulated by an increase in the transcription factor steroid regulatory element-binding proteins (SREBPs) that activate the expression of over 30 genes involved in the synthesis and uptake of fatty acids, triglycerides, cholesterol and phospholipids [22]. However, the pathway leading to changes in SREBP expression are unknown. Lipid synthetic enzymes such as pyruvate dehydrogenase, fatty acid synthase and acetyl-CoA carboxylase convert glucose to lipid under the action of insulin. Insulin inhibits lipid metabolism in adipocytes by inhibiting the enzyme hormone sensitive lipase. This enzyme is regulated primarily by decreasing cellular concentrations of cAMP by activating a cAMP specific phosphodiesterase in adipocytes [23]. Thus insulin by acting on various molecular targets brings about different activities such as differentiation, proliferation, growth, glucose uptake etc.

3. Diabetes: Consequences and Modulation

3.1. Oxidative Stress and Secondary Complications

The four most common complications of diabetes – macrovascular disease, nephropathy, retinopathy and neuropathy share numerous mechanisms by which hyperglycemia can disrupt cell and organ function, with vascular function also affecting kidneys, eyes and the nervous system. During type II diabetes uptake of glucose in response to insulin by the peripheral tissue is significantly reduced. Thus, in spite of presence of insulin, the cells fail to respond to insulin and therefore the amount of glucose in blood increases leading to hyperglycemic condition of diabetes. Persistent hyperglycemia, via several mechanisms leads to generation of oxidative stress [24]. These include increased production of mitochondrial reactive oxygen species (ROS), non-enzymatic glycation of proteins, and glucose auto-oxidation [25, 26]. Elevated free fatty acids also cause oxidative stress due to increased mitochondrial uncoupling and β -oxidation leading to increased production of ROS [27]. The combined action of increased glucose and FFA levels in turn leads to activation of stress sensitive

signaling pathways mediated by nuclear factor- κ B, p38 MAPK, JNK and PKC leading to cellular dysfunction and damage. In addition to the above, increased NAD(P)H oxidase activity in phagocytic and non-phagocytic cells also contribute to increased oxidative stress [28, 29]. Most common underlying cause for non-responsiveness to insulin is central obesity. Excess abdominal adipose tissue has been shown to release increased amounts of free fatty acids which directly affect insulin signaling, diminish glucose uptake in muscle, drive exaggerated triglyceride synthesis and induce gluconeogenesis in liver [30].

There is considerable evidence that hyperglycemia induced oxidative stress is responsible for the development of secondary complications such as nephropathy, retinopathy, neuropathy and macro and micro vascular damage [31, 32]. Several studies have demonstrated increased generation of reactive oxygen species (ROS) [33] and decreased plasma antioxidant levels like vitamin E, vitamin C, lipoic acid and glutathione (GSH) [34] in diabetes.

3.2. Molecular Targets in Diabetes

Understanding of biochemical pathways regulating the metabolism of glucose, has contributed to a great extent in disclosing molecular targets within these pathways and thereby devising therapeutic targets. The molecular targets that can be used to reduce the blood glucose level and increase insulin sensitivity are compiled in Table 1.

Table 1. Therapeutic approaches for treating diabetes

Aim	Targets	Ref
Reduction in excess glucose production	Inhibitors of glycogen phosphorylase, glucose-6-phosphatase, fructose-6-phosphatase.	[35]
	Antagonists of glucagon receptor.	[36]
Boosting glucose induced insulin secretion	Agonists of glucagon like peptide (GLP-1)	[37]
	Inhibitors of enzyme dipeptidyl peptidase IV	[38]
Modulating insulin signaling pathway	Insulin mimetics	[39]
	Sustained activation of insulin receptor by use of inhibitors of specific protein tyrosine phosphatases.	[40]
	Activators of proximal insulin signaling steps (eg: activators of PI3Kinase activity)	[41]
	Inhibitors of glycogen synthase kinase-3	[42]
Targets of obesity and lipid metabolism	AMPK activators	[43]
	Recombinant adiponectin or adiponectin mimetic compounds	[35]
Modulators of peroxisome proliferator activated receptors gamma.	Agonist of PPAR γ	[44]
Regulation of oxidative stress	Use of natural and synthetic antioxidants	[45,46,47]

3.3. Conventional Therapies for Diabetes and Use of Herbal Therapies

At present, therapy for type 2 diabetes uses several approaches to reduce the hyperglycaemia. These include compounds such as sulphonylureas (and related insulin secretagogues), which increase insulin release from pancreatic islets; metformin, which acts to reduce hepatic glucose production; thiazolidinediones, which are peroxisome proliferator-activated receptor- γ (PPAR γ) agonists, which enhance insulin action; α -glucosidase inhibitors such as acarbose, which interfere with gut glucose absorption; and insulin itself, which suppresses glucose production and augments glucose utilization. These therapies have limited efficacy, limited tolerability and significant mechanism-based side effects. Therefore plants and natural compounds have gained great importance in treatment of diabetes.

3.4. Indian Medicinal Plants in Herbal Therapy for Diabetes

Many plant extracts and natural compounds have proved their antidiabetic potential by acting at different targets such as absorption of glucose, insulin secretion, insulin receptor function, insulin signal transduction mechanism, glucose transport and metabolism, glycogen synthesis and dysregulation of fatty acid synthesis. Additionally by being strong antioxidant they help in reducing oxidative stress and minimizing the secondary complications.

Many traditional medicines in use are derived from medicinal plants, minerals and organic matter. A number of medicinal plants, traditionally used for over 1000 years named rasayana are present in herbal preparations of Indian traditional health care systems. In Indian systems of medicine most practitioners formulate and dispense their own recipes. The World Health Organization has listed 21,000 plants, which are used for medicinal purposes around the world. Among these 2500 species are in India, out of which 150 species are used commercially on a fairly large scale. India is the largest producer of medicinal herbs and is called as botanical garden of the world. This part of the current chapter focuses on medicinal plants used in the treatment of diabetes mellitus. Many Indian medicinal plant extracts showed hypoglycemic, hypolipidemic, insulin secretagogue, and antioxidant activities in different model systems, though most of the time its mode of action is not known. The activity may be because of one or two compounds from compounds or may be its synergistic effect of many compounds present in the extracts. There are some good reviews and book chapters listing Indian medicinal plants having antidiabetic and related beneficial effects (48-52). Since diabetes is associated with increased oxidative stress, plants or their components with both antidiabetic and antioxidant properties can be of great use in treating diabetes and its complications.

A recent survey of PubMed and Science Direct for the past 3 years (2007-2009) have identified the following Indian medicinal plants with having antidiabetic activities: *Aegle marmelos*, *Allium cepa*, *Aloe vera*, *Amaranthus spinosus*, *Andrographis paniculata*, *Anethum graveolens*, *Annona squamosa*, *Bauhinia purpurea*, *Begonia malabarica*, *Berberis aristata*, *Borreria hispida*, *Brassica nigra*, *Butea monosperma*, *Caesalpinia bonducella*, *Calotropis procera*, *Casearia esculenta*, *Cassia auriculata*, *Ceriops tagal*, *Chlorophytum borivillianum*, *Cichorium intybus*, *Citrullus vulgaris*, *Citrus sinensis*, *Clitoria ternatea*, *Coldenia procumbens*, *Costus speciosus*, *Costus igneus*, *Costus pictus*, *Croton klotzianus*, *Cucumis melo*, *Curcuma longa*, *Cynodon dactylon*, *Cyperus rotundus*, *Desmodium gangeticum*,

Elephantopus scaber, *Embelia ribes*, *Emblica officinalis*, *Eugenia jambolana*, *Ficus bengalensis*, *Gymnema montanum* (=sylvestre), *Hedychium spicatum*, *Heliotropium zeylanicum*, *Hibiscus rosasinensis*, *Lagenaria siceraria*, *Mangifera indica* (fruit peel extracts), *Marsdenia roylei*, *Mentha piperita*, *Momordica charantia*, *Moringa oleifera*, *Morus indica*, *Mucuna pruriens*, *Murraya koenigii*, *Musa paradisiaca*, *Nymphaea stellata*, *Ocimum sanctum*, *Phyllanthus reticulatus*, *Phyllanthus simplex*, *Pongamia pinnata*, *Punica granatum*, *Rhizophora apiculata*, *Ricinus communis*, *Rubia cordifolia*, *Solanum xanthocarpum*, *Sphaeranthus indicus*, *Swertia chirata*, *Syzygium cumini*,

Table 2. Antidiabetic and related beneficial effect of some Indian medicinal plants and their components

Plants/common/local names	Effects	Part/extract/compound	References
<i>Aegle marmelos</i> /bilva/bael	Muscarinic receptors upregulated	Leaf extract	[53]
<i>Aegle marmelos</i> /bilva/bael	Antihyperglycemic and antidiyslipidemic	Aegeline 2	[54]
<i>Allium cepa</i> /onion	Antihyperglycemic effect	S-allylcysteine	[55]
<i>Allium sativum</i> /garlic	Hypoglycemic and antilipidemic	Alliin isolated from leaves	[56]
<i>Annona squamosa</i> /sugar apple/sitaphal	Antilipidemic and Antioxidant	Aqueous extract of leaves	[57]
<i>Begonia malabarica</i> /Narayanachanjeve	Hypoglycemic and antihyperglycemic	Hexane, ethyl acetate and methanol extracts	[58]
<i>Berberis aristata</i> / daruharidra	Regulate glucose homeostasis through decreased gluconeogenesis and oxidative stress	Aqueous ethanolic extract of root	[59]
<i>Butea monosperma</i> / flame of the forest	Antihyperglycemic and antihyperlipidemic	Ethanolic extract	[60]
<i>Butea monosperma</i> / flame of the forest	Thyroid hormone and glucose regulatory efficacy	Stigmastereol	[61]
<i>Casearia esculenta</i> / saptrangi herb	Hyperglycemic effect	3-hydroxymethyl xylitol	[62]
<i>Cassia auriculata</i> / Tannre's cassia, Tarwar Herb	Antihyperglycemic and Hypolipidemic	Aqueous extract of leaves	[63]
<i>Cinnamomum zeylanicum</i> / cinnamon	Hypoglycemic and hypolipidemic	cinnamaldehyde	[64]
<i>Commiphora mukul</i> / guggul	Hypoglycemic, Hypolipidemic	guggulsterone	[65]
<i>Costus pictus</i> / spiral ginger	Antihyperglycemic and insulin secretory	Aqueous extract	[66]
<i>Costus speciosus</i> /Crape ginger, Kushta	Hypoglycemic and hypolipidemic	isolated erymanthin	[67]
<i>Costus speciosus</i> / crape ginger	Normo-glycemic and hypolipidemic	Costunolide	[68]
<i>Curcuma longa</i> /Turmeric	AMPK mediated suppression of hepatic gluconeogenesis – hypoglycemic effect	curcuminoids	[69]
<i>Curcuma longa</i> /Turmeric	Antidiabetic and antioxidant	tetrahydrocurcumin	[70]
<i>Curcuma longa</i> /Turmeric	Decreased cognitive deficit, cholinergic dysfunction and oxidative stress	curcumin	[71]
<i>Cynodon dactylon</i> / Conch grass, Doob Herb	Antidiabetic and hypolipidemic	Aqueous extract	[72]
<i>Diospyros peregrine</i> /Indian persimmon/wild mongosteen	Hypoglycemic, Hypolipidemic and Antioxidant	Aqueous extract of fruit	[73]
<i>Dodecadenia grandiflora</i> tailiya/ Newari	antihyperglycemic	Phenylpropanoyl esters of catechol glycoside	[74]
<i>Elephantopus scaber</i> / Prickly leaved elephants foot, anchuvady	Antidiabetic activity	Novel steroid/ 28Nor-22 (R)2,6,23-trienolide	[75]
<i>Emelia ribes</i> /vidanga/ false black pepper	Hypoglycemic and cardioprotective	Ethanolic extract	[76]
<i>Eugenia jambolana</i> / jamun/ black plum	Hypoglycemic and Hypolipidemic effect mediated through PPAR α , γ	Flavonoid rich extract of seeds	[77]
<i>Ficus bengalensis</i> / Banyan tree	Hypoglycemic and Antioxidant	Aerial roots- aqueous extract	[78]
<i>Gymnema montanum</i> / gurmar/ miracle fruit	Antioxidant, hypoglycaemic and Renoprotective	Ethanolic extract	[79]

Plants/common/local names	Effects	Part/extract/compound	References
<i>Gymnema montanum</i> /gurmar/ miracle fruit	Antidiabetic and antioxidant	Ethanol extract	[80]
<i>Gymnema sylvestre</i> / gurmar/ miracle fruit	Hypolipidemic and hypoglycemic	Dihydroxy gymnemic triacetate	[81]
<i>Gymnema sylvestre</i> / gurmar/ miracle fruit	Hypolipidemic, hepatoprotective etc.	Gymnemic triacetate	[82]
<i>Helicteres isora</i> /Indian screw fruit, murgshrung	Hypoglycemic, Hypolipidemic	Bark aqueous extract	[83, 84]
<i>Helicteres isora</i> / murgshrung	Antidiabetic and involvement of P13/AKT pathway	Saponins and sapogenin	[85]
<i>Helicteres isora</i> murgshrung	Glucose metabolism and gene expression (PEPCK, Glut2, ACOX, PPAR α etc)	Saponins	[86]
<i>Helicteres isora</i> / murgshrung	Protects erythrocyte membrane bound enzymes and antioxidant	Aqueous bark extract	[87]
<i>Hibiscus rosasinensis</i> / shoe flower	Hypoglycemic effect	Ethanol extract of flowers	[88]
<i>Ichnocarpus frutescence</i> / black creeper/ kalidudhi	Insulin secretagogue	Leaf methanolic and hexane extract	[89]
<i>Leucas cephalotes</i> / Dronpushpi	Antidiabetic, Antihyperlipidemic and Antioxidant	Ethanol extract of leaves	[90]
<i>Momordica charantia</i> / Bitter gourd	Increase glucose uptake and adiponectin secretion in adipose cells	Ethanol extract of fruit and seeds	[91]
<i>Momordica charantia</i> / Bitter gourd	Hypoglycemic and Augments Antihyperglycemic effect of rosiglitazone	Methanolic extract	[92, 93]
<i>Momordica charantia</i> / bitter gourd	Management of diabetes in NIDDM patients	Administration along with rosiglitazone	[94]
<i>Momordica charantia</i> / bitter gourd	Increased PPAR γ and GLUT4	In fructose fed rats	[92]
<i>Moringa oleifera</i> / drum stick tree	Antidiabetic effects	Leaf aqueous extract	[95]
<i>Mucuna pruriens</i> / Kapikachhu/ buffalo bean	Hypoglycemic	Aqueous extract of seeds	[96]
<i>Murraya koenigii</i> / curry leaf	Hypoglycemic, hypolipidemic, hepatoprotective and protection to pancreas	Leaf aqueous extract	[97, 98]
<i>Nymphaea stellata</i> / water lily/ blue lotus	Hypoglycemic and regeneration of β - cells	nymphayol	[99]
<i>Nymphaea stellata</i> / water lily/ blue lotus	Hypoglycemic	Ethanol extract of leaves	[100]
<i>Phyllanthus reticulatus</i> / potato bush	Antidiabetic		[101]
<i>Phyllanthus simplex</i> / Bhui amla	Hypoglycemic and Antioxidant	Different extracts	[102]
<i>Pongamia pinnata</i> / kranja/ Indian beech	Concomitant administration with oral hypoglycemic drug –showed synergistic effect/ antihyperglycemic activity	Petroleum ether extract of stem bark	[103, 104]
<i>Pongamia pinnata</i> / kranja/ Indian beech	Antihyperglycemic activity	Pongamol and karanjin as lead compounds	[105]
<i>Psidium guajava</i> / guava	Antidiabetic and Antioxidant	leaves	[106]
<i>Punica granatum</i> /Pomegranate	Hypoglycaemic, Antioxidant and Hypolipidemic	Aqueous extract of flowers	[107]
<i>Ricinus communis</i> / castor	hypoglycemic	Ethanol extracts of roots	[108]
<i>Salacia</i> / Marking Nut Tree	Multiple targets in diabetes and obesity – PPAR γ -mediated lipogenic transcription	Salacia roots	[109]
<i>Swietenia macrophylla</i> / Big Leaf Mahogany	Hypoglycemic and hypolipidemic	swietenine	[110]
<i>Tinospora cordifolia</i> / guduichi	Antihyperinsulinemia, antihypertriglyceridemia and antioxidant	Aqueous extract of stem	[111]
<i>Trigonella foecum graecum</i> / fenugreek	Antidiabetic and Renoprotective	Fenugreek powder/ extract	[112, 113]
<i>Trigonella foecum graecum</i> / fenugreek	Antidiabetic and antioxidant	Herbal preparation from germinated fenugreek	[114]

Tectona grandis, *Tephrosia purpurea*, *Terminalia belerica*, *Terminalia chebula*, *Tragia cannabina*, *Trigonella foenum graecum*, *Withania coagulans*, and *Withania somnifera*. Some details regarding the plants showing antidiabetic potential and related beneficial effects, their common names, parts/components used and possible mechanisms are listed as Table 2.

There are also some commonly consumed Indian plant food materials used in the management of diabetes mellitus [52]. The food materials reviewed in relation to diabetes and its complications are: *Cajanus cajan*, *Cicer arietinum*, *Phaseolus mungo*, *Phaseolus vulgaris*, *Aegle marmelose*, *Mangifera indica*, *Morus alba*, *Musa sapientum*, *Psidium guajava*, *Punica granatum*, *Syzgium cumini*, *Vitis vinifera*, *Allium cepa*, *Annona squamosa*, *Beta vulgaris*, *Cucurbita pepo*, *Ipomoea batatas*, *Momordica charantia*, *Allium sativum*, *Brassica juncea*, *Cuminum cyminum*, *Curcuma*, *Murraya koenigii* and *Trigonella foenum graecum*. In addition to these food materials black tea, green tea and red wine have also been reviewed. All these plant food materials have been reported to have varying degree of hypoglycemic and anti-hyperglycemic activity. These components of Indian diet not only possess blood glucose lowering properties but are also beneficial in decreasing the risk factors for cardiovascular and renal diseases through various mechanisms including free radicals.

The finest example of a medicinal plant derived drug is metformin. *Galega officinalis* was found to be rich in guanidine, a substance with blood glucose-lowering activity that formed the chemical basis of metformin [115]. Metformin decreases hepatic glucose production, decreases intestinal absorption of glucose, and improves insulin sensitivity by increasing peripheral glucose uptake and utilization. Unlike sulfonylureas, metformin does not produce hypoglycemia in either patients with type 2 diabetes or normal subjects and does not cause hyperinsulinemia.

3.5. Absorption of Glucose

Alpha-Glucosidase and Alpha Amylase Inhibitors

Complex carbohydrates from the diet are cleaved into oligosaccharides and disaccharides in the duodenum by pancreatic amylases. Oligosaccharides and disaccharides are not absorbed by the enterocytes of the small intestines and are required to be further digested and broken down into monosaccharides by the α -glucosidases (brush border enzymes). The process is rapid and efficient. Most carbohydrates are digested and absorbed in the upper segment of the jejunum. Slowing of carbohydrate absorption is associated with improved glycemic control, and so α -glucosidase inhibitors and α -amylase inhibitors have been developed to delay intestinal absorption of carbohydrates [116, 117]. Acarbose, miglitol and voglibose are used in diabetic patients for their inhibitory activity to α -glucosidase, by competitively binding to the carbohydrate-binding region of α -glucosidase enzymes and thereby prevent their cleavage to absorbable monosaccharides and thus reduce glucose absorption.

Many plants and natural compounds are now known for their α -glucosidase and α -amylase inhibitory activity. Indian medicinal plants such as *Azadirachta indica*; *Syzygium cumini*; *Ocimum tenuiflorum*; *Murraya koenigii*; *Linum usitatissimum* have been shown to have α -glucosidase and α -amylase inhibitory activity [118]. Phloroglucinal derivatives isolated from edible marine brown alga *Ecklonia cava*: fucodiphloroethol G, dieckol, 6,6-bieckol, 7-phloroeckol and phlorofucufuroeckol A showed potent *in vitro* α -glucosidase and

α -amylase inhibitory activities [119]. Kim et al [120] have checked different flavonoids for their enzyme inhibitory activity in which luteolin, amentoflavone, luteolin 7-*O*-glucoside, and daidzein were the strongest inhibitors of α -glucosidase activity. Diphlorethohydroxycarmalol isolated from *Ishige okamurae*, brown algae, a potent α -glucosidase and α -amylase inhibitor, was shown to alleviate postprandial hyperglycemia in diabetic mice [121]. The active compounds salacinol, kotalanol, and de-*O*-sulfonated kotalanol from Ayurvedic medicinal plant *Salacia reticulata* used in treatment of diabetes also has strong inhibitory activity against α -glucosidase. [122]. Thus by using variety of plant extracts and compounds purified from them it is possible to modulate the absorption of dietary carbohydrates for the control of hyperglycemia in Type 2 diabetes.

3.6. Insulin Secreting Stimulator

Glucose stimulates insulin secretion by triggering and amplifying signals in β -cells. Glucose transported into beta cells is metabolized to generate ATP. Elevation in the ATP/ADP ratio induces closure of cell-surface ATP-sensitive K^+ (KATP) channels, leading to cell membrane depolarization which leads to opening of cell-surface voltage-dependent Ca^{2+} channels, facilitating extracellular Ca^{2+} influx into the beta cell. A rise in free cytosolic Ca^{2+} triggers the exocytosis of insulin [123]. Many plant extracts and pure compounds act as insulin secretagogues, though their mechanism of action is not always known.

Caffeic acid and chlorogenic acid (CGA), a mono-caffeoyl esters, purified from *Cichorium intybus*, have been shown to stimulate glucose transport in muscle cells and to increase insulin secretion in a glucose-dependent manner in islets [124]. This effect is considered to be due to inhibition of glucose-6-phosphatase activity associated with regulation of insulin secretion [125]. Green tea polyphenols, epigallocatechin gallate and epicatechin gallate modulate insulin secretion by inhibiting glutamate dehydrogenase which has regulatory role in insulin secretion [126]. Anthocyanins, cyanidin-3-glucoside, delphinidin-3-glucoside, cyanidin-3-galactoside are also found to be effective insulin secretagogues [127].

3.7. Insulin Mimetic Substances

Small molecules that have insulin-like activity (insulin mimetics) have been proposed as potential therapeutic agents to supplement insulin in the promotion of glucose transport and glucose metabolism, [128,129]. One of the first insulin mimetic described, a non-peptidyl fungal metabolite (L-783,281) was found to bind the insulin receptor and stimulate glucose transport in cells and in animal models [130]. Many compounds mimicking one epitope of the insulin molecule known to be involved in binding to the insulin receptor has been reported [131,132]. Polyphenols and flavonoids also have insulin mimetic activity by binding to insulin receptor and activating insulin signaling. Two flavone glycosides, rhoifolin and cosmosiin from *Citrus grandis* (L.) (red wendun) leaves have insulin mimetic activity by acting through their enhanced adiponectin secretion, tyrosine phosphorylation and GLUT4 translocation [133]. Tannic acid was found to exhibit insulin-like activities by phosphorylation of the insulin receptor (IR) and Akt, as well as translocation of glucose

transporter 4 (GLUT 4), [134]. Natural anti-diabetic compound 1,2,3,4,6-penta-O-galloyl-d-glucopyranose binds to insulin receptor and activates insulin-mediated glucose transport signaling pathway [135]. Oligomers of grape-seed procyanidin extract activate the insulin receptor and key targets of the insulin signaling pathway differently from insulin [136].

3.8. Insulin Signal Transduction Activation

Insulin signal transduction is initiated when insulin binds to the insulin receptor which activates its intracellular tyrosine kinase domain. Activation of the insulin receptor leads to phosphorylation of several intracellular protein substrates including insulin receptor substrate 1 (IRS1), IRS2 and Shc. This initiates two major signaling cascades: the phosphatidylinositol 3-kinase (PI3K) pathway that includes activation of Akt leading to activation of glycogen synthase and the other enzymes/proteins necessary for the acute metabolic effects of insulin and the mitogen-activated protein kinase (MAPK) pathway that includes activation of ERK1 and ERK2 leading to gene transcription, protein translation and cell growth. Although PI3K is central to mediating insulin metabolic actions, other kinases such as p42/44 MAPK have also been implicated in insulin-dependent glucose uptake [128-137].

Flavonoids include a great diversity of structures, and there is accumulating evidence that such different structures might act on different targets and modify the activity of different components of the insulin signaling cascades, such as protein kinase C, PI3K and MAPK pathways [138]. In this context, myricetin acts on IRS-1-associated PI3K and glucose transporter type 4 (Glut4) activity [139]. Glycoside kaempferol 3-neohesperidoside stimulates glucose uptake via the PI3K and protein kinase C pathways [140]. Epigallocatechin-gallate (EGCG) has been reported to mimic the metabolic and vasodilator actions of insulin via the PI3K/Akt-dependent pathway [141-144]. However, recent studies have shown that EGCG blocks insulin-dependent Glut4 translocation to the plasma membrane in MC3T3-G2/PA6 adipose cells. It inhibits, as well, hepatic glucose production through an insulin-independent signaling pathway [145,146]. Tannins present in *Cichorium intybus* inhibit protein tyrosine phosphatase, nonreceptor type 1, probably in a PI3K-dependent manner [147]. Moreover, gallotannins such as penta-O-galloyl-glucopyranose from banana extract appear to be more potent and efficacious than ellagitannins in insulin receptor binding, insulin receptor activation and glucose transport induction [135]. The isoflavonoid aglycone-rich fraction of Chungkookjang enhances PPAR- γ activity and stimulates the translocation of the glucose transporter Glut4 into the plasma membrane through IRS1 and Akt phosphorylation in 3T3-L1 adipocytes [148]. Purified cinnamon polyphenols have been shown to increase the expression of the insulin receptor, tristetraprolin and glucose transporter 4 in mouse 3T3-L1 adipocytes [149]. Flavonoids from *Cephalotaxus sinensis* leaves are also the most active compounds at inducing Glut4 translocation in membrane preparations from mice adipocytes [150]. The grape-seed procyanidin extract (GSPE) requires PI3K and p38 MAPK to stimulate glucose uptake in L6E9 myotubes and in 3T3-L1 adipocytes. It also showed that GSPE, like insulin, induces Glut4 translocation to the plasma membrane [151]. Saponins and saponenin from *Helicteres isora* activate the PI3K/Akt pathway thus leading to phosphorylation and inactivation of GSK-3 α /beta with subsequent stimulation of glycogen synthesis as well as increase of Glut4-dependent glucose transport across the cell membrane [85]. Seeds of fenugreek, widely used as a spice and condiment are a rich source of fiber and its sub-

fractions are known to have hypoglycemic effects in different model systems [152]. The dialysed fenugreek extract showed the ability to sustain hypoglycemia and were able to modulate glucose metabolizing enzymes [153]. Additionally, fenugreek seed extract is known to stimulate insulin signaling pathway via PKC activation [154]. Besides its anti-diabetic effect supplementation of seeds in diet enhanced the antioxidant potential in control and diabetic rats [155].

3.9. Reduction of Excessive Glucose Production

The liver performs two different functions, depending on the level of insulin. In the presence of low levels of insulin. In the fasting state the liver produces glucose from glycogenolysis and gluconeogenesis and releases it to maintain fasting plasma glucose levels. In the presence of moderate-to-elevated levels of insulin, the liver stops producing glucose and takes glucose from the plasma, storing it as glycogen for use when plasma glucose and insulin levels decrease. Epigallocatechin-3-gallate (EGCG), a main catechin of green tea, has been suggested to inhibit hepatic gluconeogenesis. Hesperidin and naringin both play important roles in preventing the progression of hyperglycemia, partly by increasing hepatic glycolysis and glycogen concentration and/or by lowering hepatic gluconeogenesis [156]. Curcumin inhibits glucose production in isolated mice hepatocytes caused by activation of AMP kinase and inhibition of G6Pase and PEPCK activity [157].

3.10. Hyperglycemia, Oxidative Stress and Inhibition of Secondary Complications

When cells are exposed to abnormally high levels of glucose, they generally are able to reduce the transport of glucose into the cytoplasm, thus maintaining internal glucose concentrations at normal levels. However, some cells cannot quickly decrease glucose transport rates, which results in high glucose levels inside the cell. Examples of these cells include capillary endothelial cells in the retina, mesangial cells in the renal glomerulus, and neurons and Schwann cells in peripheral nerves. The results can be visual and renal impairment, and neuropathy. Hyperglycemia can produce excessive levels of superoxide in endothelial cells, which can then activate the various pathways of microvascular damage. Hyperglycemia rapidly suppresses endothelium-dependent vasodilation, probably through increased production of oxygen-derived free radicals. Increased oxidative stress is a widely accepted participant in the development and progression of diabetes and its complications. Mechanisms by which increased oxidative stress is involved in the diabetic complications include activation of transcription factors, advanced glycated end products (AGEs), and protein kinase C [158]. This suggests that substances which having antioxidant activity along with hypoglycemic play a great role in the diabetic treatment.

Antioxidant enzymes are reduced in peripheral nerves and are further reduced in diabetic nerves. Oxidative stress appears to be primarily due to the processes of nerve ischemia and hyperglycemia auto-oxidation. The antioxidant drug is potentially efficacious for human diabetic sensory neuropathy [159]. All the pathways responsible for development of diabetic neuropathy are linked to oxidative stress in one way or the other. There was marked reduction

in DNA fragmentation observed after resveratrol treatment in diabetic rats as evident from decrease in Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) positive cells in sciatic nerve sections [160]. Administration of *Ocimum sanctum* attenuated sciatic nerve transection-induced axonal degeneration, reduction of nociceptive threshold and motor in-coordination [161].

Phenolic constituents of finger millet (*Eleusine coracana*) such as gallic, protocatechuic, *p*-hydroxy benzoic, *p*-coumaric, vanillic, syringic, ferulic, *trans*-cinnamic acids and the quercetin inhibited cataract eye lens effectively [162]. Morimitsu et al [163], showed that diabetic cataract inhibitors from Japanese radish (*Raphanus sativus* L.) leaves extract, using the rat lens organ culture system. Anthocyanin monomers from the grape skin extract has also shown to have potent inhibitory activity for lens opacity.

Dietary supplementation with the antioxidant vitamin E, slowed the rate of progression of renal deterioration [164], attenuated the nephrotoxicity caused by ferric nitrilotriacetate (Fe-NTA) [165] and ameliorated the glomerulosclerosis occurring in the nephrectomy remnant kidney model in the rat [166]. Gentamicin-induced nephrotoxicity was ameliorated with garlic, known to be rich in polyphenols [167]. Green tea is considered to provide a useful therapeutic option in the reversal of oxidative stress induced cardiac dysfunction in diabetes mellitus [168].

The polyphenol rich fraction of fenugreek seeds also exhibited the ability to protect erythrocytes from oxidative damage [169]. Studies in our laboratory have shown that germinated fenugreek seed extracts exhibit high in vitro antioxidant potential [170]. Syndrex®, manufactured by Plethico laboratories, Indore, India is a commercially available anti-diabetic drug which contains powder of germinated fenugreek seeds. The soxhlet methanolic extract of this drug has high in vitro antioxidant properties and the whole drug extract improved the functionality and viability of pancreatic islet in vitro [114]. Additionally, in vivo studies of this drug also showed that pre-treatment of this drug prevented STZ induced diabetes in mice [171].

3.11. Other Targets for Treatment of Diabetes

Adipose tissue triglycerides are the predominant form of energy storage in animals. It requires a carefully regulated balance between triglyceride synthesis and hydrolysis. Dysregulation of these processes may result in metabolic disorders, such as obesity and lipodystrophy, which are associated with dyslipidemia, insulin resistance, and overt diabetes. Free fatty acids, inflammatory adipocytokines, thrombotic and antifibrinolytic factors, adiponectin, and adipokine with insulin sensitizing and anti-inflammatory actions a factors derived from adipose tissue are believed to play a central role in the development and progression of diabetes, and its vascular complications. Targeting these factors by antidiabetic agents should result in improved metabolism and in a reduction of vascular risk [172, 173].

o-Coumaric acid and rutin inhibited the expression of PPAR γ , C/EBP α and leptin and then up-regulated expression of adiponectin at the protein level and also inhibits intracellular triglyceride [174]. Hesperidin and naringin, and their aglycones hesperetin and naringenin, decrease plasma and hepatic cholesterol and triacylglycerol by inhibiting the hepatic enzymes involved in their synthesis such as 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and acyl-CoA:cholesterol acyltransferase (ACAT) in experimental animals

[175,176]. They also markedly enhanced hepatic and adipocyte PPAR γ protein expression. Chronic administration of nicotinic acid (NA) has been shown to cause a deterioration in glycemic control in diabetic patients (177). Acute use of NA inhibits lipolysis in adipose tissue suppressing circulating nonesterified fatty acid.

3.12. Use of Plant Extracts as Hypoglycemic Agent

Syndrex® is a formulated herbal antidiabetic preparation containing powder of germinated fenugreek seeds showed protective activity to mouse islets against streptozotocin insult. [114]. Ethanolic extracts of leaves of the *P. reticulatus* showed hypoglycemic effect in alloxan induced diabetic mice [101]. 3-hydroxymethyl xylitol, the compound from *C. esculenta*, possesses antihyperglycemic effect in diabetic rat [62]. The leaf extracts of evergreen plant *Ichnocarpus frutescence* showed insulin secretagogue activity in experimental diabetic rat [89]. The aqueous fractions of *Phyllanthus simplex* normalize the marked alterations in antioxidant enzymes and antioxidant parameters levels in liver and kidney of diabetic rat. Treatment with the active fractions also normalized the diabetic induced hyperlipidemia and liver glycogen [102]. *Punica granatum* aqueous extract could be used, as a dietary supplement, in the treatment of chronic diseases characterized by atherogenous lipoprotein profile, aggravated antioxidant status and impaired glucose metabolism and also in their prevention [107]. Administration of marsupisin, pterosupin, and pterostilbene, phenolic constituents of the heartwood of *Pterocarpus marsupium*, significantly lowered the blood glucose level of hyperglycemic rats [178]. Aqueous leaf and seed extracts of *Phyllanthus amarus* showed Hypoglycemic and hypocholesterolemic activities in mice [179]. Root and leaf extracts of *Withania somnifera* [180], *Tinospora cordifolia* [181] and ethanolic extracts of *Butea monosperma* [182] important medicinal plants of Indian tradition showed hypoglycaemic and hypolipidaemic effects on Alloxan-Induced Diabetic Rats.

Apart from the above details there are also some recent reviews on signaling in diabetes and oxidative stress (183-187) that pertains to newer therapeutic strategies involving insulin resistance, cardiovascular disease, insulin signaling and diabetic complications.

Conclusions

Diabetes is a major health problem involving altered metabolism of carbohydrates. The disease process involves alterations in several signaling pathways. Many natural products, plants-derived medicines have been used for the treatment of diabetes mellitus in Indian tradition. Crude extract of medicinal plants which are commonly used have been demonstrated experimental or/and clinical anti-diabetic effectiveness as a glucose lowering agent. The bioactive compounds from many of these plants are isolated and their mechanisms of action on different pathway active under diabetic condition have been worked out. Yet many plant extracts with antidiabetic activity need to be checked for their mode of action and isolation of bioactive principles from these plants. Many of them are highly effective in reducing secondary severity of secondary complications of diabetes. More clinical trials and

evidence based studies can possibly promote use of medicinal plants and their constituents in controlling diabetes.

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Chapter XIII

Indian Medicinal Plant *Curcuma Longa* Linn in Animal Model of Cerebral Stroke

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Abstract

In recent years, the use of traditional medicines has received considerable attention. The need for basic scientific investigation on medicinal plants using indigenous medical system has become imminent. Recent studies have focused on the beneficial effects of dietary antioxidants and polyphenolic compounds from plant sources, such as Ginkgo biloba, curcumin, resveratrol and blueberries in stroke. Turmeric, the powdered rhizomes of *Curcuma longa* Linn is used extensively in Ayurvedic medicine. The major chemical constituents of turmeric are volatiles; the curcuma oil, and non-volatiles; the curcumin. Some reports already indicate the utilization of curcuma oil (C.oil), such as insect repellent, anti-fungous, anti-bacterial, and anti-carcinogenic. There is almost a complete absence of studies which address the potential of the neuroprotective effect of Curcuma oil.

In the present article we have focused on the effect of treatment of C.oil obtained from dried rhizomes in two models of stroke. The major component: ar-Turmerone, α - β Turmerone and Curlone, significantly decreased the numbers of apoptotic neurons in the vitro studies but on the contrary pre treatment with all the major constituents of C.oil failed to offer protection in embolic stroke in rats. C.oil given for five continuous days in three different doses (50, 75 and 125mg/Kg) prior to ischemic insult showed a dose dependent effect in middle cerebral artery occlusion model of stroke. In our perspective, administrating C.oil should be done in a timely manner after cerebral ischemia occurs. C.oil was given post ischemia, at different time point, 2, 6 and 24hrs after ischemia showed significant reduction at 6 hrs. To explore the geographical variability, if any, the

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rhizomes were collected from twelve different regions from India. These samples showed variable chemical constituents and efficacies in neuroprotection. C.oil is significantly effective but its major constituents failed to exhibit neuroprotection in vivo model of stroke.

Neurons are produced following injuries such as targeted ablation of cortical neurons, gross trauma, or stroke. This “reactive neurogenesis” may be more successful when tissue destruction and inflammation are minimized. Does C.oil enhance recovery from cerebral ischemia through beneficial modulation of the brain vascular system and the promotion of angiogenesis? With its potent antioxidant, anti-apoptotic and anti-inflammatory effects further studies are recommended to evaluate effects of Coil on stroke animals with other co-morbidity since the animals studies most often fail in clinical trials. Since the physiological background of animals might affect the effectiveness of a treatment and determines whether clinical trials will succeed.

Keywords: *cerebral stroke, embolic stroke, ar-Turmerone, α - β Turmerone, Curlone, therapeutic time window, curcuma oil*

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Overview

The use of plants for healing purposes predates recorded history and forms the origin of much of modern medicine. At present, almost all the scientific workers in various fields have recognized the importance of plants as sources of biologically active products and have initiated active research programme either to isolate new lead compounds or to produce standardized extracts for development of future drug candidates. The indigenous knowledge of the medicinal plant along with the modern research activities should be utilized to provide a new approach; which further enhances the discovery of drugs in a much more effective way than with random collection.

Turmeric the yellow pigmented powder from rhizomes of *Curcuma longa* L. (family Zingiberaceae) is used extensively in Indian cuisine and has been used for centuries in Ayurvedic medicine for the treatment of various conditions [1]. Turmeric consists of the secondary metabolites, some of which have been identified and characterized as major components, while some newer ones are still expected to be discovered. The major chemical constituents of turmeric rhizome known so far as now are volatiles and non-volatiles. The bright yellow color of turmeric is due to the non-volatile compounds which are found to be a rich source of curcuminoids (curcumin) compounds, while its aroma accounts for its volatile oil (curcuma oil- a group of terpenoids) (Figure1 a) and both of them have been shown to be largely responsible for the pharmacological activities of turmeric powder, extracts and oleoresins. Some reports have already indicated the functionality and utilization of volatile turmeric oil, such as insect repellent [2], anti-fungous [3], anti-bacterial [4], and anti-carcinogenic [5] properties. The oil of turmeric has been shown to possess the anti-inflammatory activity and to increase the bile flow. It was found to be effective against bronchial asthma in a clinical trial [6]. Both the curcumin and the oil have been shown to

possess wound healing properties and inhibitory activities against pathogenic fungi both in vitro and vivo [7]. In carrageenin- induced edema, the volatile oil had as much anti-inflammatory activity as that of Phenylbutazone. The C.oil was also found to be potent in cotton pellet granuloma studies [8].

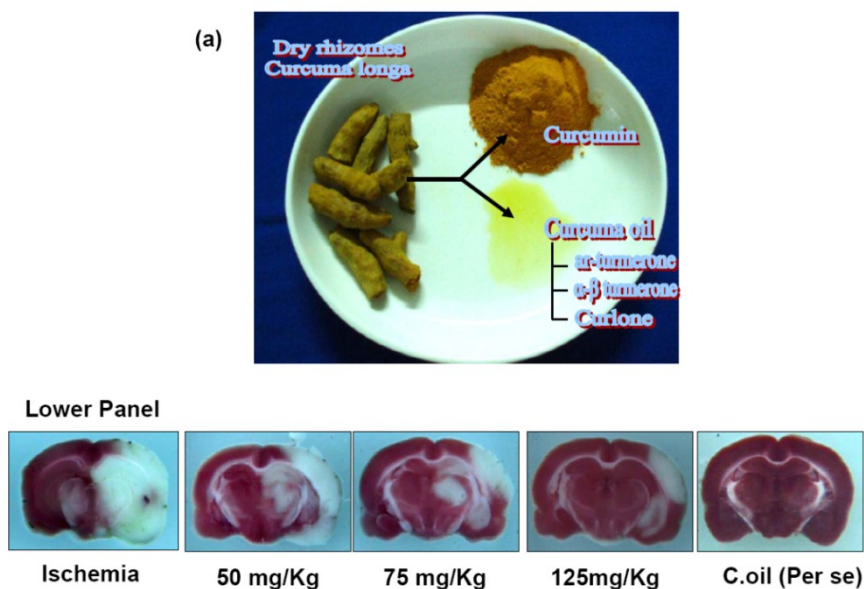


Figure 1. (a). Pictorial view of *Curcuma* rhizomes, curcuma oil and curcumin. Curcuma oil and curcumin were extracted from dried rhizomes the volatile part was Curcuma oil (C.oil) comprising of terpenoids and the non-volatile part was Curcumin comprising of curcuminoids (phenolic compounds such as monodemethoxy-curcumin, didemethoxycurcumin, curcumin). Major constituents found in C.oil were: ar-turmerone, α -turmerone and β -turmerone, curlone, four major curcumenes: curcumene, zingiberene, bisabolene sesquiphellandrene and other minor constituents like zederone, germacrone, curdione, curcumerone, zederondiol and isozederendiol. Lower Panel: Effect of multiple dosing on infarct area as seen by TTC staining. Number of animals used for infarct volume was five in each group. Study was also performed with the single dose/day of 50, 75 and 125 mg/kg body weight each for four continuous days via oral route. It was seen that 50 and 75 mg/kg of C.oil offers no protection while 125mg/kg of C.oil offers significant protection.

Curcuma oil consists mainly of terpenoids. More than 22,000 individual terpenoids (also known as terpenes or isoprenoids) are known at present, making the terpenoids the largest group of natural products [9]. Only a small percentage of terpenoids has been investigated so far and two most valuable drugs have emerged: one an antimalarial, the sesquiterpene lactone artemisinin, from *Artemisia annua* and the other the anticancer diterpene paclitaxel (Taxol), isolated from the yew. Terpenoids are water-insoluble (hydrophobic) and this lipophilic nature, which indicates how easily, a molecule is transported across a lipoproteinous membrane, is an inherent property of terpenoids.

The *C. longa* contains mostly sesquiterpinoids and its major constituents are turmerone, ar-turmerone, zingiberene, alpha-phellandrene and germacrone. The percentage of these constituents varies depending on geographical origins. Sesquiterpenoids are major constituents of turmeric oil and ar-Turmerone recognized as the major turmerone, identified by a GC-MS analysis [10]. Recently ar-Turmerone is reported to suppress the growth and the

induction of apoptosis in human leukemia cell lines [5,11]. Another interesting activity of ar-Turmerone has been recently reported, to be significantly more potent platelet inhibitor than aspirin against platelet aggregation induced by collagen but had no effect on platelet activating factor or thrombin induced platelet aggregation [12]. Recently the chemistry of major constituents of C.oil isolated from *C. longa L.* rhizome has been reported [6]. However, there are no reports on the neuroprotective effect of major constituents of C.oil. Major active compounds isolated from Curcuma oil from *C. longa L.* rhizome may be a good source for lead compound for ameliorating cerebral ischemic injury. Since most of these are largely devoid of adverse effects and have excellent pharmacological actions, they could lead to the development of new classes of possibly safer anti-stroke agents.

Therefore in the present study we investigated the effect of C.oil from various geographical origin and its major constituents viz: ar-Turmerone, α - β Turmerone and curlone were tested for neuroprotective effect in middle cerebral artery occlusion model in rat.

The idea of this article is to attract researchers to explore the therapeutic potential of curcuma oil with or without chemical modifications based in structure and biological activity relationships, in order to find new drugs that can be more effective, less toxic to humans and also can be used for the treatment of many diseases.

As of today, no treatment has been known to repair the cellular loss associated with an ischemic lesion. However, the discovery and dynamic regulation of neural stem/progenitor cells in the adult mammalian brain has open up exciting possibilities for future therapeutic interventions. Unfortunately, the results of this regenerative effort are so far limited compared to the amount of tissue loss. This could be due to the low survival of the recruited cells, but it could also be explained by insufficient activation or dysfunctional lineage selection. But if there is an alteration in the lineage selection of neural stem/progenitor cells followed by a lesion in the brain, then what are the responsible signals for their activation or whether there is a participation of these cells in post-lesion regeneration, astrogliosis or neuroprotection are still remained unclear. A greater understanding of these mechanisms is essential for finding ways to improve the endogenous regenerative capacity.

The adult brain harbors a population of neural stem cells (NSCs) throughout life in the subgranular zone (SGZ); [13] of the hippocampus and adjacent to the lateral ventricles in the subventricular zone [14]. These NSCs are self-renewing, with the potential to generate all three basic cell types of the central nervous system: neurons, oligodendrocytes and astrocytes. In vivo they provide a constant supply of new cells to particular regions of the adult brain, including the hippocampus and the olfactory bulb.

Adult neurogenesis is often studied using bromodeoxyuridine (BrdU) or other thymidine analogues to label DNA synthesis in nascent neurons during the S-phase of the cell cycle. Previous studies showed that endogenous precursors can generate new neurons in the ischemic striatum but not in the cortex, despite a large lesion burden at this site [15,16]. However, newer studies shows that the repair mechanisms may allow cortical recovery as well, [17-19], and thus cortical regeneration after ischemia remains controversial.

Functional recovery from ischemic damage is the ultimate goal of any therapeutic effort, and inspiring evidence suggests that this is possible. Behavioral evidence also suggests a recovery of brain function after growth factor treatment in ischemic animals. We observed in our previous work [20] that C.oil treated group of rats performed significantly better than compared with I/R after the insult. Our previous study thus prompted us to explore the C.oil dependent cortical recovery from transient ischemia.

Experimental Procedures

Extraction of C.Oil and its Major Constituents

Dried rhizome of *Curcuma longa* (family Zingiberaceae) was collected from Lucknow and botanically identified. A representative sample was preserved for future reference by Botany department of Central Drug Research Institute, Lucknow, India. The dried rhizomes were powdered and extracted with light petroleum ether; the solvent was removed by distillation under reduced pressure. The oily residue was collected and dried. The chemical constituents present in C.oil were identified with the help of HPLC, GC-MS. The standardization of C.oil isolation was done using HPLC method. The stability with changes in pH, physiochemical properties like photosensitivity etc has been published recently [6,21]. The major components of rhizomes were ar-Turmerone, α - β Turmerone and Curlone. These major components were separated from C.oil by chromatography (column, HPLC, GLC) (mol.wt.216, retention time 8'-08"). Detailed methodology of isolation of C.oil and major constituents has been given in a recent publication [6].

Animal Used

Male Sprague -Dawley rats (weight, 200-250 g) procured from Central Drug Research Institute, Lucknow. The rats were housed in a 12-hrs light/dark cycle and had free access to water and food. The Animal Ethics Committee of the CDRI approved animal care and the general protocols for animal use. Initially, all the rats were evaluated for behavioral neurological deficits. Rats showing any deficit were excluded from the study.

The study was divided into following groups.

Group (A): C.oil in different doses (50, 75 and 125 mg/Kg body weight) was given to un-operated rats for five consecutive days. On day 6 and 7, no C.oil was given to the rats. Untreated controls ischemic rats were given saline for 5 days and on day 6, all the groups of rats were subjected to MCA occlusion followed by 24hrs of reflow.

Group (B): All the rats were scanned for any lesion in DW-MRI before the occlusion of MCA. DW-MRI scans were done after 1, 5 and 23hrs of clot implant in all the ischemic group of rats and was repeated one hr after the C.oil (500mg/kg) was administered. C.oil was given 2, 6 or 24hrs after MCA occlusion.

Group (C): Thrombus formation was observed in sham, no treatment and C.oil treatment groups. C.oil was given 6 hrs post clot formation.

Group (D): Flowcytometric study was carried out on neuronal rich cells. The vitro studies were planned to see the effect of the major component ar-Turmerone, α - β Turmerone and Curlone prior to H₂O₂ exposure. After 1 hr of this pretreatment, the cells were washed twice with phosphate buffered saline (PBS) to remove the unabsorbed compounds. Three sets were assigned on treatment no treatment, hydrogen peroxide and hydrogen peroxide + major constituents (ar-Turmerone, α - β Turmerone and Curlone) alone or in combination. Parallel negative controls were used in all test experiments.

Group (E): The major chemical constituent, ar-Turmerone, α - β Turmerone and Curlone, were isolated from C.oil and were used alone as pretreatment in the study. After chemical

finger printing the major component α -Turmerone, α - β Turmerone and Curhone (100mg/Kg) were given alone one hour before MCAo.

Group (F): Samples of C.oil were extracted from rhizomes of *Curcuma longa* from twelve geographical regions of India. Middle cerebral artery of rats was occluded followed by 24hrs of reflow. The rats were grouped as sham operated, ischemic and C.oil treated ischemic.

Group (G): Rats were randomly divided into three groups: sham, Ischemia /reperfusion, C.oil pretreated (250 mg/kg body weight) (5 rats per group) received a twice daily of BrdU (50 mg/ kg, i.p.) after ischemia induction and were then anesthetized and perfused 24hrs after BrdU administration.

Emboic Stroke Model

Focal embolic cerebral ischemia was induced as previously reported [22,23], by injecting multiple fibrin-rich autologous clots into the external carotid artery one after another. To induce reproducible lesions, obstruction by emboli in the proximal segment of a large feeder artery should be made [22,24]. For clot formation, blood was drawn from the femoral vein of a donor rat and left aside to form a clot. The clot was fragmented by extruding it twice through a 27-gauge needle and washed and thus multiple fibrin-rich autologous clots were formed. Briefly, for producing embolic cerebral ischemia, rats were anaesthetized with chloral hydrate (350 mg/kg, i.p.). The right common carotid artery (CCA), right internal carotid artery (ICA) and the right external carotid artery (ECA) were exposed. The previously prepared multiple clots were suspended in 0.3 ml of phosphate buffered saline and were connected to Hamilton syringe through a PE 50 catheter advanced up to 17mm into the right ICA via ECA (up to the point where the middle carotid artery branches off). The injected clot was lodged into the MCA stem through the external carotid artery during which the common carotid artery was temporarily closed. Selection of appropriate size of clots and proper washing results in high fibrin content of the clots, which ensures durability of the clots and delays the spontaneous lysis of the clots. After removal of the catheter, the clips on the right CCA and the right ECA were removed and the incision was closed. The sham-operated rats underwent a similar surgical procedure; a PE 50 catheter was advanced up to 17mm in the ICA via ECA and removed without clot implant. The rectal temperature in the animals was kept at 37°C throughout the surgical procedure with the help of heating pads.

Initially all rats were evaluated for neurological deficit according to [25]. Rats showing any deficits were excluded from the study.

Filament Model of Focal Ischemia

The second set of ischemic models focal cerebral ischemia was induced by the intraluminal filament technique [26] with slight modifications. Briefly, the animal was anaesthetized with chloral hydrate 350mg/kg, intraperitoneally. To occlude the middle cerebral artery, the right common carotid artery was exposed through a midline cervical incision and gently separated from the nerves. Micro vascular clips were placed around the right common carotid artery and the internal carotid artery to prevent bleeding during the

insertion of the filament. The external common carotid artery was closed with a ligature and cut with micro scissors and electro coagulated. A heparinized 4-0 monofilament nylon suture with a rounded tip was inserted into the stump of the external common carotid artery. The filament was advanced 1.9 to 2.1 cm into the internal common carotid artery until resistance was felt, to occlude the blood flow to the MCA territory. The filament was held in place by tightening the suture around the internal common carotid artery and placing a micro vascular clip around the artery. After 60 min of MCA occlusion, the filament was removed and the external carotid artery was permanently closed by electro coagulation. The mortality in the present study was approximately 16.8%. Sham operation was undertaken in a similar manner; except the filament was not inserted and the common carotid artery was not occluded. Core temperature (rectal) was maintained around 37°C by using heating pad and lamp throughout the surgical procedure and till the animal recovered from anesthesia.

Early Detection of Lesion Area Using Diffusion-Weighted MRI (DW-MRI)

The DW-MRI scan was taken from same rat, before and after ischemia in the ischemic group. In the C.oil treated group, DW-MRI scan was taken from same rat, before and after ischemia and again 1hr after C.oil treatment. All DW-MRI experiments were carried out as described previously [27], using an animal MRI/MRI scanner (Bruker, BIOSPEC). A 69-mm circularly polarized birdcage volume resonator was used to carry out experiments at 4.7 T. MRI was used to examine ischemic rat brain after 1hr of C.oil treatment. The seven slices acquired were summed to provide the total volume of ischemic injury. The threshold value of 15% was found to be the lowest cut off value that did not include pixels in the contralateral non ischemic hemisphere [28]. The summed results of seven coronal sections from each brain of 5 rats per group have been expressed as mean $\pm$ S.E.M. A further study was planned to correlate the early lesion assessed in vivo by DW-MRI with later brain damage.

Determination of Cerebral Infarct

An overdose of chloral hydrate was given to the rats before decapitation after 24hrs of occlusion. Brains were quickly removed and placed in ice cold saline for 5 min. Seven coronal slices of 2-mm thickness were cut and incubated in 2% 2,3,5- triphenyltetrazolium chloride (TTC) for 15 min at 37°C. The stained brain sections were fixed in 4% formalin. The infarct area in each brain section was determined using image analyzer software (Biovis, Expert vision, India). Accuracy in the measurement of infarct volume is compounded by post-ischemic brain edema that may increase brain volume in the infarcted region. We calculated infarct volumes of each brain by summation of the infarcted area of all brain slices multiplied by the average slice thickness i.e. [area of infarct in square millimeters x thickness (2 mm)] from the same hemisphere. Edema correction of infarct volume was done using the equation, Volume correction = (infarct volume - contralateral volume)/ipsilateral volume. Volume of contralateral and the ipsilateral brain slices were calculated from the product of the average slice thickness (2mm) and the sum of the areas of five brain all seven-brain slices. Result of seven coronal sections each brain of 5 rats per group was used for the study. [27]

Thrombus Formation

Adult male rats (200-250gm) from the C.D.R.I. Animal house were anaesthetized with 350mg/ kg choral hydrate. The experiments were carried out as described previously [29]. A skin incision made anteriorly from the middle of the right clavicle for 1 cm and about 1cm of the jugular vein about was exposed. Five drops of 10 % formalin in 65 % methanol was dropped on the vein thereafter the skin incision was closed. After 24 hours the incision was reopened, the treated jugular vein was examined for thrombus formation which was then graded according to its presence or absence. The dark, almost black, intravascular blood clot was removed. (C.oil was given after 6 hrs of the operation given 250 mg/Kg body weight, intraperitoneally injection).

Flowcytometric Estimation of H₂O₂-Exposed Rat Neurons

Isolation of Neurons from Rat Brain

Rats were euthanized and forebrains were isolated and a homogenous suspension of neurons was prepared in PBS, (pH 7.4) by treatment of finely chopped forebrain tissue with dispase (1000 protease unit/ml) for 45 min at 37°C. After enzymatic treatment, the dissociated neurons were passed through a filter (~53 µm) to remove large neurons and tissue fragments as reported by us [20]. Confirmation of neuron population was done by labeling the neurons with NeuN (a monoclonal antibody against neuron-specific marker NeuN conjugated with PE; SC7301, Santacruz; dilution 1:50). NeuN positive neurons were gated in the dot plot and the same gating was used in the entire study.

In the present study, we chose H₂O₂ to induce oxidative cell damage in neuronal rich cell population because: 1) oxidative stress is believed to be an important mediator of neuronal cell death, and has been postulated to contribute to the pathogenesis of various neurodegenerative diseases; 2) H₂O₂ is a precursor of highly oxidizing, tissue-damaging radicals such as hydroxyl radicals, and is known to be toxic to many systems; 3) among a great variety of reactive oxygen species, H₂O₂ plays a pivotal role because it is generated from nearly all sources of oxidative stress; 4) exogenous H₂O₂ can enter the cells and induce cytotoxicity due to its high membrane permeability. In the present study, we evaluated the protective effect of major Chemical constituents of C.oil on H₂O₂-induced cell damage in neuronal rich cells. For pretreatment prior to H₂O₂ exposure, neuronal cells were pretreated with medium containing C.oil constituents. After 30 min of this treatment, the neurons were washed twice with phosphate buffered saline (PBS) to remove the unabsorbed compound. Exogenous H₂O₂ can enter the cells and induce cytotoxicity due to its high membrane permeability [30] and ability to induce apoptosis [31]. Then, buffer containing 300µM H₂O₂ was added to the cells incubated for 1 hr at 37°C and after one washing with PBS the respective fluorochromes were added. After 30 min of incubation, the cell viability/apoptosis and ROS were evaluated as described earlier [27].

Reactive oxygen species was measured with the help of the fluorochrome 2'-7' dichlorodihydrofluorescein diacetate (DCDHF-DA), which is cell permeable and non-fluorescent. It oxidizes inside the cells in presence of reactive oxygen species to produce a fluorescent compound, 2', 7'-dichlorofluorescein (DCF), which remains trapped within the

cell [27]. Isolated neurons (10^6 /ml) were incubated with $50\mu\text{M}$ 2, 7-DCDHF-DA for 30 min at 37°C . Fluorescence was determined in a flowcytometer (Becton Dickinson,UK) at an excitation wavelength of 488nm and measured at an emission wavelength of 530nm. Fluorescence was calculated by means of mean stimulatory index (SI).

Isolated neurons were washed twice with cold PBS and resuspended at a concentration of 1×10^6 cells/100 μl in staining solution containing binding buffer, FITC- labeled Annexin V and Propidium iodide (PI). Fluorescence was determined according to the manufacturers' protocol (Calbiochem, Apoptosis Detection kit). Control, ischemic as well as C.oil treated ischemic cells were stained with PI or Annexin V only or PI plus Annexin-V. After incubation at room temperature for 15 min in the dark, the cells were analyzed by FACScan analyzer at an excitation wavelength of 488nm and emission wavelength of 530 and 670nm. Acquisition and analysis were performed with the Cell Quest software package (Becton Dickinson, UK, Ltd.). When the two fluorochromes were used separately, PI positive cells fell in the 1st quadrant (upper left), Annexin-V positive cells in the 3rd quadrant (lower right). Neurons positive for none of these stains (viable) fell in the 4th quadrant (lower left). When both the fluorochromes were used simultaneously, a few cells (late apoptotic/necrotic) move to 2nd quadrant (upper right) showing affinity for both fluorochromes [27].

The Treatment with Major Components of C.Oil

The major chemical constituent, ar-Turmerone, α - β Turmerone and Curlone, were isolated from C.oil and were used alone as pretreatment in the study. After chemical finger printing the major component ar-Turmerone, α - β Turmerone and Curlone (100mg/Kg) were given alone one hour before MCAo. Ar-Turmerone was further given at 3hrs post ischemia followed by the 24 hrs of reflow. The infarct volume in the brain tissue was then evaluated to study the neuroprotective efficacy of these components in the cerebral ischemia model of rats.

Neurogenesis and Angiogenesis Studies (BrdU Labeling)

BrdU (Sigma, B5002, St. Louis, MO; 50 mg/kg i.p.) was dissolved in 0.9% saline with 1 N NaOH, filtered sterile at $22\mu\text{m}$. A series of studies was conducted first, to determine whether cells in the striatum incorporate BrdU in their proliferation or not. All the group of rats received i.p. injection of BrdU at a dose of 50 mg/ kg at 8-hrs intervals twice daily. After a survival period of 24 h, these rats were anesthetized and perfused transcardially for BrdU immunohistochemistry. The selection of a 24 h survival time was based on the fact that this time allows for the completion of at least one cell cycle by cells in S phase at the time of BrdU injection [32] and provides the window with two folds higher of BrdU labeled nuclei than those seen at a 2 h survival time [33]. Second, to monitor in situ growth of striatal cells that incorporate BrdU and to determine whether these cells survive and differentiate into neurons or glia, a detailed time course evaluation was performed. BrdU immunohistochemistry was performed on brain sections from all the group of rats to define the number and morphological characteristics of newborn cells. Brain cryosections were prepared according to Dohare et al [20]. To detect BrdU-labeled cells in tissue sections, 10- μm sections were treated with 50% formamide, 280 mM NaCl, and 30 mM sodium citrate at 65°C for 2 h,

incubated in 2 M HCl at 37°C for 30 min, and rinsed in 0.1 M boric acid (pH 8.5) at room temperature for 10 min. Sections were then incubated in 1% H₂O₂ in PBS for 15 min, and in blocking solution (0.1% bovine serum albumin in PBS) for 2 h at room temperature to reduce non-specific staining, before being treated overnight at 4°C with primary antibodies. The primary antibodies used were: mouse monoclonal anti-BrdU (B-8434, Sigma, 1:100); rabbit polyclonal anti-Double cortin (DCX) (SC-28939, Santacruz, 1:200); rabbit polyclonal anti-VEGF (SC-152, Santacruz, 1:200); goat polyclonal anti NeuN; SC-31154, Santacruz; 1:200) and rat polyclonal anti-Glial Fibrillary Acidic protein (345860, Sigma, 1:100). These brain sections were then incubated overnight at 4°C with primary antibodies mixtures described above. Sections were washed 5 times for 5min each time with PBS. After washing, sections were incubated in the dark for 4hrs at 4°C with chicken anti mouse FITC conjugated (SC-2989, Santacruz, 1:400); donkey anti rabbit PE conjugated (SC-3745, Santacruz, 1:400) goat anti-rat TR conjugated (SC-2782, Santacruz, 1:400) and donkey anti-goat PE conjugated (SC-3743, Santacruz, 1:400) according to the combinations used. Later the sections were mounted in 90% glycerol with an anti-fading agent p-phenylenediamine (PPD). For negative control, the primary antibodies were omitted. Slides were examined and images were acquired using Laser Graphics camera (Model: Personal LFR Plus), equipped with a confocal laser-scanning unit (Axiovert 200M) attached to a microscope (Zeiss, Jena, Germany). The light source was an argon and He-Ne laser ($\lambda = 488\text{nm}$), and an appropriate filter was used for microscopic detection and acquisition. Images were analyzed and processed using LSM-510 software (Zeiss, Jena, Germany) and Photoshop software (Adobe, San Jose, CA).

Effect of Pretreatment of C.Oil and its Major Constituents

The study was performed with single dosing of varying doses of C.oil (50, 75 and 125 mg/kg body weight). The volume of infarct was significantly reduced with 125mg/kg of C.oil as compared to untreated ischemic control (Figure1 Lower Panel).

Effect of C.Oil on Lesion Volume and Thrombosis

Focal ischemia was evident in all rats of ischemic group. Increased signal intensity on diffusion weighted imaging (DW-MRI) scans with hyper intense areas manifested the ischemic regions. In the ischemic group of rats a significantly hyper intense ischemic hemisphere ($32.82 \pm 1.28\%$) was observed ($2.67 \pm 0.25\%$) ($*P < 0.05$), there was a significant attenuation in the hyper intense area which represents the lesion formation in the C.oil treated groups at 2hrs and was effective up to the 6 hrs of C.oil administration after ischemia (Figure2a). This observation was further confirmed in thrombus formation studies. The thrombus in the untreated group was 2.8mg and in the C.oil treated group it was 0.75mg, showing an increase of 373.33 % in untreated group versus C.oil treated group (Figure2 b).

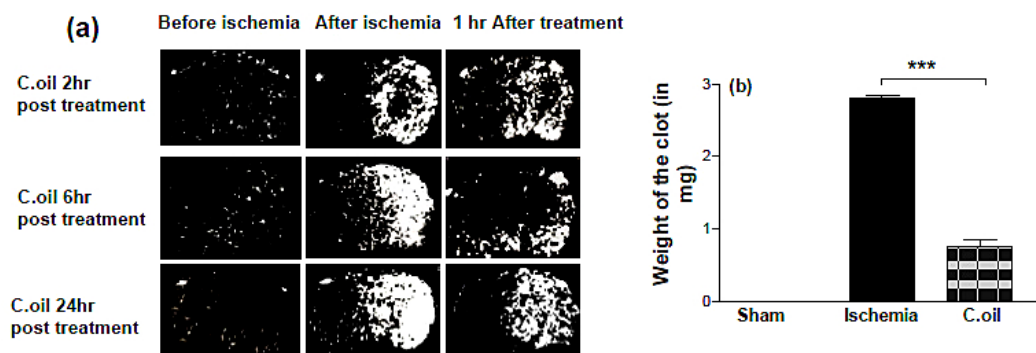


Figure 2. (a). Effect of C.oil post-treatment on lesion area of brain. The DW-MRI scan was taken from same rat, before and after ischemia in the ischemic group, in the C.oil treated group MRI scan was taken from same rat, before and after ischemia and again about 1hr after treatment. Treatment was given at three time points at 2, 6 and 24 hrs after ischemia in different groups of rats. There was a significant attenuation in the lesion area with the treatment given at 6hr. At 24hrs post treatment no reduction in the lesion area was observed compared to pre-treatment scan. The dose of C.oil was 500mg/kg post treatment. (b). Effect of C.oil on thrombosis. The weight of the thrombus in the untreated group was reduced significantly in the treated group. Number of animals used for infarct volume was five in each group. Data are expressed as mean $\pm$ S.E.M. *** $P < 0.001$ was considered highly significant when comparisons were made with the ischemic group by one way ANOVA followed by Newman Keuls post hoc test.

Effect of Pretreatment of Major Constituents of C.Oil

Isolated neurons from brain after hydrogen peroxide induced damage showed an increased level of ROS (Mean fluorescence 266.77 ± 11.7) as compared to control (Mean fluorescence 15.06 ± 3). Treatment by major chemical constituents of C.oil showed significant decrease in ROS content (Mean fluorescence 31.17 ± 1.8) (** $P < 0.001$) (Figure3 a).

Control neurons were (PI negative, Annexin negative) was observed. Both Annexin V positive (apoptotic) cells and Annexin V/PI double positive (delayed apoptotic) cells were found in the hydrogen peroxide treatment group. Significantly large number apoptotic (45.37%) neurons in comparison to other groups were found hydrogen peroxide treatment group. Neurons treated by the major components of C.oil showed significant reversal of cell death and the percent of viable cells (Annexin V negative and PI negative) was increased significantly (80.31%), with the early apoptotic cells population reduced to 7.28% (Figure3 b). *** $P < 0.001$ was considered significant.

However, pretreatment with ar-Turmerone, α - β Turmerone and Curlone in the animal models showed unexpected effects: pretreatment was unable to reduce the infarct volume significantly. To further investigate if there is any neuroprotective activity in the major components or not, the major compound ar-Turmerone was also given 3hrs after ischemia (Figure4 a, b).

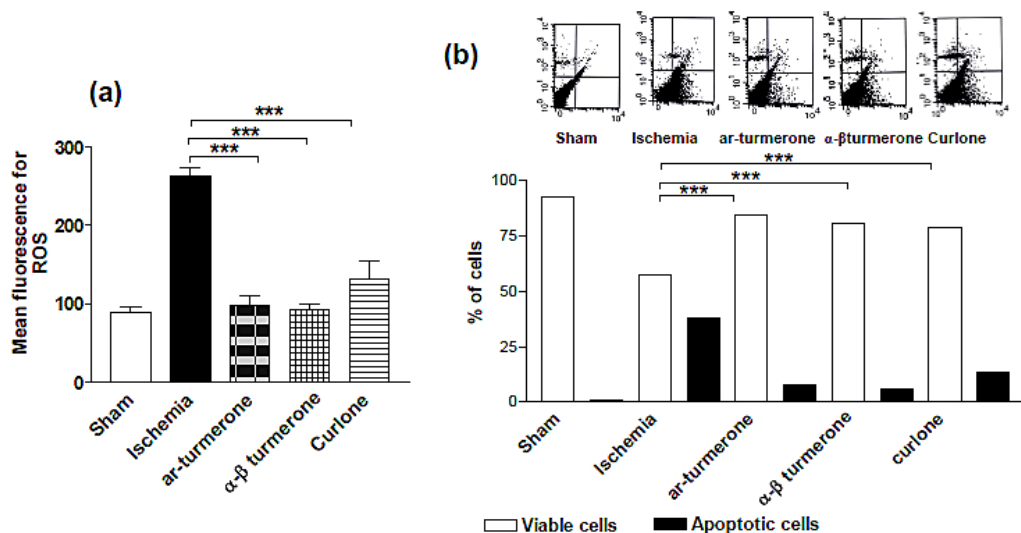


Figure 3. Mean fluorescence of neurons ($10^6/\text{ml}$) from sham, ischemic ar-turmerone, α - β Turmerone and Curlone pretreatment ischemic neurons for quantification of different parameters assessed by acquiring and analyzing in cell quest program of Becton Dickinson Flowcytometer. Data are expressed as mean fluorescence $\pm$ S.E.M of five animals per group. (***) $P < 0.001$ was considered highly significant when comparisons were made with the ischemic group by one way ANOVA followed by Newman Keuls post hoc test. (b) For ROS estimation the mean fluorescence of DHDCE-DA in isolated neurons from sham, ischemic, ar-Turmerone, α - β Turmerone and Curlone pretreatment ischemic neurons was done. (c) Histogram showing the viable and apoptotic population in neurons were obtained from sham, ischemic, ar-Turmerone, α - β Turmerone and Curlone pretreatment ischemic groups of rats. Representative dot plots for quantification of mode of death the mean fluorescence from neuron using FITC- conjugated Annexin V and Propidium Iodide (PI). Neurons ($10^6/\text{ml}$) were obtained from sham, ischemic, ar-Turmerone, α - β Turmerone and Curlone pretreatment ischemic neurons; lower left quadrant shows viable cells (Annexin V negative and PI negative), lower right quadrant shows early apoptotic cells (Annexin V positive and PI negative), Upper right quadrant shows late apoptotic/necrotic cells (Annexin V positive and PI positive) and upper left quadrant shows necrotic cells (Annexin V negative and PI positive). Data are expressed as mean $\pm$ S.E.M. (***) $P < 0.001$ was considered highly significant when comparisons were made with the ischemic group by one way ANOVA followed by Newman Keuls post hoc test.

Studies with C.Oil from Different Geographical Regions

C.oil from the different regions of India was evaluated for their neuroprotective activity. Physiochemical profile of Turmeric samples from different parts of the country showed variability in the percentage of ar-Turmerone, α - β Turmerone and Curlone. All C.oil showed neuroprotection except the one that was obtained from Indore. C.oil obtained from Bhubneshwar and Jaipur showed less neuroprotection in comparison to others. C.oil obtained from the Lucknow, Karnataka and Delhi showed significant attenuation in infarct volume (Table 1). The detailed study was carried out with the C.oil obtained from Lucknow.

Table 1. Effect of C.oil from different geographical regions upon the infarct area
Number of animals used for infarct volume was five in each group. C.oil from the different regions of India was evaluated for its neuroprotective activity. Physiochemical profile of turmeric samples from different parts of the country showed variability in the percentage of Turmerone, ar-Turmerone and Curlone. All of the C.oil showed neuroprotection except the one that is obtained from Indore. C.oil obtained from Bhubneshwar and Jaipur showed less neuroprotection in comparison to others

Code No	% Yield (oil)	Density	% ar-turmerone	curlone	Relative area (%)			Representative TTC stained brain sections
					ar-turmerone	turmerone	curlone	
1. Delhi	1.36	0.94	39.58	4.6	47.58	37.64	3.86	
2. Bhubneshwar	2.82	0.95	36.67	4.4	52.67	39.27	3.90	
3. Kolkata	1.50	0.98	28.24	3.4	39.12	45.96	2.97	
4. Indore	1.73	1.02	19.17	2.7	31.36	56.04	2.71	
5. Mumbai	1.36	1.03	21.98	3.2	27.23	61.89	2.42	
6. Karnataka	2.28	0.99	16.58	2.2	24.88	63.47	2.23	
7. Jaipur	1.63	0.95	25.09	3.1	34.09	51.86	2.62	
8. Guwahati	1.41	1.03	36.36	4.5	48.30	37.18	4.15	
9. Cochin	1.36	Vis mass	17.30	2.8	42.90	44.06	4.39	
10. Ahmedabad	1.54	0.93	40.00	7.4	36.90	47.17	4.01	

C.oil enhances accumulation of newly born cells: expression of doublecortin in brain neurons, promotes migration from proliferating region to the site of damage induces glial cell migration and angiogenesis

BrdU treated rats were made ischemic by occlusion of MCAo for 1hr and were euthanized 24 hours after reperfusion. The number of BrdU-labeled cells in sham control animals was scanty. In sham-operated rats 97% of BrdU⁺ cells were seen in the SVZ. Most of these cells were NeuN (78%) at the SVZ, Low numbers of BrdU⁺ cells were observed in the cortex.

Many BrdU⁺ cells were colabeled with doublecortin (DCX), a marker for migrating neuroblasts. In animals subjected to stroke, few DCX processes were oriented into the cortex (Figure5). In MCAo animals BrdU⁺/DCX⁺ staining was confined to the SVZ, suggesting that in 24 hours after stroke onset the distant injury triggers process extension or migration toward the injury. Most cells that expressed BrdU in the cortex (85%) after 24hrs of treatment with C.oil were also positive for DCX marker for new neuron (Figure5).

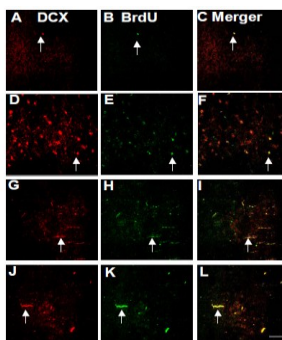


Figure 5. Photomicrographs (representative of 5 photomicrographs) showing double cortin (DCX) immunoreactivity in brain cryosections from sham group (A-C) cerebral tissue subjected to 1hr of transient ischemia followed by 24 hrs of reflow in I/R, and I/R-C.oil treated groups. Sections were immunolabeled with a monoclonal anti-BrdU antibody, which was observed as green fluorescence (B, E, H, and K) and neuroblast marker DCX shown as red fluorescence (A, D, G, and J). The merger is shown in C, F, I, and L. (A, B and C) represent sham; (D, E and F) I/R day 24 hrs; (G, H and I), (J, K and L) represent I/R-C.oil group at 24 hrs (I/R + C.oil 24hrs) rat sections. Magnification: 40X (Bar 20µm).

After 24hrs of Ischemia a robust increase in the number of newborn BrdU⁺ cells were observed in the SVZ, the corpus callosum and in the peri-infarct area. About 70% of these BrdU⁺ cells were colabeled with NeuN marker. Fewer BrdU⁺ cells were observed in the cortical sub region in ischemic animals at 24 hours post reperfusion (Figure6). BrdU⁺ cells were more numerous in C.oil treated animals than ischemic controls 24hrs postischemia. The number of BrdU⁺ cells in the ipsilateral SVZ remained suppressed. This suggests that labeled cells were depleted from the injury-proximal SVZ by proliferative dilution of the BrdU⁺ label or migration away from the SVZ. This increment was larger in C.oil treated rats (Figure6).

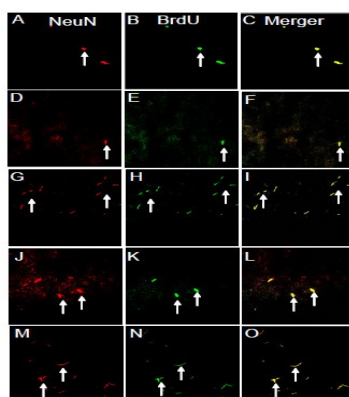


Figure 6. Photomicrographs (representative of 5 photomicrographs) showing neurogenesis. BrdU immunoreactivity in brain cryosections from sham group (A-C) cerebral tissue subjected to 1hr of transient ischemia followed by 24hrs of reflow in I/R, and I/R-C.oil treated groups. (Five animals per group were used). Sections were immunolabeled with a polyclonal anti-BrdU antibody, which was observed as green fluorescence (B, E, H, K and N) and neuron specific marker NeuN shown as red fluorescence (A, D, G, J and M). The merger is shown in C, F, I, L and O. (A, B and C) represents sham; (D, E and F) as ischemia reflow at 24 h (I/R-24h); (G, H and I), (J, K and L) represents I/R-C.oil group at 24 h (C.oil 24h), rat sections. Double-labeled newly generated migrating neuron: arrow. Magnification: 40X (Bar 20µm).

With C.oil treatment, the proliferative response observed in the peri-infarct cortex was increased (Figure7). These results show that cell proliferation occurs in the injured cortex and suggest that most newborn cells with a defined phenotype in the cortex originate in the SVZ. BrdU⁺ neurons in the CA1 region were labeled with NeuN, demonstrating that C.oil promotes generation of new neurons. By 24 hrs after the insult, some BrdU⁺ cells migrate to the damaged CA1 region of the hippocampus and express mature neuronal markers, including NeuN (Figure7).

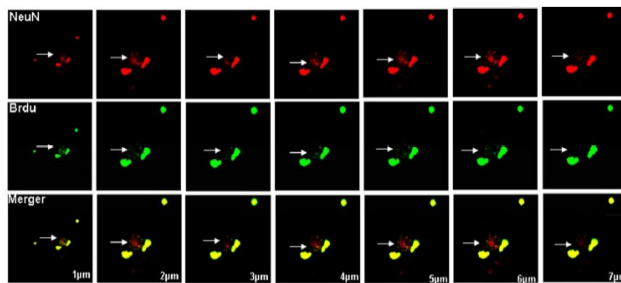


Figure 7. Effect of C.oil treatment on neurogenesis at 24 hrs. BrdU labeling (in brain cryosections) of C.oil treated I/R-24 hrs. (Five animals per group were used). 7 consecutive 1- μ m confocal images in z-dimension showing NeuN, BrdU immunoreactivity separately or as merged image. Arrows indicate double-labeled cells. BrdU labeling (in brain cryosections) of C.oil treated I/R-24hrs. Sections were immunolabeled with a monoclonal anti-BrdU antibody, which was observed as green fluorescence middle panel and neuron specific marker NeuN shown as red fluorescence upper panel. The merger is shown in lower panel. Double-labeled newly generated neuron: arrows. Magnification: 40X (Bar 20 μ m).

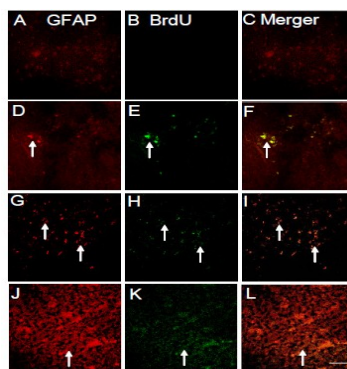


Figure 8. Photomicrographs (representative of 5 photomicrographs) showing GFAP immunoreactivity in brain cryosections from sham group (A-C) cerebral tissue subjected to 1hr of transient ischemia followed by 24hrs of reflow in I/R, and I/R-C.oil treated groups. (Five animals per group were used). Sections were immunolabeled with a monoclonal anti-BrdU antibody, which was observed as green fluorescence (B, E, H, K and N) and Glial cell marker GFAP shown as red fluorescence (A, D, G, J and M). The merger is shown in C, F, I, L and O. (A, B and C) represents sham; (D, E and F) as ischemia/reflow at 24 hrs (I/R-24hrs); (G, H and I), (J, K and L) represents I/R-C.oil group at 24 hrs (C.oil 24hrs), rat sections. Double-labeled newly generated GFAP⁺ cell: arrows. Magnification: 40X (Bar 20 μ m). VEGF expression was analyzed because this secreted protein promotes vascular development and may also act as a neural survival signal. The majority of BrdU⁺ cells co-expressed VEGF (Figure9). The number of BrdU⁺ VEGF⁺ cells increased in the ischemic and in the C.oil group increase was almost 2-fold (Figure 9).

Low-GFAP regions were also examined; no significant differences were observed between groups in the contralateral hemispheres, but the density of BrdU⁺ cells was increased in the ischemic hemisphere in C.oil treated and non-treated MCAo animals at 24hrs (Figure8). BrdU-labeled cells with high GFAP was observed in the regions of striatum and cortex. Smaller increases in BrdU⁺/GFAP⁺ cells were seen in striatum region 24hrs after stroke. With C.oil treatment after 24 hrs of reflow the BrdU⁺/NeuN⁺ cells and GFAP cells were not out numbered than MCAo untreated rats.

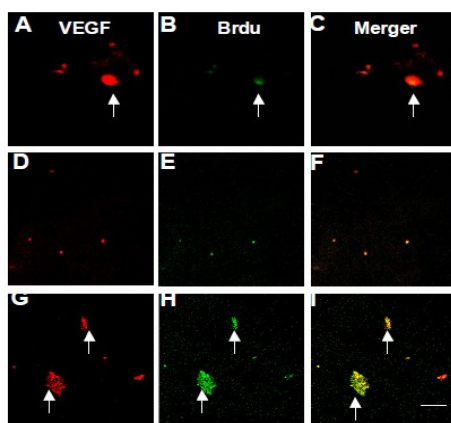


Figure 9. Photomicrographs (representative of 5 photomicrographs) showing VEGF immunoreactivity in brain cryosections from sham group (A-C) cerebral tissue subjected to 1hr of transient ischemia followed by 24hrs of reflow in I/R, and I/R-C.oil treated groups. (Five animals per group were used). Sections were immunolabeled with a monoclonal anti-BrdU antibody, which was observed as green fluorescence (B, E, H, K and N) and VEGF shown as red fluorescence (A, D, G, J and M). The merger is shown in C, F, I, L and O. (A, B and C) represents sham; (D, E and F) as ischemia reflow at 24 hrs (I/R-24hrs); (G, H and I), (J, K and L) represents I/R-C.oil group at 24 hrs (C.oil 24hrs), rat sections. Double-labeled cells: newly generated VEGF⁺ cells with healthy nucleus: arrows. Magnification: 40X (Bar 20μm).

These results are in good agreement with earlier studies where it has been shown that curcuma oil is a potent neuroprotective agent.

Discussion

In pathological conditions as in cerebral ischemia, the mitochondrial dysfunction and consequent production of reactive oxygen species (ROS) and nitric oxide due to up-regulation of inducible nitric oxide synthase, a cascade of events is initiated leading to death. Apoptosis is an important mode of cell death occurring in the penumbral region of infarct and it is the region protected by modulation of apoptosis. The majority of neurodegenerative diseases are associated with increased levels of neuronal oxidative damage and neuron death. As a result of increases in reactive oxygen species (ROS) production oxidative damage occurs to cellular macromolecules, and which promotes neuronal death. In our previous studies C.oil showed potent anti apoptotic activity in focal cerebral ischemia in rats [27]. There was reduction in the MDA levels and the activities of CAT, GSH-Px and SOD, were markedly reversed and restored to near normal levels in the groups pre-treated with C.oil. The antioxidants basically

can ameliorate these negative consequences of ROS production, thereby helping intracellular antioxidant enzymes that detoxify ROS. All of the therapeutic activities of C.oil may be attributed to its potency as active oxygen species scavengers and lipid peroxidase (a class of oxidoreductase enzymes) inhibitors. The protection offered by C.oil is evidenced by the reduction in infarct size and neurological deficit. The significant reduction in tissue levels of NO, ROS, ONOO⁻, mitochondrial membrane potential and its anti-oxidant effect greatly attenuated the delayed neuronal death and further salvage the penumbra [27]. C.oil appears to exert its neuroprotective effects by acting at multiple targets in the signaling pathways that are activated in ischemic and neurodegenerative brain diseases. The mechanisms underlying the neuroprotection against experimental cerebral ischemia by Curcuma oil has been studied previously [20]. C.oil attenuated ischemia induced elevation in [Ca²⁺] which sets into motion a cascade of ischemic injury. C.oil reduced post ischemic brain neutrophil infiltration in the ischemic area, controlled tissue NOx levels and the neuronal levels of nitric oxide, peroxynitrite and reactive oxygen species. C.oil treatment showed that the expression of nitric oxide synthase (NOS) isoforms was decreased significantly compared to the untreated ischemia group. DNA fragmentation was significantly decreased by C.oil treatment. C.oil suppressed the elevated protein level of Bax, and aided mitochondrial translocation and activation of Bcl-2 by altered mitochondrial membrane potential. It also inhibits the cytosolic release of apoptogenic molecules like cytochrome *c*, inhibits the activation of caspase-3 and the expression of p53, ultimately inhibiting apoptosis. These observations suggest that high levels of NO generated by NOS isoforms are at least partially responsible for exacerbating the neuronal damage caused by MCAo by intraluminal filament model of stroke [20].

One of our previous studies also showed the neuroprotection from C.oil in an embolic model of focal cerebral ischemia when administered after 4 h of initial ischemic insult. C.oil, when given 4 h after the ischemia suppresses post ischemic reactions which lead to an infarct after the transient ischemia, but when that C.oil given in the immediate post ischemic recovery period, blocks additional reactions of a potentially detrimental effect [34]. Based on the previous studies we further planned to study the therapeutic potential of C.oil and its major constituents on focal model of ischemia model for the study of stroke in rats.

The C.oil contains mainly ketones that are quite like turmerones. Turmerone are sesquiterpenoid cyclic ketones. Sesquiterpenoids are major constituents of turmeric oil and ar-Turmerone recognized is the major turmerone, identified by a GC-MS analysis [10]. The detailed report of the Chemistry of C.oil, used in the present study, its major constituents of C.oil; ar-Turmerone, α - β Turmerone and Curlone have been recently reported [6]. The protective effect of C.oil against the hydrogen peroxide-induced decrease in cell viability may at least partly result from its antioxidant and free radical scavenging properties [27]. When we exposed isolated neurons to hydrogen peroxide, pretreatment with ar-Turmerone, α , β -Turmerone and Curlone showed anti-oxidant, anti-apoptotic activity and hence offered a significant protection against H₂O₂-induced damage. It is possible that the protective effect of these compounds against the H₂O₂-induced damage in cell viability may at least partly result from its antioxidant and free radical scavenging properties.

In the vivo studies in the MCAo model in rat, we obtained contradictory results to what we expected; none of these major components of C.oil attenuated the infarct volume given before or after ischemia. Various reports have appeared, on the suppression by ar-Turmerone of growth and the induction of apoptosis in human leukemia cell lines [5,11]. Recently Lee et

al [12] have reported ar-Turmerone to be significantly more potent platelet inhibitor than aspirin against platelet aggregation induced by collagen.

None of the major compounds isolated from C.oil could offer protection in MCAo model of stroke. The purified compounds of C.oil are significantly effective in vitro as they can diffuse freely into the neurons but fails to offer protection in-vivo. It is not possible that they can not cross the blood brain barrier as all of the Terpenes families of molecules are small enough to penetrate the blood brain barrier. Sesquiterpene molecules have the capability of carrying oxygen, like hemoglobin does in the blood. Hence they are oxygenating to human tissue and are especially effective in fighting cancer cells which cannot tolerate oxygen.

There are some reports which shows that the terpenoid compounds seem to be significantly more active against a background of other terpenoids [35]. It is entirely possible that some underlying synergistic activity between the terpenoids might be at work, whether that is at the cellular levels or during the passage across the various compartments involved, we are unable to specify at present.

The results of the present study demonstrate that administration of different doses of C.oil before MCA occlusion in rats reduces the volume of ischemic brain damage that develops subsequently. The data therefore substantiate and extend the work that C.oil when given for four consecutive days protects against neuronal damage after MCAo has a preventive effect. The therapeutic time window of C.oil was 6hrs post ischemia.

Most of the stroke models are carried on rodents. Each model has its particular strengths and weaknesses. Mimicking all aspects of human stroke in one animal model is not possible since ischemic stroke is itself a very heterogeneous disorder. Magnetic resonance diffusion weighted (DWI) is very sensitive to early ischemic changes. DWI is able to detect ischemic lesions in the hyper acute phase of ischemia even within minutes of injury [36,37]. In animal stroke models, DWI signal abnormalities correlate highly with the location and volume of pathologically confirmed cerebral infarction [38]. The brighter the DWI lesion, the greater is the neuronal damage [39]. In the present experimental study on rats, the magnetic resonance imaging (in vivo) of rat brain showed significant attenuation in lesion volume in rats treated with C.oil after MCAo as compared with the significant brain lesions present in its own ischemic condition. Staining of the brain sections with TTC at different time points and quantifying the area of infarction further substantiated the MRI observations.

The curcuma oil from rhizomes is reported to be composed of 47 constituents, of which 24 compounds have been identified. The curcuma oil from *Curcuma domestica* contained 31 constituents on hydro distillation. The n-hexane, toluene extract and chloroform extract each contained 17 constituents. These results confirm that the type of extracting solvent affects both the chemical constituents extracted and their concentration. We have done GC-MS from twelve sample collected from different parts of India. A large variation in the major and minor components of rhizome oil from different geographical regions within India exists. This may be due to geographic divergence and ecological conditions. Recently Jain et al [6] have reported the detailed study of lipid soluble extract of *curcuma longa* collected from Lucknow, its major and minor constituents, pH dependence stability and photosensitivity. The activity of C.oil was more from Lucknow, Karnataka and Delhi and their efficacy can not be evaluated on the bases of their constituents of ar-turmerone. The C.oil contains mainly ketones that are quite like turmerones. Turmerone are sesquiterpenoid cyclic ketones. Sesquiterpenoids are major constituents of turmeric oil and ar-Turmerone recognized is the

major turmerone, identified by a GC–MS analysis [10]. We have also reported recently [6,21] that the ar-Turmerone and α - β Turmerone are the major turmerones in curcuma oil.

When C.oil was given after the onset of ischemia, the results from the neurological deficit score and the areas of the lesions were encouraging. The post treatment time of C.oil administration was gradually extended and evaluation was made at 2, 6 and 24hrs after ischemia. Up to 6hrs after onset of ischemia, a significantly reduced neurological deficit score and a reduced hyper-intense area were observed. This long therapeutic window displayed by C.oil with protection when treatment is initiated at 6 hrs after onset of ischemia, is encouraging. This study was further supported by the inhibitory effect of C.oil in thrombus formation which shows the absence of the clot formation in the C.oil treated group.

Proliferation after ischemia is advantageous only if the new cells ultimately repopulate brain regions depleted of cells as a consequence of stroke. The adult vertebrate brain retains the capacity for neurogenesis, which resides largely in selected regions: the sub ventricular zone (SVZ), especially that portion adjacent to the most rostral parts of the lateral ventricles [40,41], and the sub granular zone (SGZ) of the hippocampal dentate gyrus (DG) [42]. Some reports suggests that additional regions, such as the cerebral neocortex, may also generate new neurons in the adult, but this is disputed [43]. To study cells born after 24hrs following MCAo twice a day (50mg/kg) injections of BrdU was given post stroke. The data presented here suggest that: newborn cells are first found in the SVZ, subsequently migrate through white matter, and accumulate in the cortex and sub cortical white matter areas surrounding the infarct forming a regenerative zone with many primitive neural precursor cells immediately adjacent to the injured brain tissue.

There are reports of an endogenous neurogenic response restricted to the striatum after a large focal ischemia in the forebrain [16,19]. A slow endogenous repair process in the hippocampus after global ischemia is very well documented [44]. Cellular regeneration in the striatum and hippocampus might be a consequence of their known proximity to proliferative zones that are known to generate neurons in the adult. However, the presence of cortical regeneration is still debatable [45]. Although cortical neurogenesis is controversial, much localized lesions of neurons in this area have been shown to generate cell replacement with newly generated neurons surviving for long periods and extending axons to appropriate distant target sites. [18] Occluding the MCA at the pial surface results in ischemic injury restricted to the cerebral cortex, rapid activation of proliferation in the SVZ and migration of newborn cells into the parenchyma [17,46]. Newborn cells were recently shown to continuously form after ischemic injury involving the striatum [19]. The present study BrdU⁺/DCX⁺ double-positive cells were found to be formed. The SVZ was found to be of increased size over time and DCX⁺ cells were observed. Our study is limited because we did not perform a long time evaluation. Future experiments are required with GFP labeling and tracking these cells at the cortex from the SVZ or whether at least some emerge from other locations including the cortex, where they may reside in a quiescent form. The two recent articles that failed to detect newborn neurons at the immediate infarct border [17,47]. A contradicting observation was made that newborn progenitors were constantly present in the area close to the lesion. It is possible that factors such as inflammation, metabolic stress or local inhibitory components within the white matter surrounding the infarct prevent terminal differentiation of newborn cells into neurons. Importantly, newborn cells expressing neuronal antigens could be seen far more often in the group treated with C.oil.

Does the mere increase in the numbers of newborn cells is associated with better functional outcomes after cortical ischemia? The better neurological score, improved performance in rota rod studies support this notion. MCAo caused significant impairment in motor performance, which was evident from the increased neurological deficit score and the poor performance on the rota rod [27]. This could be because of the neuronal damage in the territory supplied by the middle cerebral artery, i.e. the caudate nucleus, the putamen, and the striatum, areas that regulate motor coordination. C.oil ameliorated the neurological deficits and poor motor coordination. Furthermore, VEGF-induced angiogenesis in the peri-infarct area, free radicals, may also contribute to improved metabolism and function of surviving neurons.

Importantly, C.oil treatment resulted in increased numbers of progenitors in the cortex after ischemia. This implies that factors related to the presence of ischemic injury are necessary to further increase neurogenesis and induce migration of newborn cells to the cortex.

Some of the recent reports show that the inducible form of nitric oxide synthase (iNOS) is linked to neurogenesis after ischemic injury [48]. MCAo in adult rats induces iNOS at high levels in the ipsilateral DG. Inhibition of iNOS activity without altering its expression attenuates the level of BrdU incorporation after ischemia. The same is true in iNOS knockout mice; however, infarct volume is also significantly decreased in these animals. C.oil in MCAo could attenuate the iNOS expression [20,34]. This makes it difficult to dissociate the decrease in cell proliferation observed with a lack of iNOS activity from a lesser extent of injury. Nevertheless, these data provide yet another potential mechanism that may be responsible for the cell proliferation observed after ischemic damage.

Taken together, our results suggest that in ischemic lesions restricted to the cortex, probably long-term stimulation with C.oil can stimulate regenerative processes that induce partial behavioral recovery. In the present study we observed BrdU⁺ neurons in the CA1 region were labeled with NeuN, demonstrating that C.oil promotes generation of new neurons.

These preliminary data encourage studies to determine the regenerative consequences of these long-term cellular responses to further define appropriate ligands that can promote recovery. The number of BrdU⁺ cells in high-GFAP regions of cortex and striatum of C.oil treated animals increased significantly above ischemic and sham animals, and this increase was maintained for 24hrs (Figure8). Recovery of DCX cells supported the conclusion that these cells were generated in the SVZ and migrated toward the area of injury in the cortical subregion umbra. Thus, many of the newborn cells observed in the infarct penumbra may have originated in the SVZ.

In the present study, slight increases in DCX⁺, and GFAP⁺/BrdU⁺ regions in C.oil treated animals was observed. Many cells expressed NeuN. Immature DCX⁺ cells may eventually mature into NeuN⁺ neurons, but the severely challenged penumbra may not provide adequate clues to allow more complete differentiation. Although some degree of spontaneous recovery after stroke is normal, the molecular and cellular mechanisms underlying recovery are poorly understood and existing treatment for stroke is generally applicable to only a small percentage of cases, or is limited in efficacy. Endogenous mechanisms of neuroprotection from ischemia, including mechanisms of neurogenesis, may hold clues for the development of improved therapy.

To conclude, the present data confirmed that C.oil had a dose-dependent neuroprotective effect in ischemic rats. It also manifests a therapeutic time window of at least 6 hrs. C.oil (125mg/Kg) administration on five consecutive days of single injection/day significantly reduced ischemic damage. The study has generated novel, valid scientific information about a plant-based compound (C.oil), its ingredients, geographical variation and finally confirmation of its activity as a neuroprotectant. Studies are continuing in our laboratory and C.oil is in an advanced stage of drug development.

The literature based on the beneficial effects of plant based compounds in various diseases shows effectively that lots of work has been done trying to find compounds with low side effects and enhanced pharmacological activities. The current increasing focus upon phytotherapies seems to be a potential way, to incorporate different natural products to be investigated, which have been commonly used in folk medicine and have efficacy against many diseases. We should realize and feel the importance of the use of herbal preparations used in India as the traditional medicines. It's already known that plants have a large number of chemical substances, which have several pharmacological actions; there should be more exploitation of natural products, which in the future could show the cure for many diseases. Finally, we hope that studies based upon phytotherapies in drug discovery will open a wide range of research upon natural products.

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Chapter XIV

Beneficial Effects of Curcumin and DHA on Human Health: Relevance to Chronic Diseases of Aging

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Abstract

Background: Nutritional and dietary factors not traditionally considered essential are now realized to play an increasingly important role in protecting against age-related diseases. Here we review the rationale for combining curcumin and the omega-3 fatty acid (n-3) docosahexaenoic acid (DHA) and overcoming barriers to translation.

Material and Methods: We focus on two nutritional factors likely to impact health where there is emerging evidence to suggest a synergy. The first is curcumin a molecule from the turmeric root, which has been used in Ayurvedic medicine for thousands of years. High intake of the second, the omega 3 fatty acids (n-3) are associated with reduced cardiovascular (CVD) and Alzheimer disease (AD) risk, and trials so far suggest a beneficial effect for AD, in those with lower genetic risk and for AD prevention.

Results: Heterogenous Indian populations typically ingest from 60-200 mg of curcumin daily and show a lower incidence and prevalence of AD and of some cancers but increased risk for type II diabetes and cardiovascular disease. At higher doses, preclinical model studies have shown curcumin protection from multiple cancers, particularly colon, as well as many neurological diseases (AD, stroke, brain trauma, multiple sclerosis and Huntingtin and Parkinson diseases).

Conclusion: Although many clinical trials with DHA have succeeded at least with patients lacking the ApoE4 allele, trials with curcumin have not been successful, despite

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stronger rationale at the basic research level. To improve success in future trials, barriers to curcumin absorption and stability in humans must be resolved. Data from our AD models argue that curcumin and n-3 may synergize to protect from synapse loss. In AD pathogenesis, curcumin exerts pleiotropic effects including antioxidant, anti-amyloid and anti-inflammatory effects, but the precise anti-inflammatory mechanism of action remains elusive.

Abbreviations

AD	Alzheimer's Disease
A β	β -amyloid peptide
AMPK	5' adenosine monophosphate-activated protein kinase
APP	Amyloid precursor protein
ApoE	Apolipoprotein E
BDNF	brain derived neurotrophic factor
CNS	central nervous system
CREB	cyclic adenosine monophosphate response element-binding protein
CRM	caloric restriction mimic
CVD	cardiovascular disease
DHA	docosahexaenoic acid
HAT	p300 histone acetyl transferase
JNK	c-jun N terminal kinase
MCAO	middle-cerebral artery occlusion
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
n-3	omega-3 fatty acid
n-6	omega-6 fatty acid
NF κ B	nuclear factor κ -light-chain-enhancer of activated B cells, transcription factor
NSAID	non-steroidal anti-inflammatory disease
PD	Parkinson's disease
SCI	spinal cord injury
TBI	traumatic brain injury

1. Introduction

Anti-inflammatory effects of the turmeric root have been proposed for nearly 5000 years, and nearly 4000 scientific articles have been published on the potential health beneficial effects on turmeric or the mechanisms of action of an active component of turmeric, curcumin, consisting of 5%-10%. However, because of poor absorption and extensive metabolism, particularly in humans, it has been difficult to extend and test benefits. Unlike diseases initiated by foreign pathogens that provide specific drug targets, most chronic diseases of aging involve dysregulation of vital biochemical pathways in which all of the players have some useful normal function, making anti-aging drug development problematic. Dysregulation of inflammatory pathways and a slow accumulation of free radical are common

common mechanisms to age-related diseases. Curcumin is a polyphenolic antioxidant with anti-inflammatory activity and a number of other properties that make it a viable candidate intervention. Properties include an outstanding safety profile with chronic use and a number of pleiotropic actions with potential for mitigating chronic age-related disease. Protective activities include promotion of neurogenesis, reducing protein misfolding and protein-aggregates, promotion of wound healing, potentiation of the heat shock response, anti-carcinogen and chemotherapeutic activities, modulation of inflammation and phagocytosis, induction of Phase II and Nrf-2 regulated defense enzymes that protect against oxidative damage and direct antioxidant activities. Many of these effects can be achieved at low micromolar or even nanomolar levels. There are a plethora of other reported curcumin effects at higher drug levels with dose-response curves that question whether or not they can be achieved *in vivo*. Nevertheless, pre-clinical *in vivo* data show curcumin's efficacy in animal models for many major diseases of aging including atherosclerosis, heart disease, diabetes, arthritis, stroke, Alzheimer's, Parkinson's and many types of cancers and other deadly and debilitating conditions. Higher levels of curcumin also have cancer chemotherapeutic utility. Despite its vast potential, two major problems have limited the translation of curcumin's preclinical success to the clinic. First, the lack of patent protection has prevented the investment required for definitive clinical trials. Second, rapid metabolism in the intestinal mucosa accompanied by high first pass metabolism contributes to poor bioavailability, particularly in humans, leading to the suggestion that its use with oral dosing may be limited to gastrointestinal disorders. Because of these concerns, we have begun to define the levels of curcumin required to reach effective blood and tissue levels. In addition, we and others are working to develop more bioavailable formulations of curcumin. The lack of critical patent protection will receive some limited help from minor patents protecting formulations, methods or manufacturing, but clinical development of curcumin will require support from foundations and governments around the world including the NIH's Aging, Cancer and Complementary and Alternative Medicine Institutes in the US.

2. Curcumin Structure, Bioavailability and Metabolism

Curcumin is a bipolyphenolic antioxidant (Figure 1). The dienone bridge renders it vulnerable to hydrolysis in aqueous solutions within minutes [1], particularly at neutral or high pH's such as in blood, but in lipid environments like the brain, curcumin is relatively stable. Curcumin can be sulfated or glucuronidated by the intestine or at first pass metabolism, where it will be rapidly cleared through the kidneys. It is possible that like estradiol, sulfotransferases sulfate curcumin in the brain, but this has not been examined. Glucuronidated curcumin cannot pass the blood brain barrier, so glucuronides are probably not found in detectable levels in the brain. In blood curcumin is less vulnerable to hydrolysis, for example because of proteins like albumin, but curcumin is vulnerable to rapid reduction via oxidoreductases such as NADPH reductase or alcohol dehydrogenase in the liver [2]. We have found that this also occurs in ex vivo human blood where curcumin is rapidly reduced to tetrahydrocurcumin and then hexahydrocurcumin in less than 4 hours (unpublished observations).

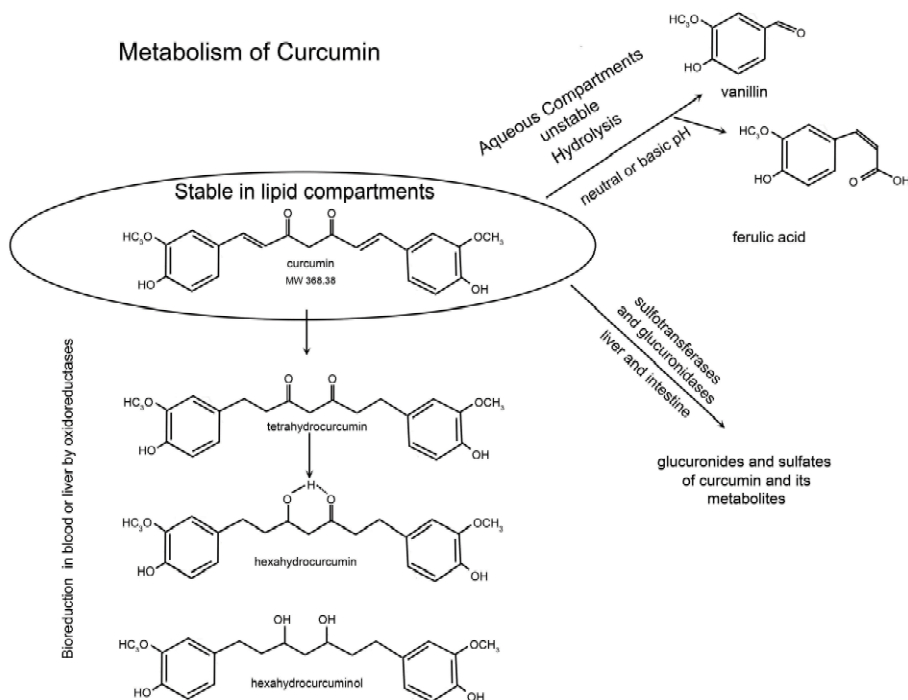


Figure 1.

Despite these barriers of rapid metabolism and poor absorption and rapid glucuronidation, curcumin is fairly well-absorbed in rodents, particularly with chronic feeding [3]. In contrast, in humans absorption appears much more difficult in part because of more extensive glucuronidation in the intestine [2]. Other reasons for poor absorption in humans are not understood [4]. Although we need more precise and accurate methods for measurement of curcumin and its metabolites and maintaining stability in stored samples, there have been recent improvements using LC/MS/MS [3, 5, 6], HPLC [7, 8] or UPLC [9].

One major problem with measurement of hydrophobic compounds in plasma, including curcumin, is that curcumin can also reside in the buffy coat or red blood cell fraction at fairly high levels (unpublished observations). Also reduction in blood left for 4 hours or more will lead to ex vivo enzymatic reduction, masking good curcumin absorption, which can be attenuated by lowering the pH in the blood collection tube or adding vitamin E, which both inhibit reductases (unpublished observations). There has been some recent progress in improving curcumin absorption through either using the glucuronidation inhibitor piperine [10], phosphatidylcholine micelles [11-13], or solid lipid nanoparticles [3, 14]. Nevertheless rigorous comparative clinical trials should be done to determine optimal formulations in humans. Some of the barriers still limiting development of better human formulations of curcumin (and other hydrophobic phytochemicals) are listed here:

1. Large doses (typically around 500 mg/kg body weight) have been used to prove formulation absorption in animals, but this is not necessarily clinically relevant, since animals absorb curcumin relatively well.

2. In determining absorption, typically only serum or plasma tissue compartments are measured, so that absorption into lipid tissues or other blood compartments could be missed.
3. All the reasons for poor absorption in humans are not understood, so confirmation in humans needs to be expedited
4. Small scale formulations based on strong rationale at basic research level, may fail to produce absorption with large-scale production, due to quality control issues with nutraceutical companies who may not have the resources to regularly check absorption of each lot made, although Good Manufacturing Practice Facilities must provide information on content of lots.
5. Big pharmaceutical companies consider that developing curcumin derivatives may be more profitable and patentable than improving absorption of a natural compound.

In summary, poor bioavailability of curcumin in humans has limited its widespread use in trials. The rodent absorbs curcumin fairly well, making modeling to predict human absorption problematic. In addition curcumin is fairly stable and may accumulate in lipid compartments, making plasma levels, a suboptimal predictor of absorption.

3. Perspective on Mechanisms Relevant to Aging

At the systemic physiologic level, aging can be defined as increased mortality arising from a reduced ability to recover from a variety of “stresses” including infectious diseases, traumatic injury and chronic age-related pathologies like heart disease, diabetes, stroke, certain cancers and AD. For example, normal aging is characterized by delayed or even failed recovery from macroscopic damage, e.g., wounds, fractures, pressure ulcers and other traumatic injuries that would lead to a more favorable outcome in younger individuals. From our perspective, many diseases of aging represent an extension to the microscopic cellular domain of this failure to heal or recover from insults that young organisms resist. The aging organism suffers from microscopic lesions whether they are endothelial injuries developing into atherosclerotic plaques or CNS injuries developing into amyloid plaques or neoplastic dysplasias or other micro-foci of emerging chronic disease. These represent small wounds or lesions and are often accompanied by a chronic low grade but unresolved inflammatory response. In atherosclerosis, AD and many cancers, these initial injuries are sustained by a failure to fully sequester or clear the irritant, which contributes to scarring and a very slow recovery or failure to heal. In this view, an acceleration of the resolution of wounds would represent a fundamental anti-aging action.

Similarly, at the molecular level aging can be seen as dysregulation of the cellular stress response that defends cells from protein denaturation with the heat shock response and from lipid peroxidation and other free radical damage with the Phase II response through Keap1, Nrf2 and antioxidant response element mediated upregulation of defense enzymes. Finally, the replacement of damaged cells from stem cell lineages is required to replace critically vulnerable post-mitotic tissues such as cardiovascular muscle and neurons. Since caloric restriction is the best studied treatment that embodies many of these anti-aging effects one can

use microarray analysis to compare the overall impact of caloric restriction on gene expression in critical tissues like heart and brain to try to help predict the impact of a treatment on aging as a potential “caloric restriction mimetic” or CRM [15, 16]. CRMs tend to reduce age-related inflammatory gene expression, promote the cellular stress response, induce the protective Phase II enzymes and control insulin signaling. The most widely-discussed CRM is the red wine polyphenol, resveratrol which this type of activity in aging mice, notably in heart [17]. These CRM effects have been hypothesized to be from activation of the sirtuin pathway, which impacts cellular defenses and lifespan in invertebrates. Based on this, resveratrol was tested in mammals and shown to extend the lifespan of obese mice fed a high fat diet [18] but this efficacy did not extend to lean mice [19]. Results from invertebrates argue that dysregulation of insulin signaling and Foxo regulated cellular stress response defenses play a major role in regulating the aging process. The metabolite of curcumin, tetrahydrocurcumin fed to middle aged male mice appears to extend lifespan [20].

4. Properties of Curcumin Relevant to Aging

Curcumin is the yellow pigment in extracts of the curry spice turmeric that is frequently used as a food preservative and coloring agent. It has medicinal properties that appear largely derived from its roles as a polyphenolic antioxidant and an anti-inflammatory agent. Curcumin’s history and many activities and medicinal potential in a wide range of preclinical disease models and small clinical trials have been the subject of several comprehensive recent reviews [21-23]. Structurally, curcumin consists of two methoxyphenol groups linked by a beta diketone bridge that confers both metal chelation and Michael acceptor activities that may participate in many of its reported actions. At the physiological level, curcumin has a long history traditional use in Indian and Chinese medicine for promotion of wound healing because it prevents scarring and accelerates recovery. As discussed above delayed wound healing epitomizes the decline in physiological repair activity during aging that leads to slow or failed recovery from common geriatric problems whether healing after falls and pressure ulcers in the skin of nursing home patients or tissues with unresolved lesions such as disease foci in various organs. The anti-oxidant, anti-inflammatory and anti-protein aggregate properties of curcumin are discussed throughout this review, but curcumin also induces the cellular defense response [24] and Nrf2 [25, 26] and Foxo [27] mediated defense pathways implicated in aging and longevity. Further, targeting the mammalian Target of Rapamycin pathway has been recently shown to increase longevity in mice [28] and curcumin also inhibits mTOR signaling [29, 30].

5. Curcumin and Diseases of Aging

Based on some of the pleiotropic anti-aging properties of curcumin, its more bioavailable metabolite, tetrahydrocurcumin, was in trials for increasing longevity in male C57 black mice beginning at 13 months of age where it appeared to modestly, but significantly prolong the survival curve [20]. Because curcumin has multiple potential anti-aging activities not shared with its metabolite tetrahydrocurcumin [31] including superior anti-carcinogen activity,

curcumin was proposed by Dr. B. Teter in our UCLA lab for chronic longevity experiments and it is now in NIA sponsored Intervention Testing Program lifespan trials in mice (R. Strong, UTHS, San Antonio). Whether or not curcumin alone is sufficient to increase the maximum lifespan of mice, it has efficacy in a large number of animal models of age-related diseases.

5.A. Cancer

Curcumin has had a long history of study in relationship to cancer with now over 1200 PubMed references. Based on a large number of successful early studies in multiple models of carcinogenesis, curcumin was placed on the National Cancer Institute's short list of promising chemopreventive agents and taken through the National Toxicology Program where its safety record was validated [32]. Curcumin has shown efficacy in animal models of major age-related cancers including prostate, breast and colon cancer, but also glioma and many other types of cancer. Because this topic has been extensively and recently reviewed elsewhere [21, 33], we will not attempt to cover the many detailed and mechanistic studies here. In general, dietary curcumin at dosing of 1,000 to 5,000 ppm, most often 2,000 ppm, significantly reduces tumor incidence, reducing mutagen induced cancer and promoting preferential apoptosis of tumor cells and reduced vascularization of tumors, through a plethora of anti-carcinogenic and anti-tumor activities. However, clinical trials have been limited and small and suffered from major bioavailability issues [2, 34] discussed elsewhere in this review.

5.B. Alzheimer's Disease (AD)

AD is the most prevalent dementia of aging and predicted to become epidemic as the world's population ages. AD is characterized by accumulations of β -pleated sheet protein aggregates of extracellular β -amyloid peptide ($A\beta$) and intraneuronal tau protein and often, α -synuclein, a protein more closely linked to Parkinson's below. AD pathogenesis involves increased oxidative damage and activation of the innate immune system and inflammation. We reported curcumin reduced AD pathology in rats CNS-infused with $A\beta$ aggregates [35] and β -amyloid precursor transgenic mice with intervention in middle-age [36] and old age [37]. Curcumin was effective in dramatically reducing plaques at late ages suggesting it might act like amyloid vaccine to stimulate plaque clearance. This has been elegantly demonstrated by Bacskai's group using in vivo multi-photon imaging to reveal a rapid shift within one week of treatment to a greater than 3-fold increase in plaque clearance along with reductions in dystrophic and aberrant neurites in remaining plaques [38]. Curcumin has multiple pleiotropic activities that block AD pathogenesis at many steps. It binds directly to $A\beta$ oligomer and fibrillar aggregates including plaques in vivo, prevents $A\beta$ aggregation and destabilizes $A\beta$ fibrils [37, 39, 40]. Curcumin inhibits oxidative damage, inflammation, protein aggregation, induces heat shock proteins and other cellular defense enzymes, limits JNK and oxidative damage induced BACE, a key enzyme in $A\beta$ production, stimulates neurogenesis, chelates metals and exhibits other neuroprotective activities [22]. More recently we have shown that it reduces accumulation of phospho-tau and phosphorylated (inactivated)

insulin receptor substrate in vitro and in a triple transgenic AD mouse model [41]. Because in that model tau pathology is driven by A β aggregates and curcumin reduces those, we have recently tested curcumin efficacy in pure tauopathy models and found efficacy, even with late intervention (unpublished results). Curcumin inhibits beta pleated sheet aggregates in multiple systems including prions [42] and synuclein [43] and possibly tau as NIH funded high throughput screens reported at PubChem find curcumin in the top 100 tau aggregation inhibitors. However, one study suggested curcumin derivatives were needed to effectively inhibit tau fibril formation in their systems [44]. Based on evidence of efficacy in preclinical models, we proposed curcumin for a pilot clinical trial with John Ringman at UCLA [45] but clinical results of this trial and another small trial run by Larry Baum in Hong Kong were negative which may be due to inadequate bioavailability as discussed below.

5.C. Parkinson's Disease

Of the common age-related neurological disorders, Parkinson Disease (PD) has the closest association with oxidative stress and damage to mitochondria [46]. Antioxidants have protective potential but curcumin has multiple additional activities that may be protective in PD, for example curcumin protects neurons and mitochondria from glutathione depletion and peroxynitrite damage to Complex I in vitro and in vivo [47, 48]. Oxidative stress-induced JNK kinase activation and downstream caspase activation and apoptotic dopaminergic neuronal death are found in models of PD including MPTP toxicity and curcumin effectively protects [49]. Both curcumin and its reduced metabolite tetrahydrocurcumin protect from dopamine loss in this model, which may be partially explained by reduced monoamine oxidase B [50] but is consistent with CNS JNK inhibition by both compounds [31]. Based on positive preclinical data and PD animal trials with JNK inhibitors showing protection, CEP-1347 an inhibitor of mixed lineage kinase upstream from JNK, was tested in a large clinical trial for PD progression but proved ineffective in this paradigm [51].

Curcumin protects from glutathione depletion, MPTP and other models of oxidative stress induced dopaminergic neuron loss, for example 6 hydroxydopamine models of PD [52] but these models don't capture another primary cause of neurodegeneration in most sporadic PD, namely accumulation of the intraneuronal α -synuclein aggregates found Lewy body inclusions. As with prions and A β , curcumin inhibits aggregation of α -synuclein in cell free systems and cultured human neuronal cells [43] and prevents long term memory deficits in human synuclein aggregate accumulating *Drosophila* model in a novel paradigm using sleep deprivation [53].

Like AD, PD involves chronic inflammation that may contribute to initiation and progression of the disease [54]. Inflammation may play a causal role. For example, a single peripheral injection of pro-inflammatory lipopolysaccharide (LPS) can induce a chronic neuroinflammatory response leading to delayed dopaminergic neuron loss [55]. Both curcumin and tetrahydrocurcumin are effective in controlling LPS-induced inflammatory responses and should be considered strong candidates for reducing neuroinflammatory stimuli driving PD [31].

5.D. Traumatic Brain Injury (TBI) and Spinal Cord Injury (SCI)

Traumatic brain injury is a common cause of neurological deficits at any age, characterized by severe axonal injury and a significant risk factor for AD and PD. It was originally as an etiologic factor identified with dementia pugilistica in boxers and later shown to interact with the major genetic AD risk factor, apolipoprotein E4 [56, 57]. In a TBI animal model, dietary curcumin protected cognition, oxidative stress and synaptic plasticity associated synapsin, BDNF and CREB [58]. Similar protection was obtained by the same group with dietary omega-3 fatty acid supplementation [59], which also protected sirtuin (*sir2 α*) and AMPK pathway deficits [60]. Dietary curcumin also protected energy homeostasis after mild fluid percussive injury [61]. Curcumin can protect from vasospasm after subarachnoid hemorrhage and reduce cerebral inflammation [62] and attenuate cerebral edema induced by TBI, possibly by limiting pro-inflammatory cytokine driven NF κ B induced aquaporin-4 [63]. Consistent with powerful protective effects from axonal injury, in a rat model of spinal cord injury (SCI) curcumin treatment beginning prior to injury inhibited apoptosis and neuron loss, quenched astrocyte activation, and significantly improved neurologic deficit 7 d after spinal cord hemisection [64]. Thus, a preventive mode curcumin pre-treatment is markedly protective against multiple adverse outcomes in several models of head injury and even remarkably protective against neuron death from completely severing axons. What remains unclear from animal models is how useful curcumin will be when administered at various intervals after the TBI or SCI and whether we can translate to humans.

5.E. Cerebral Infarct, Ischemia and Stroke

Many of the same curcumin target pathways implicated in AD (e.g., excitotoxicity, oxidative damage, neuroinflammation, JNK activation and apoptosis) also play a role in stroke, another major neurological disease of aging. Curcumin is dose-dependently protective against middle-cerebral artery occlusion (MCAO) in rats [65] where it protects neuronal apoptosis [66, 67] as well as lipid peroxidation, mitochondrial deficits and neuronal apoptosis caused by global ischemia caused from transient carotid occlusion in gerbils [68]. After CNS ischemia and reperfusion, curcumin also reduces pro-inflammatory cytokines, iNOS and nitration stress as well as damage to cerebral capillary endothelial cells and the blood brain barrier [6]. One interesting neuroprotective mechanism explored in rat models of CNS ischemia involves upregulation of the cellular defense pathways mediated by Nrf2 including hemoxygenase-1 which is seen with higher curcumin doses that also protect behavioral deficits [26]. Upregulation of Nrf2 could explain elevations in antioxidant defense enzymes [67]. There is some evidence that curcumin works even when given after the fact, for example, with treatment four hours after ischemia from a middle cerebral artery thrombus, curcumin dose-dependently reduces edema and infarct volume, as well as biochemical and neurological deficits [69]. Because most neuroprotective drugs are not effective at practical treatment times, for example four hours after the ischemia, and fail in clinical trials, it will be critical to expand work using this and other delayed treatment paradigms that may be more predictive of a possible successful clinical trial outcome.

5.F. Autoimmune And Inflammatory Diseases

Curcumin's safety and broad anti-inflammatory activity via inhibition of NFkB and AP-1 pro-inflammatory signaling and other mechanisms make it a strong candidate for treatment and prevention of recurrence of active inflammatory conditions. For example, curcumin is effective in animal models for multiple sclerosis by inhibiting STAT and IL-12 as well as IL-17 dependent Th17 driven inflammation [70, 71]. Perhaps because bioavailability is less of an issue for the gastrointestinal tract, efficacy in animal models has led to apparently successful results in small trials for inflammatory bowel disease [72]. Because many of the mechanisms are shared by autoimmune diseases, curcumin may be effective in a number of other autoimmune disorders [73].

5.G. Wound Healing and Muscle Wasting during Aging

Traditionally, curcumin has been used in Indian Ayurvedic medicine with topical application to promote wound healing in people with applications ranging from bandages and salves to acne creams [21]. Efficacy for wound healing and skin conditions including psoriasis is supported by multiple animal model studies which emphasize reduced wound-healing time, improved collagen deposition and increased fibroblast and vascular density as well as induction of pro-angiogenic and anti-inflammatory transforming growth factor-beta, [74]. This ability to promote wound healing extends to various types of tissue damage including damage to skeletal muscle [75]. Because NFkB upregulation has been implicated in many muscle diseases including wasting of aging human muscle [76], curcumin may well have therapeutic potential in conditions with significant muscle atrophy, most critical the cardiac muscle [77].

5.H. Cardiovascular Disease

Curcumin's appears to exert cardioprotective effects and efficacy in models of myocardial ischemia, myocardial infarction, cardiomyopathy, hypercholesterolemia and arrhythmia, which has recently been reviewed [78, 79]. Given that an elevated inflammatory marker, C-reactive protein, is almost as predictive of cardiovascular risk as elevated cholesterol, it is not surprising that curcumin has a protective role. For example, curcumin directly blocks C-reactive protein effects on endothelial cells [80]. Further, curcumin reduces atherosclerotic foam cell formation in cholesterol fed ApoE knockout mouse models where it favorably regulates SREBP-1 in vascular smooth muscle cells [81]. Most impressively, two very different rat models of heart failure, hypertensive, salt-sensitive Dahl rats and surgically-induced myocardial infarcts, dietary curcumin prevented deterioration of systolic function and heart failure and induced increases in both myocardial wall thickness and diameter, possibly reflecting p300 histone acetyl transferase (HAT) inhibition [82]. These results suggest possible beneficial effects in slowing down the progression of a major age-related disease.

5.I. Type II Diabetes

Insulin resistant diabetes is most often caused by a metabolic syndrome, associated with aging, obesity and chronic inflammation, that contributes to the emergence of diabetes. Because of the very high rate of obesity in the US this is an increasingly significant public health problem, compounded by an aging population. Because the insulin-producing pancreatic islet cells are also sensitive to oxidative stress, researchers have often relied on toxic agents like streptozotocin to induce oxidative stress to islet cells to create diabetes models. Given curcumin's dual anti-inflammatory and anti-oxidant mechanisms, it is perhaps not surprising that there are over 115 reports on curcumin and diabetes, primarily relying on pre-clinical model work and many showing efficacy against a wide range of endpoints, most often with toxin induced diabetes. It is outside the scope of this review to cover this already extensive literature. However, some reports clearly show protection from obesity related diabetes and mechanisms found in type II diabetes. For example, in both high-fat induced and leptin-deficient ob/ob models of diabetes, dietary curcumin limited glucose and also improved insulin tolerance and hemoglobin A1c levels. Curcumin treatment also significantly reduced macrophage infiltration of white adipose tissue, increased adipose tissue Foxo1 and adiponectin production, and decreased hepatic nuclear factor-kappaB activity, hepatomegaly, and markers of hepatic inflammation [27]. These effects are analogous to resveratrol/ Sirt1 activator-dependent upregulation of adiponectin in obesity-dependent diabetic models. In the db/db mouse model, curcumin improved insulin-resistance, lowered blood glucose and hemoglobin α chain complex 1c (HbA1c) levels, and it suppressed body weight loss and hepatic enzyme defects [83]. Curcumin can directly protect human islet cells in vitro and animal model islet cells in vivo from cytokine induced cell death [84]; one mechanism for protection of human islet cells in vitro is induction of heat shock/ cellular stress response genes [85]. Curcumin also has a direct impact on heart to prevent diabetes-induced cardiac hypertrophy, possibly through suppression of p300 HAT [86]. Multiple curcumin protective mechanisms can be invoked for metabolic syndrome and type II diabetes. For example, curcumin suppression of the proinflammatory transcription factor nuclear factor-kappa B, signal transducer and activators of transcription-3, and Wnt/beta-catenin, activation of peroxisome proliferator-activated receptor- γ and Nrf2 mediated protective pathways that result in downregulation of adipokines (TNF α , IL-6, resistin, leptin, and monocyte chemoattractant protein-1, as well as adiponectin upregulation to reduce insulin resistance, hyperglycemia, hyperlipidemia, and other symptoms of metabolic syndrome [87]. Further, JNK is one of the major kinases that can phosphorylate insulin receptor substrate to uncoupling insulin receptors and cause insulin-resistance in diabetes and in AD animal models, curcumin effectively treats this AD-relevant JNK-mediated component of insulin resistance [41]. Both curcumin and resveratrol inhibit NF κ B and have very similar protective effects on adipocyte cytokines and pro-inflammatory signaling in response to TNF α that have been implicated in obesity/ metabolic syndrome [88]. Although there have been many animal model studies, there has been insufficient clinical work to know whether any of curcumin's protective actions with diabetes will translate to humans. However, one small 8 week clinical trial in patients with diabetes mellitus used a curcumin preparation to show improvements in endothelial cell function, oxidative stress (malondialdehyde) and inflammatory cytokines in blood (NCB-02) [89].

6. Omega-3 Fatty Acids

Like curcumin, the Omega-3 fatty acid, docosahexaenoic acid (DHA), has both antioxidant activity extending to humans [90] and anti-inflammatory, anti-amyloid and neuroprotective activities in animal models that have been recently reviewed in detail [91, 92]. Neuroprotection and other protective effects from fish oil in humans are suggested by dose – dependent improved mortality after severe head injury [93]. Based on extensive evidence of omega 3 and DHA benefits to human health [94], expert panels have found that many developed countries, including the USA have suboptimal dietary intakes of omega-3. For example, DHA in the US diet is typically 80-100 mg per day but needs to be closer to 200 mg/ day or more for reduced CNS and cardiovascular disease risk. Because American diets are insufficient for reducing triglycerides, arrhythmias and sudden cardiac death rates and because fish oil has solid clinical trial data, the American Heart Association recommends fish oil supplementation for at risk groups. Both DHA [95] and omega-3 as fish oil [96] are far ahead of curcumin in advancing to successful clinical trials and there is even some hope for AD patients, at least those who lack the ApoE4 risk factor [97]. Curcumin and DHA both regulate insulin signaling relevant to aging [41, 98]. Omega-3 fatty acids have been extensively studied and have multiple health benefits. For example, anti-inflammatory activities of fish and fish oil lead to NSAID sparing activity in arthritis [99] and the cardiovascular protective effects are well documented in preclinical and clinical studies [100, 101].

7. Omega-3 and Aging

While fish consumption raises concerns about mercury and other contaminants, the health benefits in humans clearly exceed the risks, reducing coronary death by 36% and total mortality by 17% in a recent meta-analysis [102]. This is consistent with outstanding longevity in Japan, which has one of the highest fish consumption and low omega 6/ omega-3 (n-6/n-3) ratios of fatty acid intakes. In *C.elegans* deficient in long chain omega 3 fatty acid synthesis, DHA and EPA increase lifespan suggesting possible very basic mechanisms of action [103]. For example, in a clinical trial in healthy older adults, omega-3 appeared to improve insulin-sensitivity and reduce inflammatory factors associated with reduced cardiovascular risk [104]. However, attempts to increase lifespan by changing the n-6/n-3 ratio from 1 to 16 in Wistar rats, which are not prone to atherosclerosis or AD, did not show any diet related changes in mortality or lifespan [105].

8. Combining DHA and Antioxidants

Because DHA is highly unsaturated and readily oxidized, we have advocated combining DHA with antioxidants [106] and produced evidence of synergy with fish oil and curcumin in an animal model for AD [41]. We now have additional in vivo evidence showing synergism for α -lipoate and DHA in preventing synaptic marker loss in AD model mice (unpublished) adding mechanistic support for their use in combination in human clinical trials [107]. DHA

has also been combined with other antioxidants in a nutritional cocktail approach to AD [108]. Cocktail approaches combining omega 3 based supplements with dietary antioxidants are consistent with broad evidence of the health benefits of the “Mediterranean diet” which is rich in fish and antioxidants and polyphenolic nutrients.

Conclusions

Both the polyphenolic antioxidant/ NSAID curcumin and the Omega-3 fatty acid DHA have pleiotropic beneficial activities in multiple diseases of aging. They have the potential to synergistically reduce the enormous health care burden generated by chronic diseases of aging but need to be rigorously tested in clinical trials for treatment and ultimately prevention. Several new formulations are on the market claiming to improve absorption. However, proof of whether curcumin can be a useful therapy for diseases is lacking.

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Chapter XV

Potential Benefits of Curcumin in Acute and Chronic Lung Diseases

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Abstract

Occupational and environmental exposures to mineral dusts, airborne pollutants, and cigarette smoke; microbial infection, chemotherapy and radiotherapy affect airways and other parts of the lung. These factors have a tremendous impact on lowering lungs' structural and functional integrity, often failing to protect its natural repair system and giving rise to serious acute and chronic inflammatory lung diseases. Progress in our understanding of the basis of these lung diseases has discovered cellular mechanisms mediating the processes of inflammation, tissue repair and fibrosis. These studies have provided further clues as to the identification of therapeutic targets with drugs that has the potential to treat lung diseases. In this respect, the present review is concerned with the major lines of progress on benefits of curcumin on acute and chronic lung diseases. Curcumin (diferuloylmethane) is a yellow pigment present in the spice turmeric (*Curcuma longa*) that is widely used in customary Asian medicine for centuries.

Combining evidence from *in vitro* cell culture systems and *in vivo* animal models has helped to demonstrate that curcumin has an array of anti-inflammatory, antioxidant and antifibrotic effects in various lung injuries. These studies have addressed the ability of curcumin to regulate several transcription factors, cytokines, chemokines, growth factors, cell adhesion molecules and free radicals implicated in tissue injury and inflammation. While laboratory results have been supporting, curcumin has its own limitation in terms of poor solubility in aqueous solvents and low bioavailability. These functional hurdles

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need to be overcome in future before therapeutic strategies with curcumin for lung diseases can accomplish its goals in the clinic.

Abbreviations

AP-1	Activator Protein-1
BLM	Bleomycin
COPD	Chronic Obstructive Pulmonary Disease
HDAC	Histone deacetylase
IL-1 β	Interleukin-1beta
IPF	Idiopathic Pulmonary Fibrosis
NF- κ B	Nuclear Factor KappaB
ROS	Reactive Oxygen Species
TNF- α	Tumor Necrosis Factor-alpha
TGF- β 1	Transforming Growth Factor-beta1

1. Introduction

The incidence of acute and chronic lung diseases is increasing globally, leading not only to a significant social and economic burden to countries, but also a significant morbidity and mortality of affected populations. There is growing agreement that occupational and environmental exposures to toxic pollutants, particularly in industrialized nations and developing countries, as well as lifestyle factors such as cigarette smoking are driving this epidemic. In addition, the incidence of lung disease due to microbial infections, chemotherapy with anticancer drugs and radiotherapy appear at greater rates and people will face difficulty coping with this alarming increase. These increases point to a clear need for identification of factors that initiate and perpetuate the progression of lung diseases such as pulmonary fibrosis, asthma, chronic obstructive pulmonary disease (COPD)/emphysema, and to take measures for the prevention and treatment of acute and chronic lung disease.

Here, we will present an overview of the pathogenesis of fibrosis and airway inflammatory diseases followed by the therapeutic role of curcumin in acute and chronic lung disease. A detailed review of each of these aspects is beyond the scope of this article. Several timely reviews have been published on the mechanism of pulmonary fibrosis [1, 2] asthma [3, 4] and COPD [4-6]. Therefore, we have attempted to sketch some high points in the pathogenesis of these diseases, but not to cover the entirety of the subject.

2. Idiopathic Pulmonary Fibrosis

Idiopathic pulmonary fibrosis (IPF), also known as cryptogenic fibrosing alveolitis, is a chronic fibrosing interstitial lung disease that follows a wide variety of lung injuries, which may be environmental, infectious, immunologic, toxic or pharmacologic [7]. The incidence of IPF is 30 in 100,000 with 34,000 new cases annually; mean survival in IPF is 3 to 5 years

from onset of symptoms: most clinicians consider IPF to be an ultimately fatal condition and there is no known curative therapy [8, 9]. IPF is characterized by an abnormal fibroproliferative reaction to a persistent damage to the alveolar epithelium associated with hyperplasia of type 2 alveolar epithelial cells and/or epithelial–mesenchymal transition [4, 7]. Interstitial inflammation is usually patchy and consists of an alveolar septal infiltrate of lymphocytes and plasma cells; however, a role for inflammation in fibrosis is now under controversy [10]. The fibrotic areas consist largely of excess deposition of extracellular matrix proteins and proliferating fibroblasts (“fibroblastic foci”). Increasing evidence suggests that an imbalance in the expression of cytokines, chemokines, and growth factors result in proliferation and/or activation of resident structural and circulating cells, contributing to aberrant wound healing that leads to fibrosis [4, 7].

3. Asthma

Asthma is a chronic inflammatory disease, which is characterized by enormous infiltration of the bronchial mucosa by numerous cell types together with lymphocytes, eosinophils, and mast cells, and this is accompanied by increased serum IgE levels [11]. Experimental and clinical findings have shown both structural and functional derangements in asthmatic airways that include desquamation of the lung epithelium, goblet cell hyperplasia, smooth muscle hypertrophy, mucus production within the bronchioles, leading to airway remodelling, impaired airflow and airway hyperreactivity (AHR). Increased expression of inflammatory mediators, including cytokine and chemokine gene expression, and the recruitment of specific leukocyte cell types are all dominant features of asthma [3]. Evidence suggests that inflammatory reactions and airway function can be aggravated by allergens [12], respiratory tract infections [13] or environmental pollutants [14, 15]. The existence of activated eosinophils and Th2 cells [16, 17] underlines the significance of allergy in asthma pathogenesis. It is widely accepted that the Th2 cytokines (IL-4, IL-5, IL-9, and IL-13) and GM-CSF are mainly accountable for inducing bronchial asthma [18].

4. Chronic Obstructive Pulmonary Disease (COPD)

Chronic obstructive pulmonary disease, a disease of the small airways and lung parenchyma, is a major cause of respiratory morbidity and mortality worldwide. It is characterized by progressive airflow obstruction and destruction of lung parenchyma, and cigarette smoking represents a major risk factor for COPD [4-6]. Furthermore, recent studies suggest that environmental exposure to air pollutants, biomass fuel, occupational exposure to dust and fumes, prior history of respiratory tract infections and chronic asthma can also produce detrimental outcomes, which can result in the development of COPD. Disease development in COPD can result from a variety of mechanisms, ranging from production of inflammatory peptide mediators, toxic oxygen free radicals and nitrogen species, alveolar wall destruction due to elastin degradation mediated by proteases and subversion of the normal homeostatic and/or immune mechanisms [6]. The disease remains a priority health

problem due to the fact that COPD is the fourth largest cause of death in the world, indicating an urgent need for the development of new therapeutic drugs.

5. Traditional Therapies

Treatment of inflammatory and fibrotic lung diseases has long relied on the use of corticosteroids, antiinflammatory agents, immunosuppressants, exogenous antioxidants or antifibrotic compounds (penicillamine, colchicines, pirfenidone, and interferon-gamma). Despite their obvious potential, there are many disadvantages to these therapies. First, existing treatment regimens, often lead to undesirable side effects. Second, is the manifestation of resistance in patients with severe asthma, asthmatic patients who are smokers and COPD to corticosteroids. Third, there is no clear evidence indicating that immunosuppression improves mortality in patients with idiopathic pulmonary fibrosis [19-22]. How might we overwhelm these serious drawbacks? Current evidence suggests that dietary constituents and phytochemicals such as curcumin can significantly influence disease process and are becoming an increasingly alternative approach for the treatment of various acute and chronic diseases among patients. The present chapter is concerned mostly with the major lines of progress in the field of curcumin's therapeutic effect over the past 15 years. We did not include all publications concerning to the subject during this period. Older work has been displayed to help shed light on recent contributions. The scope is limited to findings pertinent to pulmonary inflammation and fibrosis.

6. Curcumin and its Properties

Turmeric, traditionally known as INDIAN SAFFRON, is derived from the root of the *Curcuma longa* plant (Figure 1). Turmeric is a yellow powder frequently used as a spice in curries and other South Asian and Middle Eastern cuisine. Of the many constituents present in turmeric, curcumin has been identified as the major pharmacological agent with health benefits. Many lines of evidence indicate that curcumin is not only a *cooking treat* but also has been used for centuries as a naturally occurring medicine for the treatment of various chronic diseases [23]. Accordingly, scientists have reported the anti-inflammatory properties of curcumin [24-26]. The chemistry, biology and pharmacology of curcumin have been the subject of intensive research in recent years. Curcumin is a bis- α , β -unsaturated β -diketone that exhibits keto-enol tautomerism. Besides having a β -diketone, it consists of two phenyl, two methoxy, two phenolic hydroxyl groups and two double-conjugated bonds. The polyphenolic structure of curcumin permits electron delocalization, imparting the ability to quench toxic free radicals and makes curcumin a potent anti-inflammatory, antioxidant, antimutagenic, antimicrobial, and anticancer agent. Commercially available curcumin (Figure 1) contains, in addition to curcumin, ~17% of demethoxycurcumin and 3% bisdemethoxycurcumin; among these constituents curcumin has been shown to be the most active principle [27].

7. Pulmonary Protective Effects of Curcumin

The protective effect of curcumin against acute lung toxicity, initially investigated in 1992 [28] has now undergone substantial experimental evaluation, and continues to expand in both pulmonary inflammation and chronic lung disease as a remarkable therapeutic compound. The efficacy and safety [29-32] of curcumin have been evaluated in several *in vitro* and animal models of pulmonary inflammation and fibrosis. Initially, we reported that curcumin can strongly influence the initiation and progression of lung injury by suppressing cyclophosphamide-induced increases in infiltration of inflammatory cells, such as neutrophils, macrophages and lymphocytes in the lung [33]. Our observations suggested a new protective mechanism that moved rapidly to testing whether paraquat or bleomycin or amiodarone-induced lung injury and fibrosis are affected by similar mechanism. These results indicated that curcumin not only influenced lung inflammatory response but also had an impact on fibrotic process [34-37]. Animal models have also been used to investigate whether administration of curcumin could ameliorate the progression of allergen-induced airway dysfunction in guineapigs. Interestingly, curcumin prevented ovalbumin-induced airway constriction and airway hyperreactivity [38].

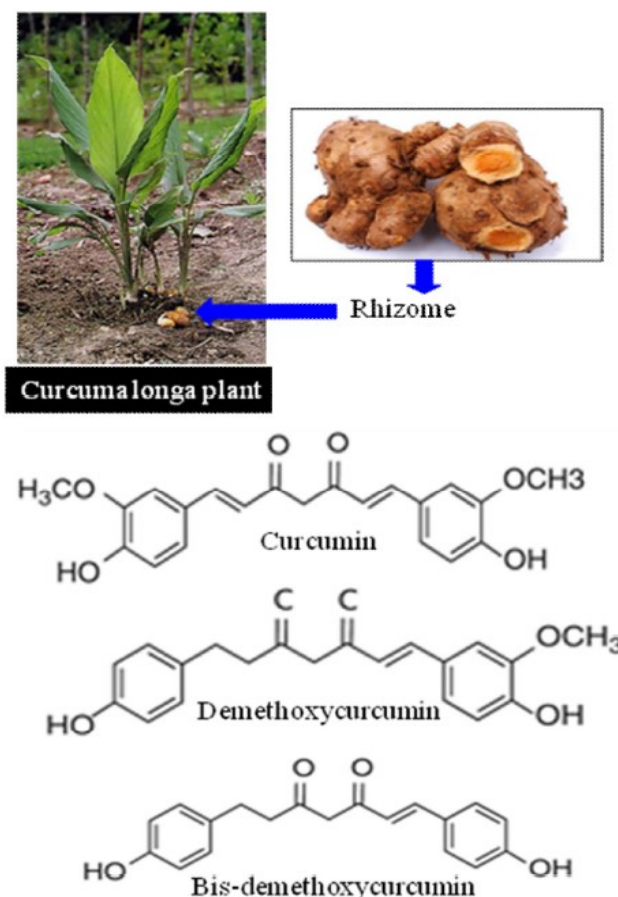


Figure 1. *Curcuma longa* plant and chemical structure of curcumin and its important metabolites.

In a study based on previous *in vitro* findings that curcumin can block cigarette smoke-induced transcription factor activation and suppression of inflammatory mediators and proteinases production [39], Moghaddam et al. [40] demonstrated that curcumin inhibits cigarette smoke-induced COPD-like airway inflammation and lung carcinogenesis in mice. This is consistent with reports from other experimental model, in which the development of pulmonary emphysema induced by elastase and cigarette smoke was attenuated by curcumin [40]. Together, these animal models have provided experimental proof that supports a role for curcumin in protection against inflammatory and fibrotic lung injuries. These models should be exploited to identify mechanisms by which curcumin therapy promotes these protective responses, and to provide guidelines for appropriate treatment strategies. The present article examines the current body of evidence to support therapies with curcumin and reviews therapeutic targets being targeted by curcumin in inflammatory and fibrotic lung injuries.

8. Effect of Curcumin on Lung Inflammation

Tissue injury results in the immediate onset of acute inflammation. It has been demonstrated that inflammatory reactions are in general characterized by spatially and temporally changing patterns of various infiltrating cells, including neutrophils, macrophages, lymphocytes, and eosinophils. To attain proper tissue homeostasis during healing, the fine balance between a complex network of various inflammatory cells and their pro- and anti-inflammatory mediators must be maintained. Evidence suggests that dysregulation of these interactions and excess accumulation of inflammatory cells cause pathologic and chronic inflammatory disease states, impairing the quality of healing [41]. This indicates that agents that target aberrant inflammatory responses and contain infiltrates of inflammatory cells are needed in restoring tissue integrity [19-21, 42].

There is mounting evidence that inflammation is the driving force in the development of several lung diseases. Recent molecular and genetic studies in acute and chronic lung injuries support strongly that inflammation plays the central role in the pathophysiology of asthma and COPD. Many studies of asthma and COPD have proposed that chronic inflammation of the airways in these diseases is mediated by the enhanced expression/activity of various inflammatory mediators such as cytokines, chemokines, adhesion molecules, enzymes and receptors [4]. Despite having inflammation, there is considerable variation in the detailed structure of inflammation, particularly those associated with airways. Asthmatic airways examined contain inflammation mainly in the larger conducting airways, but small airways also show some inflammation in more severe forms of the disease, albeit no involvement of lung parenchyma[4, 11]. In contrast, inflammatory reactions in COPD are typically found in small airways and the lung parenchyma, however, inflammation is also seen in larger airways. It has long been understood that inflammation is a critical factor during early stages of fibrosis, and studies indicated that the fibrosis observed in IPF was due to a strong alveolar and interstitial inflammatory reaction to unidentified offending agents. However, recently a considerable amount of criticism has been directed against the role of inflammation in the fibrogenesis. It appears from the information currently available that anti-inflammatory and immunosuppressive therapy failed to improve the disease process in patients with IPF and clinical measurements of inflammation did not correlate with stage or outcome in usual

interstitial pneumonia patients [10, 43]. Consequently a strong interest is evident among clinicians and scientists today, stimulated by the hypothesis that a dysregulated epithelial/fibroblastic disorder may play a key role in pathologic fibrotic process, which is independent of foregoing inflammation [44].

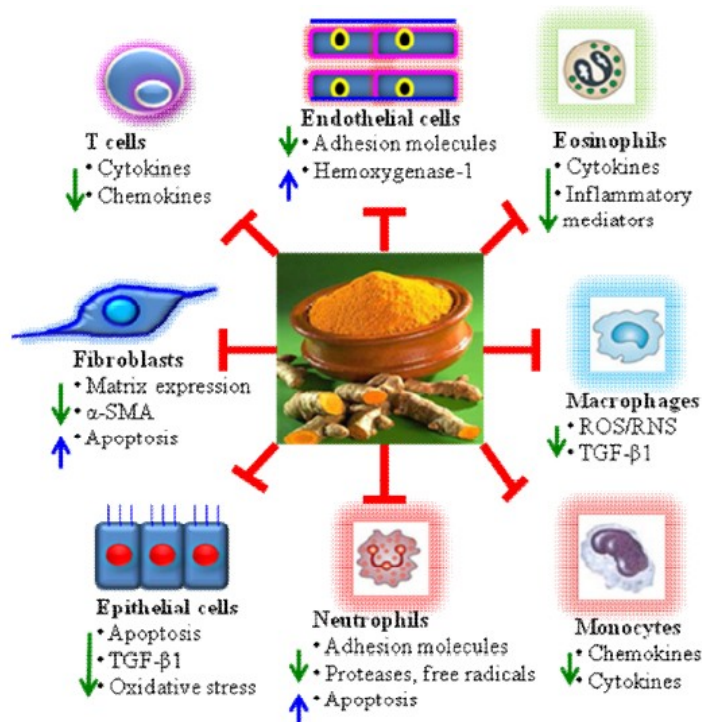


Figure 2 . A summary of the anti-inflammatory effect of curcumin on lung inflammatory and structural cells; α -SMA: alpha-smooth muscle action, ROS: reactive oxygen species, RNS: reactive nitrogen species, TGF- β 1: transforming growth factor-beta1.

There is growing understanding and documentation of the anti-inflammatory property of curcumin in a variety of disease models [45], including acute and chronic inflammatory lung diseases. Curcumin shows promise in reducing infiltration of inflammatory cells in lung lavage fluid in fibrotic and inflammatory lung disease. To investigate the general effect of curcumin on inflammatory responses (Figure 2) we have treated various drug-induced fibrotic rats with curcumin. Induction of acute or fibrotic lung injuries in rats with paraquat [35] or cyclophosphamide [33] or bleomycin [34, 36] or amiodarone [37] resulted in accumulation of inflammatory cells such as neutrophils, macrophages, lymphocytes and eosinophils in bronchoalveolar lavage fluid and in the lung interstitium. In addition, changes in vascular permeability (as measured by the accumulation of a variety of biochemical markers, derived from plasma, lung parenchymal cells and lung inflammatory cells, in lung lavage fluid) occurred in response to the above offending agents. It is noteworthy in this context that curcumin treatment reduces total cell numbers in lung lavage fluid along with a reduction in alveolar leakage and transudation of proteins from the blood stream.

In a related study, Smith et al. [46] reports that treatment with curcumin reduces inflammation and fibrosis in bleomycin-instilled mice. Although this report initially seemed

contradictory, a careful review shows that intraperitoneal administration of curcumin in bleomycin-injected mice significantly reduces (while oral administration of curcumin does not produce anti-inflammatory/antifibrotic effects) inflammatory cell accumulation in the lung interstitium. Curcumin treatment also induces improvement in survival of drug-injected mice. This investigation also helps in dissociating the bioavailability patterns of curcumin associated with different modes of administration that could influence the therapeutic outcome in bleomycin-induced lung injury in mice.

A recent study by Bansal and Chhibber [47], using an approach of acute lung infection by intranasal introduction of *Klebsiella pneumoniae* B5055 into BALB/c mice, illustrates the beneficial effects of curcumin in reducing neutrophil infiltration in bacteria-infected mouse lung. Analysis on the impact of curcumin in elastase- or cigarette smoke-induced lung injury in mice [40] showed a decreased number of neutrophils and macrophages in bronchoalveolar lavage fluid along with a reduction in air space enlargement. In these studies, curcumin (100 mg/kg, daily by oral gavage 1 h and 24 h before intratracheal porcine pancreatic elastase treatment and daily thereafter for 21 days in elastase-exposed mice; and 1 hour before each CS exposure in CS-exposed mice) treatment also increased antioxidant gene expression in elastase-instilled mice lungs. These results suggest that curcumin can attenuate both elastase and cigarette smoke-induced pulmonary inflammation and emphysema in mice.

Sun and coworkers [48] recently reported studies on the role of curcumin on lung transplantation-associated lung injury in rats. The authors showed the preventive effects of curcumin in transplantation-associated lung edema, alveolar-capillary barrier dysfunction, and leukocyte infiltration. They recognized that use of curcumin may be an alternative therapeutic strategy in the early postoperative period after lung transplantation.

Other than these antiinflammation-based effects, a recent study by Kurup et al. [49, 50] investigated the role of curcumin as an immunomodulator on mice exhibiting allergic responses to latex. The data showed that mice exposed to latex had increased serum IgG, latex-specific IgG1, IL-4, IL-5, IL-13, eosinophils and inflammation in the lungs. However, curcumin treatment (intragastric administration of 250 µg curcumin in 250 µl phosphate-buffered saline) reduced many Th2 type responses together with a reduction in lung inflammation. Moreover, curcumin treatment decreased interstitial inflammation with reduced peribronchiolar and perivascular infiltrates in latex-challenged mice. Eosinophilia in curcumin-treated mice was noticeably decreased, co-stimulatory molecule expression (CD80, CD86, and OX40L) on antigen-presenting cells was decreased, and expression of MMP-9, OAT, and TSLP genes was also reduced, suggesting that curcumin's ability to reduce inflammation, eosinophilia and Th2 cytokines may play an important role in regulating allergic responses resulting from exposure to allergens.

9. Effect of Curcumin on Cytokines and Chemokines

Cytokines are small peptide mediators of inflammation that are produced by multiple cell types. A battery of cytokines expressed by resident and migratory alveolar cells is capable of stimulating inflammatory and reparative processes [51]. These mediators are either proinflammatory or profibrotic cytokines involved in the regulation of cell proliferation,

differentiation or chemotaxis to directing wound remodelling by regulating extracellular matrix synthesis and degradation [52]. Chemokines are a group of small molecular weight cytokines (8 to 10 kD) whose production is stimulated in a variety of cells by inflammation. Members of this family of chemokines are divided into four subclasses according to their structure and by the conservation of a four-cysteine motif [1-3]; C-X-C, C-C, C, and C-X-3-C. The most important function of chemokines is their ability to mediate the recruitment of leukocytes and regulate trafficking into the tissues. For example, members of the C-X-C chemokine subfamily (IL-8 and IFN-induced protein 10, IP-10) are chemotactic for neutrophils but not for mononuclear leukocytes. On the other hand, the C-C chemokines (eotaxin, RANTES, MCP-1 to MCP-4, MIP-1 α , and MIP-1 β) attract mononuclear cells (monocytes, T cells) and granulocytes (eosinophils) other than neutrophils [53-58]. Alternatively, chemokines are also involved in the physiological leukocyte migration during hematopoiesis, antigen sampling in secondary lymphoid tissues, and immune surveillance. Chemokines mediate leukocyte trafficking under homeostatic and inflammatory conditions by binding to specific G protein linked seven transmembrane spanning receptors [53] expressed on trafficking immune cells.

Given these biological actions cytokines and chemokines are considered to have importance in the pathogenesis of acute and chronic inflammatory lung disease. Accordingly, increased expression of cytokines and chemokines has been reported in the lung during the development of an inflammatory response [59-61]. Indeed, high levels of chemokines have been shown in BAL fluid and biopsy specimens of asthmatic patients compared to control subjects [62]. Several studies have shown that CCL3 (macrophage inflammatory protein-1 α) and CC-chemokines such as CCL2 (monocyte chemoattractant protein-1), have a profibrotic role. These chemokines are produced by the macrophages and epithelial cells and it has been shown that anti-CCL3 antibodies reduced the development of bleomycin-induced lung injury in mice. In addition, studies with CC-chemokine receptor 1 (CCR1) and CCR2-deficient mice established the pathological roles of CCL3/CCL2 chemokines in pulmonary fibrosis [63-67]. Like other inflammatory lung disease, chemokines are also involved in the recruitment, activation and survival of inflammatory cells in COPD, leading to pathological changes in the airways [3, 4, 6]. It had previously been shown that IL-8 (CXC-chemokine ligand [CXCL] 8) levels were increased in induced sputum of patients with COPD compared with smokers with normal lung function and nonsmokers [68]. Subsequent investigations on humans and experimental models of COPD using gain or loss of function have also reported the role of other chemokines in the pathogenesis of COPD [3]. Together these findings have led to the notion that chemokines play critical role in pathological processes of most inflammatory and autoimmune diseases and are considered as potential therapeutic targets for the development of anti-inflammatory compounds. However, the clinical significance of anti-chemokine therapeutic approaches depends on several factors, including the degree of pleiotropy exhibited and the level of redundancy of the chemokine system. Patients with inflammatory lung diseases may not respond to treatment with an agent intended to counteract the activity of a single chemokine. However, agents that neutralize multiple types of chemokine expression/activity may prove to be potential for management of inflammatory lung disease [69]. The compound currently qualifies in this setting is curcumin.

The increasing importance of curcumin as an anti-inflammatory compound has led to investigation of the beneficial effects in bleomycin-induced pulmonary fibrosis in rats. BLM-induced pulmonary fibrosis in rats, a well-established animal model of human pulmonary

fibrosis, represents ongoing inflammation with active participation of the immune response, including increases in inflammatory cells. Among the various inflammatory cells, alveolar macrophages (AM) are thought to play a central role in the development of pulmonary fibrosis via elaboration of a wide array of inflammatory mediators, growth factors and reactive oxygen species [70]. Several lines of evidence indicate that tumor necrosis factor- α (TNF- α), a potent pro-inflammatory cytokine, released from activated macrophages drive the pathophysiology of pulmonary fibrosis, including regulation of production of other pro-inflammatory cytokines [71-73]. Significant increases in spontaneous release of these cytokines by AM coupled with upregulation of mRNA levels for TNF- α have been reported in patients with idiopathic pulmonary fibrosis and asbestosis, and in BLM-induced pulmonary fibrosis [74, 75].

Furthermore, antibodies to TNF- α and TGF- β 1 were demonstrated to inhibit BLM-induced pulmonary fibrosis [71, 72, 76]. Taken together, these studies clearly demonstrate that TNF- α is an important cytokine in the pathogenesis of pulmonary fibrosis and therefore, appears to be a potential target for therapeutic intervention.

Our work on pulmonary fibrosis showed that curcumin was very effective in suppressing bleomycin-induced inflammatory responses. Administration of curcumin (300 mg/kg 10 days before and daily thereafter throughout the experimental time period) blocked BLM-induced increases in total inflammatory cell numbers and several biomarkers of inflammatory responses in lung lavage fluid. Furthermore, curcumin showed a unique ability to block BLM-induced alveolar macrophage production of TNF- α [36]. Since TNF- α is the key regulator of the production of other inflammatory cytokines such as IL-1 β , GM-CSF and TGF- β 1, curcumin inhibition of TNF- α release by AM could abrogate this inflammatory loop and protect from lung damage. These findings suggest curcumin as a potent anti-inflammatory agent against BLM-induced pulmonary fibrosis in rats.

Exciting findings in amiodarone-induced lung fibrosis in rats also revealed that curcumin (200 mg/kg body weight after first amiodarone instillation and daily thereafter for 5 weeks) prevented amiodarone-induced increases in total cell counts such as alveolar macrophages, neutrophils and eosinophils recovered by lung lavage. In addition, TNF- α release induced by lipopolysaccharide (LPS) in the alveolar macrophages of amiodarone rats was significantly blocked by curcumin [37]. This *in vitro* demonstration of the inhibition of cytokine release is an important proof of the potential immunomodulatory properties of curcumin in fibrosis.

Literat et al. [77] reported an astounding effect of curcumin in blocking LPS-induced expression of proinflammatory cytokines and chemokines (TNF- α , IL-1 β and IL-8) by the lung inflammatory cells isolated from preterm newborns at risk for development of chronic lung disease. Curcumin was also shown to inhibit IL-8 expression by adult peripheral blood mononuclear cells, implying that curcumin may provide clinical benefits in inflammatory situations. Earlier research published from the Kobayashi lab [78] shows that curcumin can block *Dermatophagoides farinea* (Df)-stimulated interleukin-5 (IL-5), granulocyte macrophage- colony stimulating factor (GM-CSF), and IL-4 production expression by lymphocytes from atopic asthmatics. Curiously, Kobayashi et al. also found that curcumin inhibited Df-induced lymphocyte proliferation and production of IL-2. This raises the possibility that curcumin treatment could switch off cytokines affecting eosinophil function and IgE synthesis in allergic diseases.

Another good example concerns the potential of curcumin to inhibit the release of IL-10 in Der p 1-activated co-culture of eosinophils and bronchial epithelial cells. Curcumin is also effective in suppressing the release of IL-10, TNF- α and IL-1 β from eosinophils culture alone activated by Der p 1 [79]. A similar analysis by Biswas et al. [80] demonstrated that curcumin inhibited both TNF- α and hydrogen peroxide-induced IL-8 release from cultured alveolar epithelial cells (A549). More recently, *in vitro* studies [81] showed that IL-8 secretion was increased when human monocytes were exposed to cigarette smoke and curcumin treatment was associated with decreased IL-8 secretion.

Although these findings have provided us with detailed information on the ability of curcumin to inhibit cytokine/chemokine expression in various lung diseases there are still many uncertainties and gaps in our knowledge of how curcumin affects cytokine/chemokine expression and or activity.

10. Effect of Curcumin on Oxidative Stress

Many key biological processes utilize molecular oxygen (O_2) in bioenergetic or other metabolic pathways. Regardless of the energetic achievements, a serious outcome is the formation of reactive oxygen species (ROS). ROS such as superoxide anions ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH $\cdot$), are generated during respiratory bursts of activated macrophages and neutrophils as part of host defense at the site of attempted invasion. However, increased formation of ROS is pathogenic, due to tissue damage, and serves as biomarkers for organ involvement or therapeutic outcomes. Considering the fact that the respiratory tract is opened to the elements of high oxygen environment, ambient particulate matter and toxic pollutants, extensive evidence points oxidative stress as one of the key pathogenic mechanisms in fibrotic and inflammatory lung diseases.

Animal models and clinical studies have demonstrated that increased oxidative stress is capable of damaging epithelial and endothelial cells of the respiratory tract and play key roles in almost all stages of lung injury and disease development [82-85]. Eukaryotes are endowed with a variety of defense mechanisms to protect against the damaging effects of reactive oxygen species, including antioxidant molecules that function by directly quenching ROS, and numerous potent antioxidative enzymes, including superoxide dismutase, catalase, and glutathione peroxidase, all of which metabolically reduce intracellular ROS levels to forms that are readily excreted by cells.

A defect in the ability to scavenge ROS can compromise cell structure and function. Due to their reactive nature ROS can induce damage to nucleic acid bases, denaturation of proteins and intracellular molecules and lipid peroxidation resulting in alterations in the biological characteristics of membranes, including membrane leakage and tissue permeability. Peroxidation of membrane lipids produces rather stable breakdown end products such as α , β -unsaturated reactive aldehydes, such as malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), and 2-propenal (acrolein), and isoprostanes. These end products can serve as biomarkers of oxidative stress and can be measured in plasma, urine and exhaled breath condensate [86, 87]. Besides, ROS can activate lung inflammation involving recruitment of leukocytes to the injured endothelium, infiltration of these inflammatory cells into the tissue and initiation of inflammatory and fibrotic lesions.

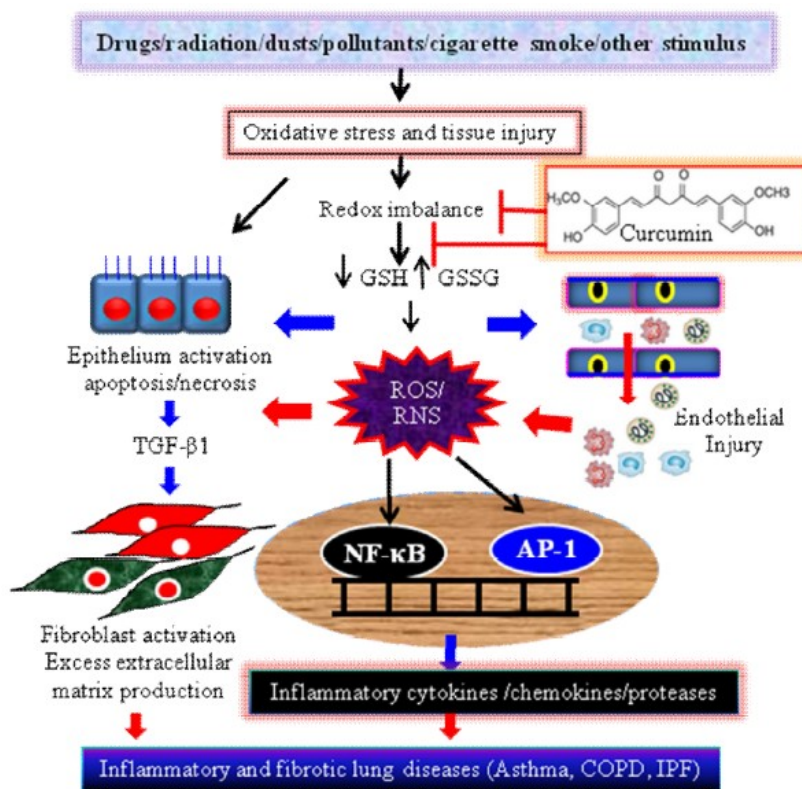


Figure 3. Simplified diagram showing possible antioxidant mechanism of curcumin. Exposure to drugs or pollutants or cigarette smoke or antigens or other stimuli cause tissue injury leading to an imbalance in the intracellular redox status and generation of reactive free radicals by inflammatory cells. These toxic reactive oxygen species (ROS) and reactive nitrogen species (RNS) injure epithelial cells and endothelial cells leading to production of proinflammatory and profibrotic cytokines and growth factors. Injured epithelial cells release TGF- β 1 that activates fibroblasts to produce excess amount of extracellular matrix proteins. Oxidative stress also activates proinflammatory transcription factors to produce inflammatory mediators. Curcumin plays a vital role against oxidative stress mediated pathological conditions by effectively scavenging reactive free radicals and maintaining the intracellular redox balance.

ROS have been implicated in the inflammatory processes in part by the activation of redox sensitive signaling mechanisms and transcription factors [89, 90]. This is associated with stimulation of protein kinases (such as the extracellular signal-regulated kinases (ERK1/2, also termed p42/44 mitogen-activated protein kinases [MAPKs], p38, JNK) Studies have reported that ROS also activate other signaling molecules and transcription factors: G-proteins ras/rac and the heterotrimeric Gi complex, NF- κ B, AP-1, Sp-1, c-Myb, p53, c-myc, EGR-1, MIF-1, GR and CREB [90]. Besides their potential role in activating various signaling molecules and transcription factors, increasing evidence indicates that ROS also function as strong signaling that regulate cell growth, aging, and transformation [91, 92].

Impressive laboratory successes in the antioxidant action of curcumin have stimulated a great deal of interest in control of the oxidative response for therapeutic purposes, not only in the field of inflammatory lung injury but also in the area of fibrotic lung injury (Figure 3). Studies on experimental alteration of oxidative response undertaken by Thresiamma and

colleagues [93] concerned with the effects of paraquat and cyclophosphamide-induced lung injury. Their results showed oxidative stress appeared to be responsible for the toxic effects of paraquat and cyclophosphamide on lung, as treatment with the antioxidant curcumin in the drug-injected mice prevented the increased formation of lipid peroxides.

Also, rats given a fibrotic dose of bleomycin could be protected from oxidative stress by pretreatment with curcumin. In rats given a curcumin dose of 300 mg/kg body weight before and after a single dose of bleomycin we showed significant inhibition of alveolar macrophage production of superoxide and nitric oxide. In addition, statistically significant suppression of malondialdehyde levels in bronchoalveolar lavage fluid and bronchoalveolar lavage cells was noted in curcumin-treated animals. Furthermore, we also made an interesting observation that antioxidant status (as measured by the levels of reduced glutathione) was restored in curcumin-treated fibrotic rats [34, 36]. We extended these observations to amiodarone-induced lung injury in rats, in which similar protective effects were noted in terms of suppression of superoxide generation by the alveolar macrophages of amiodarone rats that were treated with curcumin [37].

In view of the success of curcumin in the treatment of oxidative stress in fibrotic lung injuries, Bansal and Chhibber [47] provided evidence for suppression of malondialdehyde and nitric oxide production by curcumin in a mouse model of acute lung inflammation induced by intranasal administration of *Klebsiella pneumoniae* B5055. These authors showed that curcumin alone orally or in combination with augmentin, 15 days prior to bacterial instillation into the lungs showed a reduction in oxidative stress along with reduced myeloperoxidase activity, suggesting curcumin's role as an adjunct therapy along with antibiotics in the case of acute lung infection.

The potential of curcumin as an antioxidant was also suggested by Guzel et al. [94] who noted that curcumin decreased elevated malondialdehyde levels and prevented inhibition of antioxidant enzymes superoxide dismutase and glutathione peroxidase in aspiration material-induced lung injury in rats. In subsequent studies, it was reported that curcumin ameliorated ischemia-reperfusion induced acute lung injury by decreasing oxidative burden in rats [95]. Recognizing the role of curcumin in attenuation of oxidative damage in acute lung injury and fibrosis, it was observed by Kalpana and Menon [96] that curcumin ameliorated oxidative stress by decreasing lipid peroxidation product formation and enhancing the antioxidant status in nicotine-induced lung injury in rats.

Another set of research contribution by Sarada et al. [97] have implicated curcumin in the protective role against oxidative damage. El-Demerdash et al. [98] found that curcumin ameliorated sodium arsenite-induced oxidative damage in lungs by causing reduction in the levels of thiobarbituric acid reactive substances, as well as by enhancing the activities of antioxidant enzymes and thiol content.

No exception has been found to the rule that curcumin acts only as an antioxidant. Recent studies by Li et al. [99] concerning the effect of curcumin have revealed that curcumin does not protect RLE-6TN rat lung epithelial cells from quartz-induced oxidative DNA damage. The data indicate that curcumin induced oxidative DNA damage not only in rat lung epithelial cells but also in NR8383 rat alveolar macrophages and A549 human lung epithelial cells. Further analysis found that curcumin also enhanced the mRNA expression of the oxidative stress response gene heme oxygenase-1 (HO-1). The suggestion is made that attention should be paid while considering the use of curcumin in the prevention or treatment of lung diseases associated with quartz exposure. Data pertaining to the pro-oxidant role of curcumin have

been also noticed in studies of Kelly et al. [100]. These authors investigated the ability of curcumin to function as an antioxidant by inhibiting oxidative damage to DNA. Jurkat T-lymphocytes were pre-incubated for 30 min with 0-25 microM of curcumin and then exposed to 25 μ M hydrogen peroxide (H_2O_2). Their results showed that curcumin did not inhibit H_2O_2 -induced DNA damage. On the contrary, incubating Jurkat T-lymphocytes with curcumin alone induced DNA damage, whereas curcumin-induced damage to DNA was prevented by pre-treatment of the cells with the alpha-tocopherol, indicating that curcumin caused DNA damage via oxygen free radicals. The alleged metabolism of phenol rings of curcumin by peroxidase- H_2O_2 to form pro-oxidant phenoxyl radicals with a concomitant increase in ROS formation was considered as the cause of pro-oxidant property curcumin [101]. Taken together, these data suggest the antioxidant as well as pro-oxidant property of curcumin. However, more studies are needed to understand the mechanism by which curcumin mediates its pro-oxidant property.

11. Effect of Curcumin on Inducible Nitric Oxide Synthase and Nitric Oxide

Nitric oxide (NO), a gaseous free radical produced by various cell types, is an important physiological regulator and mediator of biological processes including smooth muscle relaxation, neurotransmission, and murine macrophage-mediated cytotoxicity for microbes and tumor cells. However, excess formation of NO is thought to mediate lung injury, tissue destruction, vasoconstriction and loss of pulmonary function in several lung diseases. There are several potential mechanisms by which NO may regulate inflammatory components and tissue injury *in vivo*: by activation of soluble guanylyl cyclase, protein nitration, and nitrosylation. NO has been suggested to play a role in human inflammatory responses by inducing tumor necrosis factor- α (TNF- α) production in neutrophils [102] and peripheral blood mononuclear cells (PBMC) [103, 104]. Importantly, the highly reactive peroxynitrite (ONOO $^-$), formed as a reaction product of NO plus superoxide causes microvascular permeability and edema formation, leading to clinical signs of septic shock [102]. As a potent oxidant, peroxynitrite reacts with lipids, thiols, DNA, and proteins and may constitute an inducer of apoptosis. Both constitutive and inducible isoforms of the NOS are present, which differ in structure and regulation. These enzymes convert arginine to citrulline and NO. Whereas constitutive isoforms of NOS (cNOS) generate nanomolar concentrations of NO in response to agonists that elevate intracellular Ca^{2+} the cytokine-inducible, or inflammatory NOS (iNOS, NOS2) produces NO in micromolar concentrations in a persistent manner even in absence of intracellular calcium elevations and is expressed mainly during inflammatory process [105, 106].

Examples of lung diseases thought to involve increased iNOS expression and NO generation include asthma, COPD, IPF, acute lung injury and acute respiratory distress syndrome. There is evidence to suggest that nitric oxide derived during allergic inflammation is involved in the expression of eosinophilic inflammation induced by chronic allergen exposure [107]. In patients with asthma iNOS expression was found to be markedly increased in airway epithelial and inflammatory cells. In the same study the authors [108] and Lane et al [109] demonstrated an association between iNOS expression in bronchial epithelium and

elevated exhaled NO levels in asthmatic children. In another study, Smith et al. [110] reported the use of exhaled nitric oxide measurements to guide treatment in chronic asthma.

There is also evidence that increased iNOS expression and NO generation is associated with the pathobiology of the airway inflammatory and obstructive process in COPD patients, as well as in a mouse model of cigarette smoke-induced inflammation [111-113]. NO has been also proposed to play an important role in bleomycin-induced lung injury in mice. Genovesse and coworkers [114] compared the response to bleomycin administration in wild-type mice (WT) and mice lacking inducible nitric oxide synthase (iNOS). The authors found that compared to bleomycin-treated WT mice, mice lacking iNOS developed a reduced degree of inflammatory and fibrotic changes, suggesting that iNOS plays an important role in the lung injury induced by bleomycin in the mice. In patients with IPF increased iNOS expression and 3-nitrotyrosine staining was observed in inflammatory and epithelial cells of fibrotic lungs [115].

Experimental work by Razavi et al. [116] shows a role for iNOS in pulmonary neutrophil infiltration in murine model of sepsis induced by cecal ligation/perforation in wild-type (iNOS^{+/+}) versus iNOS^{-/-} mice. Moreover, it has been shown by Yuba et al. [117] that iNOS play important roles in the pathogenesis of hyperoxic lung injury, whereas selective iNOS inhibitors may be useful for the treatment of hyperoxic lung injury.

Given the pathological importance of these inflammatory mediators it is understood that decreasing the levels of inducible NO synthase and NO generation may reduce the inflammatory process and slow disease progression [118, 119]. As an important extension to our finding that curcumin regulates bleomycin-induced oxidative stress and inflammation, our data also indicate that curcumin plays an important role in inhibiting NO formation induced by bleomycin. Specifically, curcumin treatment inhibited alveolar macrophage release of nitric oxide in bleomycin-instilled rats [36]. Unpublished studies from our lab show that curcumin treatment led to a marked reduction in iNOS expression in bleomycin injected rat lungs. In recent years curcumin has been also demonstrated to be effective in reducing the incidence of inflammation by blocking iNOS expression in several diseases. Along this line, Guzel et al. [94] have reported the protective effect of curcumin (200 mg/kg per day, orally twice daily for 7 days) in a model of different aspiration material-induced lung injury in rats. Curcumin's efficacy is due in part to its effect on reducing the expression of iNOS in pulmonary aspiration material-induced lung injury in rats. Curcumin administration also resulted in a significant reduction of tissue malondialdehyde content and upregulation of antioxidant enzymes, along with increases in the expression of surfactant-D protein levels in lung tissue. Aspiration of gastric contents, one of the major causes of acute lung injury, remains a worry for physicians involved in the care of critically ill patients [120, 121]. Thus curcumin might be beneficial in patients that are critically ill with different health problems with disturbance of consciousness due to drug overdose, and sometimes after aspiration of regurgitated gastric contents in patients with presence of a nasogastric tube, tracheal intubation and mechanical ventilation or impaired upper airway reflexes during induction of anesthesia, or recovery from it.

Based on the strong evidence that curcumin is an anti-inflammatory agent, Moon et al. [122] studied its effectiveness in a model of ovalbumin-induced airway inflammation in mice. In this study, the authors report that curcumin inhibited ovalbumin-induced increase in the production of inducible nitric oxide (iNOS) in lung tissue, inflammatory cytokines, and recruitment of eosinophils to lung airways, and airway hyper-responsiveness to inhaled

methacholine. Besides, exposure of A549 human airway epithelial cells to curcumin reduced IFN-gamma-stimulated iNOS and NO production. The authors concluded that curcumin may be used as an adjuvant therapy to limit airway inflammation through suppression of iNOS and NO.

Bansal and Chhibber [47] investigated the anti-inflammatory and immunomodulatory effect of curcumin in a mouse model of acute inflammation by intranasal introduction of *Klebsiella pneumoniae* B5055 into BALB/c mice. Intranasal administration of bacteria induces a strong activation of neutrophils with increased production of several inflammatory mediators, including nitric oxide. The authors further demonstrate that curcumin administration, 15 days prior to bacterial instillation into the lungs via the intranasal route, showed a significant reduction in neutrophil accumulation in the lungs and a significant decrease in the production of NO levels. Again, this study indicate that curcumin is capable of decreasing acute pneumonia-induced inflammation in mice, implying that curcumin can be used as an adjunct therapy in combination with antibiotics as an anti-inflammatory or an immunomodulatory agent in the case of acute lung infection

12. Effect of Curcumin on Histone Deacetylase

Regulation of transcription is a key event in various biological processes such as cell differentiation, proliferation, and apoptosis, and is highly regulated by numerous factors. One of its regulatory mechanisms is through acetylation of core histones that will permit transcription factors and RNA polymerase to bind to their target DNA and thereby facilitating gene transcription [123, 124]. The degree of acetylation is determined by a dynamic balance between histone acetyl transferase (HAT) and histone deacetylase (HDAC) activity. Histone acetyltransferases (HATs) play a main role in the regulation of gene expression by covalently modifying the N-terminal lysine residues of histones by the addition of acetyl groups from acetyl-CoA. On the otherhand, HDAC play important roles in deacetylating histones through recruitment of various co-repressor molecules, thus regulating gene transcription, protein function and stability. It has been shown that various coactivator molecules, such as CREB-binding protein and p300, can play critical roles in regulation of gene expression, and all of these molecules have built-in histone acetyltransferase (HAT) activity. It is becoming clear that transcription is usually up-regulated as the levels of acetylated histones increases. HDACs are a group of 11 isoenzymes that deacetylate histones and HDAC inhibition results in accumulation of acetylated nuclear histones, as well as nonhistone proteins within the nucleus.

A dysfunction of histone acetyltransferases (HATs) and histone deacetylases is often associated with the increased expression of inflammatory genes in inflammatory lung diseases, including refractory asthma and COPD. Therefore these enzymes are potential targets for anti-asthma and anti-COPD therapy. Importantly, Barnes [125] and Ito et al. [126] have reported that the expression of inflammatory genes is controlled by increased acetylation of histone-4; while HDAC2, in particular, has been associated with the suppression of inflammatory genes. For example, corticosteroids employ HDAC2 to turn off the inflammatory genes.

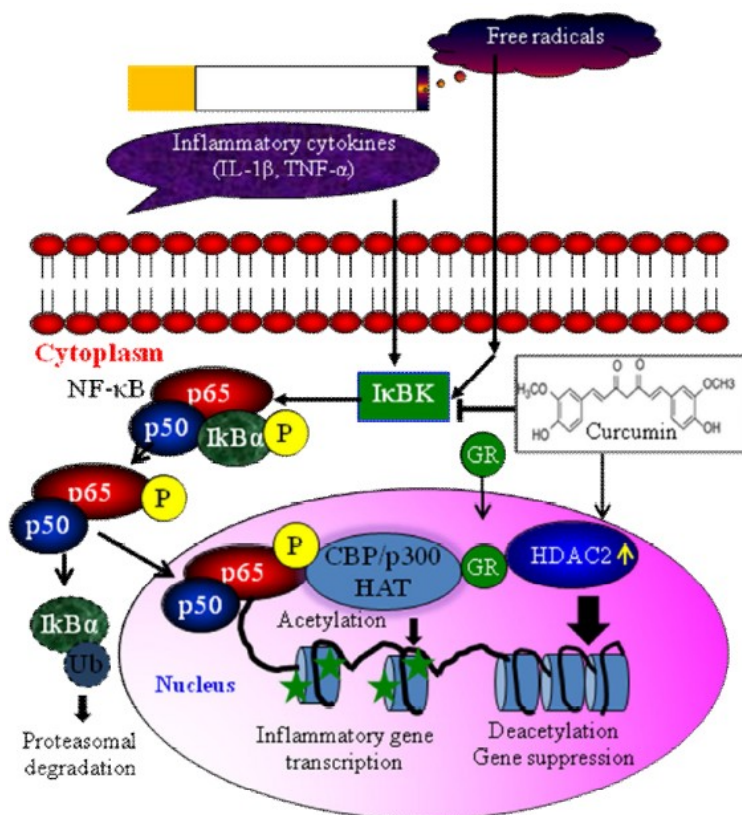


Figure 4. Possible protective effect of curcumin on restoration of histone deacetylase (HDAC) function. Inflammatory cytokines and oxidative stress activate nuclear factor kappa B (NF- κ B) to turn on histone acetyltransferase (HAT) activity causing histone acetylation and subsequent transcription of proinflammatory genes. Curcumin reverses corticosteroid resistance by inhibiting NF- κ B activation and upregulating HDAC2 activity.

Clinical studies on patients with asthma show increased acetylation of histone-4, concomitant with upregulation of various inflammatory genes [127]. Interestingly, COPD patients have an increased acetylation of histones, particularly, in association with the promoter region of inflammatory genes, such as interleukin-8 [128]. In addition, acetylation status of histones has been detected to increase with disease severity in these patients. Importantly, a reduction in HDAC activity in peripheral lung, airways and in alveolar macrophages has been demonstrated in COPD patients. The importance of HDAC activity is highlighted by the fact that the said reduction in HDAC activity has been linked to corticosteroid resistance in COPD patients.

Factors that affect HDAC activity are oxidative and nitrative stress. Increased oxidative and nitrative stress, which has been observed in COPD patients [129-131], leads to peroxynitrite formation, which then attenuates HDAC2 activity. The mechanism by which peroxynitrite produces such inhibitory effects on HDAC2 is based on the demonstration that peroxynitrite causes nitration of certain tyrosine residues on protein. Thus, as a result of protein nitration HDAC2 is poised for ubiquitination and degradation by the proteasome resulting in the reduction of low HDAC2 protein level in the lungs of patients with severe COPD.

Since oxidative and nitritative stress has been implicated in corticosteroid resistance pharmacological agents that disrupt the formation of peroxynitrite may prove to be an effective therapeutic strategy in COPD patients. Curcumin, which inhibits the formation of peroxynitrite, is one such class of therapeutic compounds. In an elegant experiment, Meja and coworkers [132] reported that curcumin at nanomolar concentrations reinstated cigarette smoke extract (CSE)- or oxidative stress-impaired HDAC2 activity and corticosteroid efficacy *in vitro* with an EC₅₀ of approximately 30 nM and 200 nM, respectively. The authors showed that CSE-mediated decrease in HDAC2 protein expression was blocked by curcumin; the reduction in HDAC2 protein expression was reversed by curcumin even in the presence of cycloheximide, a protein synthesis inhibitor. The proteasomal inhibitor, MG132, also blocked CSE-induced HDAC2 degradation, increasing the levels of ubiquitinated HDAC2. Further biochemical and gene chip analysis demonstrated that curcumin (at concentrations up to 1 μ M) exerts its effect via antioxidant-independent mechanisms associated with the phosphorylation-ubiquitin-proteasome pathway. Thus curcumin acts at a post-translational level by maintaining both HDAC2 activity and expression, thereby reversing steroid insensitivity induced by either CSE or oxidative stress in monocytes. Based on these findings the authors concluded that curcumin may be effective in reversing corticosteroid resistance that is common in patients with COPD and severe asthma (Figure 4).

13. Effect of Curcumin on Nuclear Factor- κ B

NF- κ B is a universally expressed family of transcription factors. In mammalian cells, it comprises of dimers of five family proteins namely RelA/p65, c-Rel, RelB, NF- κ B1 (p105/p50) and NF- κ B2 (p100/p52) [133]. NF- κ B forms homo- and heterodimers, with the p50–p65 being the predominant complex observed in cells, and is important for most innate and adaptive immune responses [134, 135]. In its inactive state, NF- κ B is localized in the cytoplasm by bounding to its I κ B α inhibitory protein [136].

NF- κ B is activated by various exogenous and endogenous factors, including inflammatory cytokines and oxidative stress, which in turn phosphorylates I κ B α at Ser32 and Ser36 by I κ B kinases alpha and beta. Phosphorylation of I κ B α leads to a series of biochemical events causing ubiquitination and subsequent degradation of I κ B α by the 26S proteasome complex [137–140]. Degradation of I κ B α unmasks the nuclear localization signals and discharges p50–p65 or p50–c-Rel dimers to translocate to the nucleus and bind DNA, where they regulate the expression of a battery of cellular genes involved in immunity, inflammation, cellular proliferation and apoptosis, including growth factors, cytokines/chemokines, cell adhesion molecules, complement factors, transcriptional regulators, antiapoptotic factors, and immunoreceptors.

A role for NF- κ B activation in acute and chronic lung diseases is becoming increasingly evident in asthmatic inflammation. NF- κ B is activated by factors such as allergen challenge, cytokines, chemokines, bacterial and viral infection, which in turn stimulate the expression of proinflammatory mediators, growth factors, receptors and enzymes that are involved in initiation and perpetuation of inflammatory reactions. NF- κ B activity is strongly activated in the ovalbumin-induced allergic airway disease inflammation: NF- κ B activation occurred chiefly in the epithelial cells of the conducting airways, concomitant with upregulation of NF-

κ B-mediated chemokine genes, including MIP-2 and eotaxin [141]. As a proof of principle studies demonstrated that mice deficient for p50 or c-Rel subunits of NF- κ B were protected from the development of allergic airway disease [142, 143]. In addition, Pantano et al. [144] reported that selective activation of NF- κ B in airway epithelium induces airway hyperresponsiveness and smooth muscle thickening.

When patients with COPD were examined for the expression of NF- κ B as a marker of cellular activation and inflammatory mediator formation, Di Stefano et al. [145] have observed increased expression of p65 protein, the major subunit of NF- κ B, mostly in the bronchial epithelium and the disease severity was associated with an increased epithelial expression of NF- κ B. Likewise, Yang et al. [146] have shown that cigarette smoke induced IL-8 release is associated with activation of NF- κ B activation via IKK and reduction in HDAC levels/activity in macrophages.

Many studies also indicate an important role for NF- κ B in the pathogenesis of acute lung inflammation. Analysis of BAL fluid showed increased NF- κ B activation in alveolar macrophages obtained from patients with ARDS [147]. Rats appeared refractory to the effects of IgG-ICs-induced lung inflammation and NF- κ B activation by depletion of alveolar macrophages with liposome-encapsulated dichloromethylene diphosphonate, suggesting alveolar macrophages may be central to the development of pulmonary inflammation [148]. These animal and clinical findings associate NF- κ B activation to the pathogenesis of asthma, COPD and acute lung injury, and further support the idea that inhibition of NF- κ B activation may provide benefit in the treatment of these lung diseases.

Curcumin has a remarkable NF- κ B-inhibiting activity (Figure 4) and anti-inflammatory effects that has led to its use as a treatment for various disease conditions. Of major importance is the ability of curcumin to block NF- κ B activation [149] and expression of NF- κ B-induced inflammatory mediators that subserve different roles in various pathological processes such as antiapoptosis [Bcl-2, Bcl-xL, X-linked inhibitor of apoptosis protein, and cellular inhibitor of apoptosis protein-1], proliferation (cyclooxygenase-2, cyclin D1, and c-myc), angiogenesis (VEGF and interleukin-8), invasion (matrix metalloproteinase-9), and metastasis (intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and endothelial leukocyte adhesion molecule-1)] [150-152]. Considerable experimental evidence suggests that curcumin prevents alcohol-induced liver disease in rats by both inhibiting NF- κ B activation and NF- κ B-regulated multiple genes such as TNF- α , IL-12, MCP-1, MIP-2, COX-2, and iNOS [153]. In keeping with the established anti-inflammatory role of curcumin, Biswas et al. [80] reported the ability of curcumin to inhibit both cytokine (TNF- α) and oxidative stress (H₂O₂)-induced increase in NF- κ B activation in alveolar epithelial cells (A549). During studies concerning the anticarcinogenic activity of curcumin Shishodia et al. [38] found that curcumin inhibited cigarette smoke-induced DNA binding of activity of NF- κ B in human non-small cell lung carcinoma cells. By using EMSA and NF- κ B-dependent luciferase activity, they also made the interesting observation that curcumin blocked I κ B α kinase activation, I κ B α phosphorylation and degradation, p65 nuclear translocation and CS-induced NF- κ B-dependent reporter gene expression. Curcumin was also shown to suppress NF- κ B-induced inflammatory mediator expression such as cyclooxygenase-2 and matrix metalloproteinase-9. Curcumin, therefore, provided convincing evidence that it may possibly act as an excellent antiinflammatory agent in the lung.

Liu et al. [154] reported some interesting observations in human bronchial epithelial cells that curcumin not only inhibited NF- κ B activity but also sensitized these cells to undergo

apoptosis in response to a low concentration of cigarette smoke extract. Thus, curcumin mediated sensitization of bronchial epithelial cells would prevent from DNA damage that occurs without cell death. These findings are in agreement with reports demonstrating that curcumin inhibition of NF- κ B activity sensitized cancer cells to chemotherapy, inhibited cell growth and induced apoptotic cell death in these cells [155].

In view of the role of microorganisms colonizing the injured airways of smokers, Moghaddam et al. [39] tested the effect of curcumin on lung inflammation and tumor promotion in a COPD-like model of inflammation. COPD-like airway inflammation was induced in mice by repetitive exposure to an aerosolized lysate of non-typeable (unencapsulated) *Haemophilus influenza* (NTHi). In this study, the authors were surprised to note that dietary curcumin did not block NTHi-induced NF- κ B activation, but was effective in suppressing NTHi-induced airway inflammation and lung cancer progression in mice. Therefore, it appears from these observations that additional studies are needed to analyze curcumin-mediated signaling pathways in this NTHi model of airway inflammation.

14. Effect of Curcumin on Signaling Pathways

Intracellular signaling pathways play important roles in cells by conveying environmental signals to the cytoplasm and the nucleus, resulting in altered cellular and gene expression. Various molecular, genetic and pharmacological studies have provided a great understanding of the pathophysiological relevance of signaling pathways in acute and chronic lung diseases. These investigations have resulted in the development of compounds that block several promising therapeutic targets in inflammatory and fibrotic lung injuries. It is well known that kinases play important roles in the expression and activation of proinflammatory mediators leading to structural changes in airways and lung interstitium [156]. It is documented that the 3 mitogen-activated-protein kinase (MAPK), a family of serine/threonine protein kinases, pathways including extracellular signal-regulated kinases 1 and 2 (ERK1/2), the c-Jun N-terminal kinases (JNKs), and the p38 MAPKs (p38 α , p38 β , p38 δ , p38 γ) are involved in the production of different inflammatory cytokines [157]. MAPKs can be induced by a variety of stimuli, for example, ERK1 and ERK2 are preferentially stimulated in response to growth factors and phorbol esters, whereas the JNK and p38 kinases are activated by stress stimuli, including osmotic shock and ionizing radiation and cytokine stimulation.

Early findings demonstrated p38 MAPK as a key regulator of IL-1 β and TNF- α production in human monocytes after lipopolysaccharide treatment [158, 159]. Subsequent investigations reported that stimulation of p38 MAPK controls proinflammatory cytokine production at the transcriptional and post-transcriptional levels [160, 161], projecting p38 MAPK as a therapeutic target to decrease inflammatory cytokine production [162, 163]. While different kinase pathways can turn on particular downstream transcription factors, there is substantial signaling cross-talk between the individual MAPK pathways, as well as cross-talk with secondary messenger-mediated protein phosphorylation pathways. Since multiple cytokines are involved in regulation of inflammation and structural changes basic studies and drug discovery trial approaches have reported that inhibition of signaling pathways would be more effective than targeting one mediator ligand receptor interaction.

Abnormal activation of p38 MAPK pathway has been demonstrated in all asthmatic patients, especially in patients with severe asthma where an association with decreased corticosteroid responsiveness has been suggested [164]. Data also show that costimulation-induced enhancement of ERK signaling promotes the resistance of T cells to glucocorticoids [165]. Overactivation of p38 MAPK, as judged by increased phosphorylation of p38 MAPK, has also been reported in alveolar macrophages of COPD lungs [166]. Moreover, in recent studies it was shown that SD-282, a strong inhibitor of p38- α isoform, inhibits TNF- α release from human lung macrophages in vitro [167], as well as suppresses inflammation in a smoking model of COPD in mice in which corticosteroids are ineffective [168]. There is evidence to suggest that MAPK pathways other than p38 could be involved in regulating the expression of inflammatory cytokines in alveolar macrophages, but not in blood monocytes [169]. A p38 MAPK inhibitor, SB 239063, has also been reported to reduce neutrophilia, inflammatory cytokines, MMP-9, and fibrosis in lung [170]. In addition to these studies, MAPKs are activated and associated with lung injury in patients with idiopathic pulmonary fibrosis. Yoshida et al. [171] have demonstrated increased phosphorylation of ERK, JNK and p38 MAPK in lung homogenates of patients with IPF compared with control subjects. They found activated ERK and JNK in epithelial and endothelial cells, but not in fibroblasts or smooth muscle cells of fibrotic lungs. In this work they also provide evidence to demonstrate increased activation of p38 in epithelial, endothelial, smooth muscle cells, and fibroblasts, thus indicating that MAPKs may be related to the regulation of inflammation and lung injury in IPF.

Although evidence indicates the use of p38 MAPK inhibitors to control inflammation and lung tissue remodeling, adverse side effects are a great concern for long-term use of these inhibitors. Therefore, an alternative strategy is to use natural compounds like curcumin that has shown promising results in the treatment of various proinflammatory and autoimmune conditions. Curcumin has been shown to exert anti-inflammatory and growth inhibitory effects in TNF- α -treated HaCaT keratinocyte cells through inhibition of NF- κ B and MAPK pathways [172]. Although it seems clear, at least from nonpulmonary inflammatory and autoimmune conditions, that curcumin can inhibit MAPK and other inflammatory signaling pathways, currently there is limited evidence concerning curcumin-mediated inhibition of MAPK activation in pulmonary inflammation. Therefore, studies are needed to investigate the role of signaling mechanisms in curcumin-mediated antiinflammatory effect *in vitro* and *in vivo* models of pulmonary inflammation and fibrosis.

The inhibition of p38 MAPK by curcumin might be of crucial importance for the suppression of proinflammatory reactions and gene products. However, curcumin does not always inhibit p38 MAPK activity- it also has been proposed to activate p38 MAPK in the context of promoting apoptotic activities. Polymorphonuclear leukocytes or neutrophils (PMNs) are the body's first line defense against invading pathogens and there is growing appreciation that their biological activities are determined by apoptosis. Dysregulation of neutrophil apoptosis has severe clinical consequences, which is best exemplified in acute lung injury and sepsis, where spontaneous neutrophil apoptosis is delayed [173, 174]. A decrease in neutrophil apoptosis rate is believed to play a role in enhancing tissue injury in sepsis, ARDS by promoting a large neutrophil infiltrate and unabated secretion of toxic metabolites causing tissue injury. Thus, promoting neutrophil apoptosis may provide a means to remove neutrophils from inflammatory areas with minimal injury to the surrounding tissue. In this regard, Hu et al. [175] reported the role of curcumin on both unprompted neutrophil apoptosis

and apoptosis of neutrophils following transmigration across a human lung endothelium-epithelium barrier. They found that curcumin accelerated constitutive neutrophil apoptosis and attenuated the transbilayer migration-induced interruption in neutrophil apoptosis. An interesting phenomenon that has been observed in this study was a marked increase in p38 phosphorylation and caspase-3 activity in the presence of curcumin. Conversely, treatment of p38-specific inhibitor, SB203580, suppressed both curcumin-induced apoptosis and caspase-3 activation. These findings suggest that curcumin could play a beneficial role for the resolution of the acute phase of acute respiratory distress syndrome (ARDS) by stimulating neutrophil apoptosis.

15. Effect of Curcumin on Activator Protein-1

Activator protein-1 (AP-1) is also a key transcription factor that regulates expression of proinflammatory genes both independently and synergistically with NF- κ B. AP-1 is a heterodimeric transcription factor complex of the DNA-binding proteins of the Fos (c-Fos, FosB, Fra1, and Fra2) and Jun (c-Jun, JunB, and JunD) family proteins [176]. AP-1 can be activated by proinflammatory cytokines such as TNF- α , growth factors, neurotransmitters and other intracellular signaling proteins. AP-1 regulates various cellular processes; including cell proliferation, apoptosis, and inflammation, wound healing, and cancer. AP-1 has also been proposed as a contributor to the inflammatory process by binding to glucocorticoid receptor and preventing its interaction with glucocorticoid response elements (GRE) and other transcription factors, leading to glucocorticoid resistance in asthma. Clinical studies from patients with glucocorticoid-resistant asthma indicated increased activation of JNK with higher expression of c-Fos in peripheral blood monocytes and bronchial biopsy specimens compared to samples from patients with glucocorticoid-sensitive disease [177]. Recent studies [178] in human lung type epithelial cells (A549) and normal human bronchial epithelial (NHBE) cells suggest that ox-AT (oxidatively modified α_1 -anti-trypsin) increased NF- κ B and AP-1 activity and promoted activation of JNK. Besides, suppression of the NF- κ B and the JNK/AP-1 pathways decreased production of IL-8 and MCP-1. AT is a major elastase inhibitor within the lung. Oxidation of critical methionine residues in AT generates ox-AT, which has a greatly diminished ability to inhibit neutrophil elastase. These findings suggest an important function for AP-1 in mediating the proinflammatory effect of ox-AT. Exposure of cigarette smoke extract induces nuclear expression of c-Jun and c-Fos, the two main AP-1 proteins, in human lung fibroblasts [179].

Furthermore, Eferl and colleagues [180] demonstrated a profibrotic role for the Fos-related protein Fra-2. Their studies show that ectopic expression of Fra-2 in transgenic mice caused fibrosis in many organs but predominantly in the lung. Various growth factors, chemokines, and cytokines associated with human pulmonary fibrosis were strongly expressed in fibrotic lungs of transgenic mice. Of interest, human lung specimens of pulmonary fibrosis with IPF and non-specific interstitial pneumonia (NSIP) showed strong immunoreactivity for Fra-2.

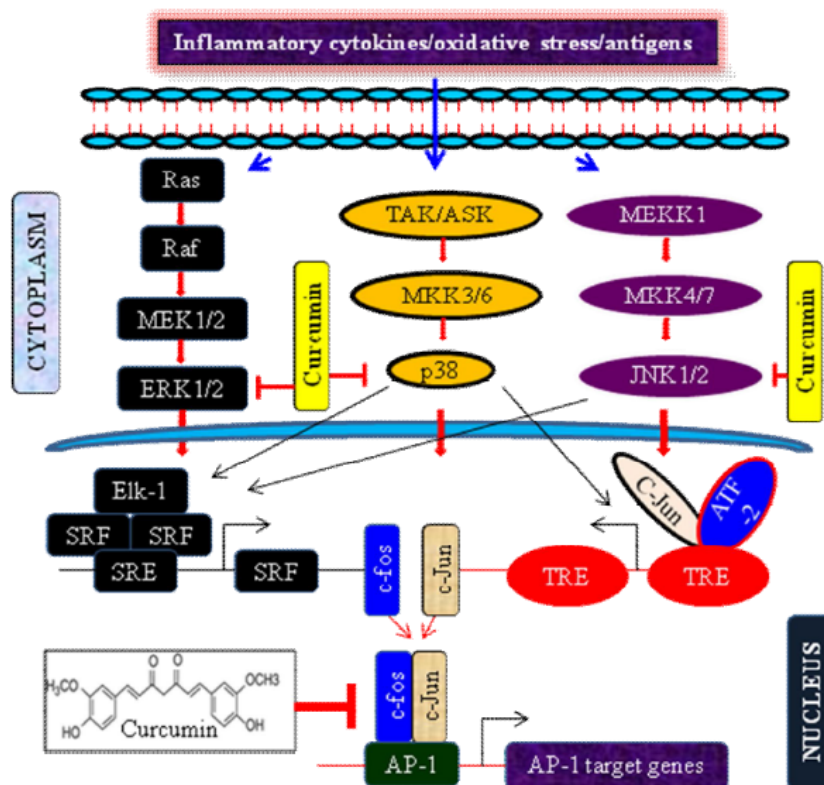


Figure 5. Possible mechanisms of modulation of activator protein-1 (AP-1) signaling pathways by curcumin. Proinflammatory cytokines, oxidative stress and other stimulus activate AP-1 by several mechanisms including through the activation of mitogen-activated protein kinase (MAPK) pathways. The MAPKs involved in the regulation of AP-1 are the extracellular signal-regulated kinases (ERK), the c-Jun NH2-terminal kinases (JNK), and p38. Serum response element (SRE), is bound by transcriptional factor complexes of serum response factor (SRF) and Elk-1. Elk-1 is activated by phosphorylation *via* ERK, JNK and p38 MAPKs, resulting in SRE-dependent *c-Fos* expression. Activation of JNK leads to the phosphorylation and activation of c-Jun and ATF-2, which then bind to TPA responsive element (TRE) to activate *c-jun* transcription. p38 activation phosphorylates and activates ATF-2. c-Fos forms heterodimer with c-Jun and binds to AP-1 response element to regulate target genes.

Although curcumin has been implicated in processes involving suppression of NF- κ B activation and expression of NF- κ B regulated gene products, little is known about the effect and mechanism of curcumin in the regulation of AP-1 (Figure 5) in inflammatory and fibrotic lung disease. Earlier studies from our laboratory showed that curcumin inhibited amiodarone-induced increases in c-Jun protein expression. Recently, Chen-Tzu Kuo et al. [181] have suggested that curcumin is involved in denbinobin (5-hydroxy-3,7-dimethoxy-1,4-phenanthraquinone)-mediated apoptosis in human lung adenocarcinoma cells via AP-1 activation, as curcumin treatment inhibited denbinobin-mediated apoptosis. Another study by Hu and coworkers [182] showed that curcumin inhibited silica induced plasminogen activator inhibitor-1 expression through an AP-1-dependent mechanism in human lung epithelial cells. Furthermore, inhibiting c-Jun N-terminal kinase (JNK) signaling by curcumin was demonstrated to block inflammation-associated alterations of hepatobiliary transporter gene expression in hepatocytes [183].

Oxidants and inflammatory cytokines like tumor necrosis factor- α (TNF- α) activate transcription factors such as activator protein-1 (AP-1), which is involved in the regulation of inflammatory responses. Might blocking AP-1 suppress the expression of inflammatory mediators? Biswas et al. [80] investigated this possibility in cultured alveolar epithelial cells (A549). The authors examined the role of curcumin on AP-1 activation using hydrogen peroxide (H_2O_2 ; 100 μM) and TNF- α (10 ng/ml) as a model. While treatment of A549 cells with hydrogen peroxide (H_2O_2 ; 100 μM) and TNF- α (10 ng/ml) significantly increased AP-1 activation and IL-8 release, curcumin repressed both H_2O_2 - and TNF- α -induced activation of AP-1, and IL-8 release, supporting an antiinflammatory role for curcumin in lung derived cells.

Bradykinin (BK) is an inflammatory nonapeptide that exerts proinflammatory effects in asthma by stimulating expression of proinflammatory cytokines, chemokines, prostanoids, and growth factors in a variety of cell types [184, 185] via AP-1 transcription factor. An obvious way to block airway inflammation and bronchoconstriction in asthma is to inhibit this transcription factor. In this study, Chien-Da Huang and colleagues [186] show that the AP-1-dependent pathway is required for BK-induced gene expression, as demonstrated by the ability of the curcumin to abrogate BK-induced IL-6 expression in human tracheal airway smooth muscle cells.

16. Effect of Curcumin on Growth Factors and Fibrotic Process

Besides production of proinflammatory cytokines, lung injury results in production and secretion of growth factors [70]. By regulating cell differentiation and activation, growth factors such as TGF- β are of clinical significance in the pathogenesis of fibrosis. The TGF- β comprises of three major isoforms, TGF- β 1, TGF- β 2 and TGF- β 3, which are 60–80% homologous to each other [187]. Of all the TGF- β isoforms, TGF- β 1 is the most powerful profibrotic [188] cytokine because (a) it stimulates the production of extracellular matrix proteins (b) it downregulates the expression of matrix degrading metalloproteinases (MMPs) and (c) it upregulates the synthesis of protease inhibitors such as tissue inhibitor of metalloproteinases. TGF- β 1 is of major importance in the transdifferentiation of fibroblasts to myofibroblasts (Figure 3). Myofibroblasts are judged as marker cells of fibrogenic process that express increased levels of α -smooth muscle actin [189]. The profibrotic effects of other cytokines such as IL-13, CTGF, endothelin, monocyte chemoattractant protein 1, and TNF- α are linked with TGF- β 1 function. In general, these cytokines exhibit fibrotic action by inducing TGF- β pathway [190-195].

TGF- β 1 is normally expressed by epithelial cells and fibroblasts, but is also highly expressed during lung injury and inflammation in other cells such as macrophages and mesenchymal endothelial and mesothelial cells of the lung [188], suggesting a role in disease process. In animal models of fibrosis and human lung pathology specimens TGF- β 1 is increased and upregulation of TGF- β 1 mRNA and protein content occur before any increase in ECM synthesis bleomycin-induced pulmonary fibrosis [196]. Consistent with a profibrotic role, Raghu et al. [197] reported that TGF- β 1 stimulated collagen synthetic activity of fibroblasts derived from normal and fibrotic human lungs. In addition, overexpression of

active TGF- β 1 induces severe and prolonged fibrosis [198]. Conversely, anti-TGF- β antibodies and TGF- β soluble receptors were effective in inhibiting excess production of ECM proteins.

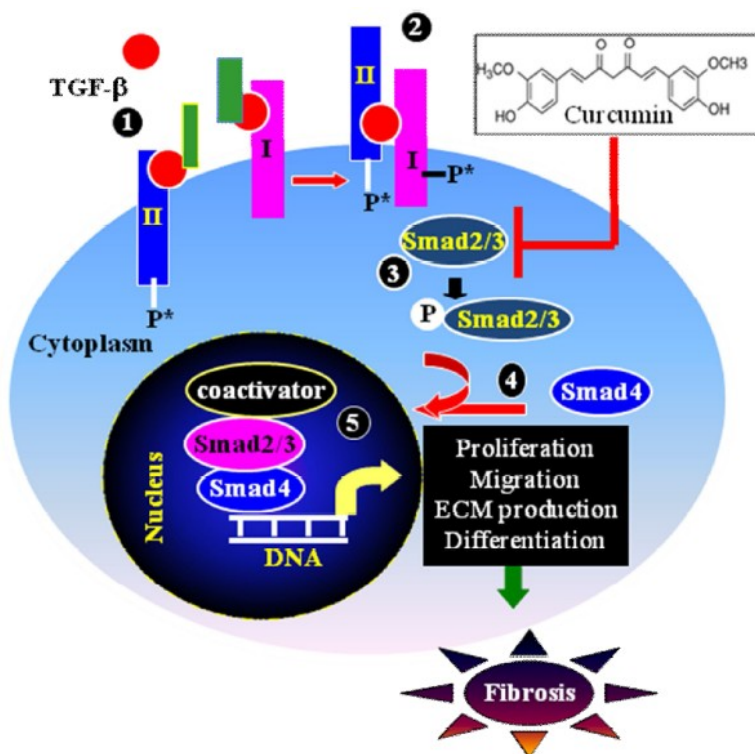


Figure 6. Lung injury results in increased TGF- β 1 expression, which induces fibrosis by activating extracellular matrix protein production, fibroblast proliferation and differentiation into myofibroblasts. Active TGF- β binds to its type II transmembrane receptor with serine-threonine kinase activity, which allows dimerization with TGF- β type I receptor and its phosphorylation. This transduces signals through recruitment of cytoplasmic proteins called Smads. Smads 2 and 3 (receptor Smads, R-Smads) are mediators of TGF- β signaling. Smad4 (Co-Smad) forms heterooligomers with R-Smads and translocates to the nucleus where they regulate the transcription of various genes involved in fibrotic process. Curcumin inhibits phosphorylation of Smads2/3 and thereby attenuates downstream pathology.

The expression of TGF- β 1 is also altered in inflammatory lung diseases, including allergic asthma and COPD. Whereas asthma is characterized by chronic inflammation and airway remodeling associated with the increased subepithelial deposition of extracellular matrix proteins [199], COPD is characterized by airway inflammation with airway wall remodeling and peribronchiolar fibrosis. Emphysema is characterized by the destruction of alveolar architecture, including inflammation, alveolar epithelial cell injury/apoptosis, and increased production of extracellular matrix (ECM) degrading proteases. In asthmatic disease TGF- β 1 is increased in a wide variety of cells, including fibroblasts, smooth muscle cells, eosinophils, macrophages and the airway ECM, with variable expression associated with epithelial cells; importantly, TGF- β 1 expression is increased in eosinophils and macrophages [200-202].

There is evidence for increased TGF- β 1 expression in the airway epithelium of smokers and in patients with COPD [203, 204]. In addition, de Boer et al. [205] have shown increased TGF- β in the small airway epithelium of COPD patients, and that its levels correlated with the severity of obstruction. Also, these authors suggested a role for TGF- β 1 in recruiting macrophages into the airway epithelium in COPD. In COPD patients plasma levels of TGF- β 1 are higher [206]. Interestingly, TGF- β 1 seems to play a role in COPD, where genetic studies have discovered TGF- β 1 as a potential link with COPD [207, 208].

The cellular effects of TGF- β 1 is conveyed by means of three types of cell surface receptors (types I, II, III), two of which are transmembrane serine/threonine kinases (Figure 6). TGF- β signaling [187, 209, 210] initiates when the TGF- β ligand binds to the TGF- β type II receptor (TGF- β RII), with subsequent recruitment of its type I receptor, activin receptor-like kinase-5 (ALK5). Phosphorylation of the type I receptor by the type II receptor activates Smad transcription factors resulting in Smad2 and Smad3 phosphorylation. Phosphorylated Smad2 and Smad3 (R-Smads) form heterodimeric and heterotrimeric complexes with Smad4 (Co-Smad) that accumulate in the nucleus and control expression of target genes in a manner specific to type I receptor and cell-type. The inhibitory Smads (Smad6 and Smad7) antagonize signaling by preventing R-Smad phosphorylation and/or by interfering with the R-Smad/Co-Smad complex. Hence, Smad family proteins are critical components of the TGF- β signaling pathway [187]. Besides, TGF- β also activates additional non-Smad pathways, including p38 MAPK pathway [211, 212]. From the above findings it is clear that TGF- β 1 activation and expression are common features in the progression of fibrosis in IPF, asthma and COPD, indicating that anti-TGF- β 1 compounds should be considered to treat these disease conditions.

The first suggestion that curcumin had any efficacy against fibrotic process came from our laboratory observations on bleomycin-induced lung injury in rats [36]. As a first step in investigating the antifibrotic property of curcumin, we demonstrated that curcumin inhibited dose-dependently the increased levels of collagen in bleomycin-fibrotic lung. This provided stimulus for extending its beneficial effects in another model of lung fibrosis, which showed that curcumin can reduce the expression of collagen type I and TGF- β 1 protein expression in rats with amiodarone-induced pulmonary fibrosis [37].

Do these *in vitro* and animal findings have any clinical relevance to the effects of curcumin on pulmonary fibrosis? The work performed by Smith and coworkers [46] to investigate the molecular mechanism of action of curcumin (Figure 6) enhances our understanding of curcumin's antifibrotic properties. This study clearly indicates that curcumin (i) inhibits proliferation of lung fibroblasts from normal subjects and patients with IPF (ii) inhibits myofibroblast differentiation (iii) inhibits TGF- β 1-induced phosphorylation of Smad 2/3 and ERK1/2 and collagen production by IPF fibroblasts. Importantly, there is evidence that *in vivo* curcumin administration significantly inhibited bleomycin-induced inflammation and collagen deposition together with a tendency to improve survival of bleomycin-instilled mice [46].

17. Challenges and Future Prospects

One of the limitations of use of curcumin is low oral bioavailability due to its metabolism through conjugation and reduction. More research is required before curcumin becomes therapeutically practical, in particular studies to enhance its bioavailability. Importantly, chemical modification of curcumin could allow the creation of structural analogs or formulations that are slowly susceptible to physiological disposition, thus increasing its bioavailability. As suggested by Aggarwal and Sung [213], curcumin can be formulated as liposomes or nanoparticles. Some investigators have found that curcumin is rather slowly eliminated when given together with piperine, with increased serum concentrations of curcumin and decreased clearance rate in rats [214]. These investigators have found that increase in serum levels of curcumin is also feasible in patients with the combined use of piperine and curcumin.

Interestingly, Anand and coworkers [215] very recently demonstrated that curcumin-loaded nanoparticles formulation had increased cellular uptake, and enhanced bioactivity *in vitro* and superior bioavailability *in vivo* over curcumin. Their results demonstrated that curcumin nanoparticles were either equipotent or more potent than curcumin in inducing apoptosis of leukemic cells and in inhibiting proliferation of various tumor cell lines. Also, compared to curcumin alone, curcumin nanoparticles strongly inhibited TNF- α -induced NF- κ B activation and suppressed NF- κ B-regulated proteins involved in cell proliferation (cyclin D1), invasion (MMP-9), and angiogenesis (VEGF). Finally, in mice, curcumin nanoparticles were more bioavailable and had a longer half-life than curcumin. These findings reinforce the idea that there is substantial room for improving bioavailability of curcumin.

Whereas curcumin is probably most frequently employed in the treatment of cancers, its use in the treatment of acute and chronic lung diseases are limited. While it is generally agreed that curcumin reduces inflammation, oxidative stress and fibrotic process, its mode of action in lung diseases is still not clearly understood. Further discoveries may bring to light new characteristics pertaining to the therapeutic properties of curcumin in pulmonary inflammation and fibrosis. Meticulous selection of animal models and analysis of laboratory observations must be executed in the manner in which the experimental models represent human inflammatory and fibrotic lung disease. Eventually, an objective of basic scientists who work in this area is to translate *in vivo* in humans from *in vitro* and animal data. An important step in this direction will be to collaborate with clinicians to develop strategies to evaluate the health benefits of curcumin in patients with lung disease.

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Chapter XVI

Garlic and its Health Claims: Molecular Aspects

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Abstract

The advent of modern pharmacology and recent analytical techniques resulted in remarkable achievements in exploring the role of botanical/herbs for public health improvement. Phytochemicals especially bioactive molecules present in plants are associated with modulation of some metabolic processes including inflammation and free radical production that are obligatory for maintaining human health. Inflammation, the second defense line system, and oxidative stress are also causative agents for the onset of various ailments i.e. cancer, cardiovascular disorders (CVD), multiple sclerosis, Alzheimer's, Parkinson's, aging and associated degenerative disorders. In the modern era, concerted efforts have been made to use diet based strategy as an intervention with an ultimate goal to control various maladies. Amongst these, garlic holds unique position to cure physiological threats attributed to its rich phytochemistry mainly dependent on sulfur containing compounds including allicin, thiosulfinates and their derivatives i.e. diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS), and ajoene. Some garlic preparations e.g. age garlic extract (AGE) are also effective in reducing the extent of several disorders. The health claims of garlic include protection against bacterial and viral infections, oxidative stress, cardiovascular complexities, cancer insurgence, and immune dysfunction. The current chapter is an attempt to synchronize the molecular basis of health promoting potential of garlic. In this context, cardio-protective effect of garlic with special reference to mechanism of cholesterol synthesis in the liver and concurrent inflammatory response has been discussed. Furthermore, its dose

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dependent effect on CVD risk factors including LDL oxidation, homocysteine, coronary arterial calcification (CAC), platelet aggregation, and acute myocardial infarction are described herein. Moreover, debate has been made on antioxidant potential of garlic and modulation of anti-oxidative enzymes through increased expression of signaling cascades. The anticancer perspectives are the limelight of the book chapter and various modules/mechanism has been discussed i.e. inhibition of carcinogen activation/formation, boost phase-II detoxifying processes, cause cell cycle arrest mostly in G2/M phase, stimulate the mitochondrial apoptotic pathway, increase acetylation of histones, and suppression of tumor proliferation through modulation of signaling pathways. Lastly, discussion and future research agenda have been presented for meticulousness of the issue.

Background

The increased medical care cost and human's desire to maintain good health through out the life span resulted in greater attention of nutritionists and public health scientists to expedite diet-health linkages. The outcomes of such studies indicated that dietary module holds promise to cure various ailments especially cardiovascular, diabetes mellitus, cancer insurgences, and immune dysfunction. However, such strategies need thorough investigations to explore nutrients dense sources, potential application, and indeed their mode of action [1]. The nature bestowed humans with ability to fight against invading cells and malfunctioning of the body owing to disturbances in metabolism. The immune system mainly comprised of three defense systems i.e. front line of defense include physical and chemical barriers like skin and mucosal membranes that inhibit the entry of the foreign particles and organisms [2], second series of defense often termed as inflammation characterized with immune cells e.g. phagocytes. There are certain chemicals that are involved in inflammatory responses also fall under this category. Third defense line comprised of complex mechanism involving specific cells, antibodies, antigens, etc. However, their specialties hide in recognizing the kind of pathogen as immune system produces cells/chemicals that are precise for particular pathogen or to carryout define functions [3].

The influence of diet in improving the body metabolism is of considerable importance as dietary molecules especially from plant source gained immense significance from immemorial times to cure various pathologies and their mechanism of action varies, depending upon source and type of disorders. However, plants or their bioactive molecules possess the ability to target at molecular level are more effective and molecular aspects include enzyme action and kinetics, cytokines release, and signal transduction pathways [4]. Array of plants and their bioactive molecules have been shown to hold pharmacological actions that include garlic, ginger, black cumin, fenugreek, ginseng, guar gum, cruciferous vegetables, berries, etc. The medicinal uses of herbs have been proven as lifesaving entities [5-6] and associated health claims are due to their rich phytochemistry [7]. Phytochemicals like Ω -3-fatty acids, dietary fibers, antioxidant, vitamins, plant sterols and flavonoids are helpful in reducing the risk of various ailments. They play a decisive role in health maintenance and promotion. Many disease processes such as diabetes, heart disease, autoimmunity, and cancer arise from defects in signal transduction pathways, further highlighting the critical importance of this mechanism [8-9].

Garlic (*Allium Sativum*) is commonly used in culinary practices for adding flavor and taste to the final product. However, garlic as whole and its components/fractions are used in medicines since long and early record depicts its presence in Chinese medicines 3000 years ago. The Egyptians fed garlic to the pyramid crews to boost their immunity thereby render safe from various maladies and improve their performance [5] and even some historians proved garlic's utilization some 5000 years ago in Indian medicines [10]. It acquired a reputation in the folklore of many cultures over the centuries as a prophylactic and therapeutic agent. The health benefits of garlic have been proven and its use as a dietary supplement is recommended in many countries. Nowadays, dehydrated garlic and its extracted oil are gaining popularity as they are being sold as dietary supplement [11-12].

The garlic organosulfur components are mainly responsible for its health promoting properties. Hundreds of research articles have been published in the last few decades aimed to explore the effects of garlic consumption against number of maladies. Healthful properties of garlic are established and its functional activities include free radical scavenging activities, immune stimulation, curing cardiovascular diseases, anti-cancer, and anti-infectious properties, etc. [13]. Its potential in combating lifestyle related disorders like hypercholesterolemia, dyslipidemia, and high blood pressure has been the focus of major research interventions. In many countries, health claims of garlic or its various preparations have been approved and are now available in the market as dietary supplements. Evidences also ensure its possible applications in cancer therapy, degenerative disorders, and certainly in immunonutrition [14-15]. This book chapter is also an attempt to elucidate the importance of garlic and its constituents against various ailments. The special attention has been paid to anti-cancer perspectives at molecular level especially modulation of carcinogen activation, induction of phase 2 enzymes, inhibition of post-translational modification and angiogenesis, induction of apoptosis, inhibition of cell cycle progression, and histone acetylation [16-18].

Chemistry of Garlic

The nutritional composition of garlic (raw and powder garlic) has been given in Table 1. Briefly, raw garlic contains water, carbohydrate, and proteins in the amounts of 58.58, 33.06, and 6.36gm/100 gm, respectively. The consumption of 100 grams of garlic provides 149 kilocalories [19]. Carbohydrates and proteins are the major components of garlic powder accounting for more than 80%, whilst its 100 grams consumption provides 331 kilo-calories. Among vitamins, thiamin is of special consideration with high bioavailability owing to some specific sulfur containing components. Regarding minerals, selenium needs special interest as its level is at least 9 ppm (parts per million); higher than almost all other plants. Appropriate selenium intake has been reported to be associated with reduced risk of prostate, rectal, and lung cancers [20-21].

The sulfur containing compounds present in garlic are of special attention as they contribute 2.3% to overall composition. Among organosulfur compounds, thiosulfates, vinylthiins, ajoenes, and water-soluble organosulfur compounds are of significance importance Allicin (diallyl thiosulphate), the principle active substance in garlic, is responsible for its typical pungent smell [5, 22].

The detailed chemistry of garlic is not yet to be discovered completely owing to large number of chemical reactions that may proceed when garlic is being processed for culinary or medicinal purposes.

Table 1. Nutritional composition of garlic

Nutrient	Units	Raw Garlic	Garlic Powder
Proximate components			
Water	g	58.58	6.45
Energy	kcal	149	331
Energy	kJ	623	1386
Protein	g	6.36	16.55
Total lipid (fat)	g	0.50	0.73
Ash	g	1.50	3.54
Carbohydrate, by difference	g	33.06	72.73
Fiber, total dietary	g	2.1	9.0
Sugars, total	g	1.00	2.43
Minerals			
Calcium, Ca	mg	181	79
Iron, Fe	mg	1.70	5.65
Magnesium, Mg	mg	25	77
Phosphorus, P	mg	153	414
Potassium, K	mg	401	1193
Sodium, Na	mg	17	60
Zinc, Zn	mg	1.16	2.99
Copper, Cu	mg	0.299	0.533
Manganese, Mn	mg	1.672	0.979
Selenium, Se	mcg	14.2	23.9
Vitamins			
Vitamin C, total ascorbic acid	mg	31.2	1.2
Thiamin	mg	0.200	0.435
Riboflavin	mg	0.110	0.141
Niacin	mg	0.700	0.796
Pantothenic acid	mg	0.596	0.743
Vitamin B-6	mg	1.235	1.654
Choline, total	mg	23.2	67.5
Vitamin A, IU	IU	9	
Lutein + zeaxanthin	mcg	16	
Vitamin E (alpha-tocopherol)	mg	0.08	0.67
Vitamin K (phylloquinone)	mcg	1.7	0.4
Lipids			
Fatty acids, total saturated	g	0.089	0.249
Fatty acids, total monounsaturated	g	0.011	0.115
Fatty acids, total polyunsaturated	g	0.249	0.178
Amino acids			
Tryptophan	g	0.066	0.121
Threonine	g	0.157	0.374
Isoleucine	g	0.217	0.414
Leucine	g	0.308	0.728
Lysine	g	0.273	0.768

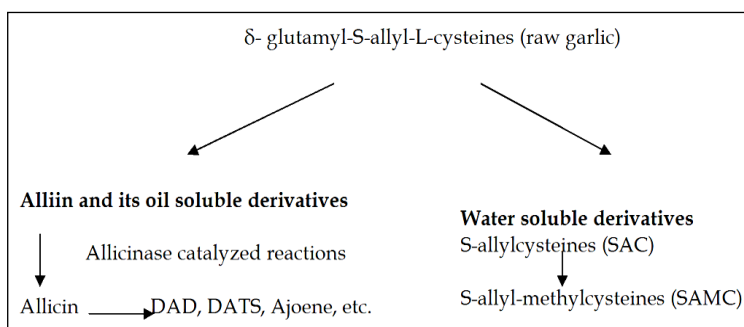
Nutrient	Units	Raw Garlic	Garlic Powder
Methionine	g	0.076	0.111
Cystine	g	0.065	0.253
Phenylalanine	g	0.183	0.525
Tyrosine	g	0.081	0.445
Valine	g	0.291	0.667
Arginine	g	0.634	3.365
Histidine	g	0.113	0.263
Alanine	g	0.132	0.485
Aspartic acid	g	0.489	1.890
Glutamic acid	g	0.805	3.658
Glycine	g	0.200	0.525
Proline	G	0.100	1.354
Serine	G	0.190	0.505

USDA National Nutrient Database for Standard Reference, Release 22 (2009).

The detailed chemical reactions are often coupled with complicated processes that are out of scope for this book chapter. However, the systematic changes that occur are described in Box-1. The phytochemicals present in garlic and its products usually categorized into two broader categories i.e. water soluble and fat-soluble components. Accordingly, Diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide and allyl methyl trisulfide, dithiins, and ajoene are fat-soluble whereas S-allyl cysteine (SAC), S-allyl mercaptocysteine (SAMC), S-methyl cysteine, and gamma-glutamyl cysteine derivatives are water-soluble in nature [23-25]. The chemical components present in both fractions are performing distinct functions. Fat-soluble fractions are mainly responsible for specific odor, aroma, and pungency of garlic. In comparison, water-soluble components are considered stable and safer (Table 2).

The major sulfur-containing compounds in intact garlic are δ - glutamyl-S-allyl-L-cysteines and S-allyl-L-cysteine sulfoxides (alliin). In natural aging process, gamma-glutamyl cysteine is converted to number of water-soluble compounds e.g. S-allyl cysteine (SAC), S-allyl mercaptocysteine (SAMC), and S-methyl cysteine derivatives.

Box 1. Systematic flow-diagram of biochemical reactions occurring in garlic during crushing and processing



However, when garlic is crushed or chopped, the membranes release some enzymes that transform alliin into allcin. It is interesting to note that most health claims of garlic are attributed to allcin but in fact, enzymatic conversion of allcin takes place to yield pungent

constituents and are mostly fat-soluble sulfur compounds. Therapeutic properties of garlic often attributed to allicin and its derivatives [5, 22-25].

Table 2. Classification of phytochemicals present in garlic

SR #	CLASS OF COMPOUNDS	EXAMPLES	CHARACTERISTICS
1	Oil-soluble compounds	Diallyl sulfide (DAS) Diallyl disulfide (DADS) Diallyl trisulfide Allyl methyl trisulfide Dithiins and ajoene	1. Odorous and pungent 2. Less stable and less safer if used in excess 3. Contains sulfur as
2	Water-soluble compounds	S-allyl cysteine (SAC) S-allyl mercaptocysteine (SAMC) S-methyl cysteine Gamma-glutamyl cysteine	Odorless Stable and safer if used in excess
3	Non-sulfur containing components	Allixin Steroid saponins	Non-pungent in nature

Source: Butt et al., (2009).

However, there exist some variations e.g. when garlic is extracted with an aqueous solution, the δ -Glutamyl-S-allyl-L-cysteines is converted into S-allylcysteines (SAC) through an enzymatic transformation with δ -glutamyltranspeptidase. These compounds act as precursors of several complexes. As stated earlier, the complexity of chemical reaction is difficult to understand but usually sulfur-containing components can be classified into different categories [26] described in Table 3. The complex chemistry of garlic makes it plausible that variations in processing can yield quite different preparations [27].

Thiosulfinates. When garlic bulbs are disrupted, it results in the formation of thiosulfinates such as allicin through the enzymatic reaction of sulfur-substituted cysteine sulfoxides with alliinase via sulfur-substituted sulfenic acids (highly reactive intermediate). Other thiosulfinates found in garlic are, allylmethyl-, methylallyl- and trans-1-propenyl-thiosulfinate and like allicin, all are unstable [28].

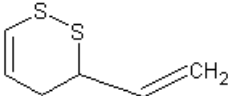
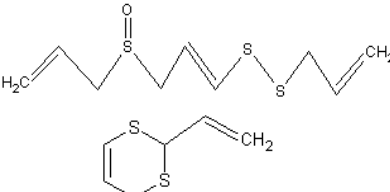
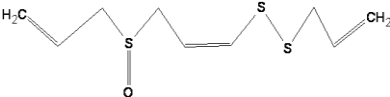
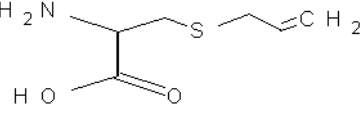
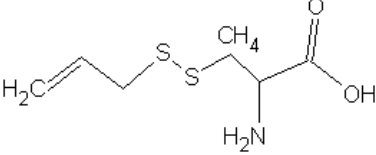
Organosulfur volatiles. Allicin usually decomposes to volatile constituents that include diallyl disulfide (DADS), diallyl sulfide (DAS), diallyl trisulfide (DTS), methylallyl disulfide, methylallyl trisulfide, vinyl dithiins, and ajoenes. Some amount of sulfur dioxide is also released during the decomposition reactions. The volatile constituents are also present in steam-distilled garlic oil and organic extracts of garlic but their concentrations vary with source. It is worth mentioning that main sulfides in garlic oil are DAS (57%), allylmethyl (37%), dimethyl (6%), mono- to hexasulfides with a meager amount of allyl 1-propenyl, methyl 1-propenyl di-, tri-, and tetrasulfides [27, 29].

Vinyldithiins. These are thermo-degradable products of allicin. Their structures were elucidated to be 2-vinyl-4H-1, 3-dithiin and 3-vinyl-4H-1, 2-dithiin based on spectroscopic studies. The formation mechanism is a type of Diels-Alder dimerization of thioacrolein derived from the β -elimination of allicin. Vinyldithiins, especially 2-vinyl-4H-1, 3-dithiin are rich in the oil macerate of raw garlic [30].

Ajoenes. Their structures are E and Z isomers of 4, 5, 9-trithiadodeca-1-oxide, 6, 11-triene-9-oxide formed by S-thioallylation of allicin, followed by copetype elimination and re-

addition of 2-propenesulfenic acid. Likewise, E-4,5,9-tritriadeca-1,7-diene-9-oxide, is another ajoene-type organosulfur compound, present in oil-macerated garlic extract [31].

Table 3. Sulfur containing constituents of garlic

Components	Compounds	Abbreviation	Chemical Formula/Structure
Thiosulfinates	Allicin		$\text{CH}_2=\text{CHCH}_2\text{-SS(=O)-CH}_2\text{CH=CH}_2$
	Diallyl sulfide	DAS	$\text{CH}_2=\text{CH-CH}_2\text{-S-CH}_2\text{-CH=CH}_2$
	Diallyl disulfide	DADS	$\text{CH}_2=\text{CH-CH}_2\text{-S-S-CH}_2\text{-CH=CH}_2$
	Diallyl trisulfide	DATS	$\text{CH}_2=\text{CH-CH}_2\text{-S-S-S-CH}_2\text{-CH=CH}_2$
	Allylmethyl sulfide	AMS	$\text{CH}_2=\text{CH-CH}_2\text{-S-CH}_3$
	Allylmethyl disulfide	AMDS	$\text{CH}_2=\text{CH-CH}_2\text{-S-S-CH}_3$
	Allylmethyl trisulfide	AMTS	$\text{CH}_2=\text{CH-CH}_2\text{-S-S-S-CH}_3$
Vinylthiins	2-vinyl-4H-1,3-dithiin		
	3-vinyl-4H-1,2-dithiin		
Ajoene	E- Ajoene		
	Z- Ajoene		
Water-soluble organosulfur compounds	S-allyl-L-cysteines	SAC	
	S-allylmercaptocysteine	SAMC	

* Critical Reviews in Food Science and Nutrition [5].

Water-soluble organosulfur compounds. Aqueous and alcoholic garlic extracts contain mainly S-allyl-L-cysteines (SAC) derived from δ -glutamyl-S-allyl-L-cysteines. SAC and trans-S-1-propenyl-L-cysteine, together with small amount of S-methyl-L-cysteine are found in garlic extract such as AGE [32].

There are number of reports suggesting the health promoting potential of garlic and its functional ingredients including antioxidant, anti-inflammatory, and anticancer perspectives [5]. The insight regarding these potential benefits are described herein.

Cardioprotective Effects of Garlic

The prevalence and pathogenesis of cardiovascular complexities has been increased in last few decades and now considered leading cause of morbidity and mortality. The coronary complexities associated with risk factors including high cholesterol, high homocysteine, hypertension, and inflammation can be tackled with healthy diet and physical exercise [33, 34].

Cardioprotective properties attributed to garlic and its functional ingredients include lowering of cholesterol and blood pressure, inhibition of cell proliferation and platelet aggregation, and enhance secretion of nitric oxide in diseases states [35]. Moreover, garlic is effective in reducing the homocysteine levels and coronary arterial calcification [5].

A. Cholesterol Lowering Potential

Management of plasma cholesterol levels continues to be a cardinal issue in cardiovascular disease (CVD) prevention as hypercholesterolemia plays crucial role in pathogenesis of heart diseases [13]. Different drugs can be used as treatment but concerning length of therapy and their reduced influence with the passage of time, natural products may act as suitable demulcent [16].

Garlic has been used as a traditional medicine to improve the lipid profile [36] and its supplementation hold favorable effect on cholesterol, LDL-cholesterol, and HDL-cholesterol, also effective in reducing blood pressure and oxidative stress in hypertensive individuals [37] in a dose dependent manner. Earlier, meta-analysis was conducted by Warshafsky et al. [38] and they recorded about 9% reduction in total cholesterol as a function of 1.5-3.0 grams daily consumption of garlic. Later, Tapsell *et al.*, [7] while elaborating health benefits of herbs further validated these results. Ackermann *et al.*, [39] observed that garlic preparations might lead to small reductions (1.2-17.3 mg/dL and 12.4-25.4 mg/dL) in total cholesterol level after 1 and 3 months respectively. Recently, Reinhart *et al.*, [40] conducted meta-analysis to study the effect of garlic on lipid parameters and observed that garlic significantly reduced total cholesterol and triglycerides. Aged garlic extract owing to SAC and SAMC holds the ability to improve the lipid profile. In this regard, Steiner et al. [41] reported that total cholesterol decreased by 6.1 to 7.0% due to aged garlic extract @ 7.2 g/day. Their findings also suggested 4% decrease in low-density-lipoprotein and 5% decrease in systolic blood pressure.

The dietary supplements of garlic are in market and in this regard, Sobenin et al., [42] investigated the effect of time-released garlic powder tablets (Allicor: 600 mg daily) in a

double-blinded placebo-controlled randomized study in 42 men aged 35-70 with mild hypercholesterolemia. According to their observation, Allicor-treated subjects showed 7.6% reduction in total cholesterol. Similarly, LDL cholesterol in treated patients decreases by 11.8%. However, the same treatment was effective in enhancing HDL cholesterol by 11.5%. The mode of action for cholesterol lowering is not efficiently understood, however, garlic has ability to inhibit enzymes involved in lipid synthesis, prevent lipid peroxidation of oxidized erythrocytes and LDL, increase antioxidant status, and inhibit angiotension-converting enzyme [43]. Furthermore, Sobenin et al., [44] observed hypocholesterolemic effects of garlic in diabetes mellitus using time-released garlic powder tablets.

Garlic extracts reduced cholesterol synthesis up to 75% without evidence of cellular toxicity likely mediated by sterol 4- α -methyl oxidase. Moreover, this process is also helpful in reducing coronary calcium progression [45, 46]. In one such study, S-allyl cysteine sulfoxide (SACS) momentarily decreased the activity of HMG CoA reductase and significantly increased the activity of LCAT in isoproterenol (ISO)-induced myocardial ischaemia [47]. As far as functional ingredients are concerned, Yeh and Liu [48] studied the effect of individual component on cholesterol synthesis. According to their observation, SAC, S-ethylcysteine (SEC), and S-propylcysteine (SPC) inhibited cholesterol synthesis by 40--60% compared with 20--35% by gamma-glutamyl-S-allylcysteine (GSAC), gamma-glutamyl-S-methylcysteine (GSMC) and gamma-glutamyl-S-propylcysteine (GSPC). Additionally, among the three S-alk(en)yl cysteines tested, only SAC appears to further decrease the activity of HMG-CoA reductase by increasing sulfhydryl oxidation of the enzyme. The garlic formulation, aged garlic extract, has also ability to reduce cholesterol synthesis by inhibiting 3-hydroxy-3-methylglutaryl-CoA reductase [33, 48] and its activity is correlated to the presence of SAC and its derivatives.

The molecular targets involve inhibition of activation of sterol regulatory element-binding protein-2 (SREBP-2). The active ingredients DADS interfered with DNA binding activity of cAMP response element-binding protein (CREB) with upstream regulatory sequences of HMGR. Moreover, Rai et al. [49] showed the effectiveness of DADS analogs in reducing the quantities of oxidized low-density lipoprotein (ox-LDL), lipid peroxidation, and NF-kappaB activity.

B. Atherosclerosis

Atherosclerosis is a chronic cardiovascular disorder characterized with accumulation of lipids in arteries especially low-density lipoprotein (LDL) cholesterol, leading to the occlusion of blood flow. The result of such fatty deposit is formation of atheroma inside the surfaces of the arterial wall. Atherosclerosis can further leads to myocardial infarction and ischemic coronary disease. Garlic could be a valuable component of atherosclerosis-preventing diets but only in optimal doses. Moreover, garlic and its formulation hypothesized to possess ability to reduce the atheroma size [50].

Hyperlipidemia is a major etiopathological factor for atherosclerosis; therefore garlic plays an important role in the prevention of atherosclerosis [51]. Various heart pathologies are often associated with atherosclerosis/ hypercholesterolemia and their prevention or cure render good heart health. The addition of garlic in diet inhibited atherosclerotic changes in the aorta wall, and is related to the homeostatic activity of antioxidative enzymes and lipid

peroxidation [52]. Inflammation is an integral part of atherosclerosis and involves adherence of leukocytes/monocytes to the endothelium that further results in sticking of the arteries. The specialized molecules or structures called cell adhesion molecules further bolster the atheroma formation. Garlic and its functional components decreased the rate of migration of cells into arteries [5].

The LDL accumulation and its oxidation is also one of the challenges/issues to be addressed by the nutritionists. Sangeetha and Darlin-Quine [47] reported the preventive effect of SAC in reducing the extent of lipid peroxidation. Oxidation of LDL results in production of various metabolites that triggers a cascade of events leading to initiation and progression of atherosclerosis [34, 53]. Oxidized LDL also damages the endothelial membrane that imparts significant changes in other body parts too. Inverse association between antioxidant effects of garlic or its components/preparation against $\text{Cu}^{(21)}$ -initiated oxidation of LDL has been reported in many studies as Ide *et al.* [54] found clear supportive data that SAC significantly prevents membrane damage, loss of cell viability and lipid peroxidation in bovine pulmonary artery endothelial cells (PAEC) exposed to oxidized LDL. Water-soluble proteins from garlic also reduced the peroxidative changes in rats due to LDL oxidation. Even allicin-free AGE and SAC have a similar effect against $\text{Cu}^{(21)}$ -initiated oxidation of LDL in human subjects consuming AGE [55]. Although, some scientists have reported controversial results that demands the efficacy of garlic and its components to be addressed through animal modeling for the meticulousness of the issues [56].

Aged garlic extract supplemented with vitamins can be more effective in reducing the extent of atheroma formation. In this context, Budoff *et al.* [57] and their research group reported total cholesterol, LDL-C, homocysteine, IgG and IgM autoantibodies to MDA-LDL and apoB-immune complexes were decreased, whereas HDL, OxPL/apoB, and Lp (a) were significantly increased in AGE supplemented groups. Their results further indicated that the AGE has the ability to reduce the plaque formation owing to decrease in cholesterol etc. [58].

C. Platelet Aggregation

Increased platelet aggregation plays a pivotal role in the etiology of cardiovascular disease, involving multiple mechanisms that include increase in free cytoplasmic calcium Ca^{2+} , thromboxane generation, activation of the fibrinogen receptor GPIIb/IIIa and other modulators such as lipoxygenase metabolites, Kinase C, cAMP and cGMP and Nitric oxide [59]. The presence of a thrombus in a stenosed coronary artery can lead to acute syndromes such as myocardial infarction and angina [60]. Platelet aggregation plays a central role in coronary thrombosis and cascade of events that includes expression of adhesion molecules on the surface of the endothelium, the oxidation of lipoproteins, monocytes invasion of the vessel wall, foam cell formation, smooth muscle phenotypic change and proliferation and platelet deposition [61]. Garlic possesses the potential to alter some of the mechanisms that are certainly beneficial for cardiovascular care [62].

It has been reported that odorless garlic not only activates fibrinolytic activity by accelerating t-PA-mediated plasminogen activation, but also suppresses the coagulation system by down-regulating thrombin formation, suggesting a beneficial role in preventing pathological thrombus formation [63]. Under disease state of hypercholesterolemia and atherosclerotic animals usually relaxation mediated by endothelium derived NO is impaired

in arteries that lead to endothelial dysfunction. Oxidative stress and increased systemic inflammation may also contribute to this phenomenon. Elimination of certain risk factors and protection of endothelial integrity is effective in maintaining hemostasis. Williams *et al.*, [64] are of the view that short-term treatment with AGE may improve impaired endothelial function in men with CAD already treated with aspirin and statin. Garlic extract modulates the production and function of both endothelium-derived relaxing and constricting factors and this may contribute to their protective effect against vasoconstriction [65, 66] Likewise, Tapsell *et al.* [7] reported that 7.2 g of AGE has been associated with anti-clotting effect as well as modest reduction in blood pressure.

D. Miscellaneous Functions

Homocysteine, a metabolite of methionine, is an independent cardiovascular disease risk factor, which causes thrombosis and oxidative-stress damage [67] often associated with atherosclerosis and higher risk of coronary heart disease, stroke and peripheral vascular disease by damaging the inner lining of arteries and promoting blood clotting. Homocysteine have linear relationship with folate deficiency and decreased NO production. AGE may partly prevents decrease in bioavailable NO and endothelium-derived hyperpolarizing factor during acute hyperhomocysteinemia [68]. Plasma homocysteine exists in two forms i.e. free and protein bound homocysteine and amount of total homocysteine is considered as marker of atherosclerosis. AGE can decrease plasma total homocysteine concentration by 30% without changing protein-bound/free homocysteine ratio [69]. Nowadays, much interest has been paid on coronary arterial calcification (CAC), a marker of plaque formation in human coronary arteries and atherosclerosis that linked with increased risk of cardiovascular events such as myocardial infarction, fatal arrhythmia, and congestive heart failure [70, 71]. Interventions that are designed to combat the process of calcification may lead to certain improvements. Budoff *et al.* [58] evaluated the ability of AGE to inhibit vascular calcification, and indicated its potential to impede the rate of progression of coronary calcification as compared to placebo over one year.

SAC derived from garlic possesses protection against cardiovascular diseases. Chuah *et al.*, [72] elucidated that SAC is responsible for this cardioprotection using acute myocardial infarction (AMI) rat models. Their results indicated that SAC significantly lowered mortality and reduced infarct size. Inhibitory action was mediated by increased activity of cystathionine-gamma-lyase (CSE), upregulated its expression via an H₂S related pathway. SAC increased left ventricular CSE activity and produced higher plasma H₂S concentration compared with control.

Garlic extracts might have some worth as angiotensin-converting enzyme (ACE) inhibitor to prevent some vascular complications of diabetes mellitus. Conjugation of active ingredients of garlic with some drugs of interest has paid some beneficial impact. Allylmercaptocaptopril is an exemplary conjugate of the ACE inhibitor captopril with allicin. According to Ernsberger *et al.* [73] allylmercaptocaptopril prevented progressive weight gain, without a detectable effect on food intake, lowered blood pressure, improved cardiac hypertrophy, as indicated by heart weight and ventricular-wall thickness. The comparative efficacy of fresh and processed garlic has been studied however, some contradictions were observed with that of common myths for not using fresh garlic owing to its problems for

gastrointestinal health. In this regard, Mukherjee et al. [74] conducted trials and enumerated that freshly crushed garlic group displayed greater phosphorylation of antiapoptotic ERK1/2 proteins, reduced Bax/Bcl-2 ratio, and reduced phosphorylation of proapoptotic p-38MAPK and JNK. Improvement in the signaling cascade ensures better efficiency of fresh garlic in improving the cardiac health as compared to processed garlic.

Cardiovascular protection is also important in people already with some other chronic diseases such as diabetic mellitus and cancer because often these maladies accompanied by high cholesterol, hypertension and changes in homeostasis of body. Ethanolic extract of garlic found to lowers lipid such as total cholesterol and triglycerides and tends to improve enzymes associated with membrane damage and transportation in diabetes mellitus [75]. AGE decreases homocysteine, lowers blood pressure, and increases microcirculation are important in diabetes whereas, micro-vascular changes increase heart disease and dementia risks [33, 76].

Some contradictions still hinder its utilization to control cardiovascular disorders but generally it is believed that garlic consumption alone can decrease serum lipids, nevertheless, cannot be used as main therapeutic agent [14]. Overall, garlic possesses plasma lipid-lowering, anti-coagulant and antioxidant properties thus improve impaired endothelial function [77] that in turn yields cardiovascular health. There is need to standardize garlic and its various fraction for the presence of active ingredients for conclusive approach drawn for their effective utilization. Moreover, techniques like micro-encapsulation can be used as safeguard to prevent its ingredients for further transformation into chemical adducts that can be potential threat for human health.

Antioxidant and Anti-Inflammatory Perspectives

Diet rich in antioxidants holds potential to scavenge free radicals being produced in the body during various ongoing processes. The phenomenon of free radical generation is proceeding during respiration and energetic processes. However, the excessive production of free radicals or reactive oxygen species (ROS) results in oxidative stress that leads to various pathological processes including chance of cancer insurgence, cardiovascular and degenerative disorders. Moreover, free radicals are implicated with disturbance in membranes and genome stability thus causing cell death [78-80].

The excessive production of free radicals in the body are often attributed to environmental factors like radiation, pollutants, excessive exercise, and even some medications. The ROS can be normalized by means of natural defense system prevailing within the body, however, antioxidant supplementation or consumption of diet rich in functional ingredients certainly is beneficial because of their role in boosting cellular antioxidant enzymes and signaling cascades [81-83].

Garlic and its functional ingredients/preparations have shown some promising results regarding their effectiveness in improving antioxidant defense capability of the body thus reducing the onset of various pathologies linked with free radical generation. Their mode of action is multidimensional ranging from free radical scavengers to activation of signaling cascade [5]. The organosulfur compounds can stimulate the antioxidants enzymes including

are glutathione peroxidase (GPx), glutathione transferase (GST), catalase (CAT), superoxide dismutase (SOD), etc. (Yin and Chang, 1998). The bioactive constituents like DAS, DADS and DAT play differential modulatory role on GSH related antioxidant system owing to increased expression of GST γ , γ b1 and γ c proteins [84, 85]. However, the degree of antioxidative efficacy of various garlic compounds or preparations differ according to variations in chemical structures and standardization procedures [27, 86]. Likewise, Wei and Lau [87], and Yamasaki and Lau [88] observed that AGE suppresses hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) generation thereby enhancing cellular antioxidant enzymes, can act as protective shield against vascular endothelial cells from oxidant injury. Later, Borek [76] confirmed the previous findings and enumerated that AGE and SAC inhibit both lactate-dehydrogenase release and lipid peroxidation induced by H_2O_2 . It is interesting for the stakeholders that AGE can increase intracellular glutathione level, glutathione disulfide reductase and SOD activity in PAECs [58]. The mechanism of action lies in its ability to inhibit emission of low-level chemiluminescence and early formation of thiobarbituric acid-reactive substances. The outcomes of therapeutic potential of AGE further include prevention of lipid peroxidation and maintaining membrane fluidity/integrity [87].

In research experiments, oxidative stress is often induced to determine the effect of selective ingredients. There are number of investigations carried out to explore the therapeutic role of garlic and its functional ingredients. The mode of induction and amelioration differed, however, they reduced lipid peroxidation, increased expression of enzymes and related proteins. In one such study, Kabasakal *et al.*, [90] provided evidence that AGE also protects kidney tissue against induced oxidative stress that includes AGE (1 mL/kg) restored the reduced GSH levels, decreased free radical production and myeloperoxidase expression. Likewise, Agarwal *et al.* [91] observed protective effect of garlic oil against Fe-NTA induced hepatic oxidative stress via shield against hepatic lipid peroxidation, generation of hydrogen peroxide, preserved glutathione level, and activity of antioxidant enzymes. Even beneficial effects of garlic oil are of significance importance in reducing the oxidative damage imparted by tributyltin (TBT) that can be transported through seafood and might be entered into food chain because of marine pollution [92]. Additionally, cytoprotective potential of DTS in cadmium-induced toxicity might be due to its antioxidant and metal chelating properties [93]. Diallyl tetrasulfide affect the reactive oxygen species induced by cadmium, and possesses a novel protective effect on the cytolethality associated with mitochondrial injury [94]. Likewise, SAC decreased CCl_4 induced liver injury by attenuation of oxidative stress [95]. The same compound holds the *in vivo* and *in vitro* antioxidant properties and ameliorates the $K_2Cr_2O_7$ induced toxicity [96]. Impaired oxidant/antioxidant balance may also take part in the cyclosporine-induced nephrotoxicity and garlic may ameliorate such toxicity [97]. The antioxidative properties further proved anti-inflammatory potential of garlic and its constituents e.g. allicin, DAS, ajoene, etc. [98-100].

The inflammatory responses are characterized with the up-regulation of some key signaling molecules e.g. tumor necrosis factor alpha (TNF- α) and interleukin-1b (IL-1b). The signal-transduction pathways that are key participants in inflammation are listed as under [101-103].

1. Oxidants as modulators of signal transduction
2. Mitogen-activated protein kinase (MAPK) pathway
3. Pro-inflammatory enzymes (COX and LOX)

4. Phosphatidylinositol-3 protein kinase (PI3) pathway
5. Transient receptor potential (TRP) channels
6. Janus kinase-signal transducer
7. Activator of transcription (Jak- STAT)
8. Nuclear factor kappa B (NF- κ B)
9. Chemokines (integrins, selectins)
10. G protein-coupled receptors (GPCRs)

Some of these processes are interdependent e.g., NF- κ B stimulation and enhanced secretions of cyclooxygenase (COX), and lipoxygenases (LOX) enzymes. The outcomes of such synergistic effects are enhanced inflammatory responses thus causing several degenerative disorders e.g. atherosclerosis, diabetes mellitus, arthritis, neurodegenerative disorders, etc. [104]. The COX/LOX further stimulate production of prostaglandins/leukotrienes that triggers inflammation. These key factors are influenced by quality of diet consumed; garlic and its products are effective in reducing the extent of these responses. Sulfur containing moieties are closely linked with increased expression of various antioxidant enzymes e.g. SOD, catalase, etc. It is generally believed that expression of COX is dependent upon presence of free radicals, whilst antioxidant supplementation can overcome such abnormalities and effect of garlic and its active ingredients are attributed to their antioxidative potential [105].

Recently, Ban et al. [106] was able to suppress ear edema, and carrageenan and mycobacterium butyricum induced inflammatory and arthritic responses using thiacremone's (bioactive molecule of garlic) topical application. The authors further hypothesized that reduced NO production as a function of bioactive molecule is possible mechanisms of action. Moreover, it holds the ability to inhibit p50 and p65 nuclear translocation thus inhibiting DNA binding activity of the NF-kappaB via interaction with the sulfhydryl group of NF-kappaB molecules. Earlier, Chauhan [107] investigated anti-amyloidogenic, anti-inflammatory and anti-tangle effects of AGE and its bioactive molecules (SAC and DADS) to check their effectiveness against Alzheimer disease. The ameliorative effects were in the order of AGE > SAC > DADS. In another investigation, Zare et al. [108] observed significant decrease as hallmark criteria of allergic airway inflammation levels i.e. eosinophil in lavage, peribronchial lung eosinophils, IgG₁ level in lavage, etc. by intra-peritoneal injection of the aged garlic extract.

Garlic can scavenge free radicals and is a successful antioxidant, has potency to enhance the activity of antioxidant enzymes. The antioxidant potential and efficacy to cure various ailments need further attention of the researchers. The dosage needs to be adjusted through controlled trails and cohort studies as excessive intake may lead to toxicity. In comparison, AGE contains higher amounts of antioxidants and most of them are water soluble in nature. Overall, AGE scavenges oxidants, enhances enzymes activity and glutathione level, inhibits lipid peroxidation and production of inflammatory prostaglandins. Garlic oil also holds promise for good health, being free radical scavenger and imparting protection against toxicity of metals and hazardous chemicals. Some other preparations such as aqueous water extract are also effective but reports narrating their limited potential [5, 33, 109].

In concluding, garlic and its various components/preparations is effective tool in amelioration of oxidative stress and related discrepancies but care should be taken not to be

used in excess quantity and consultancy of physician should be taken in account before recommending their use.

Anticancer Perspectives

Progression and pathogenesis of cancer is dependent upon many intrinsic and extrinsic factors. However, intake of proper and balanced diet can prevent 30-40% cases of cancer. The chemotherapy employing drugs or radiations are in common practice but dietary modules are gaining popularity. In this regard, plant based remedies have been thoroughly investigated and their mode of actions have been described in a comprehensive way. There exist certain ambiguities that need to be addressed by public health authorities to finalize the recommendations for perfectionism [110]. Garlic and its bioactive molecules are of significance importance in chemoprevention that eventually reported some 50 years ago for its therapeutic potential [111]. During the mid twentieth century, use of garlic in the treatment of tumors has been narrated for the first time when Weisberger and Pensky [112] indicated that garlic could inhibit the growth of malignant cells and prevents growth of sarcoma 180 ascites tumor. Since that time, several *in vivo* and *in vitro* studies have brought several mechanism of action of garlic and its components that are associated with elicitation of their anti-carcinogenic activity in a wide range of experimental systems [104, 113].

Organosulfur compounds (OSC) of garlic inhibit carcinogen activation/formation, boost phase-II detoxifying processes, cause cell cycle arrest mostly in G2/M phase, stimulate the mitochondrial apoptotic pathway, increase acetylation of histones, and suppression of tumor proliferation [114, 115]. The development of successful strategy employing dietary chemopreventive agents exists in their mode of actions. Certainly, it does not appear that a single mechanism could account for the observed protection based on the variety of carcinogens [116, 117]. The mechanism through which garlic and its functional ingredients impart anticancer effect needs further elaborations and details of some of the pathways are described herein

Cancer Initiation and Detoxification Mechanism

The initiation of cancer is dependent upon a number of factors that can be controlled e.g. ingestion of toxicants and improper diet. However, nature has bestowed human body with detoxification mechanism comprised of different enzymes and their particular receptors and sensors. The cancer insurgence can be prevented if its growth can be checked at initiation stage. Garlic and its components hold potential to reduce the expression of phase-I enzymes and enhance the activities of phase-II detoxification enzyme system [118, 119]. Administration of garlic significantly increase the level of many detoxification enzymes including GST, acid soluble sulfhydryl (-SH) cytochrome b5 and cytochrome P450. Likewise, garlic extract along with several principal sulfur constituents induces positive impact on phase II detoxification enzymes like GST and quinone reductase in mammalian tissues [120-122] and a similar impact of garlic powder was observed in experimental rat liver and mammary carcinoma [123]. Cytochrome P450s (CYPs) plays an important role in pro-

carcinogen activation and its inhibition could result in decreased formation of toxic metabolites and thus indirectly inhibiting carcinogenesis; diallyl sulfone isolated components from garlic preparations act as inhibitors of various CYPs [124]. Some OSC induce the expression of quinone oxidoreductase, an enzyme required in the detoxification of activated quinone metabolite of Benzopyrene [1998]. DADS may increase tissue activities of phase II detoxification enzymes quinone reductase (QR) and GST in some gastrointestinal organs of body thus providing protection against GIT cancers [126]. Some processed products of garlic e.g. high temperature- and pressure-treated garlic (HTPG) have been reported to enhance antioxidative and cytotoxic activities in colon carcinogenesis. HTPG significantly reduced the activity of cytochrome P450 (CYP) 2E1, while it enhanced QR and GST activity. The inhibition of phase-I detoxification mechanism and promotion of enzymes involved in phase-II detoxification system are integral part in controlling cancer insurgence at its initiation stage [127]. Administration of S-allylcysteine (SAC) showed the inhibition of tumor incidence, modulated the lipid peroxidation, and increased the reduced glutathione, glutathione-dependent enzymes, superoxide dismutase, and catalase in NDEA-induced carcinogenesis. The scientists speculate that S-allylcysteine mediates its chemopreventive effect by modulating lipid peroxidation, GST stimulation, and by increasing the antioxidants. Hence, SAC prevents cells from loss of oxidative capacity in NDEA-induced hepatocarcinogenesis [128]. The study undertaken by Das and Saha [129] indicated that garlic extract holds ability to delay skin tumorigenesis in animal modeling at initiation stage. The mechanism of action concealed in antioxidant capability as it inhibited oxidative modification of lipids, thus protecting cells from injury by the oxidized molecules.

Garlic and its bioactive molecules also hold the ability to enhance gene expression for GST thus reducing the extent of lipid peroxidation [130, 131]. In another study, administration of garlic (250 mg/kg, p.o.) effectively suppressed 4-nitroquinoline 1-oxide (4NQO)-induced tongue carcinogenesis by modulating lipid peroxidation through enhanced levels of GSH, GPx and GST [132]. The improved antioxidant defense system is mediated by the antioxidant response elements (ARE). Some transcription factors e.g. Nrf2, NF- κ B, and AP-1 have been documented to play a vital role in ARE-driven gene expression. The anticancer agents bring certain changes in the structure and these cofactors are being released. These transcription factors enhance the gene expression thereby activating phase-II detoxifying enzymes [133].

The cancer insurgence can be controlled easily at initiation stage if diagnosed properly. At initiation stage, supplementation of antioxidants is effective as it enhances the body defense mechanisms that can check the growth of tumor cells [5].

Cancer Promotion and Induction of Apoptosis

The modulation of human defense and immune system, transcription factors, upstream signaling molecules are potential target of phytochemicals. Garlic and its bioactive molecules hold wide variations in their mode of action in controlling cancer initiation and its progression. Some of OSC can effectively reduce the cancerous cell proliferation via induction of cell cycle arrest or apoptosis, inhibition of angiogenesis, induction of terminal differentiation, and inhibition of oncogene activity or DNA synthesis [134].

Programmed cell death or apoptosis is dependent upon signal transduction pathways including activation of intracellular cascades. The body in normal condition carries out the process of apoptosis to kill undesirable cells. Moreover, process of aging is also dependent upon the same phenomenon. Under normal physiological state, free radical production is essential to start the process of cell death. However, cell underwent uncontrolled mitosis during cancer insurgence. The body inhere multi-pronged strategies to remove these undesirable cells. During cancer progression and promotion, activation of anti-apoptotic and suppression of pro-apoptotic factors may also occur [135]. Apoptosis is induced via two main routes i.e. intrinsic (mitochondria) and extrinsic (activation of death receptors). The hallmark of both these remains in activation of cascade of signal transduction moieties [135, 136]. Garlic and its organosulfur constituents influence cell cycle arrest in numerous *in vitro* cancer cell models [120]. The mitochondria-mediated signaling pathways may also be effective leading to induction of apoptosis. In this regard, Nagaraj et al., [137] investigated expressions of Apaf1, cystatin B, caspase-3 and FADD (fas-associated protein with death domain) in breast, prostate, and lung cancer cells as a function of garlic's bioactive molecule (DADS). The others OSC also hold the ability to induce Bax translocation, cytochrome c release, and activation of caspase-3 and caspase-9. Furthermore, apoptosis can be induced by altering the ratio of Bcl-2 family of proteins [133].

A. Prostate Cancer

Progression of prostate cancer, third leading cause of death causalities among cancers, can be controlled through garlic and its components. The cell culture studies and animal modeling generated array of evidences to support its therapeutic use. In this regard, Chu et al. [138] demonstrated that water soluble components of garlic as SAC results in decrease production of Bcl-2 proteins and increased caspase-3 activity to cure prostate cancer [103]. Similarly, Kim et al. [139] while studying the effects of DATS in prostate cancer cell lines attributed prostate tumor suppression to ROS production that is regulated by enhanced expression for Bax and Bak proteins. They contradicted with other scientists that induction of apoptosis is not dependent on an ectopic expression of either Bcl-2 or Bcl-xL [140]. Later, Gayathri et al. [141] further strengthened the claims that DADS (25 and 40 microM concentrations) activated caspases cascade. They observed significant increase in the expression of caspases (3, 9, and 10), and proapoptotic protein Bax, while Bcl-2 protein decreased in DADS treated cells.

Recently, Herman-Antosiewicz et al. [142] claimed that DATS mediated cell cycle arrest in prostate cancer lines. They provided evidences for concentration and time-dependent accumulation of G2/M phase cells, reliant on increased S216 phosphorylation of Cdc (cell division cycle) 25C. Moreover, it can be correlated with delayed nuclear translocation of cyclin dependent kinase 1 [CDK1]. Earlier, Arunkumar et al. [143] showed that DADS inhibited the growth of prostate cancer cells in a dose dependent manner via cell cycle arrest at G2/M by down-regulating CDK1 expression. Androgen receptor (AR) protein expression and prostate-specific antigen (PSA) were significantly decreased as a function of exposure of prostate cancer cells to DATS [144, 145].

Prevention of prostate cancer can also be linked to decreased cellular proliferation through enhanced expression of cyclinB1 and securin protein and suppressed expression of neuroendocrine marker synaptophysin [146]. The decreased synthesis of messenger RNA and

inhibited synthetic androgen (R1881)-stimulated nuclear translocation are mainly responsible for reduced expression of AR proteins [145].

Prostate cancer can be cured with the use of AGE that acts through diversified mechanism including inhibition of polyamines needed for cell division, increasing breakdown of testosterone, and reducing prostate specific antigen (PSA) levels [147]. Therefore, these investigations may serve as reference that garlic and its preparations/components might be a suitable choice to be considered as therapeutic remedy for the treatment of prostate cancer [143, 148]. However, still there is need of clarity regarding the issues via meta-analysis during clinical trials.

B. Colon Cancer

The anticancer perspectives of garlic and its components are also highlighted in colon carcinogenesis. The mode of action somewhat similar to prostate cancer but there exist some variation. Using LNCaP and HCT-116 human cancer cells as a model, Xiao et al. [149] demonstrated that the Chk1-mediated mitotic arrest resulting from DATS exposure leads to apoptosis. The DATS exposure resulted in cell cycle arrest at G2 phase owing to down-regulation of cyclin-dependent kinase 1 (Cdk1), cell division cycle (Cdc) 25B, and Cdc25C leading to Tyr15 phosphorylation of Cdk1. They were of the further view that Chk1 dependent cell cycle arrest is not influenced by p53 status [150]. The study showed that administering AGE to patients with advanced cancer of the digestive system (colon carcinogenesis) improved NK cell activity, but caused no improvement in QOL [151, 152]. DADS can be effective against colon cancer through enhanced expression of signal transducer and activator of transcription 1 (STAT1) that is associated with apoptosis [153].

Histone deacetylase (HDAC) inhibitors reactivate epigenetically silenced genes in cancer cells, triggering cell cycle arrest and apoptosis [154]. Inhibition of HDAC activity by allyl mercaptan (AM), was associated with increased histone acetylation and Sp3 transcription factor binding to the promoter region of the P21WAF1 gene, resulting in elevated p21 protein expression and cell cycle arrest in colon cancer cells [155].

Song et al. [156] observed p53-independent cell cycle arrest in colon cancer cell line (HCT-116) at G2/M phase and increase of cyclin B1 with DADS treatment [157]. However, activation of p53 resulted in apoptosis that was dependent upon generation of ROS. DATS and even diallyltetrasulfide treatment of HCT116 cells led to a reduced cell viability, cell cycle arrest, and apoptosis. However, cell cycle arrest and induction of apoptosis is associated with a considerable reduction of the level of cdc25C [158]. DAS induced apoptosis in Colo 320 DM cells and promoted cell cycle arrest substantially at G2/M phase. The production of ROS and decreased activities of alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) upon DAS treatment is major culprit in antiproliferative and cytotoxic effects, respectively. The expression of NF-kappaB and caspase-3 was unregulated and suppression of extracellular regulatory kinase-2 (ERK-2) activity thus resulting in apoptosis in colon cancer cells [159]. The similar findings were also recorded when HCT-15 and DLD-1 cells were treated with DATS. Number of cells arrested at G2/M phase, the cells with sub-G1 DNA content, and the cells with caspase-3 activity were dramatically increased, showing that DATS holds the ability to disrupt microtubule network formation through specific oxidative modification [160].

C. Bladder Cancer

Bladders cancer progression can also be checked using garlic and its components. DATS inhibits phosphatidylinositol 3'-kinase/Akt activation that in turn, results in modulation of Bcl-2 family proteins, leading to enhanced apoptosis of T24 bladder cancer cells. The same component suppressed the cell proliferation that was closely associated with cell cycle arrest at G2/M stage [161]. Similarly, Lu et al. [153] studied the effect of DADS in T24 bladder-cancer cell line and they observed increased cyclin E, and decreased CDK2 gene expression that led to G2/M arrest. Additionally, same component promoted caspase-3 activity thus reducing the cell viability through apoptosis.

D. Mammary Cancer

Garlic constituent DATS suppresses the viability of cultured MCF-7 and MCF-12a cells through cell cycle arrest at G2/M phase, and induction of apoptosis. DATS-induced apoptosis was markedly elevated in MCF-7 cells compared with MCF-12a cells and is correlated with elevated levels of cyclin B1. MCF-7 cells are known to be deficient in caspase-3 [162, 163]. DATS further enhanced the expression levels of FAS and cyclin D1, but in contrast, down-regulated the expression levels of Akt and Bcl-2. Furthermore, the DATS-induced apoptosis was correlated with induction of pro-apoptotic Bax protein and p53 protein expression [163]. Earlier, Lei et al. [164] reported the anticancer mechanism of DADS. According to their observations, DADS exhibit antiproliferative properties in mammary cancer cell lines (MCF-7 cells) via inhibition of ERK and the activation of the SAPK/JNK and p38 pathways [165].

E. Lung Cancer

Wu et al. [166] summarized arguments for effectiveness of organosulfur compounds in treating lung cancer and attributed ROS generation to cell cycle arrest at G2/M and apoptosis are potential targets of DADS. Later, Wu et al. [167] review that up-regulation of extracellular signal-regulated protein kinase (ERK) is dispensable for apoptosis induction in human lung adenocarcinoma cells, provided the first evidence that Fas-mediated cell death pathway is partly involved in DADS [166, 167].

The effectiveness of DADS in suppressing lung cancer (human non-small cell lung carcinoma H1299 cells) has already been tested by Hui et al. [168]; they presented different reasons for increased apoptosis i.e. decrease in cell viability, cell cycle arrest at G2/M-phase, and transient increase of the phospho-p42/44 (phosphorylated p42/44 MAPK). However, down-regulation of cyclin-dependent kinase 1 (Cdk1), and cell division cycle 25C protein expression leading to accumulation of Tyr15 phosphorylated (inactive) Cdk1 are the potential mechanism of action of DATS [165].

F. Assorted Cancers

There are some cancers of marginal importance for researchers as their prevalence is limited over the entire globe. Shin et al. [169] described the mode of action of DAS that is helpful in reducing the extent of cancer insurgence in anaplastic thyroid cancer cells. Accumulation of sub-G1 DNA and concomitant accumulation of cells in the G2/M phase in a dose-dependent manner are of prime importance. In addition, DAS-induced apoptosis was associated with a decrease in the level of Bcl-2 expression and an increase in the level of Bax expression. Moreover, cytochrome c was remarkably released from mitochondria to the

cytosol by DAS. Furthermore, caspase-9 and caspase-3 were activated by DAS, and DAS cleaved PARP. DADS-induced apoptosis is triggered by the generation of hydrogen peroxide, activation of caspase-3, degradation of PARP, and fragmentation of DNA in human leukemia HL-60 cells [170]. Similarly, antiproliferative and apoptotic effect of SAMC on gastric cancer SNU-1 cells was associated with the induction of Bax, p53, and caspase-9, rather than the induction of Bcl-2 and p21. Mitochondrial cytochrome c activation and in vitro caspase-3 activity assay demonstrated that the activation of caspases accompanied with apoptotic effect of SAMC, which mediates cell death [171]. In comparison, DADS induced differentiation of gastric cancer cell (MGC803 cells) involved in alteration of the ERK1/2 signaling pathway [172]. However, some scientists contradict with the role of caspase-3 activation and PARP degradation in inducing apoptosis. In this context, Park et al. [173] while working on human epithelial carcinoma cells attributed caspase independent apoptosis-inducing factor (AIF) and protein kinase A (PKA) release from mitochondria as a therapeutic target of allicin to cure malignant cells.

Garlic extract induces lymphocyte proliferation triggered by potent T- cells mitogen. Proliferation of lymphocytes by increasing IL-2 and IL-4 production depends on the type and dilution of extract used [174]. Moreover, Z-ajoene another OSC has potent anti-cancer activity in HL-60 (Human promyelocytic leukemia cells) cancer cell lines. It inhibits telomerase activity and blocks G2/M phase as indicated from progressive increase in the percentage of mitotic block at G(2)/M phase owing to ability of Z-ajoene to increased cyclin B1 accumulation and decreased p34(cdc2) expression [175].

The garlic and its components have shown their effectiveness against various cancer lines e.g. skin, colon, prostate, mammary carcinoma, lungs, gastric cancer, etc. and some significant research work has been presented in Table 4.

Discussion and Future Research Agenda

Garlic and its components hold potential to cure various pathologies as it is evident from array of studies conducted in the previous few decades however, the mode of action differed with variations is disorder strata. The garlic and its constituents hold potential to reduce total and LDL cholesterol thus minimizing the chances of atheroma formation. Moreover, modulating the inflammatory responses through mechanized signaling cascade can overcome the problem associated with platelet aggregation and LDL oxidation. Some of its components are reported to release nitric oxide (NO) that mediates endothelial cell integrity thereby helps in overcoming cardiovascular disorders. The antioxidant capability of garlic is well understood as it forms thiol conjugates thus enhancing the activity of antioxidant enzymes system, render body protection again free radical production. The similar mechanism persists to eliminate the toxic effects of various intrinsic and extrinsic metabolites. The garlic and its bioactive molecules modulate the expression of various transcription factors and release certain chemokines that are effective in reducing the extent of inflammation. The mechanisms through which garlic exerts its anti-inflammatory response are highlighted in the preceding section “antioxidant and anti-inflamamtory potential of garlic”.

The cancer insurgence can be prevented or controlled through various mechanisms. However, the body inherent defense systems though different detoxification modules are

effective to stop cancer initiation. Garlic and its components hold potential to reduce the expression of Phase-I detoxification mechanism, whilst enhancing the phase-II detoxification mechanism through enzyme activation. The improved antioxidant defense system is mediated by the antioxidant response elements (ARE). Some transcription factors e.g. Nrf2, NF- κ B, and AP-1 have been documented to play a vital role in ARE-driven gene expression.

Conclusively, garlic and its components exert their influence in reducing the progression of cancerous growth. These effects are mediated through diversified mechanisms i.e. induction of cell cycle arrest or apoptosis, inhibition of angiogenesis, induction of terminal differentiation, and inhibition of oncogene activity or DNA synthesis. The OSC holds ability to enhance the expression of pro-apoptotic proteins including Bax and Bak, whilst decreasing the level of anti-apoptotic protein of Bcl-2 family.

Table 4. Garlic and its various preparation/components against cancers

Type of cancer	Functional components	Cancer type and mechanisms of action	Research work
Skin cancer	Garlic Extract	Enhanced antioxidant capability Inhibition of oxidative modification of lipids	Das and Saha [129]
	Garlic, DAS	Regulation of p21/ras protein expression	Arora et al. [176]
	DAS	Reduce number and size of tumors	Khan et al. [177]
		Recovers DNA strand breaks	Nigam and Shukla [178]
		Protein expression of PI3K/Akt and p38MAPK Reduced expression of signaling molecules including PI3K/Akt and MAPKs	Kalra et al. [179]
	Ajoene	Anti-proliferating and block the expression of α 4 β 1 integrin	Ledezma et al. [180]
		Suppressed skin tumor formation	Nishikawa et al. [181]
Prostate cancer	DATS	G2/M phase cell cycle arrest owing to differential kinetics of nuclear localization of cdk1 and cyclin B1	Herman-Antosiewicz et al. [142]
	DATS	CyclinB1 and securin protein levels, and suppression of the expression of neuroendocrine marker synaptophysin	Singh et al. [146]
	DATS	Androgen receptor (AR) protein expression and prostate-specific antigen (PSA) The decreased synthesis of messenger RNA Inhibited synthetic androgen (R1881)-stimulated nuclear translocation	Stan and Singh [145]
	DATS	Decreased cellular proliferation through enhanced expression of cyclinB1 and securin protein and suppressed expression of neuroendocrine marker synaptophysin	Singh et al. [146]
	DATS	ROS generation and regulating Bax/Bak proteins	Kim et al. [139]
	DATS	Apoptosis	Xiao et al. [140]
	Garlic and SAMC	Decreases the incidence	Howard et al. [182]
	S-allylcysteine (SAC)	Apoptosis via decrease Bcl-2 and increased caspase-3 expression	Chu et al. [138]
	DADS	Influence histone acetylation to induce apoptosis	Arunkumar et al. [144]
Colon cancer	Processed garlic	Reduced activity of cytochrome P450 (CYP) 2E1 Enhanced QR and GST activities.	Cithara et al. [127]
	Garlic extract	Enzyme modulation and apoptosis	Signups et al. [183]
	AM	Histone deacetylase inhibition triggering cell cycle arrest and apoptosis	Nina et al. [154, 155]

Table 4. (Continued)

Type of cancer	Functional components	Cancer type and mechanisms of action	Research work
	DATS	Chk1-mediated mitotic arrest leads to apoptosis Cell cycle arrest at G2/M phase	Xiao et al. [149]
	DATS	Induction of apoptosis	Hobson et al. [184]
	DADS	p53-independent cell cycle arrest at G2/M phase and increase of cyclin B1	Jo et al. [157]
	DADS	p53-independent cell cycle arrest at G2/M phase and increase of cyclin B1	Song et al. [156]
	Thiocremonone	Modulating NF-kappaB	Ban et al. [185]
	SAMC	Mitosis and Apoptosis	Xiao et al. [150]
	SAC, SAMC	Antiproliferative Cell cycle arrest at G2/M	Shirring et al. [186]
	DAS	Inhibition of aryl amine N-acetyltransferase activity	Chung et al. [187]
	Fresh garlic juice	Antiproliferation Induction of apoptosis	Ghosh et al. [188]
Lung cancer	DATS	Down-regulation of cyclin-dependent kinase 1 (Cdk1), and cell division cycle 25C protein expression	Xiao et al. [165]
	DADS	Increase intracellular ROS Cell cycle arrest at G2/M and apoptosis Up-regulation of extracellular signal-regulated protein kinase (ERK)	Wu et al. [166, 167]
	DATS	Decrease cancer cell viability, cell cycle arrest at G2/M-phase Transient increase of the phospho-p42/44 (MAPK). Increased apoptosis	Hui et al. [168]
Bladder cancer	DATS	Inhibits phosphatidylinositol 3'-kinase/Akt Cycle arrest at G2/M stage modulation of Bcl-2 family proteins to enhanced apoptosis	Wang et al. [161]
	DADS	Cell cycle arrest at G2/M Phase Induced apoptosis undergo caspase-3 activity	Lu et al. [189]
Brain tumors	DAS, DADS, DATS	Human glioblastoma T98G and U87MG cells	Das et al. [190]
Human gastric cancer	DADS	MGC803 cells	Ling et al. [171]
Hepatocarcinogenesis	SAC	Modulating lipid peroxidation GST stimulation and enhanced antioxidants status	Sundaresan and Subramanian [128]

The disturbance in the ratio of pro-apoptotic and anti-apoptotic proteins leads to cytochrome c release and activation of caspase-3 and caspase-9, eventually resulting in apoptosis. Garlic and its components are also effective in generation of reactive oxygen species ROS only in diseased state that further leads to apoptosis via decreased activity of alkaline phosphatase (ALP) and lactate dehydrogenase (LDH). However, there exist some other mechanism too like increased S216 phosphorylation of Cdc (cell division cycle) 25C, delayed nuclear translocation of cyclin, enhanced expression of cyclinB1 and securin protein, suppressed expression of neuroendocrine marker synaptophysin, histone deacetylase (HDAC)

inhibition, decreased CDK2 gene expression, extracellular signal-regulated protein kinase (ERK), caspase independent apoptosis-inducing factor (AIF), protein kinase A (PKA), etc. The biochemical transformations of the garlic's bioactive molecules to their specific adduct with thiol functional groups could be considered relevant to unveil the anticancer properties of allyl-sulfur compounds.

The congregate information presented in this chapter is certainly useful to introduce garlic and its active ingredients as a potential health remedy against various ailments especially as a chemopreventive agent nevertheless, clinical trials are mandatory to validate its usefulness.

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Chapter XVII

Garlic and Human Health: An Old Remedy with Modern Applications

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Abstract

Oxidative stress plays an important role in chronic diseases. Reactive oxygen species that damage DNA, lipids and proteins increase the risk of age related pathological conditions including cancer, cardiovascular diseases (CVD), stroke and dementia, Alzheimer's disease (AD) being its most common form. Garlic, that is rich in antioxidants, notably organosulfur compounds, flavonoids and other nutrients, is an important medicinal food with health protective and anti-aging effects, that help lower the risk of oxidant-mediated conditions and chronic diseases. Aged garlic extract (kyolic AGE), a well standardized, odor-free natural garlic preparation, rich in bioavailable water soluble organosulfur compounds, has been the preferred preparation used in research on the health benefits of garlic. Experimental and human studies have found that the intake of garlic and AGE reduces the rate of some forms of cancer and lowers risk factors for CVD, cerebrovascular and Alzheimer's disease. Garlic increased internal antioxidants including glutathione, inhibited inflammation, reduced cholesterol and platelet aggregation, inhibited arterial plaque formation; reduced homocysteine, lowered blood pressure and increased microcirculation. Studies with aged garlic extract also demonstrated that the extract and its organosulfur components prevented ischemic brain injury, that occurs with stroke and can lead to dementia, inhibited beta amyloid peptide, a hallmarks of AD, protected neurons against apoptotic death and promoted neuronal growth, increased memory capacity and learning as well as promote longevity. Garlic intake, as AGE, fortified physical endurance and reduced fatigue. In summary, a diet rich in garlic and its odor- free form of aged garlic extract promote general health and help provide protection from chronic diseases and aging.

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1. Introduction

Garlic ranks highest in popularity and efficacy among herbal remedies that are consumed for their health benefits. Garlic (*Allium sativum*), is one of the oldest plants used in medicine. It has been an important part of human life and health for centuries, across cultures and millennia. Garlic has been used to spice food, fortify soldiers for war, cure colds, heal infections, and treat ailments ranging from heart disease to cancer and even the plague.

Today, after close to 6000 years of its use in folk medicine, modern science has confirmed many of garlic's benefits. With its rich source of phytochemicals, unique organosulfur compounds and high antioxidant activity, garlic has a broad range of health benefits and anti-aging effects, helping prevent disease and age-related pathological conditions. Pre-clinical and clinical studies have shown that garlic can enhance immunity, protects against infection and inflammation and help lower the risk of cancer and cardiovascular disease. It's potential to protect against vascular dementia and Alzheimer's disease has been shown in pre-clinical studies. Scientific studies also show that garlic does not have to be eaten raw or fresh to be effective, that its potent odor is not needed for its health benefits. Research shows that aged, deodorized garlic extract (kyolic), that is highly rich in antioxidants (1-6) often works even better than fresh garlic, without causing digestive disorders and "garlic breath". Its odor-free property makes compliance easier in human studies that require a long term high intake of garlic.

2. A History of Garlic in Human Health

Garlic (*Allium sativum*) is a hardy perennial plant that belongs to the lily family. Though the exact geographic origin of garlic remains unknown, modern botanists think it originated in Central Asia, where people foraging in the fields for food and healing herbs discovered and cultivated the plant. Garlic was transported to various lands by migrating tribes and became native to the Near East, Europe, the Far East and Africa. Its characteristic white bulb is devoid of smell and flavor when intact, but when cut, macerated or chewed it acquires a pungent taste and odor, due to the enzymatic formation of allicin, an unstable and volatile compound.

Remnants of garlic have been found in cave dwellings that are over 10,000 years old. Egyptian tombs, dating back to close to 5700 years ago, were found to contain sketches and clay sculptures of the bulb. The ancient Egyptian text Codex Ebers details formulas with garlic, as a cure for heart problems, headaches, tumors and other ailments. Chinese writings dating from 2700 B.C. describe garlic for treating many ailments and for enhancing vigor. In India, Ayurvedic medicine recommends garlic as an energy booster and treatment for colds and fatigue.

In modern times garlic has become a popular health-promoting herb in the Far and Near East, Europe and the USA. In certain parts of China people eat about 20 grams of garlic a day, approximately 8 medium size cloves. In Germany, most adults take a daily garlic supplement to promote health. In the United States the use of garlic supplements has been rapidly escalating in recent years, with the most popular supplement being odorless aged garlic extract [1].

3. Beneficial Compounds in Garlic

The composition of garlic is complex, with over 200 different compounds that contribute to its effects. The most important and unique feature of garlic is its high content of organosulfur substances. Garlic contains at least four times more sulfur than other high sulfur vegetables-onion, broccoli and cauliflower. Garlic also contains carbohydrates, protein, fiber, saponins, phosphorus, potassium, zinc, moderate amounts of selenium and Vitamin C, steroidal glycosides, lectins, prostaglandins, essential oil, adenosine, vitamins B-1, B-2, B-6, and E, biotin, nicotinic acid, fatty acids, glycolipids, phospholipids, anthocyanins, flavonoids, fructosyl arginine, phenolics and essential amino acids [1-9].

3.A. Organosulfur Compounds

Depending on the conditions of its cultivation, garlic may contain at least 33 different organosulfur compounds. The allyl sulfur constituents in garlic are largely responsible for its health benefits. The major allyl sulfur content in freshly crushed/chopped/cut garlic is allicin, which is unstable and breaks down rapidly to produce odorous oil-soluble di-allyl sulfide, di-allyl disulfide, di-allyl trisulfide, and ajoene. The major allyl sulfur constituents in aged garlic extract (AGE) include S-allyl-cysteine and S-allyl-mercaptocysteine, which are water soluble and formed by the process of natural aging and bioconversion (1-6,9). From a medicinal point of view, the stable water-soluble organosulfur compounds in garlic are highly effective. Present to some extent in fresh garlic, their level increases by a process of aging.

4. Garlic Preparation on the Market

Modern scientific studies findings that confirm the medicinal properties of garlic have led to the development of commercially available garlic preparation. Among them, kyolic aged garlic extract stands out as the one most popular and best studied, with over 600 scientific publications to its name. Aged garlic extract (AGE), is produced by extracting and aging organically grown garlic, for 20 months, in an alcohol solution, at room temperature [1-4, 9]. The extraction and aging process produces a high content of water-soluble organosulfur compounds, and converts unstable components, such as allicin, to stable compounds, thus increasing the antioxidant content of AGE, compared to the fresh garlic [1-5, 9]

S-allyl cysteine, the main component in AGE is stable and highly bioavailable with an absorption of close to 98% into the circulation [1,] S- allyl cysteine is used for commercial standardization of AGE products including tablets, capsules and liquid forms. S- allyl mercaptocysteine, unique to AGE, is also a water-soluble organosulfur antioxidant compound, formed during the process of extraction and aging [1-3]; similar to S- allyl cysteine it has a wide range of health benefits. Aged garlic extract also contains some lipid soluble organosulfur compounds [1-3,9].

Non-sulfur compounds in AGE include proteins, carbohydrates (sugars, fructans, pectins), saponins, that are steroid substances recently shown to have antibacterial and antifungal actions, allixin, selenium and fructosyl arginin [4], that are important antioxidants.

Other commercially available garlic supplements include garlic powder tablets, said to contain a specified amount of the volatile allicin, oil of steam-distilled garlic, and oil macerated garlic, both containing volatile lipid-soluble diallyl sulfides [1,3].

4.A. Garlic in Modern Health Care

Research on the health benefits of garlic has increased in the last three decades, largely due to the increasing practice of alternative and complementary medicine. Garlic and some garlic supplements, notably aged garlic extract, that is the choice form of garlic in research, have been found to play a role in lowering the risk of a wide range of age-related chronic diseases and pathological conditions. These include atherosclerosis, heart disease, stroke, cancer and Alzheimer's disease; aged garlic extract has been found to help boost immunity, combat fatigue, increase endurance in exercise and protect against toxic effects of radiation and certain medications such as acetaminophen (reviewed in 1, 5-7).

5. Oxidative Stress and Antioxidants

Health and aging are influenced by genetic and environmental factors. While genes determine a hereditary susceptibility to disease, environmental factors, such as diet and lifestyle can modify genetic patterns by epigenetic means. Aging is associated with altered metabolic process accumulation of mutations and cellular death, that in the long run result in the wear and tear of the body and in a variety of age-related conditions and ailments [5-8, 10-13].

5.A. Reactive Oxygen Species

Reactive oxygen species (ROS), which include free radicals, are believed to play a significant role in age-related diseases, inflammation and aging [1,5,6,13]. Produced as by-products in metabolism and energy generation, ROS increase during infection and inflammation, exercise and stress, and following exposure to exogenous sources, such as NOx pollutants, smoking, certain drugs (e.g. acetaminophen) and radiation, including ultraviolet light from sunlight and other sources. [1] The presence of high levels of ROS in the body and inadequate levels of antioxidants to neutralize them leads to a state of oxidative stress that can trigger and promote age-related pathological conditions; these include cancer, cardiovascular and cerebrovascular diseases, Alzheimer's disease and other neuro-degenerative conditions. Oxidative damage accumulates with age and its consequences depend on the molecules that are attacked and on the prevailing levels of protective antioxidants, which are depleted by ROS and fall to lower levels with age [6, -8,12]. Oxidative stress may affect acquired immunity, by activating nuclear kappa B, which governs gene expression involving cytokines and cell adhesion molecules [13].

5.B. Types of Oxidative Damage

The detrimental consequences of ROS damage depend on the molecules involved. When DNA is the primary target ROS- induced mutations can lead to cancer [8]. Oxidation of lipids and proteins, damage cell membranes, increase the fragility of blood vessels, injure immune cells, modify enzymes, including protective enzymes and many other molecules [6, 7,8,13]. Oxidative modification of LDL cholesterol increases the risk of atherosclerosis, cardiovascular and cerebrovascular disease. In the brain, free radical-producing beta amyloid peptides (A β), trigger apoptosis in neurons, thereby increasing the risk of brain atrophy and dementia, including AD. Stroke, triggered by an atherosclerotic clot, can lead to dementia, as brain cells are deprived of oxygen and blood (ischemia), during blockage and die by apoptosis [10-12,14]

5.C. Apoptosis

Apoptosis, known as programmed cell death, is regulated by the Bcl-2 family of proteins that include the pro-apoptosis Bax and others and the anti-apoptosis Bcl-2 proper, Bag, and others [14]. The pro-apoptotic proteins activate a mitochondrial-dependent pathway, with the release of cytochrome C, followed by activation of cysteine proteinases (caspases) with caspase 3 leading cells to apoptotic death. Alternately, apoptosis can proceed by other pathways that are mitochondrial-independent pathway, such as the ligation of death receptors CD95 (Fas/Apo1/) and the subsequent recruitment of caspases [14].

5.D. Oxidative Stress and Disease

The link between oxidative stress and age-related ailments is largely observed in studies showing that a high intake of antioxidant-rich plant products, including garlic, is associated with a reduced risk of many chronic diseases and aging [1, 7, 8]. Antioxidants neutralize and destroy ROS, and prevent reactive radicals from causing genetic changes and apoptosis [7, 13] While internal antioxidant such as glutathione, superoxide dismutase, catalase and peroxidases provides some defense against oxidative damage, a diet rich in plant products that contain essential nutrients and phytochemicals with antioxidant activity protects against free radical- mediated conditions. Garlic represents an important source of antioxidant phytochemicals and has been shown to help prevent age related diseases and apoptosis [1,5,6] Antioxidant supplementation can also reverse some age-associated immune deficiencies, increasing levels of killer cell activity, enhancing antibody response to antigen stimulation, decreasing lipid peroxidation and prostaglandin synthesis [1, 13]

6. Garlic and Cardiovascular Disease (CVD)

Several clinical reports, including meta-analyses, have shown the cholesterol-lowering effects of garlic supplementation in humans. Garlic and aged garlic extracts have impart

cardiovascular benefits through multiple mechanisms, reducing cardiovascular risk factors [1,5, 6, 15-36]. Garlic and AGE have been shown to lower blood pressure [23], inhibit the aggregation and adhesion of platelets (thus helping prevent blood clots) [18,19], lower homocysteine [16,17], reduce LDL cholesterol (the bad cholesterol) and elevate HDL (the protective cholesterol) [15,20]. Garlic compounds prevent LDL oxidation that exacerbates atherosclerosis [22]. Studies with S- allyl cysteine, the main water soluble compound in aged garlic extract, have shown a mechanism underlying the lowering of cholesterol, with S allyl cysteine deactivating the cholesterol synthesizing enzyme, HMG-CoA reductase, by 30-40% [28] and having an additive effect with lipid lowering statins.

6.A. Garlic Increases Antioxidant Capacity

A recent study on elderly subjects found that ingestion of garlic leads to decreased oxidation reactions, which plays a part in the beneficial effects of garlic. Supplementation of garlic extract improves blood lipid profile, increased the antioxidant enzymes glutathione peroxidase and superoxide dismutase, significantly lowered LDL cholesterol and reduced systolic and diastolic blood pressure. It also leads to a decrease in the level of peroxidation product (MDA) in the blood, which demonstrates reduced oxidation reactions in the body [29].

Another human study investigated the effects of ingesting garlic extract on plasma and erythrocyte antioxidant parameters of atherosclerotic patients. The patients ingested 1 ml/kg body weight of garlic extract daily for 6 months. Blood samples obtained from healthy subjects served as the controls. In the patients with atherosclerosis plasma xanthin oxidase and MDA levels, marker of oxidant stress, were higher, but plasma and erythrocyte glutathione peroxidase activities were lower, relative to those of the control group. Ingestion of garlic extract leads to significantly lowered plasma and erythrocyte MDA levels in the patients, even in the absence of changes in antioxidant enzyme activities. This study shows the prevalence of oxidant stress in blood samples from patients with atherosclerosis and the ability of garlic to prevent oxidation reactions by eliminating this oxidant stress and “have beneficial effects in atherosclerotic diseases” [35].

6.B. Garlic Reduces Blood Lipids, Blood Pressure and Inflammation

A double blind, crossover, placebo controlled trial was performed to evaluate the effect of aged garlic extract on blood lipids, in 41 moderately hypercholesterolemic men (cholesterol 220-290 mg/dL). Following a 6 months investigators found that a daily ingestion of 7.2 g aged garlic extract, resulted in a 6.1% or 7.0% reduction of total cholesterol, over baseline, LDL and blood pressure were decreased by aged garlic extract, 4% when compared with average baseline values and 4.6% in comparison with placebo. In addition, the intake of aged garlic extract also resulted in a 5.5% decrease in systolic blood pressure and a modest lowering of diastolic blood pressure [36].

Garlic, in the form of AGE has been found to reduce smoking- related oxidative damage and lower the production of prostaglandins involved in inflammation [6,30] Aged garlic extract has also been found to reduce levels of homocysteine, a risk factor for CVD

[17,20,21,32,33] and reduce the levels of nuclear factor kappa B that plays a role in oxidation and inflammation linked to atherosclerosis [34].

6.D. Garlic Inhibits Coronary Artery Calcification

A double blind placebo controlled randomized clinical study, with aged garlic extract, showed that garlic inhibits the progression of coronary artery calcification and potentially reduces the risk of heart attacks [20]. Twenty-three patients, who were at high risk for heart disease, received either 1200 mg/day aged garlic extract, for one year, or a placebo. As all patients were on statin and aspirin therapy, any improvement seen under the influence of the extract would be an additional benefit to statin treatment. Using a non-invasive electron beam computed tomography to measure calcification, that is a measure of atherosclerosis, investigators found that in the placebo group coronary artery plaques progressed at a rate of 22% a year, while the addition of aged garlic extract to the diet reduced progression to 7.5%. The garlic extract also elevated HDL and decreased LDL, triglycerides and homocysteine, as compared to placebo.

In another, placebo-controlled double blind, randomized trial, 65 patients at intermediate risk for CVD were treated daily, for one year, with placebo capsule or a capsule containing aged garlic extract (250mg) plus vitamin B12 (100 micrograms), folic acid (300 micrograms), vitamin B6 (12.5 micrograms) and L arginine (100mg). All patients underwent coronary artery calcium scanning to determine coronary artery atherosclerotic progression as well as tests for a variety of high risk factor. The results showed that calcification progression was significantly lower in the AGE plus supplements as compared to placebo. Total cholesterol, LDL, homocysteine and oxidation biomarkers were lower in the AGE group, compared to the placebo group, while HDL and other protective factors were significantly increased [37]. A recent study confirmed the strong relation among markers of oxidative stress, vascular dysfunction, coronary artery calcification and progression of atherosclerosis [38], thus further confirming the cardio-protective effects of aged garlic in its ability to reduce these biomarkers in human subjects.

6.E. Garlic Protects Endothelial Function

Garlic has been shown to protect endothelial cells from oxidative damage, increase blood flow and improve microcirculation [1,15,17]. These effects are most significant in aging, as arteries stiffen with age and microvasculature is damaged, increasing the risk of CVD as well as dementia. Experimental and clinical studies with aged garlic extract show that garlic increases the production of an endothelial relaxing factor, cellular nitric oxide (NO), that improves endothelial function and helps reduce hypertension [17, 25].

An increased plasma homocysteine, above 12 $\mu\text{mol/L}$, is associated with elevated oxidant stress, depleted endogenous antioxidants, such as glutathione, and endothelial dysfunction, thus enhancing the risk of CVD (17). Aged garlic extract has been shown to increase glutathione levels and stimulate NO generation in endothelial cells. A placebo-controlled, blinded, crossover trial, in healthy subjects found that aged garlic extract protected endothelial cells from homocysteine damage, in part, by preventing a decrease in bioavailable

NO and other factors, during acute hyperhomocysteinemia [17]. In another randomized, double blind placebo-controlled trial, 15 men with coronary artery disease were treated with 2.4 grams/day of aged garlic extract or placebo, for two weeks. The results showed that garlic treatment increased endothelial function by 44% (increasing vessel dilation and blood flow), compared to placebo, indicating that even a short term treatment with the garlic extract improves endothelial function that has been impaired by coronary artery disease [31].

7. Garlic in Cancer Prevention

7.A. Cancer, Oxidative Stress and Aging

Cancer, a major cause of human death increases in frequency with age [8]. All cancers involve the malfunction of genes that control cell growth and division. About 5-10% of these diseases are hereditary, whereby, inheriting defective genes predispose a person to a particular cancer. The remainder cancers are thought to result from multiple mutations in DNA and damage to other molecules, which accumulate with age, following oxidant – induced mutations and cell death. Exogenous oxidants, such as tobacco, chemicals, and radiation, including sunlight, as well as internal factors such as hormones, infections, immune reactions and inflammation contribute of cancer development. As free-radical oxidative stress plays an important role in the initiation and promotion of cancer, a high intake of antioxidants-containing plant foods, notably garlic, reduces the risk of certain cancers [7, 8, 39-44].

7.B. Mechanisms of Garlic in Cancer Prevention

Garlic and its allyl sulfur constituents have been reported to exert their cancer preventive effects in animal and in vitro studies by several mechanisms, including inhibition of carcinogen-induced DNA adduct formation , blockage of cell growth, blockage of cell proliferation, and blockage of angiogenesis, induction of differentiation and/or apoptosis, enhancement of carcinogen-detoxifying enzymes, suppression of carcinogen-activating enzymes, inhibition of cyclooxygenase-2 expression, scavenging carcinogen-induced free radicals, and inhibition of lipid peroxidation. Pre-clinical studies have shown that garlic, aged garlic extract and their components, inhibit the growth of mammary, colon, stomach and other cancers and induce apoptosis in the cancer cells [1, 44-45]. S-allyl cysteine and, S-allyl mercaptocysteine, have powerful radical scavenging ability [1-4], a capacity to increase the cellular antioxidant glutathione and to inhibit lipid peroxidation, that is linked to cancer development [38]; some lipid soluble organosulfur compounds, in garlic and in aged garlic extract have also been found to be cancer preventive [44,50], in part, due to their ability to scavenge ROS and decrease lipid peroxidation [51].

7.C. Molecular Studies

Molecular studies on cancer preventive effects of garlic have been carried out largely with aged garlic extract and its component. Among the potential chemopreventive mechanisms of organosulfur compounds, cell-cycle regulation and apoptotic signaling have received considerable attention. S allyl mercaptocysteine, was found to exert antiproliferative effects through a direct binding of sulfur- containing metabolites to redox-sensitive sites, on signal protein or transcription factors, arresting cells in mitosis and activating other pro-apoptotic signaling pathways [47] Experiments showed that within 10 minutes after entry S-allyl mercaptocysteine interacted with tubulin and initiated microtubuler depolymerization. Within the following 15 minutes S- allyl mercaptocysteine induced the c-Jun NH-terminal kinase pathway that leads directly to the activation of caspase-3, poly(ADP-ribose) polymerase, and to other events that mediate the early phase of apoptosis. Concurrently with these events, S-allyl mercaptocysteine interfered with the assembly of mitotic spindles, arresting cells in mitosis within the first 12–24 h. This arrest triggered spindle-checkpoint events that culminated in late-phase apoptosis. In addition, other pathways became activated by S- allyl mercaptocysteine such as ERK1/2 and p38 MAP kinase [47]

Studies using flow cytometry analyses of DNA found that some S-allylsulfides can prevent certain tumor-cell lines from progressing through the G1/S phase while other tumor-cell lines are blocked in G2/M [47]. Aged garlic extract was also found to inhibit angiogenesis, that is essential for tumor growth [48] and stimulate immune function such as proliferation of lymphocytes, cytokine release, enhanced natural killer (NK) cell activity and phagocytosis [49]. Other molecular studies have found that, while inducing apoptosis in cancer cells, an important determinant in successful cancer treatment, AGE protects normal cells from the apoptotic effects of various modalities used in cancer therapy, including radiation and chemotherapeutic agents, such as methotrexate [46]. The viability and apoptosis of intestinal cells were examined in the presence of methotrexate and /or AGE. The results showed that while methotrexate decreased cell viability in a dose related fashion, the presence of aged garlic extract at a low concentration of 0.1% almost completely prevented the methotrexate –induced cell death. The apoptotic characteristics of chromatin condensation, DNA fragmentation, caspase-3 activation and cytochrome c release, seen in cells exposed to methotrexate, were preserved to the control levels by AGE. Similarly, AGE prevented a decrease in intracellular GSH, caused by methotrexate. Cells in the G2/M stage were markedly decreased 72 h after methotrexate. AGE restored them to control level [46].

7.D. Garlic and Human Cancer

The efficacy of garlic as a cancer preventive food has been shown in epidemiological observation, largely in China, the United States and Italy as well as in clinical studies. A high consumption of garlic was found to reduce the risk of stomach and colorectal cancer, sometimes by 50% [1, 40,41,43]. A trend towards an association between garlic consumption and reduced risk of prostate and breast cancer has also been reported [41].

Colorectal cancer is the 3rd leading cause of death from cancer for both men and women in the United States. Most colorectal cancers are thought to develop from slowly growing adenomas that are benign polyps. A review of case- control/ cohort studies conducted in the

last 10 years found a protective effect of garlic against colorectal cancer [40]. One randomized controlled trial reported a statistically significant 29% reduction in both size and number of colon adenomas in colorectal cancer patients taking aged garlic extract [40, 52]. Five of 8 case control/cohort studies suggested a protective effect of high intake of raw/cooked garlic and 2 of 8 of these studies suggested a protective effect for distal colon. A meta-analysis of 7 of these studies confirmed the inverse relationship, with a 30% reduction in relative risk [40]. The significance of the randomized trial lies also in the fact that while fresh garlic intake has been known for some time to lower the risk of gastrointestinal cancer [40–43], the efficacy of a garlic supplement in humans remained unknown. The trial with aged garlic extract was a double-blind, randomized clinical trial, in which patients with pre-cancerous colorectal adenomas received a high-dose of kyolic aged garlic extract (2.4 ml/day), while other patients, serving as controls, received a low-dose (0.16 ml/day). The number and size of adenomas were measured, by colonoscopy, before garlic intake and at 6 and 12 months after intake. Following 12 months of treatment the investigators found that in control subjects the number of adenomas increased in linear fashion compared to baseline, at the onset of the study. By contrast, the group given the high dose aged garlic extract showed a significant 29% reduction in developing at least 1 new adenoma in these patients, with a reported relative risk of 0.71. There was a reduction in both the number and size of adenomas, ($P=0.04$), potentially inhibiting the progression of adenomas to colorectal cancer [52].

8. Garlic and Dementia

Various types of dementia, including Alzheimer's disease (AD) its most common form, are devastating age-related forms of mental deterioration. During the past decade, a growing volume of data has shown that vascular factors, such as homocysteine, play a role in Alzheimer's disease [53] and that people with risk factors for CVD and a history of stroke have an increased risk of vascular dementia and Alzheimer's disease [5, 54].

The antioxidant actions of garlic and aged garlic extract and their ability to reduce CVD risk factors may play a significant role in lowering the risk of vascular dementia and AD. The role of garlic compounds as potential preventive agents against neurodegenerative conditions includes the ability of garlic to lower cholesterol, inhibit inflammation, reduce homocysteine, prevent oxidative brain injury following ischemia, protect neurons against apoptosis triggered by oxidative stress, and prevent neurotoxicity by beta amyloid peptide, the hallmark of AD [5]. In addition, aged garlic extract was found to prevent atrophy in the frontal brain of early senescence mice models, improved learning and memory retention and increased longevity [55]; other studies showed that allixin, a component of garlic and AGE enhanced the survival of neurons and increase the branching points in axons of hippocampus neurons [56].

8.A. Garlic and Ischemic/Reperfusion Brain Damage

Ischemia occurs in heart diseases, transient ischemic attacks, cerebrovascular accidents, and peripheral artery occlusive disease. The heart, brain and kidneys, are the most sensitive to inadequate blood supply. Ischemia in the brain tissue, due to stroke or head injury, results in a

cascade of events in which proteolytic enzymes and ROS destroy neurons by apoptosis, sometimes leading to dementia. Restoring blood flow following ischemia increases damage, as the replenishment of oxygen enhances free radicals production, resulting in reperfusion injury and necrosis that contribute to the development of dementia following a stroke.

Garlic and its organosulfur compounds protect neurons from ischemia/reperfusion injury and increase their survival. In focal ischemia, aged garlic extract, water-soluble S-allyl cysteine, lipid soluble allyl sulfide or lipid soluble disulfide was administered 30 min prior to the ischemic insult. Three days after the insult, investigators assessed the degree of ischemic damage by measuring the water content of the ischemic and contra-lateral hemispheres. Treatment with aged garlic extract or S- allyl cysteine attenuated ROS, reduced infarct volume and prevented brain injury. None of the lipid- soluble compounds had a protective effect [59]

8.B. Garlic and Homocysteine and Dementia

Elevated homocysteine, a risk factor for cardiovascular and cerebrovascular diseases [57], is an independent risk factor for Alzheimer's disease and other forms of dementia [53,58]. The association between high homocysteine and dementia, observed in epidemiological studies, has been confirmed in case-control studies, where people with vascular dementia and AD had higher levels of homocysteine, than did healthy people. The study provided compelling evidence for a direct link between high homocysteine and loss of cognition. It showed that in adults with intact cognition, a long-term increase in plasma homocysteine was associated with an increased incidence of dementia, including AD [53].

Garlic can protect against homocysteine-related vascular damage [16,17]. In a clinical trial, aged garlic extract reduced macro-and microvascular endothelial dysfunction, during acute hyperhomocysteinemia [17]; a pre-clinical trial showed that while homocysteine stimulated the uptake of oxidized LDL into macrophages, a step in early atherosclerosis, AGE inhibited the uptake, thus preventing the formation of early atherosclerotic lesions [16].

Other pre-clinical studies suggest that AGE may serve as an added treatment in hyperhomocysteinemia, a condition that is largely associated with a depletion in folic acid [21]; In one study homocysteine levels were examined following a 4 week feeding of a folate deficient diet that contained AGE, compared to a diet that was contained both folic acid and AGE. The results showed a 30% lower homocystein level in the group that was fed the folic acid deficient diet with AGE, compared to a diet that had folate and AGE.

8.C. Garlic and Neuroprotection

Garlic has been shown to act as a neuroprotective agent, potentially defending against Alzheimer's disease [5], a disease in which oxidative damage and inflammation, result in neuronal apoptosis. Extra-cellular fibrillar aggregates of beta amyloid peptides [A β], that produce free radicals, are major constituents of senile plaques found in the brains of AD patients and have been linked to AD neurotoxicity. In experimental studies, cells exposed to A β show a significant increase in reactive oxygen species, produced by A β , supporting the notion of the role of ROS in A β -mediated neurotoxicity and apoptosis [60];

Garlic and S- allyl cysteine protect neuronal cells against Abeta toxicity and apoptosis [60-63] and prevent cognitive decline [64-68]. The studies show that the garlic extract and S-allyl cysteine prevent Abeta- induced neurotoxicity, by suppressing ROS generation, attenuating the activation of the apoptotic enzyme caspase 3, in a dose related manner, and attenuating DNA fragmentation and apoptosis. [61-63]. S allyl cysteine also provided protection by binding to Abeta, inhibiting Abeta fibrillation and destabilizing pre-formed Abeta fibrils. [67] Feeding of aged garlic extract to mice with accelerated Abeta formation prevented deterioration of hippocampal- based memory tasks, suggesting that aged garlic extract may have a potential ability to prevent AD progression. [68]

Preclinical studies in models that are genetically prone to early senescence show that garlic has protective effects on brain function. [64,65]. Treatment with aged garlic extract or S-allyl cysteine, prevented the degeneration of the frontal lobe, improved learning and memory retention and extended life span. Isolated neurons from the hippocampus, grown in the presence of aged garlic extract or S- allyl cysteine, showed a unique ability to grow and branch out, which may be linked to the efficacy of the extract in increasing learning and cognition [65].

A recent study, in the Swedish double mutant mouse model for AD (Tg2576), investigators studied the anti-amyloidogenic, anti-inflammatory and anti-tangle effects of aged garlic extract (2%) and compared its efficacy with that of its major constituent, water soluble S-allyl-cysteine (SAC), at 20 mg/kg and with the lipid-soluble di-allyl-disulfide (DAD), at 20 mg/kg. The protective action of the dietary interventions were in the order of AGE>SAC>DADS [69]. The authors suggest that dietary intervention with herbal alternative such as aged garlic extract, which has a wide range of benefits, including cholesterol reduction, antioxidant effects and anti-inflammatory actions and has no adverse side effects, may provide greater therapeutic benefit over a single-ingredient synthetic pharmaceutical drug, which may have serious side effects in treating Alzheimer's disease [69].

9. Garlic Enhances Endurance and Reduces Fatigue

Fatigue and reduced energy often accompany aging and certain ailments. Traditional medicine and practices in antiquity used garlic as a remedy to help increase energy levels [1]. Recent studies have shown, that indeed, garlic, in the form of aged garlic extract, can increase endurance in exercise, and reduce fatigue [70,71]. Though the underlying mechanisms remain unclear, the efficacy of garlic in increasing vasodilation and blood flow and consequently oxygen supply may play an important role in its anti-fatigue effects.

Conclusion

A large number of studies confirm the benefits of garlic in human health. Rich in organosulfur compounds, that provide most of its medicinal effects, as well as other antioxidants, garlic has been shown to help prevent certain chronic diseases and conditions associated with aging, in humans, notably cardiovascular disease and certain cancers. The

protective actions of garlic are largely due to its ability to reduce oxidative stress and inflammation, prevent oxidative damage to DNA, lower cholesterol, triglycerides and homocysteine, inhibit coronary artery plaque formation, prevent platelet aggregation, increase vasodilation and blood flow, lower blood pressure, prevent neuronal cell death by apoptosis and increase memory and cognition, boost immunity, increase glutathione, an important internal antioxidant, increase physical endurance and ameliorate fatigue. Among the many studies on the health benefits and anti-aging effects of garlic, the most consistent results are from preclinical and clinical research with aged garlic extract (kyolic) [72,73], a highly standardized supplement, prepared by a long term extraction and aging of fresh garlic, which enriches its content of antioxidant and renders its stable water soluble compounds, such as S-allyl cysteine, highly bioavailable [72]. Taken together, a diet rich in garlic its odor- free form AGE has the potential to promote human health, and helps provide protection from chronic diseases and aging.

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Chapter XVIII

Biological Activity and Mechanism of Action of Plant Polyphenols: Relevance to Human Health and Disease

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Abstract

A number of studies have shown that consumption of fruits, vegetables, wine and tea may give protection against development and progression of several chronic human diseases such as cancer, diabetes, Alzheimer's and cardiovascular diseases. Polyphenolic compounds including flavonoids and stilbenes, produced as the result of secondary metabolism of plants represent an important source of antioxidants in the diet. More than 8000 different polyphenols have been described and classified into different groups as a function of the number of phenol rings that they contain and on the basis of structural elements that bind these rings to one another. The mechanism(s) by which these active compounds elicit their effects, is/are still less and inconclusive. In the present chapter we have tried to provide a current update on the mode of action of plant polyphenols including their antioxidant, anti-estrogenic, anti-inflammatory and anti-proliferative activities.

Keywords: *Polyphenols, Metabolism, Biological activity, Human diseases*

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Introduction

Polyphenols are active plant metabolites and are ubiquitous major constituents of our daily diet. A wide variety of dietary plants and plant products including grains, legumes, fruits, vegetables, tea and wine contain polyphenols in abundant amount (Manach et al., 2004). Plant and plant products have been used for good health without any side effects since ancient times, the beneficial effects being attributed to the presence of vitamins. However, later experimental evidence revealed that there are other compounds having the six-carbon aromatic ring(s) and two or more phenolic hydroxyl groups, known as polyphenols and that these polyphenols are responsible for most of the health beneficial effects of plant/plant products (Stevenson and Hurst, 2007). Polyphenols are synthesized in plants via the major biosynthetic pathway that starts with phenylalanine, which is converted into cinnamic acids and then elaborated into the various other classes of compounds, ending with the anthocyanins (Ross and Kusum, 2002).

In recent years there has been a remarkable increment in scientific knowledge dealing with the beneficial role of polyphenols. Epidemiological studies have repeatedly shown an inverse correlation between consumption of polyphenols or polyphenol rich diet and development and progression of many chronic human diseases (Yang et al., 1997; Scalbert et al., 2005). A number of studies have demonstrated that the consumption of polyphenols limits the development of cardiovascular diseases (CVDs). Supplementation of drinking water with de-alcoholized wine, pomegranate juice, catechins, or quercetin reduced the size of these lesions in apoE-deficient mice (Hayek et al., 1997). Tea catechins are known to inhibit the invasion and proliferation of smooth muscle cells in the arterial wall, a mechanism that may contribute to slowing down the formation of the atheromatous lesion (Maeda et al., 2003). Grape seed polyphenols have hypocholesterolemic effect on rats fed a high-cholesterol diet (Tebib et al., 1994). It is reported that consumption of flavanones by normolipemic or hyperlipemic rats for 2–6 weeks reduced total plasma cholesterol, LDL-cholesterol, and triglycerides (Bok et al., 1999). Polyphenols can improve endothelial dysfunction, an early event in atherogenesis. The endothelial-dependent vasorelaxing activity of isolated polyphenols, such as wine anthocyanins, soy isoflavones, resveratrol, quercetin, and cocoa proanthocyanidins has been observed (Scalbert et al., 2005).

Polyphenols are also known as potent anticancer agents with the ability to block the development of cancer at all three stages (Aggarwal et al., 2004). Polyphenols, when given to rats or mice before and/or after the administration of a carcinogenic agent or the implantation of a human cancer cell line, are most often protective and induce a reduction in the number of tumors or of their growth (Yang et al., 2001). These effects have been observed at various sites, including mouth, stomach, duodenum, colon, liver, lungs, mammary gland or skin. Many polyphenols, such as quercetin, catechins, isoflavones, lignans, flavanones, ellagic acid, resveratrol and curcumin, were tested; all of them showed protective effects in cancer (Scalbert et al., 2005).

Several reports confirm that moderate consumption of red-wine is associated with a lower incidence of Alzheimer's disease. Studies now in existence show that polyphenols especially resveratrol present in red-wine is responsible in prevention of neuro-disorders (Anekonda, 2006). Tea catechins are also reported to elicit neuro-protection. EGCG at 1 μ M concentration was able to reduce neuronal cell death in serum-starved cells and to promote

neurite outgrowth, suggesting a capacity to reduce or even reverse neuro-degeneration (Stevenson and Hurst, 2007). Curcumin is reported to have a strong neuroprotective activity (Rossi et al., 2008). Results of epidemiological studies suggest that the significant lower occurrence of Alzheimer's disease in India compared to the United States may be due to the higher consumption of turmeric (Ganguli et al., 2000). Polyphenols also show anti-diabetic effects. Green tea polyphenols, resveratrol, quercetin, and recently myricetin are reported to have protective effects in diabetic patients (Rizvi et al., 2005; Pandey et al., 2009a). Isoflavones with weak estrogen-like activity have attracted much attention as a possible alternative treatment to prevent osteoporosis (Anderson and Garner, 1997). Anti-aging effects of polyphenols are very recent entry in the field of beneficial effects of polyphenols. Protective role of plant polyphenols has also been reported in many other altered physiological conditions such as hypertension and stress (Pandey and Rizvi, 2009a). In the present review we provide a current update on polyphenol research and discuss the major mechanisms by which polyphenols elicit such diverse health beneficial effects. In the last part of this review, clinical trials of polyphenols on humans are also covered.

Sources and Different Classes of Polyphenols

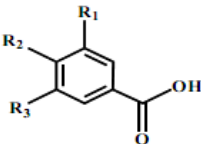
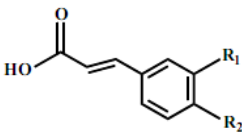
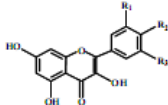
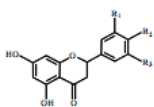
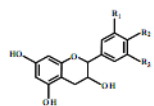
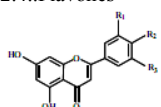
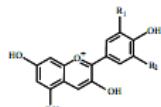
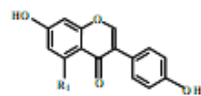
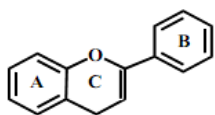
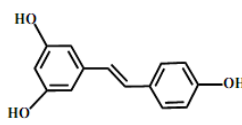
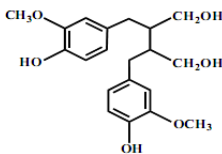
Polyphenols comprise largest groups of bioactive compounds of plant origin. All food products obtained from plants including fruits, vegetables, cereals, beverages and spices contain polyphenols in more or less amount (Naczki and Shahidi, 2006). Polyphenols are secondary metabolites of plants, essential to plant physiology, contributing to their pigmentation, growth, reproduction, and resistance to pathogens and predators. They are also responsible for the astringency and bitterness of plant derived food and beverage, polyphenols also prevent oxidation during processing or storage (Scalbert et al., 2005).

More than 8000 phenolic compounds of plant origin have been discovered, ranging from simple molecules to highly polymerized compounds and have been classified into different groups as a function of the number of phenol rings that they contain and of the structural elements that bind these rings to one another. The main classes include phenolic acids, flavonoids, stilbenes and lignans (Spencer et al., 2008). Flavonoids (di-phenyl propanes, two aromatic rings linked through three carbons forming an oxygenated heterocycle) being the most representative in abundance (table 1). On the basis of their molecular structure, flavonoids are further divided into six subclasses: flavonols, flavones, flavanones, flavanols, anthocyanins, and isoflavones. Individual differences within each group arise from the variation in number and arrangement of the hydroxyl groups and their extent of alkylation and/or glycosylation (Spencer et al., 2008; Pandey and Rizvi, 2009a).

Phenolic acids account for about one-third of the total phenols and are divided into two subclasses; hydroxybenzoic acids and hydroxycinnamic acids. Caffeic acid, gallic acid are very common examples of phenolic acids, well known for their antioxidant effects. Quercetin is one of the most studied flavonoid belongs to the flavonol, subclass. Catechins, the green tea polyphenols are flavanols and an infusion of green tea could contain 1 g/L catechins, whereas in black tea, their content is reduced to about half of this value due to their oxidation into more complex polyphenols during fermentation (Yang et al., 1997). The main catechins of

green tea are epigallocatechin-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin (EC), and (-)-epicatechin gallate (ECG) (Figure1).

Table 1. Examples, sources and representative structures of different classes of polyphenols

Polyphenols	Common Chemical Structures	
1. Phenolic acids Hydroxybenzoic acids Sources: Blueberries Cereals, Oilseeds Examples: Gallic acid Protocatechuic acid Hydroxycinnamic acids Sources: Apricots, Carrots, Citrus fruits Examples: Caffeic acid, Ferulic acid	 	2.1. Flavonols  Examples: Quercetin, Myricetin 2.2. Flavanones  Examples: Naringenin, Hesperetin 2.3. Flavanols  Examples: Catechins, Theaflavins 2.4. Flavones  Examples: Apigenin, Luteolin 2.5. Anthocyanins  Examples: Cyanidin, Delphinidin 2.6. Isoflavones  Examples: Daidzein, Genistein
2. Flavonoids Sources: Apples, Grapes Onions, Soya beans		
3. Stilbenes Sources: Grapes, Peanuts, Pomegranate, Berries Examples: Resveratrol Piceids, Astringins		
4. Lignans Sources: Buckwheat Flax seed, Sesame seed, Barley Examples: Matairesinol Secoisolariciresinol		

Anthocyanins are pigments of red fruits such as berries, grapes, and strawberries, and their contents could vary from 0.15 to 4.5 mg/g in fresh fruit. Flavones are comparatively less common and identified in sweet red pepper (luteolin) and celery (apigenin) (Hertog et al., 1993). The citrus fruits are main sources of flavanones, hesperidin from oranges being the most widely consumed. The main source of isoflavones is soy, which contains around 1 mg of genistein and daidzein per gram of dry bean (Mira et al., 2002).

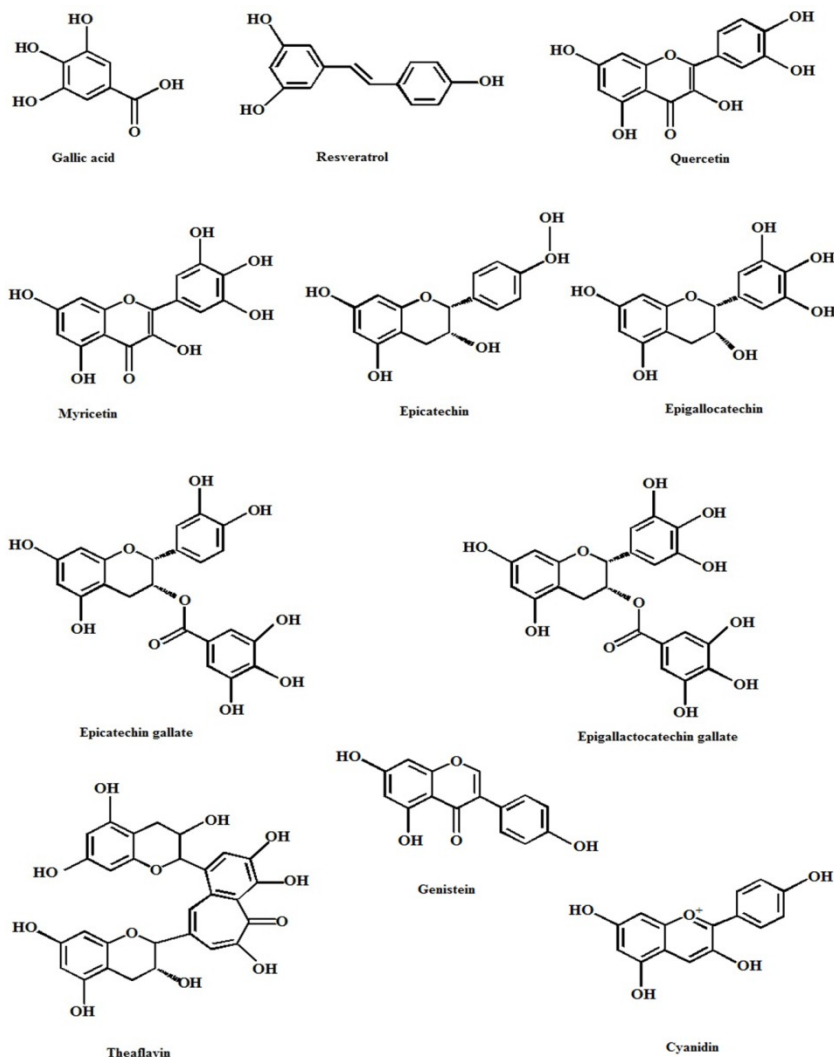


Figure 1. Structures of some common polyphenols.

Stilbenes are not widely found in food plants, however their presence are abundantly reported in beverages and berries. Resveratrol (Figure1) is a well known example of stilbenes. Researchers have found that resveratrol possess a dynamic health beneficial properties including anti-aging (Harikumar and Aggarwal, 2008; Pandey and Rizvi, 2009b). Lignans are mainly present in flaxseed and flaxseed oil and are recognized as phytoestrogens due to their estrogen-like effect.

Mechanism of Action of Polyphenols

An array of mechanisms of action has been put forward to explain the pleiotropic biological effects of polyphenols. Investigations have revealed that several complimentary actions work in concert for a particular biological effect.

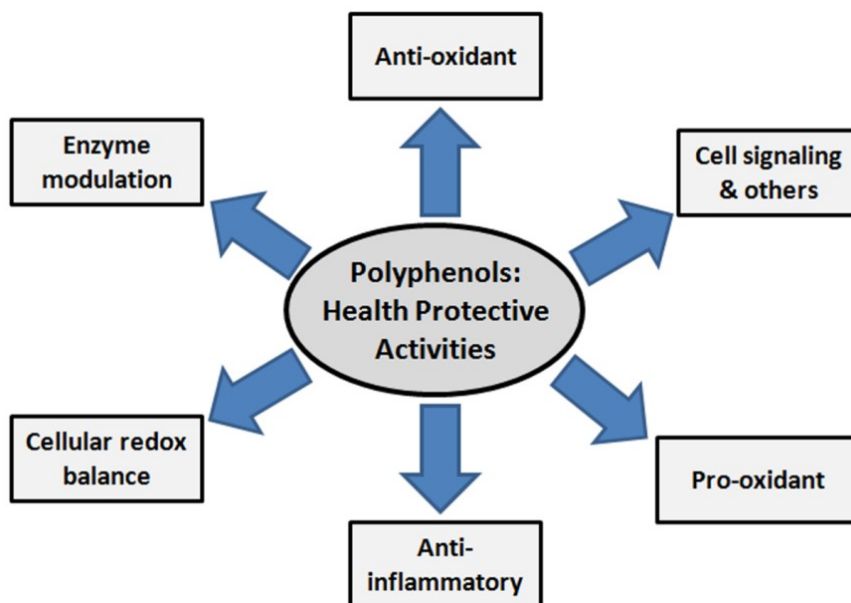


Figure 2. Mechanisms involved in pleiotropic health protective effects of polyphenols. Polyphenols are reported to elicit their effects through an array of mechanisms, however shown activities are the major mechanisms comprise for most of the effects exhibited by polyphenols.

Antioxidant Activity

Antioxidant activity is the most documented action of plant polyphenols, responsible for most of the effects shown by them. Reactive oxygen species (ROS) are continuously produced inside all the aerobic cells as by-product during their normal metabolic processes including the electron transport in mitochondria. ROS are highly hazardous, they oxidize membrane polyunsaturated lipids, proteins and nucleic acids, thus disrupting their structures, and impairing their function, and interfering with many other aspects of normal cell metabolism (Halliwell and Gutteridge, 2006). ROS are counteracted in biological systems by a large set of endogenous antioxidants, including enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) as well as other compounds such as reduced glutathione (GSH), Vitamin C and E. However when the ROS are produced in excess, beyond the inherent capacity of biological systems to counteract them due to exposure of various environmental factors, life style or weak endogenous defense systems, a condition develops known as oxidative stress. Now it has been well explored that oxidative stress is one of the main causes in development of many chronic human diseases such as cancer, CVDs,

diabetes, neuro-disorders and aging (Halliwell and Gutteridge, 2006, Pandey and Rizvi, 2010a).

Mounting evidence suggests that polyphenols counteract oxidative damage and thus slow/prevent the onset of many serious diseases. The antioxidant activity of polyphenols is correlated with the number of hydroxyl groups present on the aromatic rings, and with the presence of a C2– C3 double bond: the most active ones contain between 3 and 6 hydroxyl groups (van Acker et al., 1996; Rossi et al., 2008). The antioxidant mechanism is based on the donation of a hydrogen and the formation of a phenoxyl radical that undergoes stabilization either by release of a further hydrogen, or by reaction with another radical. The ability of flavones and flavanol polyphenols to chelate redox-active transition metal ions, such as iron or copper, depends on the presence of their carboxylic and hydroxylic groups and may contribute to their antioxidant activity, because it prevents metals from catalyzing free radical formation (Rossi et al., 2008).

Catechins can exert their antioxidant activity through various mechanisms, one being by chelating metal ions such as copper(II) and iron (II), and therefore prevent the generation of potentially damaging ROS (Levites et al., 2003). It has been reported that catechins may also exert their antioxidant effects through the ultra rapid electron transfer to ROS-induced radical sites on DNA or by forming stable semiquinone free radicals (Singh et al., 2008). Moreover, after the oxidation of catechins by ROS, a dimerized product is formed which has an increased iron-chelating potential and ability to scavenge superoxide anions. The prevention of oxidative-induced damage by catechins is very effective as catechins have ability to inhibit the ROS-induced damage by a wide variety of initiators including H₂O₂, iron, or radiolysis (Singh et al., 2008).

In several studies, resveratrol has been recognized for its powerful antioxidant properties (Pandey and Rizvi, 2009c; Shakibaei et al., 2009). There is evidence to support that resveratrol is a potent inhibitor of the oxidation of polyunsaturated fatty acids (PUFA) that play a major role in cardio-vascular problems (Miller and Rice-Evans, 1995). Compared to flavonoids, resveratrol has been shown to be more potent in protection against copper catalyzed oxidation (Franke et al., 1993). Our recent findings indicate that resveratrol, at the concentration from 1-100 μM showed the strong antioxidant activity as evidenced by the decreased level of protein oxidation and lowered lipid peroxidation in human erythrocytes (Pandey and Rizvi, 2009c). The antioxidant property of resveratrol is also reported to be based on its ability to maintain the cellular status of antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase (O'Byrne et al., 2002; Baur and Sinclair, 2006). Indeed resveratrol has been shown to increase reduced glutathione (GSH) levels, which leads to lowered ROS production (Pandey and Rizvi, 2010b).

The intake of rutin enhances the antioxidant status of mouse liver tissue and Mn-superoxide dismutase activity (Gao et al., 2002). It has been documented that anthocyanins from elderberry extracts protect endothelial cells against several oxidative stressors, including H₂O₂, AAPH (2,2'-azobis(2-amidinopropane), and Fe/ascorbic acid (Youdim et al., 2000). Curcumin, in the micro to millimolar range was also shown to scavenge ROS, such as superoxide anion, H₂O₂ and nitric oxide (NO), both *in vitro* and *in vivo* (Rahman et al., 2006).

Anti-Inflammatory and Enzyme Modulatory Effects

There is substantial evidence that polyphenols also elicit their effects as anti-inflammatory agent.

Inflammation is thought to be implicated in the patho-physiology of many serious diseases including Alzheimer's disease (AD) (Rossi et al., 2008). Investigations into the mechanism of action of these molecules have thrown light to the fact that polyphenols may not merely exert their effects as free radical scavengers, but may also modulate cellular signaling processes during inflammation or may themselves serve as signaling agents (Aggarwal and Shisodia, 2004). Direct impact of specific polyphenolic compounds on inflammation has been studied both *in vitro* and *in vivo*. It has been reported that catechins can exert anti-inflammatory effects by scavenging nitric oxide radical, the peroxynitrite anion, or to reduce the activity of nitric oxide synthase (NOS) (Tedeschi et al., 2004). The neuronal nitric oxide synthase (nNOS) and the inducible nitric oxide synthase (iNOS) isoforms could also be inhibited by catechins and this effect of catechins is likely to involve the inhibition of the transcription factor NF- κ B as the κ B sequence is present in the promoter region of the iNOS gene (Lin and Lin, 1997). On the contrary, catechins could induce the endothelial isoform eNOS activity, a vasodilator-inducing enzyme (Singh et al., 2008).

Resveratrol, the stilbene, is documented as potent anti-inflammatory agent. In 2005, Birrel et al. demonstrated that *in vivo*, resveratrol can inhibit inflammatory cytokine expression in response to lipopolysaccharide (LPS) challenge in rat lungs. Furthermore, in both monocytic U937 cells and alveolar epithelial A549 cells, resveratrol inhibits NF- κ B and activator protein-1 (AP-1) activation (Donnelly et al., 2004). Resveratrol is found to inhibit cyclooxygenase-2 (COX-2) (Von Knethen et al., 1999). Like iNOS gene, COX-2 is also known to be regulated by NF- κ B activation and on this basis it may be inferred that resveratrol suppresses COX-2 and iNOS expression by inhibiting NF- κ B activation. Besides COX-2, various other genes such as matrix metalloproteinase-9 (MMP-9) and cell surface adhesion molecules are also regulated by NF- κ B. It is believed that the anti-carcinogenic properties shown by resveratrol may be via suppression of NF- κ B-mediated expression of such genes and their corresponding translated equivalents (Rahman et al., 2006).

Anti-inflammatory effect shown by curcumin is due to its inhibitory effect on lipoxygenase and COX-2, both enzymes being responsible for the synthesis of the pro-inflammatory leukotrienes, prostaglandins, and thromboxanes (Rao, 2007). There are also reports that curcumin is a suppressor of iNOS and a potent inhibitor of NF- κ B and AP-1 activation (Bengmark, 2006; Singh et al., 2008). Curcumin has been demonstrated to down regulate other NF- κ B-regulated genes involved in inflammation and cellular proliferation such as leukotrienes, PLA2 (Rahman et al., 2006). From above experimental evidences polyphenols thus appear to modulate a myriad of inflammatory signaling pathways and as such it is difficult to attribute the anti-inflammatory properties to one particular pathway.

Regulation of Cellular Redox System

Several factors influence the pathogenesis of diseases such as degradation of biomolecules, alterations in physiologically crucial enzymes and modulation of cell signaling, causing imbalance in overall cellular redox system. Polyphenols are reported to play an

effective role in balancing the cellular redox system via different pathways. An alternative mechanism may be that polyphenolic components of dietary plants may increase the endogenous antioxidant defense potential and thus modulate cellular redox state. Recently it has been proposed that some polyphenols have ability to stimulate Nrf2 as this important cellular redox dependent transcription factor regulates the expression of several genes involved in the modulation of inflammatory processes (Rahman et al., 2006). Nrf2 is a member of the “cap ‘n’ collar” family of transcription factors which bind to nuclear factor erythroid derived 2 (NF-E2) binding sites (GCTGAGTCA) that are essential for the regulation of erythroid specific genes. The NF-E2 binding site is a subset of the antioxidant response elements (ARE) that have the sequence GCNNNGTCA. Nrf2 is expressed in a wide range of tissues, many of which are sites of expression for phase 2 detoxification genes (Dinkova-Kostova et al., 2002; Rahman et al., 2006). Curcumin is documented to stimulate the expression of Nrf2 in a concentration as well time dependent manner (Balogun et al., 2003). Similarly, other polyphenols, such as resveratrol, have also been reported to stimulate Nrf2 in PC12 cells through mitogen activated protein (MAP) kinase signal transduction pathways (Chen et al., 2005).

Thiol groups in the cell play several crucial roles from neutralization of stressors to maintenance of overall cellular redox balance (Reglinski et al., 1988). There is experimental evidence that polyphenols also maintain the cellular redox balance by means of protecting the thiols groups being oxidized (Rizvi et al., 2005; Pandey et al., 2009b). A very recent study done by authors on human erythrocytes and plasma revealed that resveratrol has a very potent ability to protect oxidation of thiols in dose as well as time-dependent manner (Pandey and Rizvi, 2010b; Pandey and Rizvi, 2010c).

Eukaryotic cells contain a plasma membrane redox system (PMRS) which contains a group of multiple oxido-reductase enzymes (Rizvi et al., 2009; Hyun et al., 2006). The PMRS is now known to incorporate an ascorbate free radical (AFR) reductase which works to reduce extracellular AFR to ascorbate (ASC) using electrons derived from intracellular ASC through PMRS. The concerted action of erythrocyte PMRS and AFR reductase provides a mechanism for recycling of ASC between intracellular and extracellular compartments and acts as a complementary/protective mechanism which get activated during aging and in stress conditions (Rizvi et al., 2009). Recently it has found that some polyphenolic compounds possess unique property to activate this system via donating their electron from inside the cells, to maintain the ASC level in plasma and thereby minimize oxidative stress (Fiorani and Accorsi, 2005; Rizvi and Pandey, 2010). Since ascorbic acid is primary antioxidant present in plasma, however humans are unable to synthesize this vital molecule inside the body due to lack of functional L-gulonolactone oxidase, the final enzyme of the ASC biosynthetic pathway (Nishikimi et al., 1994). The role of polyphenols in recycling ASC in the plasma and thereby maintaining cellular redox balance may thus exert a major effect on health protection.

Pro-Oxidant Activity

Contrary to the above mentioned antioxidant effects, a pro-oxidant nature of polyphenols have also been reported, this is perhaps another mechanism by which polyphenols may exert their health protective effects. It has been proposed that such a pro-oxidant action could be a common mechanism for anticancer and chemopreventive properties of plant polyphenols

(Shamim et al., 2008). Resveratrol is cited to elicit pro-oxidant property, leading to oxidative breakage of cellular DNA in the presence of transition metal ions such as copper. However this property of resveratrol depends upon the concentration of resveratrol and the cell type (de la Lastra and Villegas, 2007). Fukuhara and Miyata (1998) first reported the pro-oxidant activity of resveratrol in a plasmid-based DNA cleavage assay in the presence of transition metal ions such as copper. DNA degradation by resveratrol in the presence of copper (10–100 μM) or alone (200 μM) (in the absence of added copper) has also been shown in a cellular system of peripheral lymphocytes isolated from human blood. The cytotoxic mechanism of resveratrol probably involves mobilization of endogenous copper ions, possibly chromatin bound copper (Azmi et al., 2005).

Curcumin has also been demonstrated to exhibit pro-oxidant effect in view of its failure to prevent single strand DNA breaks by H_2O_2 , a damage that was prevented by Vitamin E (Kelly et al., 2001). The pro-oxidant property is believed to be due to the generation of phenoxyl radicals of curcumin by the peroxidase– H_2O_2 system, which co-oxidizes cellular glutathione or NADH, accompanied by O_2 uptake to form ROS (Galati et al., 2002).

Like resveratrol and curcumin, EGCG and other tea polyphenols have also been shown to act as pro-oxidants. The relatively short half-life of EGCG, 0.5–2 h *in vitro*, could be prolonged by the addition of SOD, suggesting that the superoxide radical may be responsible for the short half-life. Since the pO_2 in a cell culture system is much higher (160 mmHg) in comparison to that in the blood or tissues (<40 mmHg), it is not yet clear whether the pro-oxidant properties of EGCG can also be envisaged at relatively lower pO_2 conditions *in vivo*. Under appropriate conditions EGCG and other catechins can be oxidized to form phenolic radicals, superoxide radicals, and hydrogen peroxide, thus contributing to cell apoptosis ((Hong et al., 2002; Rahman et al., 2006).

Others

There are many other pathways by which plant polyphenols are reported to show their health beneficial effects. Modulation of apoptosis pathway is one of them. It has been reported that the main green tea flavonoid; EGCG has ability to modulate apoptosis pathways to protect cells against oxidative stress (Singh et al., 2008). The effects of catechins on apoptotic pathways could be divergent. For instance, on PC12 cells EGCG, at low doses (1–10 μM), could inhibit caspase-3 activity or activate the PI3K/Akt pathway, which promote cell survival (Koh et al., 2004). On the other hand, catechins could modulate apoptosis by altering the expression of anti-apoptotic and pro-apoptotic genes (Levites et al., 2002). EGCG is also reported to promote cell survival by restoring the protein kinase C activity, a critical regulator of cell proliferation and survival. However, at high doses, EGCG (50–500 μM) can induce pro-apoptotic properties by increasing Bax, Bad, and caspase-6 activity while decreasing Bcl-x and Bcl-2 activity (Singh et al., 2008). Curcumin is reported to induce heme oxygenase-1 (HO-1) through the ARE (Hayes and McMahon, 2001).

Induction of cell-cycle arrest and apoptosis is another mechanism by which polyphenols could exert their effect against tumour growth. Anti-proliferative and anti-apoptotic effects of resveratrol in cancer cell lines have been reported (Yu et al., 2003). Some polyphenols have unique property to make the tumour cells sensitive towards other inducers of apoptosis, thus being indirectly involved in the chemoprevention. Resveratrol is very good example of such

effect (Fulda and Debatin, 2004). Some Polyphenols have been reported to act as a phytoestrogen in various systems, and it has been suggested that this property might mediate cardio-protective effects (Kopp, 1998).

It is well-known that reducing food intake or caloric restriction extends lifespan in a wide range of species. Recently, it has been found that resveratrol can mimic dietary restriction and trigger sirtuin proteins (Harikumar and Aggarwal, 2008)). The sirtuin enzymes are a phylogenetically conserved family of enzymes that catalyze NAD-dependent protein deacetylation. In yeast, sirtuin is essential for lifespan extension by caloric restriction and a variety of other stresses, including increased temperature, amino acid restriction, and osmotic shock (Anderson et al., 2003). Activators of sirtuins can be a key to extending lifespan and overcoming a variety of stresses in higher organisms. Resveratrol induced the highest activity of sirt1 and increased the lifespan of yeast by nearly 70% (Howitz et al., 2003). Polyphenols are reported to be beneficial to patients suffering from asthma and other inflammatory and immune diseases such as rheumatoid arthritis with resistance towards therapeutic effects of corticosteroids (Rahman et al., 2006).

Clinical Trials on Humans

At present, a large body of evidence from *in vitro* and animal studies indicates that polyphenols may be beneficial to many aspects of human health. However results from *in vitro* studies cannot be directly correlated from the actual impact of polyphenols *in vivo*. To translate such promising findings into clinical reality, studies on the potential toxicity, pharmacokinetics and bioavailability of polyphenols in humans, followed by interventional clinical trials are clearly needed. Tea catechins showed beneficial effects when tested on humans, after 42 days of the consumption of 2 cups of green tea, containing approximately 250 mg of total catechins, a significant increase in plasma total antioxidants was observed, whereas the plasma peroxide level decreased (Erba et al., 2003). The beneficial anti-inflammatory effect of polyphenols was demonstrated by a Finnish study involving over 10,000 participants, wherein a significant inverse correlation was observed between intake of polyphenols and the incidence of asthma (Knekt et al., 2002). Similar beneficial associations were also observed for chronic obstructive pulmonary disease (COPD) in a study encompassing over 13,000 adults. This study reported that increased polyphenol intake correlated with improved symptoms, as assessed by cough, phlegm production and breathlessness and improved lung function as measured by forced expiratory volume in 1 sec (FEV1) (Tabak et al., 2001). Human epidemiological and nutritional studies suggest a beneficial role for polyphenol flavonoids in preventing neurodegeneration. An epidemiological study has shown reduced risk of Parkinson's disease associated with consumption of two cups/day or more of tea and two or more cola drinks/day (Checkoway et al., 2002).

Pace-Asciak et al. (1996) reported that trans-resveratrol can be absorbed from grape juice in biologically active quantities and in amounts that are likely to cause reduction in the risk of atherosclerosis. In 1999, Blaridi et al. reported the stimulation of endogenous adenosine release by oral administration of quercetin and resveratrol in man. He documented that thrombin, at a concentration of 4 mg/kg/day, inhibits ADP-induced platelet aggregation in

humans and rabbits, despite having no effect on serum lipid levels. Resveratrol also causes an increase in plasma adenosine levels and blood nucleosides in human subjects. Gautam et al. (2000) found that *ex vivo* purging of contaminating tumor cells may reduce the incidence of relapse in patients undergoing bone marrow transplantation. In this study, they demonstrated that resveratrol exhibits anti-leukemic activity against mouse and human leukemic cell lines by inhibiting cell proliferation. Long-term exposure to resveratrol also inhibits the clonal growth of normal hematopoietic progenitor cells, but at a higher IC₅₀ than for most of the leukemia cell lines tested. The inhibitory effect of resveratrol on hematopoietic progenitors is partially reversible, whereas the effect on leukemia cells is largely irreversible. The inhibition of leukemia cells by resveratrol involves nucleosomal DNA fragmentation (Aggarwal et al., 2004).

In continuation of studies, in 2005, Zern et al. reported the decrease in level of plasma tumor necrosis factor TNF- α , plasma lipids (cardio protective effect), and reduced oxidative stress in pre and post menopausal women by using lyophilized grape powder. A recent phase I dose escalation pharmacokinetic study in healthy volunteers performed by Boocock et al. (2007) emphasizes resveratrol as a potential cancer chemopreventive agent. They have reported that consumption of resveratrol did not cause serious adverse events. Pharmacologically, curcumin has been found to be safe and human clinical trials indicated no dose-limiting toxicity when administered at doses up to 10 g/day (Cheng et al., 2001). Curcumin showed low oral toxicity in man but also low oral bioavailability. 500–1000 nM curcumin was reported in the plasma after 8 g/day oral administration (Shoba et al., 1998). However, consumption of curcumin along with piperin, an active ingredient in peppers which is a known inhibitor of intestinal and hepatic β -glucuronidation of curcumin, may improve the oral bioavailability 20-fold but it still has a short half-life of only 40 min (Shoba et al., 1998).

Thus, the results of these clinical trials are exiting however more studies are required to test a range of polyphenol concentrations *in vitro* and then apply those doses *in vivo* to a wide variety of human diseases.

Conclusion

Polyphenols in diet affect human health by virtue of their ability to act as powerful antioxidants and modulate several cellular processes such as signaling, proliferation, apoptosis, redox balance, and differentiation. Predominantly, reports on the beneficial effects of polyphenols have been obtained from *in vitro* studies and more detailed investigations are still required to extrapolate these results to *in vivo* conditions. This is particularly relevant in view of the fact that polyphenols are known to undergo various biochemical transformations which affect their bioavailability as well as bio-efficacy. Although attempts have been made to estimate the relative contribution of polyphenols to total antioxidant capacity in plasma, insufficient knowledge on the nature and concentration of circulating polyphenol species render these results only speculative. The mechanism of action of polyphenols by which they elicit their biological effects are diverse and complex. In view of their pleiotropic biological activities, polyphenols hold great promise as potential therapeutic/prophylactic agents in chronic human diseases.

Conflict of Interest

Authors show that there is conflict of interest.

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Chapter XIX

Perspective and Direction for Future Research on Phytochemicals

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Abstract

Phytochemicals not only modulate cellular differentiation, proliferation, oxidative stress, inflammation and apoptosis, but also regulate activities of proteins and enzymes that are involved in these processes at the molecular level. In addition, phytochemicals also modulate immune function and changes in metabolism. Inflammation and oxidative stress impact upon almost all acute and age-related chronic and progressive disorders in human physiology. Phytochemicals produce their effects by retarding oxidative stress, inflammation, and carcinogenicity in chronic and age-related progressive disorders and their animal models. In neurological disorders, the use of phytochemicals not only improves neuronal and cognitive functions, and ocular health, but also protects genomic DNA integrity. The efficacy of phytochemicals for treating age-related chronic diseases and cancer may not only depend on their optimal doses, bioavailability (absorption in the digestive tract), half-lives, and ability to cross the blood brain barrier, but also on potential ability of optimal doses of phytochemicals to effectively reach various subcellular particles and their synergistic actions

1. Introduction

The rising prevalence of chronic diseases worldwide and the corresponding rise in health care costs have resulted in a propelling interest among investigators for multiple health benefits related to phytochemicals (phytonutrients), which are colored or colorless organic, naturally occurring, and non-nutritive compounds found in fruits, vegetable and other plant

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products, such as legumes, whole grains, and nuts. Unlike vitamins and minerals, phytochemicals are not required for sustaining cell viability, but they play a vital role in protecting us from chronic age-related diseases [1]. It has been suggested that there is a relationship between the type of food people eat, their health and their life expectancy. The consumption of colored vegetables and fruits may protect against cancers, cardiovascular disease, and neurodegenerative disorders [2]. Phytochemicals have been shown to induce diversified physiological and pharmacological effects. They not only inactivate cancer-causing substances (antitumor effects), and stimulate the immune system, but also protect humans from arterial damage in cardiovascular and cerebrovascular systems, and neurodegenerative diseases [3] (Figure1). In addition, phytochemicals also activate adaptive cellular stress response pathways and protect skin from premature aging. They also delay or retard the onset of many chronic neurodegenerative diseases, such as Alzheimer disease (AD) [2]. Some of the well-known disease fighting phytochemicals are lycopene in tomatoes, isoflavones in soy, allyl sulfides in onions, leeks, and garlic, carotenoids in fruits and carrots, flavonoids in fruits and vegetables, terpenes in citrus fruits and cherries, and polyphenols in tea and grapes. Some phytochemicals may not act as antioxidants *in vivo*, but regulate signal transduction processes through their prooxidants, cellular redox tone and oxidizing redox sensitive site activities [4].

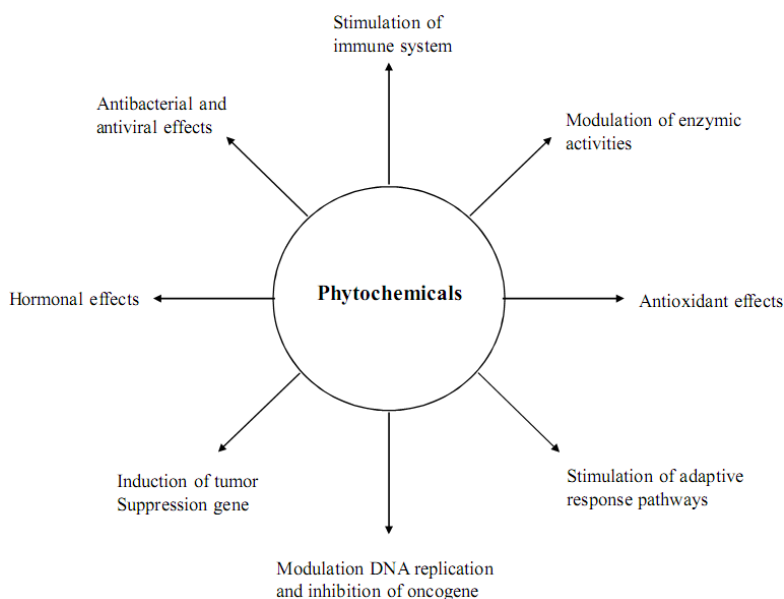


Figure 1. Modulation of various functions by phytochemicals.

Many phytochemicals present in plant foods are poorly absorbed by human subjects, and undergo rapid excretion, but they do exert their biological effects (anti-inflammatory, antioxidant and anticarcinogenic) at realistic doses that may contribute to putative benefits of plant foods such as berries, colored fruits, brassica vegetables, nuts, herbs, and tea. Although, the use of synthetic drugs, especially those targeting inflammation and oxidative stress is an attractive and reasonable approach to retard chronic human diseases, but traditional fruits, nuts, herbs, and spices also exert protective effects through the modulation of signal transduction pathways associated with inflammation and oxidative stress [2-4].

2. Molecular Aspects of Oxidative Stress, Inflammation, and Carcinogenicity

Oxidative stress is a cytotoxic process that occurs in cells when antioxidant mechanisms are overwhelmed by the production of reactive oxygen species (ROS) [5]. ROS include superoxide anions, hydroxyl, alkoxyl, and peroxy radicals, and hydrogen peroxide. Several mechanisms are involved in ROS production. They include mitochondrial dysfunction, enzymic and non-enzymic oxidation of polyunsaturated fatty acids [6], iron-mediated peroxidation of unsaturated fatty acids, and activation of many enzymes, such as enzymes of mitochondrial electron transport chain, xanthine oxidase, cyclooxygenase, lipoxygenase, NADPH oxidase, and nitric oxide synthase (NOS). At low levels, ROS function as signaling intermediates in the regulation of fundamental cell activities such as growth and adaptation responses. At higher ROS concentrations, reaction between ROS and proteins or unsaturated lipids in the plasma membrane leads to a chemical cross-linking of membrane proteins and lipids and a reduction in membrane unsaturation. This results in decreased membrane fluidity and decreased activity of membrane-bound enzymes, ion-channels, and receptors [7].

ROS stimulates NF- κ B, a transcription factor, which is present in the cytoplasm in a repressed form attached to its inhibitory protein, I- κ B. ROS-mediated stimulation of NF- κ B-I κ B complex facilitates the release of NF- κ B, which is translocated to the nucleus where it mediates the transcription of more than 150 genes that not only influence the survival of neural cells, but also maintain their normal functional integrity. NF- κ B also induces many genes implicated in inflammation, oxidative stress, and immune responses (Figure 2). These genes code for many enzymes, such as sPLA₂, COX-2, NOS, and SOD, intracellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), E-selectin, cytokines (TNF- α , IL- β), and matrix metalloproteinases (MMP) [6]. ROS also promote apoptotic cell death through changes in mitochondrial permeability and poly(ADP-ribose)polymerase activation, which is accompanied by the depletion of NAD. Depletion of NAD causes reduction in ATP leading to cell death [8]. In summary, oxidative stress is mainly caused by an imbalance between the activity of endogenous prooxidative enzymes (such as NADPH oxidase, xanthine oxidase, or the mitochondrial respiratory chain) and anti-oxidative enzymes (such as superoxide dismutase, glutathione peroxidase, heme oxygenase, thioredoxin peroxidase/peroxiredoxin, catalase, and paraoxonase) in favor of the former. The high demand for molecular oxygen, presence of polyunsaturated fatty acids at the sn-2 position of neural membrane phospholipids, and relatively low abundance of antioxidant defense enzymes make brain tissue vulnerable to oxidative stress. Excessive production of ROS is an important mechanism underlying the pathogenesis of chronic neurological and visceral diseases. In brain, production of high levels of ROS and persistent inflammation is associated with pathophysiology of neurodegenerative diseases such as AD, Parkinson disease (PD), Huntington disease (HD), and amyotrophic lateral sclerosis (ALS) [9]. This process is initiated and maintained by the expression of oxidative stress and neuroinflammation inducing genes (Figure 2). Similarly in visceral tissues, ROS have been implicated with the pathogenesis of rheumatoid arthritis [10], systemic lupus erythematosus [11], sickle cell anemia [12], cardiovascular disease [13], and various forms of cancer and tumor formation [14]. In contrast, oxidative stress and inflammation-mediated damage at each stage of carcinogenesis not only causes DNA damage and subsequently mutations, but also stimulate

proliferation of initiated cells and even metastasis and angiogenesis [5,15,16]. A high level of ROS in cancer cells may lead to a variety of biological responses, such as cell adaptation, genetic instability, and resistance to some drugs used in anticancer therapy.

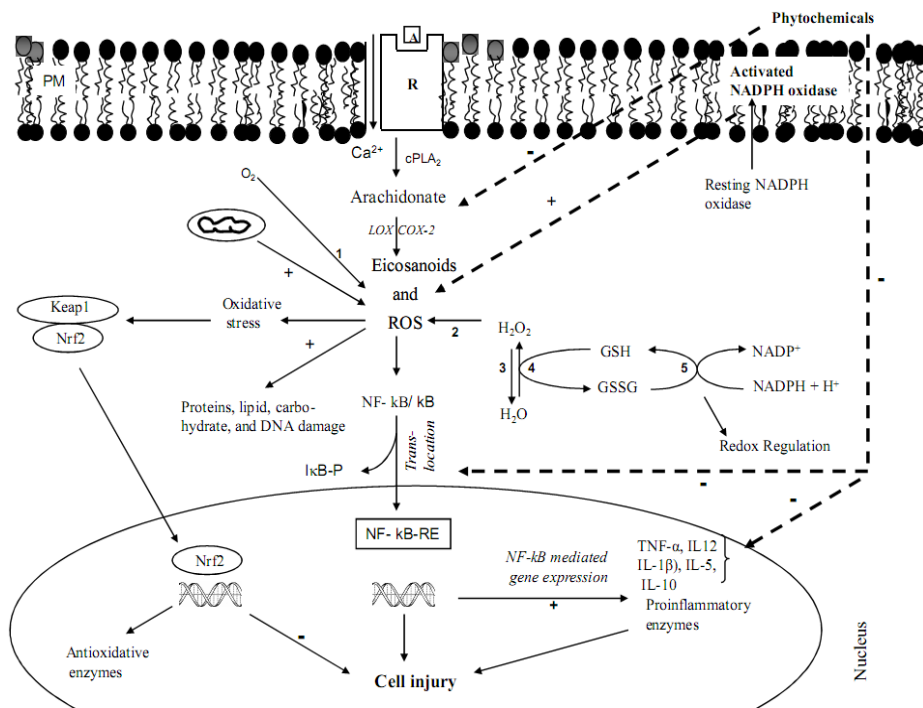


Figure 2. Modulation of antioxidant, antiinflammatory and immunomodulatory activities by phytochemicals. NADPH oxidase (1); superoxide dismutase (2); catalase (3); glutathione peroxidase (4); 5, glutathione reductase; cytosolic phospholipase A₂ (cPLA₂); cyclooxygenase (COX); lipoxygenase (LOX); reactive oxygen species (ROS); cytokines, TNF- α and IL-1 β ; reduced glutathione (GSH); oxidized glutathione (GSSG) and hydrogen peroxide (H₂O₂). nuclear factor κ B (NF κ B); nuclear factor κ B-response element (NF κ B-RE); inhibitory subunit of NF κ B (I κ B); nuclear factor-erythroid-2-related factor 2 (Nrf2); kelch-like erythroid Cap'n/Collier homologue-associated protein 1 (Keap1); Plasma membrane (PM); proinflammatory enzymes include sPLA₂, COX-2, and NOS in the nucleus. The expression of cytokines (TNF- α and IL-1 β) up regulates activities of cPLA₂, sPLA₂, COX-2 and NOS through a positive loop type of mechanism in cytoplasm and neural membranes. Positive sign indicate stimulation and negative sign indicate inhibition.

Inflammation is a protective mechanism that isolates the injured cells from uninjured cells, destroys injured cells, and repairs the extracellular matrix [17]. The main mediators of inflammation are eicosanoids and platelet activating factors. In addition, the chronic activation of macrophages induces cellular damage through the release of potentially cytotoxic molecules such as proinflammatory cytokines, reactive oxygen intermediates (ROS), proteinases and complement proteins [17]. The reaction between superoxide anion and nitric oxide results in the generation of peroxynitrite. This metabolite may be one of the major cytotoxic metabolite during inflammation-mediated cell death [17] (Figure 2). Two types of inflammation, acute and chronic, occur in the body tissues. Acute inflammation develops rapidly with the experience of pain, whereas chronic inflammation develops slowly. Chronic inflammation is below the threshold of pain perception. As a result, the immune

system continues to assault the tissue and chronic inflammation lingers for years, ultimately reaching the threshold of detection [17]. Chronic inflammation is associated with slow progressive neurodegenerative diseases. In contrast, acute inflammation is involved in ischemia, head injury, and spinal cord trauma. Acute inflammation is a short-lived process characterized by a neutrophilic infiltration and complete resolution. In contrast, chronic inflammation represents a long-lasting phenomenon associated with mononuclear infiltration, tissue hyperplasia, and progressive cavitation in the tissue [17].

The nuclear factor-erythroid-2-related factor 2 (Nrf2) regulates inflammatory and immune responses by controlling oxidative stress. Under the normal conditions, Nrf2-dependent transcription is repressed by a negative regulator kelch-like erythroid Cap'n'Collar homologue-associated protein 1 (Keap1). During oxidative stress, high levels ROS promote the dissociation of Nrf2 from Keap1. Free Nrf2 translocates to the nucleus, where it binds and activates antioxidant responsive element (ARE). ARE-driven genes include direct antioxidants (e.g., glutathione peroxidase), thiol metabolism-associated detoxifying enzymes (e.g., glutathione S-transferase), stress-response genes (e.g., heme oxygenase-1), and proteasome subunit beta type-5 and gamma-glutamylcysteine synthetase (γ -GCS). These genes are closely associated with maintenance of cellular redox homeostasis. In addition, Nrf2 has recently been recognized as a key factor regulating an array of genes that defend cells against the deleterious effects of environmental insults [18].

Another protective mechanism involves sirtuins 1 and 2 (silent information regulator1 and 2 or SIRT1 and SIRT2), a group of enzymes that hydrolyze acetyl groups from proteins in the presence of NAD^+ and add it to the ADP-ribose part of NAD^+ to form O-acetyl-ADP-ribose [19,20]. Sirtuins promote cell survival under oxidative stress when it is expressed in the nucleus and have emerged as critical regulators for aging and longevity [19,20]. Reduction in food intake (caloric restriction) increases the life-span of many species. This is due to the protective effects of SIRT1, which is involved in the regulation of gene silencing, DNA repair, rDNA recombination, and aging, apart from regulating programmed cell death. *In vitro* studies indicate that overexpression of SIRT1 protects cells against amyloid-beta ($\text{A}\beta$)-induced ROS production, DNA damage, and apoptotic death. Moreover, caloric restriction-mediated overexpression of SIRT1 rescues neurons from chronic neurodegenerative diseases, such as AD and HD [19,20].

3. Modulation of Oxidative Stress, Inflammation, and Carcinogenicity by Phytochemicals

It is clear from information presented in various chapters of this book that the consumption of phytochemicals (polyphenols, flavonoids, and carotenoid) induces the expression of enzymes involved in both cellular antioxidant defenses and elimination/inactivation of electrophilic carcinogens. Nrf2 plays a crucial role in the coordinated induction of those genes, which encode many stress-responsive and cytoprotective enzymes and related proteins. These include NADPH oxidase, heme oxygenase-1, glutamate cysteine ligase, glutathione S-transferase, glutathione peroxidase, thioredoxin [21,22]. As stated above, in resting cells, Nrf2 occurs in the cytoplasm as an inactive complex with the repressor Keap1 (Figure 2). Phytochemical-mediated dissociation

of Nrf2 from its repressor is achieved through the oxidation or covalent modification of critical cysteine sulfhydryl groups in Keap 1 resulting in the release, translocation, and accumulation of Nrf2 in the nucleus, where Nrf2 forms a heterodimer with other transcription factors, such as small Maf, which in turn binds to the 5'-upstream cis-acting regulatory sequence, termed antioxidant response elements (ARE), located in the promoter region of genes encoding various antioxidant and phase 2 detoxifying enzymes [22,23]. In addition, phytochemical-mediated phosphorylation of specific serine or threonine residues in Nrf2 by upstream kinases may also facilitate the nuclear localization of Nrf2. Collective evidence suggests that multiple mechanisms may be involved in the phytochemical-mediated activation of Nrf2 through one or more of the upstream kinases, such as mitogen-activated protein kinases, phosphatidylinositol-3-kinase/Akt, protein kinase C, and casein kinase-2. Thus, the consumption of phytochemicals may produce beneficial effects through Nrf2-Keap1 system-mediated expression of cytoprotective genes [22].

Resveratrol (trans-3,4',5-trihydroxystilbene), a polyphenolic compound found in red wine, not only acts as potent anti-cancer agents, but also provide protection against cardiovascular disease and neurological disorders. *In vitro* studies have revealed that resveratrol acts on multiple intracellular sites that modulate cell growth, inflammation, apoptosis, angiogenesis, and invasion and metastasis [24]. Targets for the molecular action of resveratrol include tumor suppressors p53 and Rb; cell cycle regulators, cyclins, CDKs, p21WAF1, p27KIP and INK and the checkpoint kinases ATM/ATR; transcription factors NF- κ B, AP-1, c-Jun, and c-Fos; angiogenic and metastatic factors, VEGF and matrix metalloproteinase 2/9; cyclooxygenases for inflammation; and apoptotic and survival regulators, Bax, Bak, PUMA, Noxa, TRAIL, APAF, survivin, Akt, Bcl2 and Bcl-X(L). In addition to antioxidant action, resveratrol exhibits prooxidant activity under certain experimental conditions, causing oxidative DNA damage that may result in cell cycle arrest or apoptosis [25]. Consumption of resveratrol increases the activity of SIRT1 and rescues neurons from age-related diseases, such as stroke, AD, HD cardiovascular disease, and cancer [26,19]. Resveratrol also modulates the activity of numerous other proteins, such as peroxisome proliferator-activated receptor coactivator-1 α (PGC-1 α), the FOXO family, Akt (protein kinase B) and NF- κ B [27].

In vitro ischemic preconditioning enhances SIRT1 activity at 48 hours, while resveratrol preconditioning increases SIRT1 activity at 1 hour but not 48 hours after treatment in hippocampus. Resveratrol significantly reduces mitochondrial uncoupling protein 2 (UCP2) levels by 35% compared to sham-treated rats [28]. Treatment with SIRT1-specific inhibitor, sirtinol, blocks resveratrol preconditioning-mediated the neuroprotection and the decreases UCP2 levels. Furthermore, resveratrol preconditioning significantly increases the ADP/O ratio in hippocampal mitochondria reflecting increase in ATP synthesis efficiency [28]. Collective evidence suggests that neuroprotective effects of resveratrol are similar to ischemic preconditioning-mediated neuroprotection [29] and inhibition of SIRT1 abolishes ischemic preconditioning-mediated neuroprotection in CA1 region of the hippocampus. Since resveratrol and ischemic preconditioning-mediated neuroprotection requires the activation of SIRT1-mediated mitochondrial uncoupling of protein 2 pathway, therefore this may provide targeted therapeutic treatment for stroke and other neurological diseases [29].

Berries contain a variety of phytochemicals (anthocyanins, flavonols, and proanthocyanidins; substituted cinnamic acids and stilbenes; and triterpenoids such as ursolic acid

and its esters) that provide protective effects to various tissues. These phytochemicals act by mechanisms that not only inhibit oxidative stress and decrease inflammation through reduction in ornithine decarboxylase and decreased expression of matrix metalloproteinases, but also through the inhibition of cyclooxygenase and modulation of macromolecular interactions and expression of genes associated with age-related diseases [30]. Supplementation of diet with strawberry, blueberry or spinach extracts in rat and mice reverses age-related deficits in several neuronal and behavioral parameters including: oxotremorine enhancement of K^+ -evoked release of dopamine from striatal slices, carbachol-stimulated GTPase activity, calcium buffering in striatal synaptosomes, motor behavioral performance and Morris water maze performance [31,32]. Similarly, dietary supplementation with blueberry extract enhances the survival of dopamine-producing neurons in a model relevant to PD therapy [33]. Although, the molecular mechanism involved in berry-mediated neuroprotection is not fully understood, but it is proposed that phytochemicals in berries enhance neuronal communication via increasing the neuronal signaling and decreasing the stress signals- mediated by oxidative/inflammatory stressors (e.g. NF- κ B). Collective evidence suggests that a greater intake of high-antioxidant foods (such as berries) may increase "health span" and enhance cognitive and motor functions in aging [34].

Curcumin (diferuloylmethane) is a polyphenol present in the spice turmeric, which not only has ability to scavenge free radicals, such as superoxide anion and nitric oxide, but can also modulate important signaling pathways mediated via NF- κ B and mitogen-activated protein kinase pathways. Curcumin also decreases the expression of proinflammatory mediators, such as matrix metalloproteinases, adhesion molecules, and growth factor receptor genes. Extensive research over past 50 years indicates that curcumin is highly pleiotropic molecule with numerous pharmacological activities, including antioxidant, anti-inflammatory, and antimicrobial properties. Curcumin interacts with a variety of proteins and inhibits the activity of various kinases. By modulating the activation of various transcription factors, curcumin modulates the expression of inflammatory enzymes, cytokines, adhesion molecules, and cell survival proteins [35]. Curcumin inhibits cyclin D1, cyclin E and MDM2; and activates p21, p27, and p53. Various preclinical cell culture and animal studies indicate that curcumin has potential as an antiproliferative, anti-invasive, and antiangiogenic agent; as a mediator of chemoresistance and radioresistance; as a chemopreventive agent; and as a therapeutic agent in wound healing, diabetes, AD, PD, cardiovascular disease, pulmonary disease, and arthritis [36-39]. Because of curcumin's rapid plasma clearance and conjugation, its therapeutic usefulness has been somewhat limited, leading researchers to investigate the benefits of complexing curcumin with other substances to increase systemic bioavailability. The anticancer potential of Curcumin is severely affected by its limited systemic and target tissue bioavailability and rapid metabolism [40].

Epigallocatechin gallate (EGCG), a phytochemical found in green and black tea, inhibits cancer cell survival by suppressing androgen receptor expression and signaling via several growth factor receptors [41-43]. Cell cycle arrest or apoptosis involves caspase activation and altered Bcl-2 family member expression. EGCG-mediated inhibition of telomerase activity leads to telomere fragmentation. EGCG and flavins reduce nitric oxide production by suppressing inducible nitric oxide synthase via blocking nuclear translocation of the NF- κ B. In addition, green tea polyphenols also modulate a number of key enzymes, including mitogen-activated protein kinases and protein kinase C, and alter protein/mRNA levels of

cyclins, oncogenes, and tumor suppressor genes [41-43]. Metastasis is inhibited via effects on urokinase and matrix metalloproteinases. These polyphenols suppress angiogenesis, in part by decreasing vascular endothelial growth factor production and receptor phosphorylation. It is also reported that EGCG inhibits dihydrofolate reductase activity, which modulates nucleic acid and protein synthesis. EGCG also acts as an aryl hydrocarbon receptor antagonist by directly binding the receptor's molecular chaperone, heat shock protein 90. Collective evidence suggests that phytochemicals in green and black tea act at numerous points regulating cancer cell growth, survival, and metastasis [41-43].

Components of green tea components (polyphenon E (PE): 63% of epigallocatechin-3-gallate, 11% of epicatechin, 6% of (-)-epigallocatechin and 6% of (-)-epicatechin-gallate) activate adaptive cellular stress responses and protect neurons against a range of oxidative and metabolic insults. Thus, green tea components provide neuroprotection of dopaminergic neurons from 6-hydroxydopamine-mediated injury in a rat model of PD [44]. Similarly, green tea components reduce the misfolding of mutant huntingtin and neurotoxicity in HD animal models [45]. Finally, green tea component not only protect neurons from A β toxicity [46], but also provide protection against A β -mediated cognitive impairment in a rat model of AD [47].

Collectively, information presented in this book suggests that nutritional interventions, by increasing dietary intake of phytochemicals, can not only retard or even reverse age-related declines in brain function and cognitive performance in animal models, but also provides beneficial effects in chronic visceral diseases. The mechanisms through which dietary supplementation with phytochemicals prevents age-mediated chronic diseases is related to their ability to counteract toxic production of both ROS and RNS, along with the upregulation of vitagenes, such as heme oxygenase-1, antioxidant enzymes, and phase II enzymes [48]. The most prominent dietary factor that affects the risk of many different chronic diseases is energy intake - excessive calorie intake increases the risk of age-related diseases, while decreasing energy intake through caloric restriction or intermittent fasting increases lifespan and protects various tissues from chronic diseases [49].

4. Unsolved Problems and Phytochemical Therapy of Chronic Diseases

Despite limitations in their bioavailability, experimental and epidemiological data on human subjects suggest that the consumption of phytochemicals produces beneficial effects in chronic neurodegenerative and visceral diseases. The use of phytochemicals as antiaging agents for correcting the fundamental oxidant/antioxidant and proinflammatory/ anti-inflammatory imbalance is recommended from childhood to old age. Although, information on optimal doses of phytochemicals in humans remains controversial, but it is becoming increasingly evident that consumption of phytochemical remains an attractive and reasonable approach to retard aging and chronic human diseases. It is suggested that the consumption of phytochemicals may also delay the onset of age-related chronic diseases and cancer if consumption is started before the onset or at the earliest stages of the disease [50]. However, once the onset of chronic age-related diseases and cancer has occurred, reversal through the use of phytochemicals may be mechanistically improbable.

The efficacy of phytochemicals for treating age-related chronic diseases and cancer may not only depend on their optimal doses, bioavailability (absorption in the digestive tract), half-lives, and ability to cross the blood brain barrier, but also on potential ability of optimal doses of phytochemicals to effectively reach various subcellular particles and their synergistic actions [51]. Thus, for the treatment of age-related chronic diseases and cancer, a localized and controlled delivery of phytochemicals at the site, where cell are dying is preferred because it reduces toxicity of phytochemicals and increases treatment efficiency. The concept of using naturally occurring foods with phytochemicals as potential therapeutic agents has advanced the field dramatically [52-54]. Throughout the years, many phytochemicals present in natural foods and products have been evaluated using various experimental models and number of them progressed to early clinical trials [24]. More recently, the focus has been directed towards molecular targeting of phytochemicals to identify mechanism(s) of action of these newly discovered bioactive compounds. Moreover, it is recognized that single phytochemical may not always be sufficient to provide therapeutic efficacy, and, therefore, the combination of phytochemicals through the consumption of whole fruits, vegetables, and grains has become an increasingly attractive area of research [55]. The failure of phytochemical therapy in age-related chronic diseases may be due to the disruption of signal transduction process caused by the accumulated abnormal protein aggregates, creating a downstream block that cannot be overcome by upstream modulation of receptor function by phytochemicals [6]. This view is supported by studies on cell culture models of chronic diseases. Another factor is the heterogeneity of age-related chronic diseases. Many if not all age-related chronic diseases are heterogeneous with respect to their clinical, biochemical, and genetic features. It is likely that these variants of these chronic diseases may have different courses and responses to phytochemical therapy [56].

Conclusion

Oxidative stress and chronic inflammation are closely associated with the pathogenesis of chronic neurological and visceral diseases. Although, key issues of phytochemical therapy such as bioavailability, metabolism, dose/response and toxicity of phytochemicals have not been well established yet; but based on epidemiological studies, it is speculated that targeting oxidative stress and inflammation with phytochemicals may boost the endogenous signal transduction mechanisms associated with cell survival. This may provide protection against chronic neurological and visceral diseases. Thus, dietary flavonoids, polyphenol, carotenoids, and theophylline and epigallocatechin-3- gallate found in curcumin, resveratrol, green tea components protect tissues through the modulation of kinases and transcription factors that induce the expression of genes encoding for antioxidant enzymes, protein chaperones, phase-2 enzymes, neurotrophic factors, and other cytoprotective proteins involved in the sirtuin-FOXO pathway, the NF-kappaB pathway, and the Nrf-2/ARE pathway. In addition above phytochemicals also regulate genes for glutathione biosynthesis, chromatin remodeling and inflammatory gene expression.

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