Comprehensive

Bioactive Natural Products

Vol 4

Antioxidants & Nutraceuticals

V K Gupta
Anil K Verma

Studium Press LLC

Comprehensive
Bioactive Natural Products

Volume 4
Antioxidants & Nutraceuticals

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Ed. V.K. Gupta

Vol. 4* : *Antioxidants & Nutraceuticals
Eds. V.K. Gupta & Anil K. Verma

Vol. 5* : *Immune-modulation & Vaccine Adjuvants
Ed. V.K. Gupta

Vol. 6* : *Extraction, Isolation & Characterization
Eds. V.K. Gupta, S.C. Taneja & B.D. Gupta

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About the Series

Nature abounds with a rich potential heritage of bioactive natural products as that have been exploited for effective and beneficial use either as a therapeutic resource against a human disease or as a chemopreventive, antioxidant, nutraceutical or diet supplement. A natural product is a substance or a chemical compound produced by a living organism found in nature that usually has a pharmacological or biological activity. Some of these agents are derived from terrestrial plants, whereas, others are obtained from microorganisms, marine or freshwater organisms and even the higher animals.

Past few decades have witnessed an unprecedented worldwide growth in the interest of bioactive natural products based medicines, both in developed and developing countries, and offer a resource-pool for lead identification in drug discovery owing to the reason that isolation of a novel molecule is easier than *de novo* synthesis, especially when the molecular structure of the compound is very complex. On account of such advantages, pharmaceutical companies are opting for bioactive natural products based drugs. More than 20 new drugs, launched world over between 2000 and 2005, originate from natural products. Scrutiny of medical indications by source of compounds has demonstrated that natural products and related drugs are used to treat 87% of all categorized human diseases (infectious and non-infectious). The development of new bioassay techniques, biotechnology methods, screening and high performance analytical methods have introduced new concepts and possibilities of rational drug design and drug discovery.

Interest in bioactive natural products research is growing more strongly and can be attributed to several factors, including remarkable diversity of natural products, unmet therapeutic needs, the development of novel and sensitive techniques to detect, isolate, purify and structurally characterize these active constituents. This has opened opportunities for multidisciplinary research that joins the forces of natural product chemistry, molecular and cellular biology, synthetic and analytical chemistry, bio-chemistry, bio-technology, general biology, and pharmacology to exploit the vast diversity of bioactive natural products.

Taking cognizance of these facts, we have initiated efforts in editing the present series "**Comprehensive Bioactive Natural Products**" and brought eight volumes (1 to 8) providing edited information from over 139 original research and review articles by eminent scientists and researchers

from India and abroad, representing 40 countries of the world on wide range of topics in the area of natural products categorized under the themes *viz.*,

1. *Potential & Challenges*
2. *Efficacy, Safety & Clinical Evaluation I*
3. *Efficacy, Safety & Clinical Evaluation II*
4. *Antioxidants & Nutraceuticals*
5. *Immune-modulation & Vaccine Adjuvants*
6. *Extraction, Isolation & Characterization*
7. *Structural Modifications & Drug Development*
8. *Quality Control & Standardization*

These volumes critically evaluate the present state-of-art, current status and future prospects in a well-illustrated manner. It is hoped that this series, which reflects the contributor's research results as well as world wide reviews will be widely read and used by all interested in these fields and shall open new vistas of research and academic pursuits for those engaged in the fields of bioactive natural products.

I would like to take this opportunity to thank each of the authors who have contributed in the preparation of this book series.

Jammu, India

V.K. Gupta
Series Editor

AMITY INSTITUTE FOR HERBAL
AND BIOTECH PRODUCTS DEVELOPMENT

- An Institution of Ritnand Balved Education Foundation - Thiruvananthapuram

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(Former Director, NBRI, Lucknow)

08-06-2009

Foreword to the Series

Medicine among the ancient civilizations was a blend of Religion and Science. Natural products played an important role throughout the world in the prevention and treatment of diseases. These natural products originated from varied sources such as plants, animals, micro-organisms, marine organisms, terrestrial vertebrates and invertebrates. In man's quest for discovering new and potent remedies for diseases, he keenly observed his immediate neighbourhood and experimented not only with organic substances but also with in-organic materials such as metals, minerals, ores etc. Over the years, the accumulated medical wisdom was compiled into excellent treatises and these have later developed into the various classical systems of medicine associated with the ancient civilizations and cultures of the world. Apart from the classical systems of medicines, which by its nature and practice had universal applications, there existed another stream of medicine in the countryside, among the less privileged class of people, among the forest dwellers and among the nomadic tribes which were location specific, confined to small communities, tribes and families. These medical systems were mostly perpetuated by oral traditions. Nevertheless, the value of the oral traditions cannot be overlooked as these localized knowledge systems are veritable treasures of invaluable information on the properties and uses of hundreds of plant, animal and mineral products.

Ethno medical information is now increasingly being utilized in the search for novel bioactive molecules from natural products. Several well known plants such as Licorice (***Glycyrrhiza glabra***) myrrh (***Commiphora sps***) and poppy capsule latex (***Papaver somniferum***) were mentioned in the clay tablets dating back to 2600 B.C, obtained from Mesopotamia. These plants and their derivatives are still used in various forms as herbal drugs as well as pure isolates in different systems of medicine. For instance,

codeine, papaverine, morphine etc isolated from ***P. somniferum*** are still used in modern medicine. Similarly hemisuccinate carbenoxolone sodium, a semi synthetic derivative of glycyrrhetic acid isolated from licorice is used for the treatment of gastric and duodenal ulcers. Plants, especially those with ethno medical history have been the major source of bio active molecules. According to a study by Fabricant and Famsworth (2001), out of 122 plant derived drugs, 80% had their origin from plants with ethno medical use. Since secondary metabolites from plants are products of biosynthetic reactions occurring in living systems, bioactive molecules from natural sources are excellent drug candidates as they are perceived to show more biological friendliness than synthetic drugs.

The ever increasing popularity and acceptance of natural products have evinced an extra ordinary interest and scope in the subject in recent years. Compendium of Bioactive Natural Products- a series of eight books edited by Dr. V.K. Gupta (Volumes 1,2,3 and 5) and Dr. V.K. Gupta, Dr. S.C. Taneja, Dr. B.D. Gupta and Dr. A.K.Verma (Volumes 4,6,7 and 8), published by M/S. Studium Press LLC, U.S.A are an excellent compilation, giving comprehensive and reliable data on the current development in the field of bioactive natural products. This series contain 139 original research and review articles contributed by a galaxy of eminent scientists and academicians from 40 countries around the world. The editors have taken care to invite eminent scholars in the relevant areas to contribute each chapter in the series. The series deals with wide ranging topics related to natural products such as extraction, isolation and characterization, structural modifications and drug development, quality control and standardization and, immune modulation and vaccine adjuvants, antioxidants and nutraceuticals, efficacy, safety and clinical evaluation (Parts-1 and 2) and potentials and challenges. The editors of this series Dr. V.K. Gupta, Dr. S.C. Taneja, Dr. B.D. Gupta and Dr. Anil K. Verma are known to me for more than three decades and they are all stalwarts in natural products and related areas.

It is gratifying to know that this series is dedicated to the memory of Col. Sir Ram Nath Chopra, the Founder Director of Regional Research Laboratory, now known as Indian Institute of Integrative Medicine (IIIM), Jammu.

Col. Sir R.N. Chopra (1882-1973) was a pioneer of systematic studies of indigenous drugs, promoter of Indian Systems of Medicine and Patron of Pharmacy in India. Most of the medicinal plants, he studied were in use, in Ayurveda for thousands of years. The major fields of Cot. Chopra's research were general pharmacology, chemotherapy, indigenous drugs, drug addiction and drug assays. At the Calcutta school of Tropical Medicine, he developed a pattern of research in studying the action of medicinal plants which influenced an entire generation of pharmacologists. Pharmacists and physiologists in India. In the words of Dr. G.V. Satyawati, Former Director General of Indian Council of Medical Research, the credit of kindling the interest of Indian Chemists and Pharmacologists in Medicinal Plants should rightfully go to

Sir Ram Nath Chopra who, has been claimed as the Father of Indian Pharmacology'.

I had the privilege and good fortune of meeting Col, Sir Ram Nath Chopra along with Prof. Dr. E.K. Janaky Ammal at his residence in 1969 immediately after my joining Regional Research Laboratory as a Scientific Assistant. My meeting with Col. Sir R.N. Chopra had left a lasting impression of a great scientist in my memory.

It is a fitting tribute to Col. Sir. R.N. Chopra that the editors have decided to dedicate these eight volumes to his memory. I congratulate the editors for bringing out this series which is timely, relevant and important to all those who are engaged in the study of natural products and development of therapeutically useful bioactive molecules. I am sanguine that these volumes will receive wider acceptance among the scientific community, especially among those engaged in Natural Product research.

(P. Pushpangadan)

About the Editors

Dr. Vijay Kumar Gupta, Ph.D., FLS, London (born 1953-) Deputy Director & Head, Animal House, Indian Institute of Integrative Medicine (CSIR), Jammu, India. He did his M.Sc. (1975) and Ph.D. (1979) in Zoology both from University of Jammu, Jammu-India. His research capabilities are substantiated by his excellent work on histopathology, ecology and reproductive biology of fishes, turtles, birds and mammals, which has already got recognition in India and abroad. Dr. Gupta has to his credit more than 75 scientific publications and review articles which have appeared in internationally recognized Indian and foreign journals. Founder fellow, life member and office bearer of many national societies, academies and associations. He has successfully completed a number of research/consultancy projects funded by various governments, private and multinational agencies. His current areas of interest are histopathology, toxicology, pre-clinical safety pharmacology and reproductive efficacy studies of laboratory animals. He is also Editor-in-chief of the books 1) Utilisation and Management of Medicinal Plants 2) Medicinal Plants: Phytochemistry, Pharmacology and Therapeutics 3) Perspectives in Animal Ecology and Reproduction (Vols.1-6). The Editor-in-chief of the American Biographical Institute, USA, has appointed him as *Consulting Editor* of *The Contemporary Who's Who*. Dr. Gupta also appointed as Nominee for the *Committee for the Purpose of Control and Supervision of Experiments on Animals* (CPCSEA, Govt. of India). Recently the *Linnaean Society of London, U.K.* has awarded fellowship to him in November 2009 in recognition of his contribution towards the cultivation of knowledge in Science of Natural History.

Dr. Anil K. Verma, Ph.D., M.N.A.Sc., FLS, London (born 1963-) Sr. Asstt. Professor, Department of Zoology, Govt. College for Women (P.G.), Gandhi Nagar, Jammu, J&K State, did his M.Sc. in Zoology (1986) from University of Jammu, Jammu. He has undergone his M.Phil. (1988) and awarded first rank and Ph.D. (1993) in the field of animal reproduction at the same University and has published about 50 research papers and review articles in reputed journals and books. He is also a member Editorial Board of the book series "Advances in Fish and Wildlife: Ecology and Biology". In recognition of his standing in greater scientific community, the Board of Directors of the American Association for the advancement of science (AAAS) New York, Washington, has awarded membership to him. Recently the *Linnaean Society of London, U.K.* has awarded fellowship to him in October 2006 in recognition of his contribution towards the cultivation of knowledge in Science of Natural History.

Preface

The growing interest in the substitution of synthetic food antioxidants by natural antioxidants and the health implications of antioxidants as nutraceuticals has fostered research on natural sources and the screening of raw materials for identifying newer natural bioactive molecules with enhanced health promoting potential. Educating health-conscious consumers on the vital contribution of antioxidant-rich foods and nutritional supplements, and about their health and longevity, is of great importance in today's world. Clinical studies suggest that increasing the antioxidant status of our blood serum shall result in reduced risk of many chronic degenerative diseases. It is well known that populations consuming a large proportion of plant-based foods, including fruits, vegetables, whole grains and cereals or those with a high intake of seafoods, have a lower incidence of cardiovascular diseases and certain types of cancer. Thus, research efforts to identify antioxidants, nutraceuticals and bioactive components from many natural sources including plants, animals, microorganisms, and marine organisms have been intensified during the past decade.

Antioxidants have, therefore, received much attention recently, as they are bioactive compounds that have a potential to reduce the levels of oxidative stress. Several epidemiological studies also suggest that a high intake of food rich in natural antioxidants can moderately reduce the oxidative stress and thus may help in the prevention of degenerative conditions such as cancer and heart diseases. Oxidative damage to biomolecules is believed by many to be a significant factor in the etiology of many degenerative diseases and the aging process itself. Oxidative damage to cellular DNA is also an underlying element in the initiation of cancer. Similarly, oxidative damage to low-density lipoproteins in the blood is a causal agent in the development of atherosclerotic plaque in cardiovascular disease. It has been suggested and supported by various types of evidence that consuming antioxidants may provide greater protection against the deleterious effects of oxidative damage.

Free radical levels in the body rise as we age, so a continuous intake of antioxidants is important to assure us protection. Free radicals are produced by normal body metabolism and by factors such as exposure to radiation and environmental pollutants. A high-fat diet increases free-radical reactions, as does eating heated and processed oils. However, antioxidants both made in the body and supplied by the diet, prevent oxidative damage and protect the body from the harmful effects of free radicals. Green and yellow vegetables, fruits, nuts and seeds all contain antioxidants. A healthy body will produce healthy cells, which is the best strategy for protection against free-radical damage.

Despite all the evidences from the scientific literature about the relationship between the oxidative stress and disease progression, particularly the chronic ones; the administration of antioxidant products to patients (antioxidant therapy) is considered not relevant in the therapeutics methodologies. One reason to explain such tendency is that the regulatory health agencies do not consider antioxidants as drugs, instead they are classified as "nutritional supplements or natural products for health" since oxidative stress is not considered as a therapeutic category.

The most extended myth about oxidative stress may be its relation with a large number of diseases. Such association raises doubts in medical authorities concerning the efficacy of antioxidant therapy in the prevention or reduction of diseases progression. The oxidative stress is not considered as a disease because it is not possible to directly correlate its association with a specific syndrome as the diabetes, hepatopathies or hyperlipidemies do. This is another element that reinforces the misgivings about antioxidant therapies.

Therefore, understanding the occurrence, chemical composition and therapeutic activities of such natural products are extremely important in order to demonstrate the benefits of antioxidants and nutraceutical therapy associated with the improvement of person's quality of life and in the treatment of some diseases. The first and foremost activity in this direction is the publication of books and/or monographs of the bioactive natural products that would provide a systematic and updated account regarding their therapeutic and pharmacological profiles as an antioxidant or a nutraceutical and this volume ***"Antioxidants and Nutraceuticals"***, in the series **"Comprehensive Bioactive Natural Products"** brings together the latest scientific documentation of the advanced investigations from the outstanding scientists, from across the globe. It presents 20 research and review papers in the core areas. First fourteen chapters are exclusively on antioxidants and free radical sacvenging activities derived from natural products, phytochemicals, non alcoholic beverages, *Allium* species, fruits, berries and vegetables, *Aquilegia vulgaris*, medicinal plants, *Amaranthus tricolor*, *Limonia acidissima*, *Sorghum arundinaceum* and the rest chapters are on neutraceuticals related with Pseudocereals, fruits and vegetables etc.

The aim is the enthuse the reader with this active and exciting area of research and to lay a solid foundation on which further study of its various facts may be based and the authors will consider themselves to be amply rewarded if this humble piece of work proves to be useful for those it is meant. Finally, we would always remain debtor to all our well-wishers for their blessings, without which this book series would not have come into existence.

Jammu, India

V.K. Gupta
Anil K. Verma

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1

The Challenges of Antioxidant Therapy with Natural Products

A.J. NÚÑEZ SELLÉS[1,*] AND G. GARRIDO GARRIDO[1]

ABSTRACT

Antioxidant Natural Products (ANPs) and their use in clinical practice still remain as an unsolved problem. Antioxidant natural therapy, meaning the use of ANPs for improving the patient's health status, is controversial within the common medical practice as related to synthetic antioxidants like vitamins (A, C, E), carotenoids or lipoic acid, just to mention few examples. Furthermore, the recent publication (2007) of a meta-analysis about clinical trials results (more than 200,000 patients within the period 1970-2005) with those synthetic antioxidants has been confusing. The major factors affecting those results were: (i) not well-defined criteria for patient's inclusion in trials; (ii) trial's length was not uniform; (iii) different oxidative stress markers to evaluate patients progression in blood, urine and/or other biological fluids; and (iv) lack of validated protocols to evaluate patient's quality of life. The fact is that basic mechanisms and biochemical pathways of the oxidative stress generated in human beings, and its correlation with a disease progression, are topics not well understood by the medical community yet. The challenges for the right use of antioxidant products (including ANPs) in clinical practice, commonly known as Antioxidant Therapy, is considerably high starting from the basic concepts. Therefore, the manuscript describes the results from recent clinical trials with ANPs and reviews the present state-of-the-art of oxidative stress.

Key words : Antioxidant therapy, Natural health products, Reactive oxygen species, Oxidative stress (OS), OS biochemical markers

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INTRODUCTION

Flavonoids and other phenolic substances have become familiar since an European epidemiological study on cardiovascular diseases risks was conducted in the 80's, leading to the phrase known as *"The French Paradox"* (Reanaud *et al.,* 1992) based on the apparent compatibility of a high fat diet with a low incidence of coronary atherosclerosis attributed to the regular consumption, by the French, of red wine and/or grape juice, both with a high flavonoid content (Dembow *et al.,* 1994).

Flavonoids, and other phenolic substances contained in red wine, are assigned with antioxidant properties (Jovanovic *et al.*, 1994) which lower the oxidation of low density lipoproteins (LDL) and consequently, the risk of atherogenic diseases (Frankel *et al.*, 1993; Fuhrman *et al.*, 1995). Other examples are the correlation observed between the aging process of the human body and the increase of free radicals due to the drop in the oxygen used by metabolic processes (Cutler, 1991), and the initiation and promotion of cancer and tissue injury by free radicals (Pitot *et al.*, 1996; Kehrer, 1993; Halliwell, 1996; Hertog *et al.*, 1995), which has induced the intake of antioxidant products as chemical factors that prevent the onset of the disease.

Cell degradation processes may lead to a partial or total loss of the functions of the physiological systems of the body. Currently, the incidence of free radical imbalance on the onset and evolution of more than 100 diseases (cardiovascular, neurological, endocrine, respiratory, immune and self-immune, ischaemia, gastric disorders, tumor progression and carcinogenesis, among others) has been demonstrated (Burr, 1994; Miller *et al.*, 1997; O'Brien *et al.,* 1996; Heliovarra *et al.,* 1994; Sharma *et al.,* 1996; Ebadi *et al.,* 1996; Portal *et al.,* 1995). However, the relationship between oxidative stress and the progression of diseases has not attracted the attention of the scientific and medical communities until recent years. Most of the physicians have considered the *"antioxidant therapy"* of secondary importance or not relevant for therapeutics. One of the contributing factors to that situation is the regulatory environment, where antioxidants can not be considered as drugs but food or dietary supplements, because the oxidative stress has not been described as a syndrome and it is not a pharmacological or physiological disorder. Marketers have taken advantage of that situation (there is no need of clinical trials) and new terms for antioxidants and other natural products as well, have appeared as *nutraceuticals* (nutritional supplements with pharmaceutical applications), *functional foods* (foods with one or more additives with therapeutic effects), *cosmeceuticals* (cosmetics which may improve skin health), and *natural health*

products (natural products with claimed but not proved effects on health). Promotion and publicity have attributed to these products therapeutic properties, sometimes miraculous, which are not supported on strong scientific evidences, from companies more interested on profits and sales than health improvement.

Perhaps, the largest challenge of the oxidative stress is the claim of nutritionists and natural products researchers in hundreds of articles that it is related to more than 100 diseases, and a lot of oxidative stress markers in biological fluids have been described. Medical authorities are reluctant to that claim and ask for long-term randomized double-blind clinical trials on thousands of people to assure the effectiveness of antioxidant therapy for a specific medical application. These are the extremes of the present situation about antioxidant therapy. Nevertheless, there are an increasing number of controlled clinical trials which are demonstrating the relevance of the antioxidant therapy in diseases.

Supplementation with vitamin C (1,000 mg/day) and vitamin E (400 IU/day) has reduced the incidence of pre-eclampsia in women at risk and this was associated with improvement in a range of biochemical markers of placental insufficiency and oxidative stress supporting the rationale of prophylactic use of antioxidants (Kelly, 2002). Other studies revealed that antioxidant protection on bone and cartilage may not work directly on the damaged tissue by reactive oxidative species (ROS) but may instead shift cytokine balance, like glycosaminoglycans, carotenoids, essential fatty acids and flavonoids (Levin, 1998). Oral supplementation with glucosamine sulfate has been examined in osteoarthritis and subjects reported a significant reduction in pain and decrease limitations on active and passive movements than all other treatments (Tapadinhas, 1982). A clinical trial on the effect of chronic administration of vitamin E (600 IU/day) on the cardiac autonomic nervous system was investigated over a period of 4 months in 50 type-2 diabetic patients with neuropathy. Vitamin E significantly improved measures of diabetes (glycosylated hemoglobin and plasma insulin), oxidative stress (catechol amines) and hearth functions (Manzella *et al.*, 2001). The effect of a high dose of vitamin A (25,000 IU) and vitamin E (500 IU) supplementation on corneal re-epithelization time, visual acuity and haze using two groups of 20 patients (treated and placebo) who underwent photorefractive keratectomy was studied in a 1-year trial. Patients supplemented with vitamins A + E were significantly improved ($p<0.05$) as compared to the placebo group suggesting that antioxidant therapy may accelerate the healing process in these patients (Vertugno *et al.*, 2001).

However, most of the publicized clinical trials with antioxidant products have been conducted with synthetic products (mainly vitamins). Many natural products, assumed to have strong antioxidant properties (ANPs, antioxidant natural products), with a large ethnomedical and historical practice in Latin America and other economical non-developed countries, are lacking funds for adequate scientific and clinical research. That is a sound challenge for governments and scientific bodies from non-developed countries in the attempts to develop their own pharmaceutical development. No less important is the practice of most research groups and pharmaceutical companies from the West World (developed countries) to consider only valid a single-purified compound from a natural product extract better than a standardized whole-crude natural extract with a large number of components to be developed as a new product for therapeutics. Asian medicine and its millenary practice have demonstrated the contrary.

But first we have considered appropriated to present a brief overview of concepts about the oxidative stress and the redox balance of the human organism.

Reactive Oxygen Species (ROS)

ROS (also called oxygen free radicals) are defined chemical species, which have one or more unpaired electron(s) and a high reactivity to form other free radicals by a chain reaction (Elejalde Guerra, 2001). They are quite unstable and have the ability to react very quick with chemical compounds, making a difference with other species (ions), which have an electric charge (positive or negative).

Under the term ROS are included not only free radicals but neutral molecules with a high reactivity, like hydrogen peroxide and hypochlorous acid, able to produce free radicals in the human body (Scandalios, 1992; Mohanakumar *et al.,* 2002; Spatz *et al.,* 1992; Young *et al.,* 2001). The most common ROS of high biological relevance are *singlet oxygen* ($^1O_2^{\bullet}$), *hydroxyl* ($HO^{\bullet}$), *peroxyl* ($RO^{\bullet}$), *superoxide anion* ($O_2^{\bullet -}$), *hydrogen peroxide* (H_2O_2), *hypochlorous acid* (HOCl), *nitric oxide* (NO), and *peroxynitrite* ($NO_2O^{\bullet}$). Other group of free radicals has the unpaired electron on other atoms different from oxygen (carbon, nitrogen or sulphur), but in terms of simplicity all these species will be called as ROS hereafter.

ROS in the Human Organism

ROS may be generated by endogenous processes like mitochondrial respiration ($^1O_2^{\bullet}$), the activation of polymorphonuclear leukocytes

(HOCl, $^1O_2$, HO$^\bullet$, and H_2O_2), arachidonic acid metabolism ($O_2^{\bullet-}$), enzymatic functions ($O_2^{\bullet-}$, H_2O_2, and NO) and iron- or cupper-mediated catalysis (HO$^\bullet$) among others (Barry *et al.,* 1997; Ahmad, 1995; Forman *et al.,* 1997). The human organism produces these ROS as a need of its function and harmonic balance of several physiological processes. Alterations or disorders in that ROS production might lead to a pathological condition or immune disorder, which may evolve to a disease progress (Montagnier *et al.,* 1998; Kumpulainen *et al.,* 1999; Oldham *et al.,* 1998; Cross *et al.,* 2006).

The overproduction of ROS, mainly caused by exogenous processes, implies an excess of free radicals in the human organism leading to a redox imbalance. Within these factors it is worth to mention environmental pollution (atmospheric, aquatic and terrestrial), radiations (ultraviolet, gamma, hertzian), toxic habits (drugs, alcohol, tobacco), inadequate habits of food consumption, exposition to toxic substances (fertilizers, pesticides), drug metabolism, and a high physical or psychological stress. Some of them might be controlled, like food consumption, toxic habits, or physical exercises, but others are out of control from the person (environmental pollution and exposition to radiations). This means that the human organism is continuously exposed, without distinctions, to uncontrolled exogenous factors which lead to an overproduction of ROS (Beck, 2000; Lorgeril *et al.,* 1994; Clarkson, 1995).

Other source generating ROS in the human organism is by the structural modification of essential cell macromolecules (DNA, proteins and lipids) owing to the occurrence of non-reversible chemical reactions on cell membrane (Murray, 1996). These reactions generate compounds like malonyldialdehyde (MDA) or organic hydroperoxides (ROOR) which are able to propagate the oxidative damage to other cells and tissues. Although it is still controversial, is generally accepted that this third route of ROS production is a consequence of genetic alterations (hereditary or not) or physiological disorders caused by a disease. Other researchers have considered the contrary from their experimental evidences (Whitton, 2007). That means that the cause of genetic alterations or physiological disorders is the overproduction of ROS, endogenously or exogenously, which favor DNA fragmentation and cell membrane disruption. Therefore, the topic of *oxidative stress* should have a higher relevance and importance for therapeutics than it has been for the medical community.

Oxidative Stress

Oxidative stress is the imbalance between the necessary endogenous

generation of ROS and the body defense mechanisms against their overproduction caused by exogenous factors. The oxidative balance within the human organism, which means the production of ROS without trespassing certain limits, is essential for the metabolic regulation, metabolic energy control, activation/inactivation of certain biomolecules, signal transduction, cell exchange, gene expression, and endothelium-related vascular functions.

The antioxidant body mechanisms against the excess of ROS or oxidative stress may be classified as follows (Martínez *et al.,* 2003).

Preventive Mechanism

Proteins which have a coordinated nucleus, like iron and copper, or with the capacity to bind those metals, like albumin, metallothionein, ceruloplasmin (copper), and ferritin, transferrin, myoglobin (iron), which prevent the overproduction of $HO^{\bullet}$.

Repairing Mechanism

Enzymes which repair or eliminate damaged biomolecules by ROS like glutathione peroxidase (GP), glutathione reductase (GR), and methionine-sulphoxide reductase (MSR).

Scavenger Mechanism

Enzymes with capacity to scavenger excesses of ROS like superoxide dismutase (SOD), GP, catalase, and other metalloenzymes, together with chemical entities with scavenging capacities like polyunsaturated fatty acids, vitamins (C and E), uric acid, bilirubin, carotenoids, and flavonoids.

Fig 1 shows a scheme of redox system in the human organism. When these body defense mechanisms fail or are not enough to avoid the excess of ROS, the administration of antioxidant formulations becomes a need within the therapeutic strategy for patient treatment. This is known as the *antioxidant therapy*, which means the reduction of patient's oxidative stress when the human body has not been able to eliminate through its endogenous defense mechanisms.

The relationship between oxidative stress and diseases might be well understood if it is considered the effect of an excess of ROS at the cellular level. A cell attacked by ROS may (Cuter *et al.,* 2002):

(i) *modify* its gene expression by the modification of DNA structure and/or the destruction of base pairs.
(ii) *repress* gene expression by the inhibition and/or destruction of transcriptional factors.

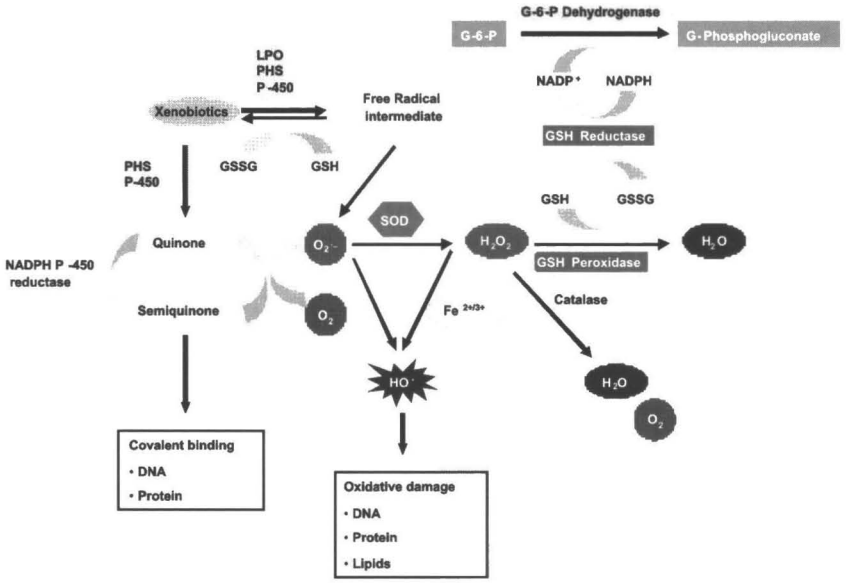

Fig 1. Diagram of the reduction-oxidation (redox) balance from the human organism. The formation pathways of the different Reactivate Oxygen Species (ROS) induced by xenobiotics can be appreciated, which are regulated by diverse enzymatic processes that regulate the endogenous mechanisms of antioxidant defence. The rupture of this redox balance in favour of the ROS overproduction is known like *oxidative stress*

(iii) *loose* its integrity by rupture of the cell wall by lipid oxidation.
(iv) *modify* its functions by the accumulation of oxidized low-density lipoproteins (LDLs).
(v) *activate* or *inactivate* key enzymes for cell function.

All or part of those cell degradation processes would influence physiological body systems, which support the correlation between oxidative stress and disease progress. Neurodegenerative disorders are among the most studied, although still it is not clear the cause of neuronal death (Sayre *et al.*, 2001).

A recent report evidenced that the dysfunction of glutathione metabolism by the oxidative stress may be an important factor in the pathogenesis of Alzheimer's disease (AD). Strong evidence that oxidative stress is involved in the pathogenesis of AD comes from a clinical study showing that oral vitamin E intake delayed progression in patients with moderately severe impairment from AD (Sano *et al.*, 1997; Schulz *et al.*, 2000). The role of oxidative stress in AD is further supported by increased levels of thiobarbituric acid-reactive substances (TBARS) and 4–hydroxynonenal (HNE). Recently, a new

biomarker for AD in the silent phase of the disease characterized by mild cognitive impairment (MCI) has been reported as 8,12-isoPGF2α-VI. MCI patients were found to have significantly higher levels of the isoprostanoid in cerebrospinal fluid, plasma and urine as compared to cognitively normal elderly subjects (Praticò *et al.*, 2002; Lovell *et al.*, 1995; Marcus *et al.*, 1998). Induced neurodegeneration in AD by the oxidative stress is reduced by a gene in a transgenic animal model, where neuron cellular replication was inhibited (Klein *et al.*, 2002). Moderate oxidative stress may specifically inhibit several types of genes through the influence of ROS on transcriptional factors in the central nervous system (Morel *et al.*, 1999).

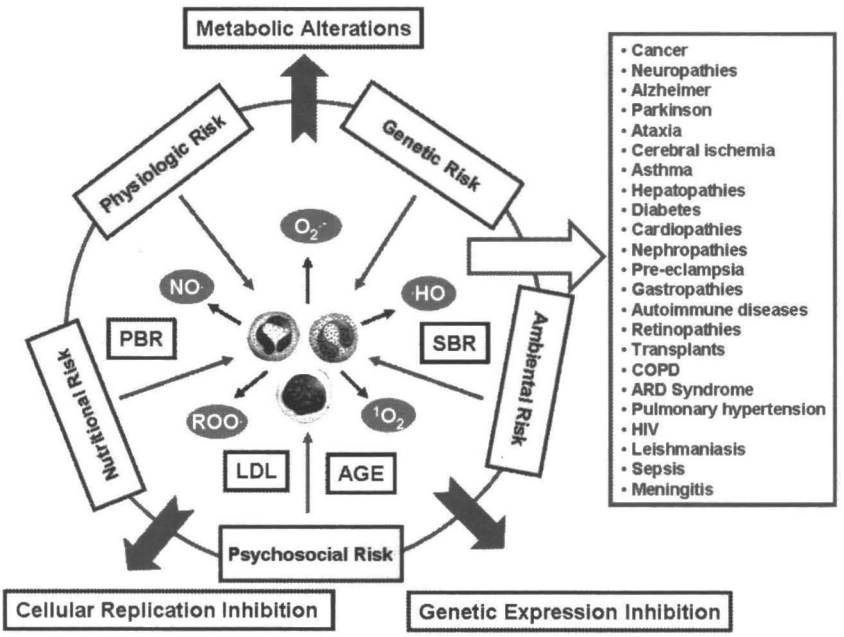

Fig 2. Different types of stress constitute the factors that induce the overproduction of ROS (oxidative stress). The ROS can produce the fragmentation of protein chains and DNA [peptidic bound rupture (PBR), cysteinic sulphur bridge rupture (SBR) and the loss of bases], the degradation of carbohydrates (mainly of oligo- and poly-saccharides), the lipid oxidation (production of LDL oxidised), and the production of advanced glycosilation end-products (AGE). Those chemical reactions can induce metabolism alterations, inhibition of the cellular replication and genetic alterations that are related with the physiopathology of a high number of diseases. COPD – Chronic obstructive pulmonary disease, ARD – Acute respiratory distress, HIV – Human immunodeficiency virus

Fig 2 shows the connections between oxidative stress and disease progression. ROS excess induces their attack to cells (DNA, proteins and lipids), with a lot of subsequent chemical reactions, leading to an increase in disease progression. Nevertheless, some authors have considered that is exactly the opposite: the oxidative stress is the cause and not the consequence of disease progression (Juranek *et al.*, 2005). This question still remains unanswered being a strong challenge for the scientific community.

Antioxidant Products

The best antioxidant product is that able to *prevent* the excess of ROS, *stimulate* the endogenous antioxidant repairing mechanism, and *provide* a large amount of chemical entities to increase the endogenous antioxidant scavenger mechanism. However, the continuous exposure to environmental and other risks, which are unavoidable by most of the people, would lead to an oxidative stress in a larger or lesser extent, but mainly present.

The first option to prevent the oxidative stress is to have healthy habits. It would be better always to prevent a disease than to cure it. Therefore, the following recommendations are valid in order to avoid or to lower the effects of oxidative stress:

- Consume fresh products instead canned or processed (fast) foods. Fruits (fresh or juices), vegetables (fresh or boiled) and legumes (without cooking) give natural polyphenols, flavonoids and minerals to fight against the oxidative stress.
- Avoid the consumption of fats and fried products. Even vegetable oils become harmful when are used to fry (*i.e.* potatoes) because of double-bond saturation while heating.
- Eliminate toxic habits like excessive alcohol (one cup of wine in the meals is always welcome), and smoking. Forbidden drugs must be eliminated completely.
- Practice physical exercises at least three times a week. 1/2 h walking daily (at least) would help highly the cardiovascular system.
- Look at life and the daily routine with optimism and a positive point of view. Enjoy the music, read a book, go to the theater or a concert, etc. Both good physical and psychological conditions are basics to have not an increase in the body redox conditions.

However, there are situations where the above recommendations would be not enough and a supplementation with ANPs (better than the synthetic ones) is recommended strongly. People with immunodeficiencies (like HIV seropositives), hereditary and chronic diseases (like cancer or neurodegenerative disorders, and the elderly

have proven to have a severe oxidative stress and ANPs supplementation would help to improve their redox status and quality of life. The main problem (and challenge) is to select the most appropriated antioxidant treatment for each condition, and the adequate biochemical markers to follow the progression of their health status. The challenge is to design a tailor-made antioxidant treatment with none or few practical evidences of the patient's redox status.

Today, there are about ten antioxidant products in the market with proven experimental evidences, both pre-clinical and clinical, of their preventive, repairing or scavenging ROS effects. Few of them are shown in Table 1. However, a lot of doubtful "antioxidant" formulations are available in the market, without a comprehensive experimental evidence of their biological and toxicological effects, under acute or chronic administration. Most of them are extracts from natural products commonly, which have not been investigated by the producers and distributors.

Nevertheless, few exceptions to the above mentioned rule are worth to be mentioned. Cat´s claw (*Uncaria tomentosa*) was probably the first antioxidant natural product (ANP) to be available in the market (Sandoval-Chacon *et al.*, 1998; Heitzman *et al.*, 2005), and a lot of formulations (capsule, tablet, powder, syrup, etc) are widely distributed. Grape seed extract (*Vitis vinifera*) has been tested extensively as ANP (Orallo, 2006) as well as an extract from the stem bark of a California pinus, sold under the brand name *Pycnogenol* (Packer *et al.*, 1999). The extract of *Ginkgo biloba* has attracted the attention of both the scientific and medical communities (Mahadevan & Park, 2008) overall for its capacity to improve the Mild Cognition Impairment (MCI) in the elderly. Recently, a comprehensive review of experimental evidences about the antioxidant, anti-inflammatory, analgesic and immonumodulatory of a mango stem bark extract (*Mangifera indica* L.), sold under the brand name *Vimang*, has been published (Nuñez-Sellés *et al.*, 2007).

Perhaps, the challenge to increase R&D on ANPs will be one of the most difficult to face because of the present perception of national health regulatory bodies. It is not the same to deal with high complex mixtures of natural extracts, with a lot of chemical compounds, than with a defined structure of a single compound (isolated or synthesized). Whilst the medicine based on evidence will prevail above that based on practice, the present situation of ANPs, and phytodrugs as well, will continue to face that challenge, which is lacking of attention and financial resources, overall in the non-developed countries where are the larger sources of that called "green gold".

Table 1. Antioxidant products with large scientific evidences

Antioxidant product	Administration route	Medicinal uses (Published in literature)	Reference(s)
Selenium	Oral, parenteral	Chemopreventive	El-Bayoumy *et al.*, 1995
		Antitumoral	Patrick, 2004
		HIV	Baum *et al.*, 1997
		Ictus	Yamaguchi *et al.*, 1998; Ogana *et al.*, 1999
		Rheumatoid arthritis	Peretz *et al.*, 2001
		Bronchial asthma	Gazdik *et al.*, 2002
		Infertility	Hawkes & Turek, 2001
		Cataracts and macular injury	Karaküçük *et al.*, 1995
Alpha-lipoic acid	Oral, parenteral	Diabetes	Packer *et al.*, 2001; Henriksen, 2006
		Polyneuropathy	Sachse & Willms, 2006
		HIV	Dworkin *et al.*, 1990; Fuchs *et al.*, 1993
		Hepatitis C	Berkson, 1999
		Cataracts	Maitra *et al.*, 1995
		Cognitive disorders	Stoll *et al.*, 1993
Vitamins	Oral, parenteral	Atherosclerosis	Thomas & Stocker, 2000
		Coronary diseases	Steinberg, 1993
		Ischemia	Gey *et al.*, 1993
Cat's claw	Oral	Osteoarthritis	Piscoya *et al.*, 2001; Harding, 2007
Pinus sp.	Oral	Vasodilatation	Nishioka *et al.*, 2007
		Attention deficit hyperactivity disorder	Dvoráková *et al.*, 2006
		Climacteric syndrome	Yang *et al.*, 2007

Table 1. *Contd.*

Antioxidant product	Administration route	Medicinal uses (Published in literature)	Reference(s)
		Analgesic	Kohama *et al.*, 2004
		Endometriosis	Kohama *et al.*, 2007
Grape seed	Oral, topical	Platelet function and reactivity	Shenoy *et al.*, 2007; Polagruto *et al.*, 2007
		Arteriosclerosis	Sano *et al.*, 2007
		Cancer	Katiyar, 2007
Ginkgo biloba	Oral	Alzheimer's dementia	Mazza *et al.*, 2006
		Cancer, Cardio-vascular diseases	Mahadevan & Park, 2008
Cocoa	Oral	Platelet and leuko-cyte function	Heptinstall *et al.*, 2006
		Vascular effects	Wang-Polagruto *et al.*, 2006
Green tea	Oral, topical	Photoprotection	Katiyar, 2003
		Inflammation	Katiyar *et al.*, 1999
Mango stem bark	Oral, topical	HIV	Gil del Valle *et al.*, 2002
		Dermatological diseases	Guevara *et al.*, 2007
		Analgesic	Garrido-Suárez *et al.*, 2007

Oxidative Stress Biomarkers (OSBs)

Around 100 clinical trials including more than 250 000 patients have been reported in the last 30 years with antioxidant products, mainly vitamins and other synthetic products. The most critical point of all those trials has been the inadequate selection of OSBs in biological fluids (plasma, blood, urine, etc.) as evaluation criteria for the trial end-point. First clinical studies, between the 70´s and 80´s, took the antioxidant concentration in the biological fluid(s), which did not bring any significant information about the oxidative damage within the organism. At the end of the 80´s began the measurement of more specific OSBs, which directly or indirectly did give information about

ROS concentrations in the human body. Moreover, several reports have shown the high probability of specific OSBs according to the disease related to OS progression.

The increase of OSBs in biological fluids has been strongly related, in a non-specific way, to the increase of pathological conditions in a large number of diseases. Those biomarkers are claimed to be relevant variables to be considered within disease prevention, diagnosis or treatment, and also as control variables of the OS status connected to their follow-up. Thus, OSBs measurement would be one of the crucial steps in order to avoid or slow pathological conditions like atherosclerosis and cardiovascular events as the most significant.

OSBs may bring information about three progressive stages of the disease as:

1. Measurable quantitative parameters of the oxidative damage to biomolecules (*i.e.* DNA, proteins, lipids, and carbohydrates).
2. Functional markers (*i.e.* cognitive and respiratory functions).
3. Specific markers of disease progression, mainly connected to the common biochemical markers in biological fluids (*i.e.* hemoglobin, transaminase and cholesterol).

They may be used also for the pre-symptomatic or symptomatic disease diagnosis in order to establish a therapeutic strategy, including the administration of antioxidants for the patient's treatment. OSBs might be highly useful in order to provide early indications of the disease and its progression. An acceptable OSB should be a chemical entity with some or preferably all the following characteristics (Polidori *et al.*, 2001):

- produced as a result of a severe oxidative damage, which can be related, without any doubt, to the disease onset and progression.
- accessible on a target issue or organ which indicates quantitatively its modification.
- specific for the ROS under study and which can not be interpreted as a factor derived from food consumption or food supplements intake.
- detectable at low concentrations in a specific, sensitive, and reproducible way according to the available equipment or technique.
- present in the biological fluid with a low dispersion (high precision) in a way that inter-sample variations must be lower than inter-patients ones.
- stable and with non- or low-susceptibility to form artifacts or be lost on sample handling.

The high complexity of diseases related to OS makes it almost impossible to choose only one OSB in order to assess or confirm the clinical diagnosis. Therefore, a set of OSBs is essential for a better understanding of disease diagnosis or its progression control. Although here are analytical techniques to make a direct determination of ROS in the body and/or biological fluids, like the electronic paramagnetic resonance (EPR) and the spin trapping method (STM), they are not commonly used in clinical practice (Miura *et al.,* 2000; Mason, 1996). Routine practices are the indirect measurement through OSBs in a way that a ROS is trapped by a chemical in order to form a stable chemical entity, which is further detected and quantified by gasometric, spectrophotometric, chromatographic or immuno-enzymatic techniques. As examples, hydroxylation of salicylic acid (Halliwell *et al.,* 1997), deoxyribose assay (Biaglow *et al.,* 1997), reduction of cytochrome c (Kutham *et al.,* 1982), and reduction of NO to nitrite (Amano *et al.,* 1995).

Quantitative parameters of OSBs are commonly called as the patient's "oxidative fingerprint". Specific end-products of ROS interaction with biomacromolecules and Low Molecular Weight Antioxidants (LMWAs) are measured from the individual's biological fluid(s) giving an unique information of the patient "oxidative status". The detection of those oxidation end-products are a confirmation or not of the presence of ROS in the subject.

The biomacromolecules of significant biological relevance for the OS diagnosis are DNA and lipids. High pressure liquid chromatography (HPLC) and high resolution gas chromatography (HRGC) coupled to mass spectrometry (HPLC/MS and HRGC/MS) techniques have been applied to the analysis of 8-oxo-2-deoxyguanosine (8-oHdG) after DNA enzymatic hydrolysis and simple-cell electrophoresis (Comet Assay) are two of he most used specific techniques to evaluate oxidative damage through the detection of base-pair or -adduct of DNA (Fairbarian *et al.,* 1995). Other techniques determine DNA single- or double-chain breakdown, which lead to the quantification of DNA-oxydized adducts (Sutherland *et al.,* 2001). DNA-aldehyde adducts, like those formed with MDA (Zhang *et al.,* 2002), or 4-hydroxynonenal (HNE) (Sodum *et al.,* 1989), are examples of these techniques.

Lipid peroxidation (LPO) is a complex process with three stages: initiation, propagation and termination, strongly related to atherosclerosis, inflammatory processes and mitochondrial cell functions. Each LPO stage has several available techniques which afford its quantification at each one. LPO produces unsaturated fatty acids (UFAs) at the initiation; therefore determination of UFAs by HRGC/MS in blood and/or urine gives an indication of LPO at

this early stage. During propagation, oxygen is consumed at a high rate; thus oxygen measurement in blood through electrometric techniques with oxygen electrodes (oxymetry) gives an indication of LPO progression. A different approach to measure LPO progression is through the quantification of RO· radical in blood as an indication of fatty acid oxidation. A hydrogen is substracted from the fatty acid, which structure is re-ordered leading to a free radical which further forms a conjugated diene. This is later detected and quantified by spectrophotometric techniques (Halliwell *et al.,* 1999). At the termination stage, peroxides are decomposed to form aldehydes, like MDA and HNE, which are detected by a colorimetric technique. Thiobarbituric acid is commonly used to form the colored complex and those chemicals are known as thiobarbituric acid reactive species (TBARS). Chemiluminescence and fluorescence techniques are highly sensitive methods to measure OS (Albertini *et al.,* 1998; Hammer *et al.,* 1988). Light emissions are produced through the interaction of aldehydes, from LPO, with amino groups from aminoacids, proteins or DNA-bases, which lead to obtaining Schiff´s bases.

Oxidative damage can be also measured by the carbonyl group technique (Levine *et al.,* 2000). These groups are formed after the attack of a ROS to a terminal protein aminoacid or even the peptide bond. They can be determined specifically by gel electrophoresis. Hydroxyl, peroxyl, and sulphydryl groups may be also liberated by a ROS attack on the DNA, and detected by gel electrophoresis, giving a more complete information about the OS damage and/or extension in different tissues and organs.

Antioxidant enzymes and LMWAs are two main components of endogenous mechanisms against OS. Some techniques may evaluate directly the activity of OS-related enzymes by biochemical, spectrophotomeric, and immuno-cytochemical techniques. Generally, it would be preferable those techniques able to correlate the ratio oxidant:reducing agents, *i.e.* oxidized glutathione (GSH:GSSG), oxidized dehydrogenase (NADH:NAD), and ascorbate:dehydroascorbic acid (Motchnik *et al.,* 1994)

Perhaps, the most used OSB at present is the so-called total antioxidant status (TAS) because of its simplicity and market availability. It determines the total concentrations of LMWAs instead of a single molecule and is clear indicative of how the endogenous antioxidant defense mechanisms are working all together (Berry *et al.,* 1999). About a dozen of LMWAs has been recognized at present but only few of them are known (*i.e.* ascorbic and uric acids). It is preferable to detect the effect of the LMWAs mixture than a single unknown LMWA.

Other procedures to determine the total LMWA concentration are based on the antioxidant capacity of their reaction products obtained either by oxidation or reduction. Direct techniques for this objective are based on electro- and coordination-chemistry. The electrochemical techniques use electrodes which determine the redox pair within the biological fluid, which are measured by potentiometry, voltametry or titration using one or both methods (Skoog *et al.,* 1988). Complexometric techniques measure the physical properties of both oxidized and reduced chemicals, which are quite different and possible to detect and quantified separately, *i.e.* the ability to reduce Fe III, known as the ferric-reducing antioxidant power (FRAP) technique, where the reaction products of the pair Fe II-Fe III with LMWAs are measured by spectrophotometry at 593 nm (Benzie *et al.,* 1999). These techniques provide information about the OS status taking into account all LMWAs, lypophyllic and hydrophyllic, and can be determined on cell cultures, tissues and biological fluids. Indirect techniques are those measuring the oxidation products from a ROS attack, which are detected and quantified by spectrophotometric or fluorescence techniques. Other indirect techniques are based on the quantification of the oxidation inhibition, which has been provoked by the addition of a free radical promoter followed by a scavenger antioxidant. The inhibition can be measured by routine instrumental laboratory equipment. The Trolox assay, known as Trolox equivalent antioxidant capaciy (TRAP) (Lissi *et al.,* 1995), and the oxygen-radical absorbance capacity (ORAC) (Prior *et al.,* 1999) are good examples of indirect determinations of the OS status in the human organism.

The objectives of all above described techniques in order to assess the human OS conditions (mild, moderate or severe), according to the analytical results derived from the determination of OSBs, are directed to three main targets:

i. Modified molecules by ROS attacks, like MDA, HNE, and 8-oHdG, where their concentration can be related directly to
ii. Enzymes and LMWAs which are associated to ROS endogenous metabolism, like GSH, nitric oxide synthase
iii. Transcriptional factors which can be modified by ROS attack, like the nuclear-transcriptional factor κB (NFκB).

The main drawback of all these techniques is their inespecificity when dealing with a defined disease. They contribute to assess the OS status as a measure of the whole human organism functions, but not a specific and direct indication of disease progress; although it may be accepted as an indirect measure of it. Therefore, R&D efforts are focused at present on the development of specific OSBs, which can be related directly to the disease onset and progression. Table 2

summarizes a list of OSBs recently reported, and this list is increasing every day.

An adequate design of a clinical protocol with and ANP (or synthetic) must first consider which OSBs will describe better the disease progression under study, according to the treatment strategy (prophylactic or therapeutic), and the individual characteristics of trial subjects. It means that antioxidant clinical trials must be tailor-made designed and therefore can not be compared one to another or only in terms of the clinical end-point of the clinical research. That has been the subject of a lot of recent medical publications (comparison of antioxidant clinical trials), which have conduced to misleading interpretations and messages to both the scientific and general public audiences. A basic understanding of Chemistry and Biology underlying the OS, and its connection to disease progress–also related to the human conditions–, is the first step in the attempts to make antioxidant therapy a well-defined and accepted procedure in clinical and primary health practices.

Antioxidant Therapy (AT)

AT is at present a highly controversial topic as above mentioned. Most of the physicians consider AT as the administration of formulations (oral, topical, rectal, vaginal, and/or parenteral) containing antioxidant products. This antioxidant administration may be the only choice or concomitant to prescribed standard drugs to the disease treatment. Physicians (and patients as well) accept that the progression of many diseases are related, in some way, to the OS of the individual, but most of them do not known the basic aspects of how they are connected. Moreover, the majority of clinical practices and research protocols classified under AT have not considered, or even worst discard, the inter-subject variability of OS. Determinations of OSBs are commonly limited to R&D labs or research clinical protocols, but at present it is not an established routine when considering the design of a strategy for the diagnosis and follow-up of the disease.

In spite of all scientific experimental evidences about the connection between OS and disease progression or pathological conditions, overall in neurodegenerative diseases, the AT is considered a present as a "supplementation" therapy and, therefore, of secondary relevance. In our opinion, two factors are contributing to that appreciation: first, the insufficient knowledge of the chemical and biological mechanisms underlying the OS; and second, the regulatory scenario, where antioxidant formulations –except vitamins– are possible to be registered before health authorities as *food supplements*

Table 2. Recent reported specific oxidative stress biomarkers

Disease	Biomarker	Reference(s)
Cardiovascular system		
Instable angina	F2-IsoPs	Dalle-Donne *et al.*, 2006
Atherosclerosis	MDA, HNE, acrolein, F2-IsoPs,	Dalle-Donne *et al.*,
	NO2-Tyr, Cl-Tyr, Di-Tyr	2006
	HO-1,	Sanchez *et al.*, 2005
	NF-κB,	Baldwin, 2001
	COX-2	Sengupta, 1999; Turini & DuBois, 2002
Hypercholesterolemia	F2-IsoPs, NO2-Tyr	Dalle-Donne *et al.*, 2006
Hyperlipidemia	S- Glutathionylated proteins	Dalle-Donne *et al.*, 2006
Ischemia-reperfusion injury	F2-IsoPs,	Dalle-Donne *et al.*, 2006
	HO-1	Sanchez *et al.*, 2005
Coronary artery disease	F2-IsoPs, NO2-Tyr, Cl-Tyr	Dalle-Donne *et al.*, 2006
Cardiovascular disease	HNE, acrolein, F2-IsoPs, Decrease in GSH concentration and/or GSH:GSSG ratio, NO2-Tyr, Cl-Tyr,	Dalle-Donne *et al.*, 2006
	NF-κB	Marczin *et al.*, 2003
Myocardial infarction	F2-IsoPs,	Dalle-Donne *et al.*, 2006
	HO-1, COX-2	Sanchez *et al.*, 2005 Sengupta, 1999
Myocardial inflammation	NO2-Tyr	Dalle-Donne *et al.*, 2006
Heart failure	F2-IsoPs	Dalle-Donne *et al.*, 2006
Hypertension	HO-1	Sanchez *et al.*, 2005
Platelet aggregation	HO-1	Sanchez *et al.*, 2005
Vascular injury	HO-1	Sanchez *et al.*, 2005
Gastrointestinal system		
Inflammatory bowel disease	HO-1,	Sanchez *et al.*, 2005
	NF-κB,	Baldwin, 2001
	COX-2	Sengupta, 1999

Table 2. *Contd.*

Disease	Biomarker	Reference(s)
Intestinal ischemia-reperfusion	HO-1	Sanchez *et al.*, 2005
Crohn disease	F2-IsoPs, NO2-Tyr, Carbonylated proteins	Dalle-Donne *et al.*, 2006
Intestinal ischemia	HO-1	Sanchez *et al.*, 2005
Liver		
Acute and chronic alcoholic liver disease	F2-IsoPs, Decrease in GSH concentration and/or GSH: GSSG ratio	Dalle-Donne *et al.*, 2006
Hepatotoxicity	HO-1	Sanchez *et al.*, 2005
Hepatic perfusion	HO-1	Sanchez *et al.*, 2005
Hepatic shock	HO-1	Sanchez *et al.*, 2005
Hepatobiliary function	HO-1	Sanchez *et al.*, 2005
Hepatic ischemia-reperfusion	HO-1	Sanchez *et al.*, 2005
Cirrhosis	F2-IsoPs (primary biliary and hepatic)	Dalle-Donne *et al.*, 2006
Autoimmune diseases		
Arthritis	Carbonylated proteins (juvenile chronic arthritis), (psoriatic and reactive rheumatoid arthritis,), Decrease in GSH concentration and/or GSH:GSSG ratio, NO2-Tyr, Cl-Tyr	Dalle-Donne *et al.*, 2006
	NF-κB,	Baldwin, 2001
	NO	Blanco García *et al.*, 2005
	COX-2 (rheumatoid arthritis)	Sengupta, 1999; Turini & DuBois, 2002; Li *et al.*, 2000
Amyotrophic lateral sclerosis	MDA, Decrease in GSH concentration and/or GSH: GSSG ratio, NO2-Tyr, Carbonylated proteins	Dalle-Donne *et al.*, 2006
Multiple sclerosis	F2-IsoPs, NO2-Tyr	Dalle-Donne *et al.*, 2006
Systemic sclerosis (scleroderma)	F2-IsoPs	Dalle-Donne *et al.*, 2006
Cystic fibrosis	F2-IsoPs, NO2-Tyr, Cl-Tyr, Di-Tyr, Carbonylated proteins	Dalle-Donne *et al.*, 2006

Table 2. *Contd.*

Disease	Biomarker	Reference(s)
Systemic lupus erythematosus	F2-IsoPs	Dalle-Donne *et al.*, 2006
Osteoarthritis	F2-IsoPs, NO2-Tyr,	Dalle-Donne *et al.*, 2006
	NO	Blanco García *et al.*, 2005
Osteoporosis	F2-IsoPs,	Dalle-Donne *et al.*, 2006
	COX-2	Turini & DuBois, 2002
Psoriasis	Carbonylated proteins	Dalle-Donne *et al.*, 2006
	NO	Namazi, 2006
Chronic fatigue syndrome	Carbonylated proteins	Dalle-Donne *et al.*, 2006
Infections by microorganisms		
Chronic hepatitis C	Carbonylated proteins	Dalle-Donne *et al.*, 2006
	NO	Maeda & Akaike, 1998
Infection and inflammation by	Carbonylated proteins	Dalle-Donne *et al.*, 2006
Helicobacter pylori		
HIV	S-Glutathionylated proteins, Decrease in GSH concentration and/or GSH:GSSG ratio	Dalle-Donne *et al.*, 2006
	HO-1	Sanchez *et al.*, 2005
	NO	Maeda & Akaike, 1998
	NF-κB	Baldwin, 2001
Cutaneous leishmaniasis	MDA	Dalle-Donne *et al.*, 2006
Meningitis	Carbonylated proteins	Dalle-Donne *et al.*, 2006
Acute autoimmune myocarditis	Carbonylated proteins	Dalle-Donne *et al.*, 2006
Pancreatitis	F2-IsoPs, Carbonylated proteins	Dalle-Donne *et al.*, 2006
Sepsis	Carbonylated proteins	Dalle-Donne *et al.*, 2006

Table 2. *Contd.*

Disease	Biomarker	Reference(s)
	NO	Maeda & Akaike, 1998
	NF-κB	Baldwin, 2001
Immune System		
Autoimmune diseases	HO-1	Sanchez *et al.*, 2005
Mastocitary activity modulation	HO-1	Sanchez *et al.*, 2005
Basophil activation	HO-1	Sanchez *et al.*, 2005
Neutrophil chemotaxis	HO-1	Sanchez *et al.*, 2005
Leukocyte adhesion	HO-1	Sanchez *et al.*, 2005
Complement cascade	HO-1	Sanchez *et al.*, 2005
Respiratory system		
Asthma	MDA, F2-IsoPs, Decrease in GSH concentration and/or GSH:GSSG ratio, NO2-Tyr, Cl-Tyr, Carbonylated proteins	Dalle-Donne *et al.*, 2006
	HO-1,	Sanchez *et al.*, 2005
	NF-κB,	Baldwin, 2001
	NO	Matera, 1998
Lung injury	NO2-Tyr, F2-IsoPs (cardio-pulmonar bypass)	Dalle-Donne *et al.*, 2006
Bronchopulmonary dysplasia	NO2-Tyr (severe in neonates), Carbonylated proteins	Dalle-Donne *et al.*, 2006
Interstitial lung disease	F2-IsoPs	Dalle-Donne *et al.*, 2006
Chronic obstructive pulmonary disease	HNE, F2-IsoPs, NO2-Tyr, Carbonylated proteins	Dalle-Donne *et al.*, 2006
	HO-1	Sanchez *et al.*, 2005
Idiopathic pulmonary fibrosis	Decrease in GSH concentration and/or GSH:GSSG ratio, Carbonylated proteins	Dalle-Donne *et al.*, 2006
Pulmonary hypertension	F2-IsoPs	Dalle-Donne *et al.*, 2006
Respiratory distress syndrome	F2-IsoPs, Decrease in GSH concentration and/or GSH: GSSG ratio, NO2-Tyr, Cl-Tyr, Di-Tyr, Carbonylated proteins	Dalle-Donne *et al.*, 2006
Hyperoxia	HO-1	Sanchez *et al.*, 2005
Hypoxia	HO-1	Sanchez *et al.*, 2005
Allergic rhinitis	HO-1	Sanchez *et al.*, 2005

Table 2. *Contd.*

Disease	Biomarker	Reference(s)
Asbestosis	Decrease in GSH concentration and/or GSH:GSSG ratio	Dalle-Donne *et al.*, 2006
Renal system		
Chronic kidney disease	F2-IsoPs, Di-Tyr, Carbonylated proteins	Dalle-Donne *et al.*, 2006
Chronic renal failure	Cl-Tyr, Carbonylated proteins	Dalle-Donne *et al.*, 2006
Acute renal injury	HO-1,	Sanchez *et al.*, 2005
	COX-2	Turini and DuBois, 2002
Renal ischemia-reperfusion	HO-1	Sanchez *et al.*, 2005
Glomerulonephritis	HO-1	Sanchez *et al.*, 2005
Uremia	Carbonylated proteins	Dalle-Donne *et al.*, 2006
Uremia associated with peritoneal haemodialysis	S-Glutathionylated proteins	Dalle-Donne *et al.*, 2006
Penis erection	HO-1	Sanchez *et al.*, 2005
Aceruloplasminemia	Carbonylated proteins	Dalle-Donne *et al.*, 2006
Spherocytosis	S-Glutathionylated proteins	Dalle-Donne *et al.*, 2006
Sickle cell disease	F2-IsoPs,	Dalle-Donne *et al.*, 2006
	NF-κB	Baldwin, 2001
Nervous system		
Ataxia	S-Glutathionylated proteins (Friedreich), Decrease in GSH concentration and/or GSH:GSSG ratio (telangiectasia)	Dalle-Donne *et al.*, 2006
Spinal cord injury	F2-IsoPs	Dalle-Donne *et al.*, 2006
	HO-1	Sanchez *et al.*, 2005
Mild cognitive impairment	HNE, acrolein	Dalle-Donne *et al.*, 2006
Alzheimer's disease	MDA, HNE, F2-IsoPs, Decrease in GSH concentration and/or GSH:GSSG ratio, NO2-Tyr, Carbonylated	Dalle-Donne *et al.*, 2006

Table 2. *Contd.*

Disease	Biomarker	Reference(s)
	proteins	
	HO-1	Sanchez *et al.*, 2005
	COX-2	Sengupta, 1999; Turini & DuBois, 2002
Creutzfeldt–Jakob disease	F2-IsoPs	Dalle-Donne *et al.*, 2006
Huntington disease	F2-IsoPs	Dalle-Donne *et al.*, 2006
Parkinson's disease	HNE, Decrease in GSH concentration and/or GSH: GSSG ratio, Carbonylated proteins	Dalle-Donne *et al.*, 2006
Ischemic brain	Decrease in GSH concentration and/or GSH:GSSG ratio	Dalle-Donne *et al.*, 2006
	NO	Bashkatova & Rayevsky, 1998
Down syndrome	F2-IsoPs	Dalle-Donne *et al.*, 2006
Ictus	NF-κB,	Baldwin, 2001
	COX-2	Turini & DuBois, 2002
Others		
Cancer	Decrease in GSH concentration and/or GSH:GSSG ratio, NO2-Tyr (lung), Carbonylated proteins (lung), S-Glutathionylated proteins (renal cell carcinoma)	Dalle-Donne *et al.*, 2006
	HO-1,	Sanchez *et al.*, 2005
	NF-κB,	Baldwin, 2001
	NO,	Maeda & Akaike, 1998
	COX-2	Sengupta, 1999; Turini & DuBois, 2002; Chun & Surh, 2004
Diabetes (types 1 & 2)	MDA, F2-IsoPs, Decrease in GSH concentration and/or GSH:GSSG ratio, S-Glutathionylated proteins, NO2-Tyr, Carbonylated proteins	Dalle-Donne *et al.*, 2006

Table 2. *Contd.*

Disease	Biomarker	Reference(s)
	NF-κB,	Baldwin, 2001
	COX-2	Turini & DuBois, 2002
Preeclampsia	MDA, Decrease in GSH concentration and/or GSH: GSSG ratio, NO2-Tyr, Carbonylated proteins	Dalle-Donne *et al.*, 2006
	HO-1	Sanchez *et al.*, 2005
Hyperhomocysteinemia	F2-IsoPs	Dalle-Donne *et al.*, 2006
Cataracts (genesis)	Decrease in GSH concentration and/or GSH:GSSG ratio, S-Glutathionylated proteins, Carbonylated proteins	Dalle-Donne *et al.*, 2006
Retinopathies	Decrease in GSH concentration and/or GSH:GSSG ratio (of prematurity)	Dalle-Donne *et al.*, 2006
	COX-2 (diabetic)	Turini & DuBois, 2002
Sarcoidosis	Carbonylated proteins	Dalle-Donne *et al.*, 2006
Progeria	Carbonylated proteins	Dalle-Donne *et al.*, 2006
Werner syndrome	Decrease in GSH concentration and/or GSH:GSSG ratio, Carbonylated proteins	Dalle-Donne *et al.*, 2006
Systemic amyloidosis	Carbonylated proteins	Dalle-Donne *et al.*, 2006
Obesity	F2-IsoPs	Dalle-Donne *et al.*, 2006
Caquexia	NF-κB	Baldwin, 2001
Zellweger syndrome	F2-IsoPs	Dalle-Donne *et al.*, 2006
Organ and tissue transplants	HO-1,	Sanchez *et al.*, 2005
	COX-2	Turini & DuBois, 2002

MDA- malondialdehyde; HNE- 4-hydroxy-2-nonenal; GSH- reduced glutathione; GSSG- glutathione disulfide; F2-IsoPs- F2 isoprostanes; NO2-Tyr- 3-nitrotyrosine; Cl-Tyr- 3-chlorotyrosine; HO-1- hemo oxygenase 1; NF-κB- Nuclear transcription factor κB; NO- Nitric oxide; COX-2- Cyclooxygenase-2.

or *health products* (naturals or synthetics). The problem is that OS is not considered a disease but a human organism condition (Nuñez-Selles, 2005).

OS is an alteration of the redox balance within the organism leading to a ROS overproduction above the needs of its functions, but the following questions are relevant:

- Which ROS is affecting the redox balance?
- Which one of the endogenous defense antioxidant mechanisms are failing?
- Which antioxidant product(s), synthetic or natural, would be the best according to the beforesaid answers?

That means that it is not enough to detect and quantified the OS, according to its biomarker(s), but to design a "tailor-made" design of AT for the patient, which makes a high difficulty for clinical protocols according to the existing standards. All publications about AT have followed those standards of regulatory bodies, which impair to their results (since the protocol conception) a severe limitation. For example, a recent report (Bjelakovic *et al.,* 2007) arrived to the conclusion that AT did increase the patient mortality by around 5%, except when selenium-containing formulations were administered, where a similar decrease of mortality was observed. The study was a meta-analysis of 68 clinical trials with different antioxidant products conducted on 230000 patients in the last 20 years.

The first evidences of the relation between OS and disease risk factors were reported for the cardiovascular system. However, after 25 years of that publication (*the "French" paradox*), still there considerations as follows: "The evidence from these observational studies suggests that increased intake of antioxidants is associated with a reduced risk of cardiovascular disease. However, because of inconsistencies among the studies, difficulty accounting for confounding variables, a reliance on food questionnaires, and a lack of validation of historical data or vitamin intake with objective laboratory evaluations, we should view these studies as preliminary observations of effects that need to be further addressed in randomized controlled trials" (Hasnian *et al.,* 2004).

A recent review about AT in the pathological conditions within the Central Nervous System (CNS) (Gilgun-Sherki *et al.,* 2002) may illustrate the above mentioned opinion and can be summarized as follows:

1. Brain damage is produced basically by the attack of superoxide anion and nitric oxide.
2. Results of clinical trials in order to prevent brain-vascular

infarct or patient's improvement after the event through the administration of vitamins C or E, β-carotene, N-acetylcysteine, ubiquinone (co-enzyme Q10), and lipoic acid were contradictory and did not allow to have definitive conclusions. An interesting recommendation was to apply the AT only where the OS condition was evaluated as mild. New clinical trials were also recommended and results were only considered as preliminaries.

3. Only one antioxidant product (*Ebselen*, an organic selenium salt) showed a significant effect both as neuroprotector (prophylactic) and to improve patient recovery within the next 24 h of brain infact, and follow-up oral administration during the next 30 days.

The main recommendation was the future use of an antioxidant cocktail instead of a single antioxidant, with a high probability of synergic effects, which would be the case of ANPs considering that they contain a crude extract of natural origin with several components, some of them antioxidants. The study also concluded to work in the molecular design of new synthetic antioxidants considering the molecular basis of the relationship between OS and brain damage.

The main controversy in the AT clinical practice, as reflected in scientific publications, can be found in cancer treatment. Whereas some researchers claim that AT should be avoided during radio- and chemo-therapy, which would lower the efficacy of those treatments (D'Andrea, 2005), others said the contrary and recommend the AT as part of the therapeutics and even the prophylaxis of cancer (Prasad *et al.,* 2006). Cancer chemoprevention by selenium administration is widely accepted by the medical community today (Combs *et al.,* 1998; Rayman, 2000), reduction of side-effects of radio- and chemo-therapy by AT has been reported (Hasnian *et al.,* 2004) and excellent results have been obtained when a combination of radio- and/or chemotherapy with AT has been followed, overall to reduce the side-effects of conventional cancer treatments (Ladas *et al.,* 2004). A recent report from a team at the "Thomas Jefferson" University has concluded: "Synthetic antioxidant cytoprotectants are routinely used by oncologists to attenuate the toxicity of chemotherapeutic agents and radiation therapy while preserving the effectiveness of such therapy...This further lends support to the argument that natural antioxidant supplementation could safely be combined with chemotherapy and radiation therapy" (Anon, 2006).

Finally, there is the possibility that besides or together with the antioxidant effect of a natural product, an ANP may show other anti-cancer effects like anti-proliferative, anti-angiogenic and apoptosis-induction on tumor cells. Recent results from our research group on

a mango stem bark extract have shown those experimental evidences (*in vitro* and *in vivo*), overall in solid tumors (Delgado *et al.,* 2007) which need an extensive clinical evaluation in the future.

An increasing number of well-designed clinical protocols are showing the relevance of AT in diseases like diabetes (Manzella *et al.,* 2001), prostate disorders (Pasqualotto *et al.,* 2000), arthritis (McAlindon *et al.,* 2005), infertility (Agarwal *et al.,* 2002), and keratitis (Vertugno *et al.,* 2001), just to mention the most recent ones. However, controlled clinical trials with ANPs from traditional- and ethno-medicine are still lacking, which faces a second challenge or paradox besides AT: the necessity to declare a single-unique active principle within the natural crude or purified mixture. The largest limitation would be probably found in the lack of financial resources, overall in non-developed countries with the larger sources of plants, for pre-clinical and clinical research. The sad truth is that companies involved in natural products production and distribution allocate better funds to publicity and exaggerated promotion campaigns, most of the time without any ethics and scientific evidences of "health" claims.

Nevertheless, the need of more and better-designed clinical research is a fact that should be faced in the near future in order to demonstrate the efficacy and importance of AT. That will be a great challenge to be solved for both the scientific and medical communities for the benefit of the world population.

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2

Antioxidant Activity of the Phytochemicals

BRATATI DE[1,*] AND ARCHANA BANERJEE[2]

ABSTRACT

The oxygen radicals such as superoxide radical ($O_2^{-\bullet}$), hydroxyl radical ($^{\bullet}OH$) and non free radical species such as H_2O_2 and singlet oxygen ($^1O_2$) are various forms of activated oxygen generated in many redox processes. These radicals may induce some oxidative damage to biomolecules thus accelerating aging, cancer, cardiovascular diseases, neurodegenerative diseases, inflammation etc. Antioxidant nutrients vitamin E, vitamin C and β-carotene may play a beneficial role in the prevention of several chronic disorders. Flavonoids, tannins, anthocyanins and other phenolic and some nonphenolic constituents present in foods and drugs of plant origin are potential antioxidants. Role of these phytochemicals as antioxidants has been reviewed and in some cases structure related activity/relationship has been discussed.

Key words : Antioxidant, phytochemicals, phenols, vitamins, free radical, oxidative damage

INTRODUCTION

Free radicals are species that contain unpaired electrons. The oxygen radicals such as super oxide radical ($O_2^{\bullet-}$) and hydroxyl radical ($^{\bullet}OH$) and non free radical species such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) are various forms of reactive oxygen species (ROS) generated in many redox processes (Tournaire *et al.*, 1993; Gulcin *et al.*, 2002; Yildirim *et al.*, 2000). They are trapped and

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destroyed by the specific enzymes such as superoxide dismutase, catalase and glutathione peroxidase. Overproduction of free radicals, together with A, C and E avitaminosis and a reduced level of the above-mentioned enzymes are considered as the main factors for the oxidative stress (Ellnain-Wojtaszek *et al.*, 2003). During the course of normal oxidative phosphorylation between 0.4 and 4% of all oxygen consumed is converted into free radical $O_2^{\bullet-}$ (Evans *et al.*, 2002). Xanthine oxidase (XOD) mediates generation of $O_2^{\bullet-}$during oxidation of hypoxanthine to uric acid. Molecular oxygen acts as electron acceptor during the reoxidation of XOD to generate $O_2^{\bullet-}$ (Fridovich, 1970). Functioning of immune system such as phagocytosis stimulates activation of NADPH oxidase, an enzyme normally inactive in resting cells and production of $O_2^{\bullet-}$ (Devasagayam & Sainis, 2002).

$O_2^{\bullet-}$ is then converted to H_2O_2 by superoxide dismutase (SOD). H_2O_2 is either detoxified to H_2O and O_2 by glutathione peroxidase (in the mitochondria) or diffuses into cytosol and is then detoxified by catalase in peroxisomes. Hydroxyl radicals are generated by Fenton reaction in presence of reduced transition metals such as Cu and Fe and by Haberweiss reaction. Among these radicals $^{\bullet}OH$ is the most reactive (Gutteridge, 1984). These oxygen radicals may induce some oxidative damage to biomolecules such as carbohydrates, proteins, lipids, DNA (Kellog & Fridovich, 1975; Lai & Piette, 1977; Halliwell & Gutteridge, 2007).

Oxidative stress mediated damage of biomolecules accelerates aging, cancer, cardiovascular diseases, neurodegenerative diseases, inflammation (Ames, 1983; Stadtman, 1992; Sun, 1990; Van der Hagen *et al.*, 1993; Markesbery & Carney, 1999); diabetes (Wolff, 1993; Halliwell, 2002). Oxidation of low-density lipoprotein (LDL) plays a critical role in atherogenesis (Aviram *et al.*, 1996). Several lines of evidence suggest that overproduction of ROS is implicated in neurotoxicity (Esposito *et al.*, 2002). Free radicals alter a cell's genetic make up, causing the cell to divide more frequently (Loft & Poulsen, 1996). Metabolic activities of carcinogen are free radical dependent reaction. The carcinogens include tobacco smoke, environmental pollutants and oxidants; toxic substances in food. Excessive production of free radicals; metabolic activation of carcinogens, xenobiotics, lipoxygenase and cycloxygenase pathways cause damage to DNA (Dizdaroglu *et al.*, 1991, Poulsen *et al.*, 1998; Palli *et al.*, 2001; Tiwari, 2004). Oxidative stress is produced under diabetic conditions (Baynes & Thorpe, 1999) because hyperglycaemia depletes natural antioxidants and facilitates production of free radicals (Penckofer *et al.*, 2002). The altered balance of the antioxidant enzymes and the decreased activities of CAT and SOD may be a response to increased production of H_2O_2 and O_2 by the autoxidation of glucose and non-enzymatic

glycation (Chang *et al.,* 1993, Biessels *et al.,* 1994), Diabetics characteristically exhibit signs of oxidative stress in the retina (Doly *et al.,* 1992). A number of studies have suggested that enhanced oxidation is the underlying abnormality responsible for some of the complications in diabetes (Hasanain & Mooradian, 2002) such as retinopathy and atherosclerotic vascular diseases. Epidemiological studies indicate that a number of factors like exposure to herbicides, industrial chemicals, stress metals, cyanide, organic solvents, Co and carbon di sulphide may increase the risk of developing Parkinson's disease (Olanow & Tatton, 1999). Majority of these increase ROS and oxidative stress (Tiwari, 2004; Zhao, 2005). ROS produce inflammatory symptoms (Libby, 2006), kidney damage (Baud & Ardaillon, 1993), pulmonary diseases and asthma (Greene, 1995; Brown *et al.,* 1996; Shaheen *et al.,* 2001; Nagel & Linseisen, 2005), multiple sclerosis (Calabrese *et al.,* 1994), dysfunction of the reproductive process (Fujii *et al.,* 2003, 2005; Sheweita *et al.,* 2005), macular degenerations and cataracts (Doly *et al.,* 1992; Lu *et al.,* 2006; Evans, 2006).

Antioxidants are defined as a substance that in small quantities are able to prevent or greatly reduce the oxidation of easily oxidisable molecules such as fats (Chipault, 1962). According to Halliwell and Gutteridge (1995), antioxidant is the substance that when present at low concentration compared to those of an oxidisable substrate significantly delays or prevents oxidation of that substrate. Later they simplified the definition of antioxidant to a substance that delays, prevents or removed oxidative damage to a target molecule (Halliwell & Gutteridge, 2007).

Antioxidants exert their effects by differrent mechanisms (Tiwari, 2004):

1. Suppressing formation of active species
2. Scavenging active free radicals
3. Sequestering metal ions
4. Repairing and/or clearing damage
5. Inducing biosynthesis of other antioxidants or defence enzymes

Body possess several antioxidant systems which include

- Enzymatic: Superoxide dismutase, Catalase, Glutathione peroxidase
- Non-enzymatic: Vitamin E, Vitamin C, albumin and bilirubin, thiols, glutathione

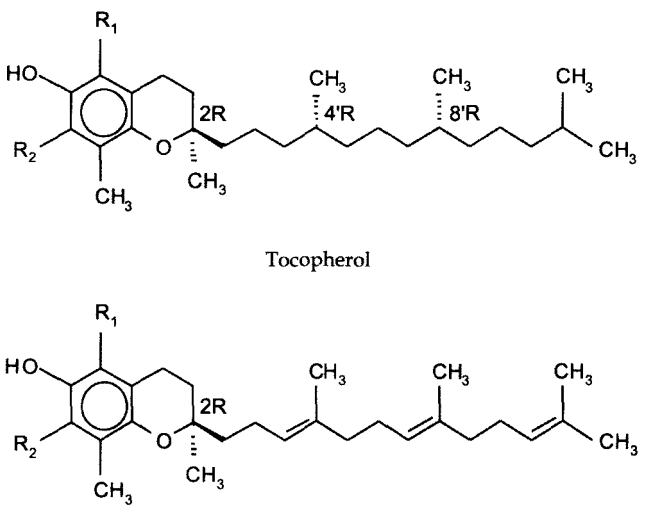
Epidemiological studies have consistently shown that consumption of fruits and vegetables has been associated with reduced risk of chronic diseases such as cardiovascular diseases and cancers (Kris-Etherton *et al.,* 2002; Serafini *et al.,* 2002; Gerber *et al.,* 2002) and

neurodegenerative diseases including Parkinson's and Alzheimer's diseases (Di Matteo & Esposito, 2003) as well as inflammation and problems caused by cell and cutaneous aging (Ames *et al.*, 1993). Studies to date have demonstrated that phytochemicals in common fruits and vegetables can have complementary and overlapping mechanisms of action, including scavenging oxidative agents, stimulation of the immune system, regulation of gene expression in cell proliferation and apoptosis, hormone metabolism, and antibacterial and antiviral effects (Waladkhani & Clemens, 1998). Here different phytochemicals reported to have antioxidant activity have been reviewed.

ANTIOXIDANT PHYTOCHEMICALS

Vitamin E

Natural vitamin E includes two groups of closely related fat-soluble compounds, tocopherols and tocotrienols (Fig 1), each with the 4 α, β, γ and δ analogues (Ricciarelli *et al.*, 2001). The compounds are widely distributed in nature. The richest sources are latex lipids (85% w/v) followed by edible plant oils. Sunflower seeds contain almost exclusively α-tocopherol (59.5 mg/g of oil). Soybean oil contains the γ-, δ-, and α-tocopherol (62.4, 20.4, and 11.0 mg/g oil). Palm oil contains high concentrations of tocotrienols (17.2 mg/g oil) and α-tocopherol (18.3 mg/g oil) (Ricciarelli *et al.*, 2001).



	R_1	R_2
α	CH_3	CH_3
β	CH_3	H
γ	H	CH_3
δ	H	H

Fig 1. Chemical structure of tocopherol and tocotrienol analogues

Vit E is the major hydrophobic compound that prevents the propagation of free radical reactions in the lipid counterpart of membranes, vacuoles and plasma lipoprotein (Ricciarelli *et al.*, 2001). Vit E supplementation has been shown to have beneficial effects for numerous disorders particularly atherosclerosis, ischaemia, heart diseases (Practico *et al.*, 1998; Keaney *et al.*, 1999), diabetes (Halliwell, 2002), neurodegenerative diseases (Kontush & Schekatolina, 2004) and development of different types of cancers (Sigonnas *et al.*, 1997; Bianchini *et al.*, 2000). Vitamin E protects against photooxidative stress (Havaux *et al.*, 2005).

Pro-oxidant Activity

In contrast to the described antioxidant property of Vit E, lipid peroxidation of LDL is faster in presence of α-tocopherol *in vitro* or *in vivo* (Bowry *et al.*, 1992; Upston *et al.*, 1999). It was proposed that peroxidation is propagated within lipoprotein particles by the vit E radical (α-tocopheroxyl radical) unless it became reduced by vit C (Stocker, 1999). Phenolic antioxidants also recycle α-tocopherol (Liu *et al.*, 1999).

Ascorbic Acid (AA)

Ascorbic acid (Fig 2) is an essential ingredient of the human diet and known to be a free radical scavenger. Ascorbic acid treatment arrested the decline in the activities of superoxide dismutase and glutathione peroxidase, glutathione contents and inhibited the radiation-induced lipid peroxidation in the skin of mice (Jagetia *et al.*, 2003). Ascorbic acid can protect flavonoids from oxidative degradation that reveal antioxidant synergies between ascorbic acid and the compounds (de Souza & De Giovani, 2004) and synergistic effects with other antioxidant vitamins (Truscott, 2001).

The measurement of concentration of lipid hydroperoxides in LDL showed that ascorbic acid inhibited peroxidative modification of LDL (Sakuma, 2001).

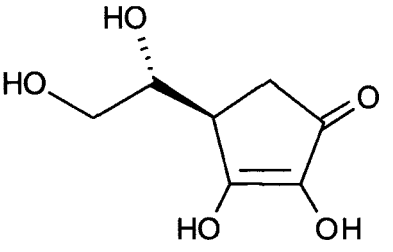


Fig 2. Ascorbic acid

Arsenic compounds have shown to exert their toxicity chiefly by generating reactive oxygen species, A significant increase in the level of lipid peroxidation and decrease in the levels of antioxidants and in the activities of mitochondrial enzymes were observed in arsenic intoxicated rats. Co-administration of arsenic treated rats with ascorbic acid and alpha-tocopherol showed significant reduction in the level of lipid peroxidation (Ramanathan *et al.,* 2003). On the contrary, ascorbic acid has prooxidant effect (Podmore *et al.,* 1998) in the presence of iron *in vitro* (Premkumar & Bowlus, 2004).

Carotenoids

Carotenoids (Fig 3) are phytochemicals having a 40-carbon skeleton of isoprene units and one of nature's most widespread pigments and occur widely in plants, animals and microorganisms. The structure may be cyclised at one or both end and have various hydrogenation

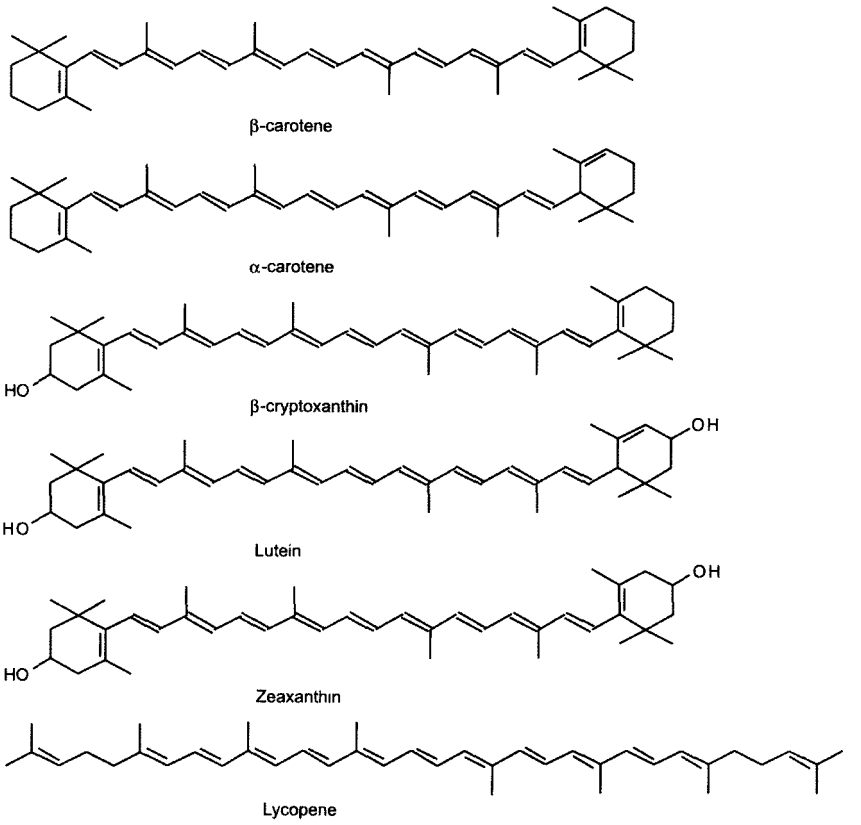


Fig 3. Carotenoids

levels or possess oxygen containing functional groups. Carotenoids act as photoprotectants in both plant and human tissues. This protective role originates from the ability of carotenoid pigments to quench and inactivate ROS such as singlet oxygen formed from exposure to light and air (Britton, 1995). Carotenoids can react with free radicals and become radical themselves. Carotenoid radicals are stable owing to delocalisation of unpaired electrons over the conjugated polyene chain of the molecules. This delocalisation allows addition reactions to occur at many sites of the radical (Britton, 1995). At relatively low O_2, this process would consume peroxyradicals and the carotenoid would act as a chain breaking antioxidant. Alternate pathways are possible, especially at higher oxygen concentration when a carotenoid radical could react with oxygen to generate carotenoid peroxy radical, CAR-OO. The carotenoid peroxyradical could act as a prooxidant promoting peroxidation of unsaturated lipid (LH) and hence causing damage (Lieber, 1993). The situation *in vivo* is not clear (Britton, 1995).

Phenols

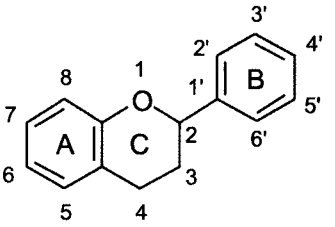
Phenolics are products of secondary metabolism of plants and are ubiquitous in all plant organs. Interest in food phenolics has increased because of their antioxidant and free radical scavenging abilities. Natural plant phenolics may be of different types.

Flavonoids

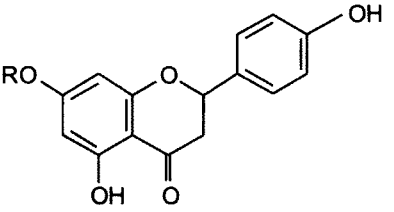
Flavonoids can be subdivided into 9 classes (Harborne, 1998). These are anthocyanin, proanthocyanidins, flavonols, flavones, glycoflavons, bi-flavonoids, chalcones and aurones, flavanones, isoflavones. Major classes of flavonoids are shown in Fig 4.

Flavonoids are almost ubiquitous in plant foods (vegetable, cereals, legumes, fruits, nuts etc). Since 1936 over 6000 flavonoids have been identified in plants (Harborne & Williams 2000, Godjevac *et al.,* 2004) and activities of many of them against different oxidants have been studied (Fukumoto & Mazza, 2000; Luximon-Ramma *et al.,* 2002; Antolovich, 2004).

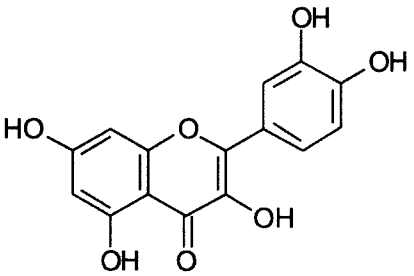
Many flavonoids and polyphenols can exhibit antioxidant function as their conjugated π-electron system allow ready donation of electron or hydrogen atoms from the hydroxyl moieties to free radicals (Bors & Saran, 1987). The antioxidant efficacy depends on structural features such as the number and position of the hydroxyl moieties on the ring systems and the extent by which the unpaired electron in the oxidised phenolic intermediate can delocalise throughout the



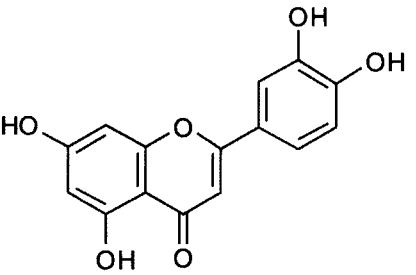
Basic structure



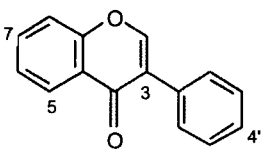
Naringenin: R = H
Naringin: R = rhamno-glucosyl
(Flavanones)



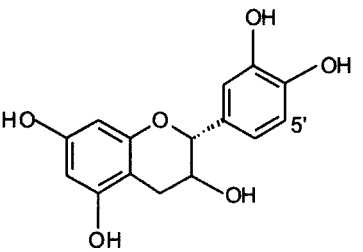
Quercetin (Flavonols)



Luteolin (Flavone)



Isoflavones



Flavan-3-ols

	5	7	4'
genistein	OH	OH	OH
genistin	OH	Oglc	OH
daidzein		OH	OH
daidzin		Oglc	OH

	3	5'
(+)-catechin	βOH	
(-)-epicatechin	αOH	
(-)-epigallocatechin	αOH	OH

Fig 4. Major classes of flavonoid

molecule (Lugasi *et al.*, 2003). Flavonoids are more potent antioxidant than vitamin C and E (Rice –Evan *et al.*, 1995).

Most phenols, specially, flavonoids are very effective scavengers of •OH, peroxyl radicals and $O_2^{\bullet-}$ radicals (Manach *et al.*, 1996; Tewari, 2001). Flavonoids are chelators of metals and inhibit the Fenton and Haber-weiss reactions, which are important sources of active oxygen radicals (Shahidi & Wanasundara, 1992). In addition, flavonoids retain their free radical scavenging capacity after forming complexes with metal ions (Afanasev *et al.*, 1989). The electron or H^+ donating capacity of flavonoids seem to contribute to termination of lipid peroxidation chain reaction based on their reducing power (Jovanoic *et al.*, 1994; Van Acker *et al.*, 1996). 15-Lipoxygenase participates in the oxidation of LDL and several flavonoids inhibit this enzyme (Samuelson, 1999).

Structural Determinants for Radical Scavenging Property and Antioxidant Potential

Bors *et al.* (1990) proposed the structural determinants for effective radical scavenging properties by flavonoids. As shown in Fig. 5 three structural groups are important determinants for radical scavenging and/or antioxidative potential: (1) the O-dihydroxy (catechol) structure in the ring B, which is the obvious radical target site for all flavonoids with a saturated 2, 3 bond; (2) the 2,3 double bond in conjugation

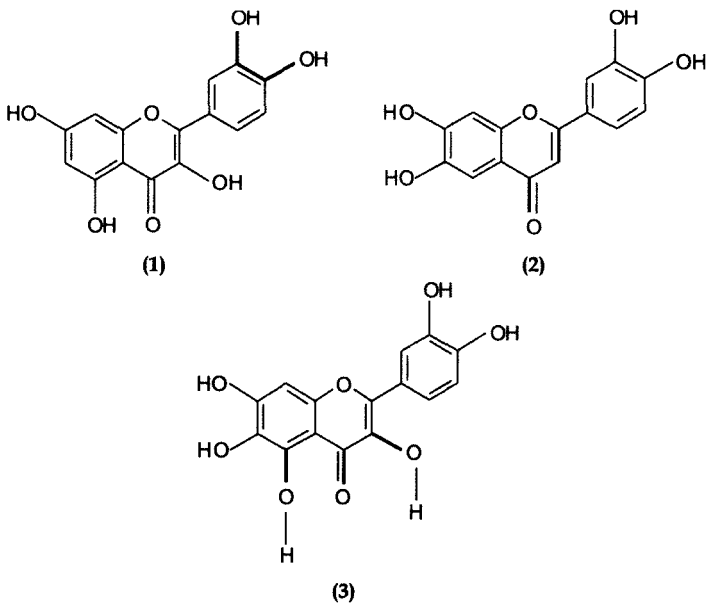


Fig 5. Structural determinants for radical scavenging (Bors *et al.*, 1990)

with α 4-oxo function, which is responsible for electron delocalization from the B ring and (3) the additional presence of both 3-and 5-hydroxyl groups for maximal radical scavenging potential and strongest radical absorption.

According to Pietta (2000) a number of flavonoids efficiently chelate trace metals and the proposed binding sites for trace metals to flavonoids are the catechol moiety in ring B, the 3-hydroxy, 4-oxo groups in the heterocyclic ring and the 4-oxo, 5-hydroxyl groups between the heterocyclic ring and the A ring (Fig 6).

Flavonoids usully give rise to semiquinone free radical in alkaline solution. The semiquinone free radicals or aroxyl radicals may react with the second radical aquiring a stable quinone structure (Pietta, 2000) (Fig 7). The activities of the antioxidants are related to the stability of the free radicals formed after they react with active radicals. Flavonoids with O-tri or O-dihydroxyl in the B ring and/or in the ring A form stable free radicals. This is an important feature with flavonoid compounds due to which many are better antioxidants than antioxidant nutrients Vit C, A and β carotene. These antioxidant nutrients do not form stable radical and dependent for the scavenging/ transport on other systems (Yoshida *et al,* 1989; Tiwari, 1999).

$O_2^{\bullet-}$ -radicals are of interest because it is involved *in vitro* in different conditions. In majority of the cases, this radical is generated enzymatically. Xanthine oxidase (XOD) mediated generation of $O_2^{\bullet-}$ radical has been extensively studied. Oxidation of hypoxanthin to uric acid with simultaneous generation of $O_2^{\bullet-}$ radical and H_2O_2 has been observed to play a critical role during myocardial ischaemia, respiratory injury, gout, rheumatoid arthritis and many other inflammatory conditions. Molecular oxygen, which is easily available

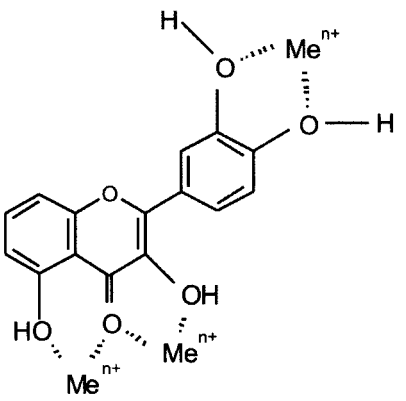


Fig 6. Binding sites in flavonoids for trace metals

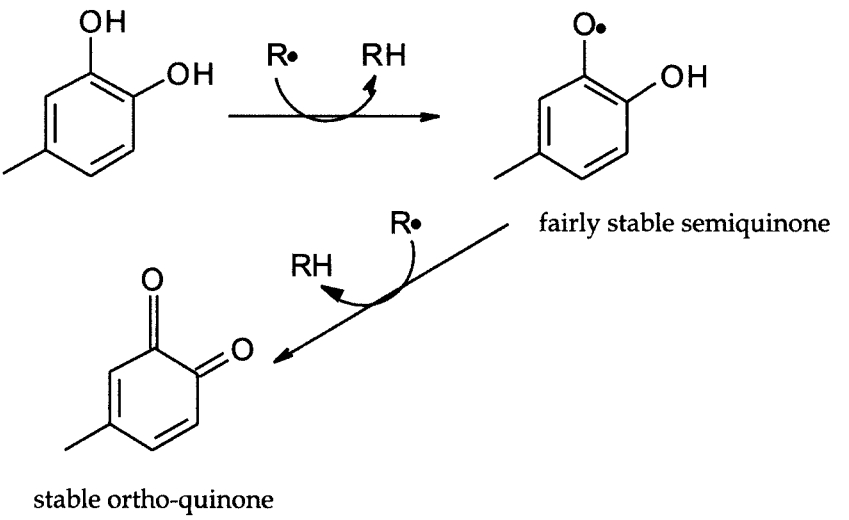


Fig 7. Scavenging of ROS(R) by flavonoids and formation of stable structure (Pietta, 2000)

in vivo, acts as an electron acceptor during the reoxidation of XOD generating $O_2^{\bullet-}$ and H_2O_2 (Fridovich, 1970).

Detailed study of Cos *et al.* (1998) categorised flavonoids into different classes based on their structure and biological activity related to XOD inhibition and/or $O_2^{\bullet-}$ scavenging.

1. Flavonoids that can scavenge only $O_2^{\bullet-}$ without inhibitory activity in XOD, such as (+) taxifolin, (-) epicatechin, (-) epigallocatechin.
2. Flavonoids that can effectively inhibit XOD activity but cannot scavenge $O_2^{\bullet-}$ radicals such as kaempferol, morin and isorhamnetin.
3. Compounds which posses both the $O_2^{\bullet-}$ scavenging activity as well as XOD inhibitory capacity *i.e.* quercetin, 7-neohesperidosylluteolin, 4′, 7-dimethyl quercetin, 3-rutinosyl kaempferol.
4. Compounds that possess XOD inhibitory activity but may become prooxidants and increase the generation of $O_2^{\bullet-}$ such as luteolin and apigenin.
5. Compounds with marginal effect on XOD inhibition along with prooxidant properties such as 7-hydroxyflavone.
6. Flavonoids with neither XOD inhibitory nor $O_2^{\bullet-}$ scavenging capacity such as 4′-hydroxyflavanone, 3-hydroxyflavone, cirsimarin, 6-glucosyl-8-xylosylapigenin.

Based on the above categorisation the following stuctural criteria for a flavonoid have been proposed:

- Flavonoids with both XOD inhibitory and $O_2^{\bullet-}$ radical scavenging properties possess in common OH- groups either at C-5, C-3 or C-3´ and C-4´.
- To possess strong XOD inhibitory activity, flavonoids should have hydroxl groups at C-5 and C-7 with a double bond between C-2 and C-3.
- To scavenge $O_2^{\bullet-}$ effectively, on the other hand, a hydroxyl group at C-3´ in ring (B) and at C-3 position is essential.

Anthocyanins

Anthocyanins (Fig 8) as natural food colour have antioxidant potential (Bridle & Timberlake, 1997; Gabrielska *et al.*, 1999; Lila, 2004). Because of their structure they are efficient antioxidants, more oxidisable on opening of the C ring (flavylium cation). Hypothetical reaction mechanism concerning superoxide anion is shown in Fig 9. This reacton cannot be extra polated to other species of uncharged free radicals (Saint-Crick de Gaulejac *et al.*, 1999).

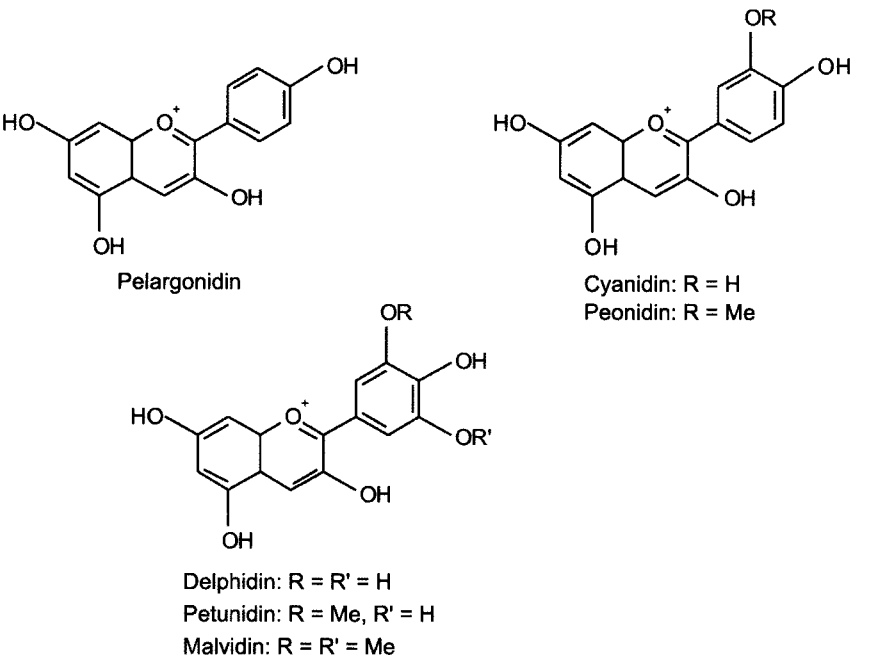


Fig 8. Anthocyanins

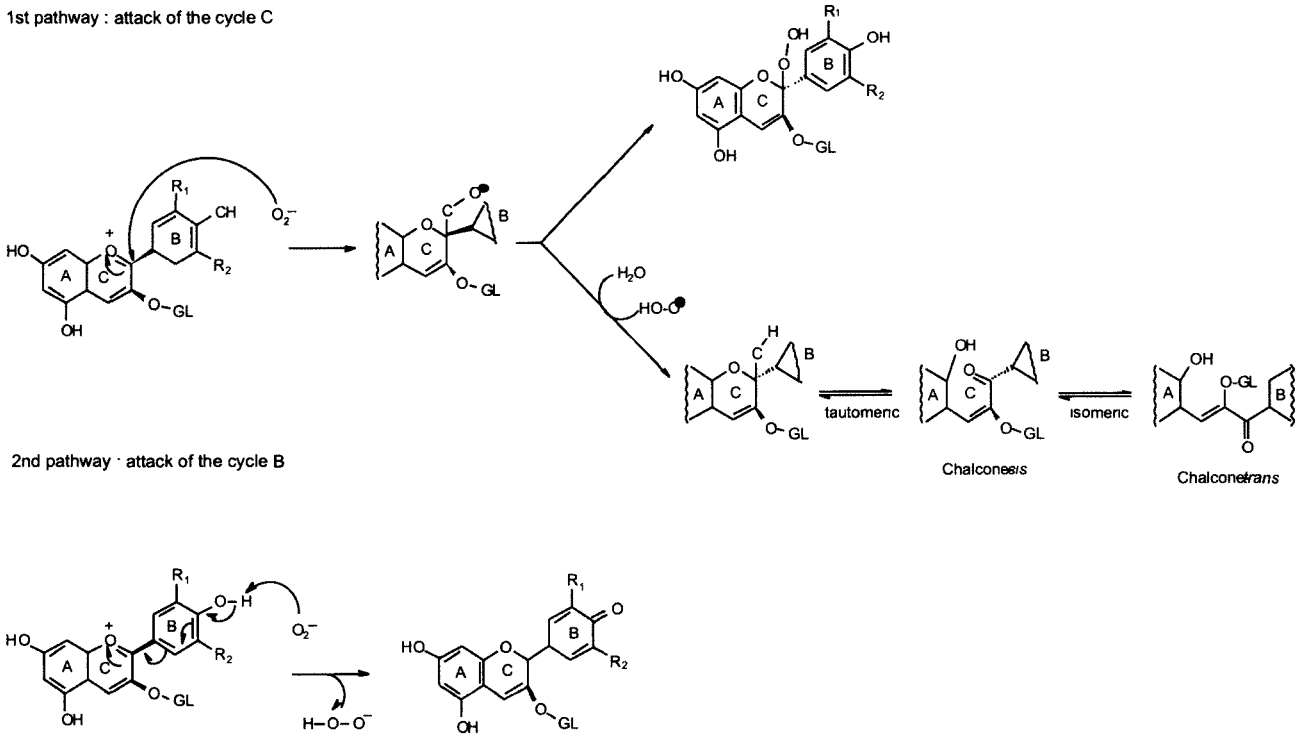


Figure . Diagram of the different hypothetical pathways of oxidation of the anthocyanins by the superoxide O2 radical. As the radical is negatively charged, pathway A (attack of the flavylium cation) may be more probable.

Fig 9. Hypothetical pathways of oxidation of the anthocyanins by the superoxide radical (Saint-Crick de Gaulejac *et al.*, 1999)

The different substitutions between anthocyanins have an influence upon their ability to trap oxygen radicals. A lesser efficiency for malvidin and paeonidin could be due to the presence of methoxy groups in the lateral ring. Free anthocyanin fractions are more effective than isolated molecule. This explains the synergistic effects of anthocyanin molecules (Saint-Crick de Gaulejac *et al.,* 1999).

Anthocyanidins inhibited Fenton reagent, $^{\bullet}OH$ generating system possibly by chelating with ferrous ion, scavenged $O_2^{\bullet-}$ in a dose dependent manner but did not scavenge NO effectively (Noda *et al.,* 2002). Cyanidin and cyanidin 3-O-beta-D-glucoside showed a protective effect on DNA cleavage, free radical scavenging activity and significant inhibition of xanthine oxidase (Acquaviva *et al.,* 2003). Anthocyanin mixtures show synergistic effect (Stintzing *et al.,* 2002).

Phenolic acids

Plant phenolics have received considerable attention because of their potential antioxidant activity (Lopez-Velez *et al.,* 2003). Phenolic compounds are the major contributors of antioxidant activity in vegetable and fruit juices (Gardner *et al.,* 2000; Vinson *et al.,* 2001; Lee *et al.*, 2003) and are effective hydrogen donors, which make them good antioxidants (Rice-Evans *et al.*, 1995; da Silva Porto, 2003; Siquet *et al.,* 2006).

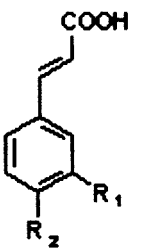
Gallic acid is a strong antioxidant (Stroka & Cisowski, 2003). The activity is more than that of Vitamin C and other phenolic constituents such as quercetin, epicatechin, catechin, rutin and chlorogenic acid (Kim *et al.,* 2002). The relative Vitamin C Equivalent Assay of phenolic standards were as follows: gallic acid > quercetin > epicatechin > catechin > vitamin C >rutin > chlorogenic acid > Trolox with the DPPH radical assay (Kim *et al.*, 2002). The radical scavenging activity of other phenolic acids on DPPH decreased in the order caffeic acid > Sinapic acid > ferulic acid > p-coumaric acid (Kakuzaki *et al,* 2002). Protocatechuic acid and caffeic acid showed a potent inhibitory effect on iron induced oxidative DNA damage (Lodovici *et al*, 2001). In *in vitro* copper catalysed human LDL oxidation assay, the antioxidant activity of the monomeric hydroxy cinnamates decreased in the following order: caffeic acid > sinapic acid > ferulic acid > p-coumaric acid (Andreasen *et al.,* 2001). Chlorogenic acid, the ester of caffeic acid with quinic acid, is one of the most abundant polyphenols in the human diet. Antolovich *et al.* (2004) studied and examined for the oxidation of a range of phenolic acids: 5-cinnamic acids, 2 benzoic acids using two oxidation systems; periodate oxidation and the Fenton oxidation. Reaction product identified as various quinone dimers and aldehydes, but the nature of the products differed between the

oxidation systems. All cinnamic acids in the study reacted with the Fenton reagent to produce benzaldehydes as the main products with the exeption of 5-caffeoyl quinic acid. Quinone formation observed in the two compounds caffeic acid and 5-caffeoylquinic acid possessing o-hydroxy groups is shown in Fig 10.

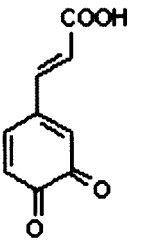
Anthraquinones and xanthones

Anthrone and dihydroxy anthraquinones reported in families Liliaceae (*Aloe* spp.), Polygonaceae (*Rheum* spp.), Caesalpiniaceae (*Cassia* spp.) (Bruneton, 1995) are potential antioxidants (Yen *et al.*, 1999).

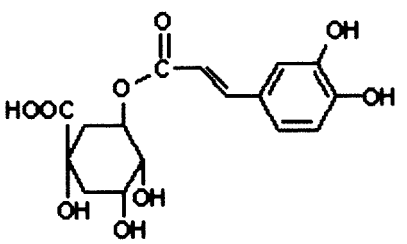
Xanthones show antioxidation activities (Minami *et al.*, 1994; Jiang *et al.*, 2004; Lin *et al.*, 2005). Mangiferin (Fig 11), a xanthone from *Mangifera indica* shows excellent antioxidant and neuroprotective (Sanchez *et al.*, 2000; Martínez *et al.*, 2001) activity.



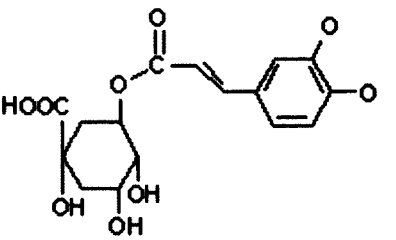
Compound	R_1	R_2
caffeic acid	OH	OH
3-hydroxycinnamic acid	OH	H
p-coumaric acid	H	OH
ferulic acid	OCH_3	OH



quinone of caffeic acid



5-caffeoylquinic acid (chlorogenic acid)



quinone of 5-caffeoylquinic acid

Fig 10. Oxidation of phenolic acids in Fenton system

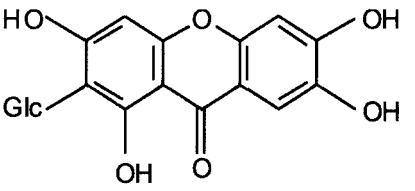


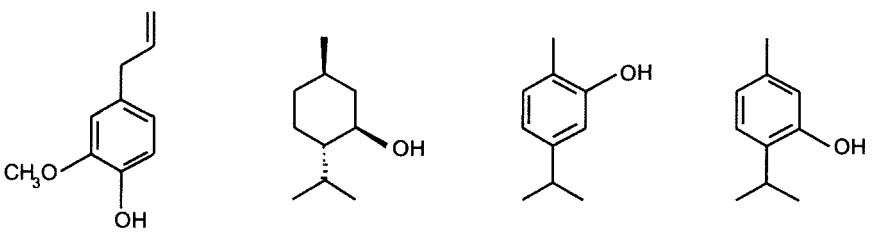
Fig 11. Mangiferin

Tannins

Tannins are water-soluble polyphenols present in many foods. Tannins have been recognised as antioxidants. Polyphenols and tannins reported to have protective action against DNA damage (Casalini *et al.*, 1999; Giovannelli *et al.*, 2000). Tea polyphenols and many tannin components are suggested to be anticarcinogenic (Okuda *et al.*, 1992; Chung *et al.*, 1998; Lin *et al.*, 2001; Li *et al.*, 2003; Cos *et al.*, 2004). Many carcinogens and/or mutagens produce oxygen free radicals for interaction with cellular macromolecules (Lin *et al.*, 2001). The antioxidative properties of tannins are important for protecting against cellular oxidative damage. The generation of $O_2^{\bullet -}$ radicals was inhibited by tannins and related compounds. But toxic effects were also observed.

Essential oils

Aroma extracts isolated from some plants (Fig 12) have shown good antioxidant activities (Lee *et al.*, 2000; Ruberto & Baratta, 2000; Mohammad *et al.*, 2004). Eugenol most efficiently scavenged reactive oxygen species (Opoku *et al.*, 2002; Fujisawa *et al.*, 2002). *O. tenuiflorum* shows good antioxidant activity with anthocyanin and β-caryophyllene (Simon *et al.*, 1999). Eugenol, thymol, carvacrol, and 4-allylphenol showed stronger antioxidant activities than did the other components tested in basil and thyme. They all inhibited the oxidation of hexanal (Lee *et al.*, 2005).



Eugenol (-)-Menthol Carvacrol Thymol

Fig 12. Some essential oil constituents

Resveratrol

The stilbene resveratrol (Fig 13) is an antioxidant, antiinflammatory and anticancer agent found in *Arachis, Cassia, Eucalyptus, Polygonum, Veratrum,* grapes (Evans, 2002).

Pterostilbene, chemically related to resveratrol and a strong antioxidant isolated from *Pterocarpus marsupium,* fight off and reverse cognitive decline (Suh *et al.*, 2007).

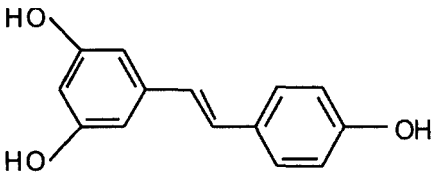


Fig 13. Resveratrol

Curcumin

Curcumin is a polyphenol derived from turmeric (Fig 14). It is a potent antioxidant at neutral and acidic pH (Sharma *et al.*, 2005), inhibits cellular reactive oxygen species generation and low density lipoprotein oxidation, through H-atom abstraction from the phenolic groups (Chen *et al.*, 2006).

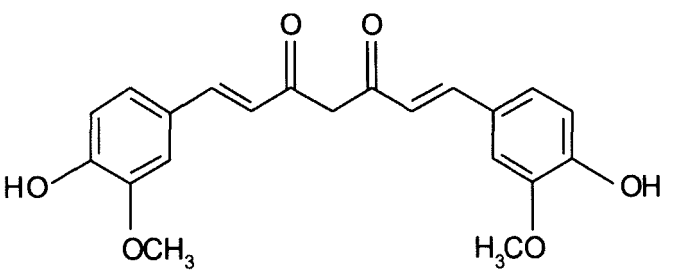


Fig 14. Curcumin

OTHER PHYTOCHEMICALS

Phytic Acid

Phytic acid suppresses iron-catalyzed oxidative reactions, inhibits lipid peroxidation, oxidative spoilage, such as discoloration, putrefaction (Graf & Eaton, 1990).

Glucosamine

D-Beta Glucosamine is the most abundant amino sugar obtained from the *Aloe barbadensis* plant. Chinese Foxglove GlcN possessed excellent antioxidant activities as manifested by strong chelating effect on ferrous ions and protection of macromolecules such as protein, lipid, and deoxyribose from oxidative damage induced by hydroxyl radicals (Yang *et al.,* 2007).

Vitamine B (Thiamine)

Thiamine inhibits lipid peroxidation in rat liver microsomes and free radical oxidation of oleic acid *in vitro.* Thiamine interacts with free radicals and hydroperoxides and is oxidized to thiochrome and thiamine disulfide. The antioxidant effect of thiamine is probably related to successive transfer of $2H^+$ from the NH_2 group of the pyrimidine ring and H^+ from the thiazole ring (after its opening) to reactive substrates (Lukienko *et al.,* 2000).

Vitamin B-6 (Pyridoxine) 6-Hydroxypyridoxine

Pyridoxine was comparable to polyphenols such as (+)-catechin, rutin and gallic acid in the antioxidative activity though the DPPH radical-scavenging activity was somewhat lower than that of the polyphenols (Tadera *et al.,* 2003).

Proteins

The storage protein from *Dioscorea batatas* tuber was shown to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, $^{\bullet}OH$ radical, inhibit lipid peroxidation and there was a positive correlation between scavenging effects against radicals and amounts of dioscorin which are comparable to those of glutathione at the same concentrations (Hou *et al.,* 2001, 2002). Patatin, a tuber storage protein of potato, shows exellent antioxidant activity (Liu *et al.,* 2003; Kudoh *et al.,* 2003). A compound isolated from *Azadirachta indica* seed kernel found to be a potent inhibitor of lipoxygenases (Rao *et al.,* 1998).

Uric Acid

Urate is a strong reducing agents (electron donor) and potent antioxidant. UA is considered a potent peroxinitrite scavenger shown to be neuroprotective during oxidative stress conditions both *in vitro* and *in vivo,* may be a marker of oxidative stress, and may have a potential therapeutic role as an antioxidant. Uric acid can also act as a prooxidant, particularly at elevated levels (PMID 16375736, "Uric Acid." Biological Magnetic Resonance Data Bank, 2008).

Melatonin

The indoleamine melatonin was found to be ubiquitous in the animal kingdom and in some lower and higher plants. This compound gives antioxidant protection (Machackova & Ramanov, 2002).

Betalains

Betacyanin and betaxanthins are natural nitrogen containing pigments characteristic of the order Centrospermae (exeption: Families Caryophyllaceae and Molluginaceae). These are red or violet betacyanin and the yellow betaxanthin that occur as water soluble glycosides. These are a class of compounds with antioxidant and radical scavenging activities (Zakharova & Petrova, 1998; Kanner *et al.*, 2001; Cai *et al.*, 2003; Stintzing *et al.*, 2004; Tesoriere *et al.*, 2004).

Alpha-Lipoic Acid (ALA)

ALA has been identified as a powerful antioxidant (Packer, 1995; Kim *et al.*, 2006) found naturally in our diets (first detected in *Lactobacillus*) and as endogenous antioxidant that interrupts cellular oxidative processes in both its oxidized and reduced forms. ALA protects against LDL oxidation (Wollin & Jones, 2003; Lexis, 2006). It is an effective $^{\bullet}OH$ radical quencher, the sulphur bond being the reactive part of the molecule. It also scavenges peroxyl, ascorbil and chromamoxyl radicals (Tiwari, 2002). In addition to ROS scavenging, ALA has been shown to be involved in the recycling of other antioxidants in the body including vitamins C, E and glutathione (Wollin & Jones, 2003).

Linoleic Acid

Dietary conjugated linoleic acid reduces lipid peroxidation (Kim *et al.*, 2005), alpha linolenic acid in *Juglans regia* (Anderson *et al.*, 2001), *Olea europea* (Ninfali *et al.*, 2005) shows antioxidant activity.

Alkaloids

Some alkaloids are reported to have free radical scavenging activity and antioxidant capacity in diabetes (Jang *et al.*, 2000). *Scoparia dulcis* plant extract was reported to be rich in an alkaloid-6-methoxybenzoxazolinone and terpenoids (scoparic acids A, B, C and scopadulcic acids), which may be responsible for scavenging free radicals (Pari & Latha, 2004). A quinoline alkaloid from rice aleurone layer has shown antioxidant activity (Chung & Woo, 2001). Boldine ([s]-2,9-dihydroxy-1, 10-dimethoxyaporphine) is a major alkaloid found

in the leaves and bark of boldo (*Peumus boldus* Molina), and has been shown to possess antioxidant activity and anti-inflammatory effects (Jang *et al.,* 2000).

Pectin

Pectin exhibited protection against hydroxyl radical-mediated DNA damage and low-density lipoprotein peroxidation tests. Antioxidant and antiradical activities are correlated with degrees of esterification values of pectin (Yang *et al.,* 2004).

Food as Antioxidant

The isolated pure compound either loses its bioactivity or may not behave the same way as the compound in the whole food. So it is not wise to take mega doses of purified phytochemicals as supplements before sufficient scientiffic evidence supports this (Liu, 2003). The additive and synergistic effects of phytochemicals in fruits and vegetables are responsible for their potent antioxidant and anticancer activities and that the benefit of diet rich in fruits and vegetables is attributed to the complex nature of phytochemicals present in whole foods (Eberhardt *et al.,* 2000; Chen *et al.,* 2002; Sun *et al.,* 2002).

Epidemiological studies have shown that consumption of fruits and vegetables as well as grains has been strongly associated with reduced risk of chronic diseases such as

- **Cardiac diseases** (Joshipura *et al.,* 2000; Knekt *et al.,* 1996, 2002; Kris-Etherton *et al.,* 2002; Hertog *et al.,* 1997; Bazzano *et al.,* 2002; Vogel, 2006).
- **Cancer** (Block *et al.,* 1992; Willett, 1995; Knekt *et al.,* 1997; Hertog *et al.,* 1994; Murphy *et al.,* 2000; Kris-Etherton *et al.,* 2002; Seraffini *et al.,* 2005).
- **Diabetes** (Akkus *et al.,* 1996; O'Brien *et al.,* 1996; Dandona *et al.,* 1996; Sabu & Kuttan, 2002; McCune & Johns, 2002).
- **Gastrointestinal diseases** (Bulger *et al.,* 1998);
- **Neurodegenerative diseases** (Zhao *et al.,* 2005).
- **Alzheimer's disease** (Di Matteo & Esposito; 2003; Behl, 2005; Kim *et al.,* 2006b), age related diseases (Ames, 1993; Temple, 2000).

Phytochemicals common in fruits and vegetable can have complementary and overlapping mechanisms of action including modulation of detoxification enzymes, scavenging oxidative agents, stimulation of the immune systems, regulation of gene expression in cell proliferation and apoptosis, hormone metabolism and antibacterial and antiviral effects (Dragsted *et al.,* 2004). Antioxidant of leafy

vegetables have been reported (Dasgupta & De, 2007). The change in dietary behaviors such as increasing consumption of fruits, vegetables and grains is a practical strategy for significantly reducing the incidence of chronic diseases (Harold *et al.*, 2000; Liu, 2003).

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3

Determination of Antioxidant Capacities of Non Alcoholic Beverages Prepared from Three Wild Fruits of Zimbabwe

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ABSTRACT

The study was carried out to evaluate the antioxidant capacities of beverages prepared form three wild fruits of Zimbabwe namely, Parinari curatelifolia, Strychnos spinosa and Adansonia digitata and comparing them to orange juice and baobab nectar a commercial beverage. Methanolic extracts of the beverages were investigated for their ability to scavenge free radicals by the DPPH and superoxide radical scavenging assays whilst the β-CLAMS and inhibition of phosholipid peroxidation were used as model systems. Results showed that the beverages in this investigation were capable of acting as antioxidant sources as they displayed radical scavenging properties. Adansonia digitata had the highest antioxidant activity in comparison to the other beverages used in the study. There was a positive correlation between antioxidant activity and phenolic compounds content but there was no clear relationship between anthocyanidin content and antioxidant activity.

Key words : *Parinari curatelifolia, Strychnos spinosa, Adansonia digitata,* antioxidant activity

INTRODUCTION

Free radical damage has been associated with many degenerative diseases common to the human body. However, antioxidant substances

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that scavenge the free radicals and detoxify the organism can block the harmful effects of free radicals. Observational epidemiological studies have consistently shown that a diet rich in fruit and vegetables is associated with a lower risk of specific cancers and of cardiovascular disease (Pen-neira *et al.*, 1997). Oxidative stress and oxidative damage are considered to play a role in the early stages of the pathophysiological processes of both diseases. Many studies have already explored the potential of selected nutrients and bioactive compounds, such as phenolic compounds, present in fruit and vegetables, on a range of biomarkers of *in vitro* oxidative stress and oxidative damage. Significant and possibly relevant effects have been reported, especially for the antioxidant vitamins C and E and A (Pinalo *et al.*, 2000; Tabernero *et al.*, 2006). However, there is a discrepancy between the outcome of the observational and experimental studies and the few controlled intervention studies investigating the effect of high dose supplementation on cancer or cardiovascular disease mortality and morbidity reported so far. This discrepancy may partly be explained by the fact that in the intervention studies, synthetic compounds were given in relatively high dosages compared with the level present in natural food. Moreover, other compounds in the food matrix may have a health beneficial effect, not necessarily associated with an antioxidant action. One complicating factor in interpretation of the experimental studies is lack of knowledge with respect to the critical pathophysiological processes and the consequent questions with respect to validity and relevance of the various biomarkers used (Alonso *et al.*, 2004).

In the past few years, an increasing interest in plant polyphenols, which are common components of the human diet, has manifested. In fact, most of the antioxidant capacity of a fruit or vegetable may be from compounds other than vitamin C, vitamin E, or β-carotene. Plant polyphenols such as flavonols, flavanols, anthocyanins, and phenylpropanoids also act as antioxidants or as agents of other mechanisms contributing to anticarcinogenic or cardio protective action (Rice-Evans *et al.*, 1995). The antioxidant potential of these compounds is dependent on the number and arrangement of the hydroxyl groups and the extent of structural conjugation, as well as the presence of electron-donating and electron-withdrawing substituent in the ring structure (Cao *et al.*, 1998).

The search for natural antioxidants have grown over the past decade due to the ever increasing concerns by consumers about the addition of synthetic additives to food which have been thought to be toxic (Chun *et al.*, 2005). Recent studies have attempted to quantify

the antioxidant capacity in foods, and significant antioxidant activity was demonstrated to be exerted, *in vitro* experimental systems of many natural plant products.

Beverages of plant origin are good sources of biologically active compounds such as vitamins, fiber and phenolic compounds. There is a large body of literature on the phenolic composition and content of plant beverages. Because of the complexity of this wide group of plant metabolites, however, many polyphenols remain unidentified. Moreover, it is difficult to compare data within the literature, owing to the lack of agreement on an appropriate method to analyze phenolic compounds. As a result, information in the literature on the content and composition of polyphenols is not complete but sometimes also contradictory and difficult to compare (Aruoma *et al.*, 1993). Polyphenols are products of secondary metabolism of plants and ubiquitous in all plant organs.

In this study we aimed at determining the antioxidant capacity of methanolic extracts of beverages prepared form three wild fruits of Zimbabwe in comparison with a beverage from a domestic fruit and a commercial fruit beverage.

MATERIALS AND METHODS

Chemicals

All the reagents used were of analytical grade. Nitro blue tetrazolium salt (NBT), 1, 1- diphenyl – 2 picrylhydrazyl radical (DPPH$^{\bullet}$), phenazine methosulphate (PMS), ascorbic acid, trichloroacetic acid (TCA) and potassium ferricyanide, sodium phosphate (monobasic), sodium phosphate (dibasic), metaphosphoric acid, ferrous sulphate, β-carotene, linoleic acid, sodium carbonate, sodium hydrogencarbonate, Folin-Ciocalteu, gallic acid, catechin, vanillic acid, tween 80, metaphosphoric acid, ferric chloride were obtained from Sigma – Aldrich Chemie (Steinheim, Germany). Reduced nicotinamide adenine dinucleotide (NADH) was obtained from Boehringer, Manheim, Germany. The chemical standards used were all of analytical grade. Chloroform, methanol, butanol, hydrochloric acid (HCl), and were obtained locally.

Sample Procurement and Preparation

Fresh ripe samples (Table 1) of *Strychnos spinosa* and *Adansonia digitata* fruits were collected from Buhera area about 160 km outside

Samples Used

Table 1. List of fruit samples used

English name	Shona name	Latin name
Baobab	Mauyu	*Adansonia digitata*
Mobola plum	Hacha	*Parinari curatelifolia*
Monkey orange	Matamba	*Strychnos spinosa*
Orange	Ranjisi	*Citrus sinensis*
Baobab nectar	–	–

Harare. *Parinari curatelifolia* was collected in Harare in Hatfield. Oranges and baobab nectar were bought in a Spar retail outlet. Fruit samples were washed in running water upon arrival in the laboratory. The *Parinari curatelifolia* sample was processed to obtain pulp and the collected pulp was stored in a freezer at – 20°C. Some of the pulp was reserved to determine water content.

Preparation of Fruit Beverages

Strychnos spinosa

The shell of the fruit was broken on a hard surface to collect the pulp and the seeds. Pulp and seed were collected into a clean bowl and then pressed in a clean cotton cloth to obtain the juice.

Adansonia digitata

Fruit shell was broken to obtain pulp and seed. Pulp was separated from seed by manually scrapping off the powdered pulp and the resulting powder was stored in a cool dry place. The powder (10 g) was diluted with different volumes of water to determine an appropriate consistency and taste. A dilution of 10% in water was considered appropriate.

Parinari curatelifolia

Peels of *Parinari curatelifolia* were removed manually and the pulp was scrapped off the seed into a clean container. The (10 g) pulp was weighed and diluted with 50 mL of water. The mixture was then squeezed through a clean cotton cloth to obtain the juice.

Citrus sinensis

Oranges were cut cross-sectional and the juice was squeezed using a lemon/orange electric squeezer.

All the beverages were stored in 10 mL aliquots in a – 20°C deep freezer for analysis.

Extraction of Phenolic Compounds

Total phenolic compounds were extracted from the beverages using the method described by Makkar (1999). The sample of the beverage (2 mL) was extracted with cold 50% methanol (8 mL). The solution was centrifuged at 3000 rpm for 10 min and transferred into small bottles for analysis. The extracts were kept in the refrigerator.

Folin-Ciocalteau Assay for Total Phenolics

Distilled water (950 µl) was added to samples (50 µl) to make up to 1 mL. Folin-Ciocalteau reagent (500 ml) was added followed by 2 % sodium carbonate (500 µl). After incubation at room temperature for 40 min absorbencies were measured at 725 nm on a Spectronic 20® Genesys™ spectrophotometer. Gallic (0.5 mg/mL) was used as a standard at varying concentrations.

Radical Scavenging Assays

DPPH Radical Scavenging Activity

The radical scavenging activity was determined following method by Kuda *et al.* (2005) 1.0 mL methanolic solutions of DPPH (1.250 mg/ 100 mL) were put into a cuvette and 80 µl of sample was added. Absorbance at 517 nm was read on a Spectronic 20® Genesys™ spectrophotometer over 40 min. Ascorbic acid (0.1 M) was used as the positive control.

Superoxide Anion Radical Scavenging Activity

Anion radical scavenging activity of the extract was determined following the method by Kuda *et al.* (2005). The sample (up to 80 µl) was mixed with phosphate buffer (0.5 mL, 0.1 M pH 7.2), NADH (125 µl, 2 mm) and NBT (25 µl, 120 µm). The blank was set by reading the absorbance at 550 nm on a Spectronic 20® Genesys™ Spectrophotometer before the addition of PMS. After three min of incubation with PMS absorbance was measured. A control was also set up with the sample replaced by phosphate buffer; ascorbic acid was used as a positive control. Percentage activity was calculated as:

$$\% \text{ activity} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Reducing Power Effects

The method described by Kuda *et al.* (2005) was used to determine the reducing power of the extracts with some modifications. Up to 80 µl of sample or ascorbic acid was mixed with phosphate buffer (0.2 mL, 0.2 M pH 7.2) and 1% potassium ferricyanide (0.2 mL). The mixture was incubated at 50°C for 20 min. After incubation trichloroacetic acid (0.2 mL, 10%) was added followed by distilled water (0.4 mL) and (0.064 mL, 0.1%) ferric chloride ($FeCl_3$). Absorbance was measured at 655 nm using a Spectronic 20® Genesys™ Spectrophotometer.

Inhibition of Phospholipid Peroxidation

Inhibition of phospholipids peroxidation by the extracts of the beverages was investigated using a modified method of Wangensteen *et al.* (2004). Female Sprague Dawley rats (*Rattus norvergicus*) were obtained from the Animal house, University of Zimbabwe and dissected in the physiology department to obtain the rat liver. The rat liver was stored at 85°C until used. Homogenization of rat brain (2 g) was done in chloroform: methanol mixture (2:1, v/v) followed by centrifugation at 3000 rpm for 5 min. The supernatant obtained was used as the source of phospholipids. The test run contained phospholipid solution (50 µl), fruit beverage extract, (0.5 mL) 50% methanol and ferrous sulphate ($FeSO_4$, 0.2 mL). The blank contained the phospholipid solution (50 µl) mixed with distilled water (0.5 mL) instead of the sample and 50% methanol (0.2 mL). Ascorbic acid (5%) was used as the positive control. Incubation of the test mixture and the blank at 37°C was followed by the addition of thiobarbituric (0.5 mL) acid and trichloroacetic acid (4 mL) and the solution was heated in a boiling water bath for 15 min. After cooling the sample on ice, absorbance was read at 532 nm on a Spectronic 20® Genesys™ Spectrophotometer.

β-Carotene Linoleic Acid Model System (β-CLAMS)

A modified method of Gorinstein *et al.* (2004) was used to determine the inhibitory effects of the extract in β-CLAMS. β-Carotene (2 mg) was dissolved in 10 mL of chloroform. (1 mL) of this solution was put into an evaporating dish and the β-carotene solution was evaporated on boiling water. Tween 80 (40 µl) and 400 µl were added to the β-carotene and the mixture was immediately diluted with 100 mL of

distilled water and the mixture was transferred to a volumetric flask. The β-carotene solution was agitated vigorously until an emulsion was formed. The emulsion (3 mL) was added to a test tube and the sample (200 µl) was added to the emulsion. The test mixture was shaken and incubated in water bath at 50 °C for 2 h of which absorbencies at 470 nm were measured at 5 min intervals during the incubation. The blank contained distilled water whilst the positive control had butylated hydroxyanisole (1%) and the negative control contained 50% methanol instead of sample. % activity was calculated as:

$$\frac{\text{Absorbance } (T_0)}{\text{Absorbance } (T_x)} \times 100$$

Where T_0 is time 0 and T_x is any time after wards (5 min intervals).

Determination of Ascorbic Acid Content

The method-designed by University of Zimbabwe Biochemistry Department was used to determine the ascorbic acid content of the beverages. Standardization of the method was done by titrating 20 mL of DCPIP solution (26 mg dissolved in 100 mL distilled water containing 21 mg of sodium hydrogen carbonate) against ascorbic acid (50 mg in 50 mL metaphosphoric acid). Titration was done in not less than 30 seconds and not more than 2 min. Fruit beverage (10 g) was diluted with distilled water (50 mL). This solution (25 mL) was pippeted into a volumetric flask and metaphosphoric acid (20 mL, 5%) was added. The solution was left to stand for 20 min after which the volume was topped to 50 mL with distilled water. The solution was filtered. The beverage solution was titrated against 1 mL of DCPIP.

Butanol-HCl Assay

The butanol-HCl assay was carried out using the method described by Makkar (1999). Sample methanolic extract (0.5 mL) was added to tubes followed by butanol-HCl reagent (3 mL, 95:5 v/v) and ferric reagent (0.1 mL, 2 g ferric ammonium sulphate dissolved in 100 mL distilled water containing 16.6 mL of HCl). The tubes were vortexed, covered with a glass marble and heated in a boiling water bath for an h. The tubes were cooled and absorbance was read at 550 nm using Spectronic 20® Genesys™ Spectrophotometer. The blank was prepared for each sample where the same reagents were added in the same quantities the difference being that the blanks were not boiled. The absorbance was expressed as % leucocyanidin equivalence by the formula:

$$\% \text{ leucocyanidin equivalence} = \frac{\text{Absorbance (550nm)} \times 78.26}{\% \text{ Dry mass}}$$

Vanillin-HCl Assay

The vanillin-HCl assay was determined by the method described by Makkar (1999). To the sample (500 µl), vanillin reagent (2.5 mL, 1 g in 100 mL-distilled water) and methanol-HCl reagent (2.5 mL, 1:1 v/v) were added and the tubes were incubated at room temperature for 20 min after which absorbance was read at 500 nm using Spectronic 20® Genesys™ Spectrophotometer. Catechin (4 mg/mL) was used as a standard. The blank contained 50% methanol instead of sample. The content of flavanols in the beverages was expressed as mg catechin equivalents per 100 mL of sample.

RESULTS AND DISCUSSION

Variation of antioxidant activity may depend upon, which free radicals or oxidant is used in the assay. Each method of determination of antioxidant activity is based on the reactivity of different scavenging radicals and also dependent on pH of the system (Cano *et al.*, 1998). Antioxidant activities of fruit extracts also depend on polarity of extracting solvent, isolation procedures, purity of active compounds and the assay technique and substrate used (Chun *et al.*, 2005; Arnao, 2000). The amount of total phenolic compounds in an extract may also contribute to the overall antioxidant capacity of an extract (Arnao, 2000). It was therefore, important to determine the phenolic content of extracts, since they have been implicated in antioxidant activities.

Total Phenolic Content

The extracts showed differences in the amounts of phenolic compounds contained by beverages. The phenolic content ranged between 12 and 58 mg GAE/100 mL.

Citrus sinensis beverage contained the highest phenolic content than the other three samples. *Adansonia digitata* beverage had phenolic content of 51.15 ± 2.4 mg GAE/100 mL. The phenolic content of *Citrus sinensis* (57.35 ± 2.5 mg GAE /100 mL) was not very different from that of *Adansonia digitata* (51.15 ± 2.4 mg GAE /100 mL) and also in agreement with those stated by Murillo (2002), which are about 64.2 ± 7 mg GAE/100 mL serving. *Parinari curatelifolia* had the lowest phenolic content of 17.85 ± 1.2 mg GAE /100 mL although it has been assumed that *Parinari curatelifolia* is rich in phenolic compounds. This discrepancy can be explained in terms of the type of phenolic compounds present, most of the phenolic compounds in the pulp of *Parinari curatelifolia* could be structural phenolic

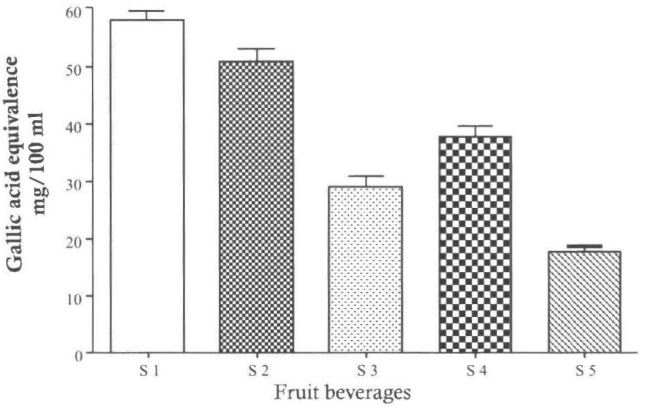

Fig 1. Total phenolic content expressed as garlic acid equivalence (GAE) as determined by Folin-C assay for three beverages prepared from wild fruits *Adansonia digitata* **(S2)**, *Strychnos spinosa* **(S4)**, *Parinari curatelifolia* **(S5)** and two beverages from wild commercial and domestic sources, *Citrus sinensis* **(S1)**, Baobab nectar **(S3)**

compounds. Structural phenolic compounds are not usually extracted by the methods of extraction available hence these compounds are not accounted for (Goristein *et al.*, 2004). The phenolic compound of a fruit is dependent on environmental factors such as soil type, soil richness, available moisture and temperature, and thus may vary from tree to tree, place to place for the same type of fruit.

DPPH Radical Scavenging Assay

The percentage decrease in absorbance of DPPH due to the presence of antioxidants in the beverages is shown in Fig 2. Activity of the extracts decreased with an increase in time. A steep gradient shows high abilities of the sample to quench the DPPH radicals.

The model of scavenging the stable DPPH radical is a widely used method to evaluate antioxidant activities in a relatively short time compared to other methods (Baumann *et al.*, 2002). Ascorbic acid, which was used as the positive control, showed a sharp decrease in absorbance showing that ascorbic acid rapidly quenches the DPPH radical. This observation was possible since ascorbic acid is thought to be a reducing agent hence readily donates a hydrogen atom to the DPPH radical. Upon acceptance of the hydrogen atom the DPPH radical changes color from deep violet to yellow and this color change can be observed at a wavelength of 517 nm (Chang *et al.*, 2002). The percentage decrease in absorbance is directly proportional to the quenching of the DPPH radical. *Adansonia digitata* and *Citrus sinensis* had almost the same trend of radical depletion although that of

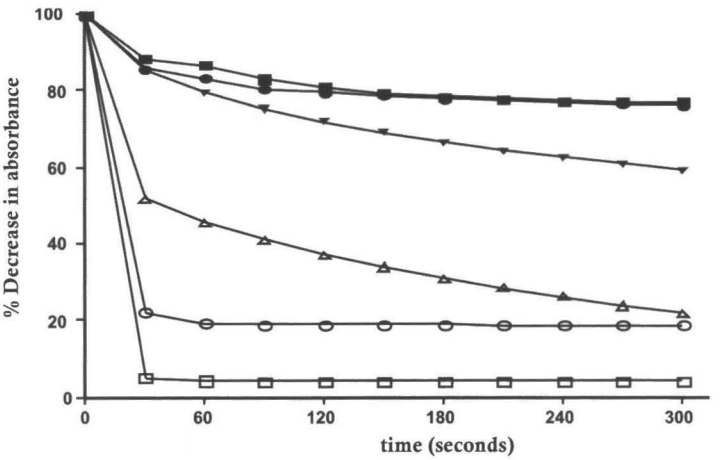

Fig 2. Free radical scavenging activity of three beverages prepared from wild fruits, *Strychnos spinosa* (•), *Adansonia digitata* (Δ), *Parinari curatelifolia* (▲) and two beverages from wild commercial and domestic sources, baobab nectar (▪) *Citrus sinensis* (O) and ascorbic acid control (□) by DPPH

Citrus sinensis showed a slightly greater capability for hydrogen donation. The ascorbic acid content of the orange and that of *Adansonia digitata* could have also contributed to their antiradical activity. *Strychnos spinosa* and the baobab nectar showed almost the same trend in radical depletion activities. The activity of *Strychnos spinosa* can not be attributed to its phenolic content alone; the level of phenolic compounds in the *Strychnos spinosa* beverage extract were low compared to that of *Adansonia digitata* and *Citrus sinensis* beverage extracts hence its low capability to donating hydrogen atoms to the DPPH radical. The traces of vitamin C in the *Strychnos spinosa* beverage extract could have contributed to the hydrogen donation activity. *Parinari curatelifolia* showed low hydrogen donation ability that also corresponds to its low phenolic compounds, its traces of ascorbic acid could also have been responsible for the antiradical activity.

The DPPH radical scavenging assay needs assays to back it since it does not quantify the antiradical activity. However, it is important in showing trends on how the antioxidant is depleted.

Superoxide Radical Scavenging Assay

Increase in superoxide radical scavenging activity with an increase sample volume was observed in Fig 3. An initial steep increment in activity was observed between 20 and 40 µl after which the graphs levels off.

The method is based on the capacity of the beverage extracts to inhibit the reduction of nitroblue tetrazolium by NADH (Dasgupta & De, 2004). A mixture of NADH and PMS generate superoxide radicals. The samples effectively scavenged the superoxide radical in a concentration dependent manner as shown in Fig 3. The reduction of NBT produces a blue color but in the presence of antioxidants the blue color does not appear or is of less intensity. The percentage radical scavenging activity increased with an increase in concentration of the sample. This may be due to the increase in the number of molecules capable of reacting with the superoxide radicals to produce stable compounds. Baobab nectar had the least antioxidant activity as shown by Fig 3 where its activity was below 40% even at 80 µl. *Adansonia digitata* had the highest activity at 80 µl of about 72% followed by ascorbic acid and *Citrus sinensis*, 66% and 64% respectively. *Strychnos spinosa* and *Parinari curatelifolia* had activities of about 54% and 51% respectively at 80 µl. Initially there was some sharp increase in activity from 20 µl to 40 µl which was followed by little increase in activity from 40 µl onwards.

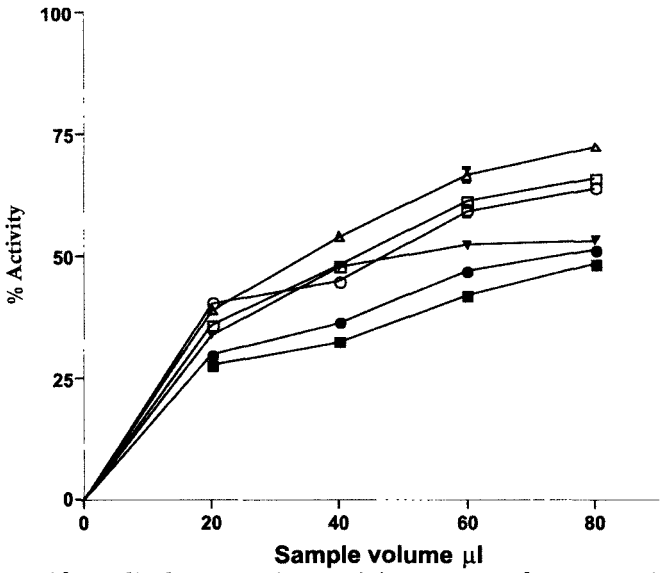

Fig 3. Superoxide radical scavenging activity expressed as % activity of the extracts of the beverages, *Strychnos spinosa* (▲), *Adansonia digitata* (Δ), *Parinari curatelifolia* (•) and two beverages from wild commercial and domestic sources, baobab nectar (▪) *Citrus sinensis* (○) and ascorbic acid control (□)

Reducing Power Effects

Reducing power effects of the extracts of the beverages increased with an increase in sample concentration as shown in Fig 4. Absorbances increased with an increase in sample concentration.

The reducing power effect is measured on the basis of the ability of the antioxidants in the beverages' extracts to reduce the Fe^{3+}/ ferricyanide complex to the ferrous (Fe^{2+}) form. Fe^{2+} can be monitored by the formation of Perl's Prussian blue at 720 nm (Chun *et al.*, 2005). Reducing power is mainly associated with the presence of reductones which have been shown to exert antioxidant action by breaking the free radical chain by hydrogen donation. Reductones are also reported to react with certain precursors of peroxide thus preventing peroxidation formation (Chung *et al.*, 2002).

The general trend of the reducing power effects of the beverages is an increase in the reducing power with an increase in the concentration of the sample. The reducing power of the samples showed a characteristic of dose dependence. Initially, up to about 50 µl there was an increase in the reducing power effects of the extracts with increases in sample concentration and small increases in the reducing power was achieved after a volume of 75 µl. This is in agreement with results obtained by Makris *et al.* (2003). *Adansonia digitata* and *Citrus sinensis* had the highest reducing power abilities compared to other extracts. *Strychnos spinosa* and *Parinari curatelifolia* had similar reducing properties as shown by the closeness of their curves in Fig 4 above. The reducing properties of the extracts are also in correlation with the antioxidant activities of the extracts of the beverages. The correlation may be supported by the point that the ability to donate electrons or hydrogen atoms can function in

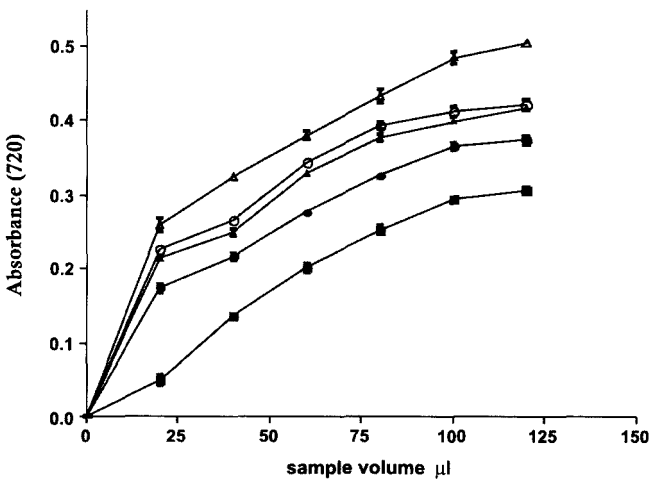

Fig 4. Reducing power effects of three beverages prepared from wild fruits, *Adansonia digitata* (Δ) *Strychnos spinosa* (▲), *Parinari curatelifolia* (•), and two beverages from wild commercial and domestic sources, baobab (▪) nectar *Citrus sinensis* (○) and a control reducing agent ascorbic acid (□)

terminating radical chain reactions by converting radicals to stable products (Yen & Duh, 1993) as in the DPPH assay and β-CLAMS. Reducing power effects are directly correlated to the phenolic content of the extracts as increase in extract concentration also increases the concentration of phenolic compounds which is related to an increase in the reducing power ability.

Inhibition of Phospholipid Peroxidation

Activity of the samples varied with *Adansonia digitata* having the greatest activity.

In biological systems, lipid peroxidation generates a number of degradation products such as malonaldehyde (MDA). MDA is found to be important cause of cell membrane destruction and cell damage (Pin-Der-Duh *et al.*, 1999). MDA has been measured as an index of lipid peroxidation and as a marker of oxidative stress (Wangensteen *et al.*, 2004). The abilities of the extracts of the beverages to inhibit the peroxidation of lipids from the rat are shown in Fig 3. Iron sulfate ($FeSO_4$) was used to induce lipid peroxidation by forming hydroxyl radicals. *Adansonia digitata* showed the greatest ability of inhibiting the formation of MDA as low absorbencies were observed whilst baobab nectar showed least inhibitory effect. The efficiency of the extracts in preventing lipid peroxidation is inversely proportional

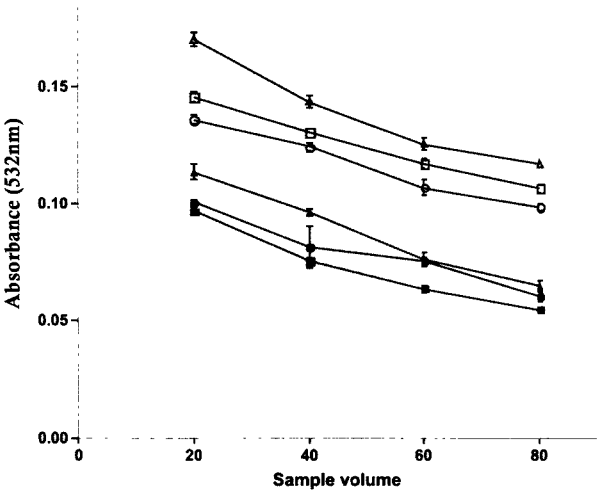

Fig 5. Inhibition of phospholipids peroxidation of three beverages prepared from wild fruits, *Adansonia digitata* (Δ) *Strychnos spinosa* (▲), *Parinari curatelifolia* (•), and two beverages from wild commercial and domestic sources, baobab (■) nectar and *Citrus sinensis* (○) and a control reducing agent ascorbic acid (□)

to the amount of MDA formed. The more the MDA formed the less efficiency of the beverage extract to work as an inhibitor of phospholipids peroxidation. Inhibition of lipid peroxidation was dependent on the concentration of the samples; high sample concentration resulted in limited MDA molecules being formed. The inhibition of phospholipids peroxidation shows the ability of antioxidant components in the extract of the beverages to act as chain breakers. Chain breaking properties are as a result of hydrogen and electron donation which was observed in the reducing power effects and the DPPH radical quenching abilities. The chain breaking activities is correlated to the total phenolic content as increase in phenolic content increased the extent of inhibition of peroxidation. Using phospholipids obtained from a rat liver, is important in depicting what happens in biological systems as the oxidation and radical chain initiation occurs in the body.

β-Carotene Linoleic Acid Model System (β-CLAMS)

In Fig 6 the absorbencies decreased with time though the minimum percentage decrease did not go below 50%.The decrease in absorbance was gradual and tending to be constant after 70 min on average

At elevated temperatures linoleic acid is oxidized, during oxidation, an atom of hydrogen is abstracted from the active bis-allylic methylene group of linoleic acid located on carbon-11 between two double bonds (Kim *et al.*, 2006) generating peroxides. The pentadienyl free radical so formed then attacks highly unsaturated β-carotene molecules in an effort to reacquire a hydrogen atom. As the β-carotene molecules lose their conjugation; the carotenoids lose their characteristic orange color. Fortunately, this process can be monitored spectrophotometrically. The presence of a phenolic antioxidant can hinder the extent of β-carotene destruction by "neutralizing" the linoleate free radical and any other free radicals formed within the system (Jayaprakasha *et al.*, 2001). Hence, this forms the basis by which plant extracts can be screened for their antioxidant potential.

The antioxidant activities of the beverage extracts and standard antioxidants as measured by bleaching of β-carotene are presented in Fig 6. The rate of decrease in absorbance is indirectly proportional to the activity of the beverage extract. A slow decrease in absorbance shows that the beverage extract is a good antioxidant. Generally the beverage extracts tested had the ability to quench peroxyl radicals formed from oxidation of linoleic acid thus retarding the decolorisation of the β-carotene by the peroxyl radical. *Adansonia digitata* and *Citrus sinensis* extracts had similar trends of retarding the decolorisation of β-carotene although the activity *Citrus sinensis* had greater activity than *Adansonia digitata* during the first 60 min. After 60 min the

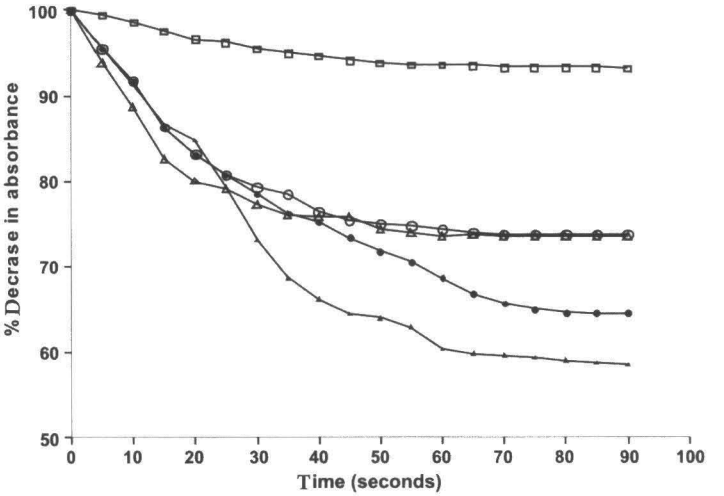

Fig 6. The capacity to prevent β-carotene oxidation in a model system of three beverages prepared from wild fruits, *Strychnos spinosa* (▲), *Adansonia digitata* (Δ), *Parinari curatelifolia* (•) and two beverages from wild commercial and domestic sources, baobab nectar (▪) and *Citrus sinensis* (○) and a control antioxidant Butylated hydroxyanisole (□)

activity of *Adansonia digitata* and *Citrus sinensis* were the same. If 100% is considered to be the initial concentration of β-carotene, then after 90 min *Adansonia digitata* and *Citrus sinensis* extracts retained 75% of the β-carotene by preventing its peroxidation.

Baobab nectar had lower activity than *Adansonia digitata* and *Citrus sinensis* extract as after 80 min it managed to retain about 55% of the β-carotene. Baobab nectar showed 55% efficiency in quenching peroxyl radicals formed by the oxidation of linoleic acid. Activity of the baobab nectar can be attributed to synthetic antioxidants used. *Strychnos spinosa* had slightly more ability of quenching peroxyl radicals than Baobab nectar. *Strychnos spinosa* retained about 59% of the β-carotene, about 4% more than Baobab nectar did. *Parinari curatelifolia* had a radical quenching ability about 64% of the peroxyl radical. The retardation of β-carotene decolorizing ability of the beverages' extracts is related to the phenolic content. *Adansonia digitata* and *Citrus sinensis* that had the highest total phenolic compounds also showed higher activity in retarding the decolorisation of β-carotene. Butylated hydroxyanisole was found to have antioxidant activity of 93.4% after 80 min of incubation, a result similar to that obtained by Shon *et al.* (2003). Butylated hydroxyanisole can thus be said to be able to prevent the oxidation of β-carotene while the beverages studied in this investigation retard the oxidation of β-carotene.

Ascorbic Acid Content

Ascorbic acid content varied with the samples and it was present in all the beverages prepared from fruits and absent in baobab nectar.

Ascorbic acid content varies with geographical distribution as well as environmental conditions such as nutrient availability, water and temperature. High nitrogen fertilizers lower vitamin C content while proper potassium levels increase the vitamin C levels. The maturity state of the fruit also affects vitamin C content as vitamin C decreases with ripening. Position on the tree affects the vitamin C levels in a fruit because sunlight increases vitamin C levels thus fruits on the outside, southward have higher levels. *Citrus sinensis* juice had the highest ascorbic acid content of about 51.26 ± 3.16 mg/100 mL, which is in close relation with the values obtained by Lo Scalzo *et al.* (2004) of 57.68 ± 5.3 mg/100 mL. In oranges only 26% of vitamin C content is found in the juice. *Adansonia digitata* had the second highest levels of ascorbic acid of 37.41 ± 3.89 mg/100 mL. The ascorbic acid content of *Adansonia digitata* beverage is lower than that of *Citrus sinensis* juice, the reverse for the fruit pulps where *Adansonia digitata* has six times greater ascorbic acid content than *Citrus sinensis*. The reason for this difference could be that the *Adansonia digitata* pulp had been diluted ten times to obtain the beverage while the *Citrus sinensis* beverage was straight juice from pulp with no dilutions.

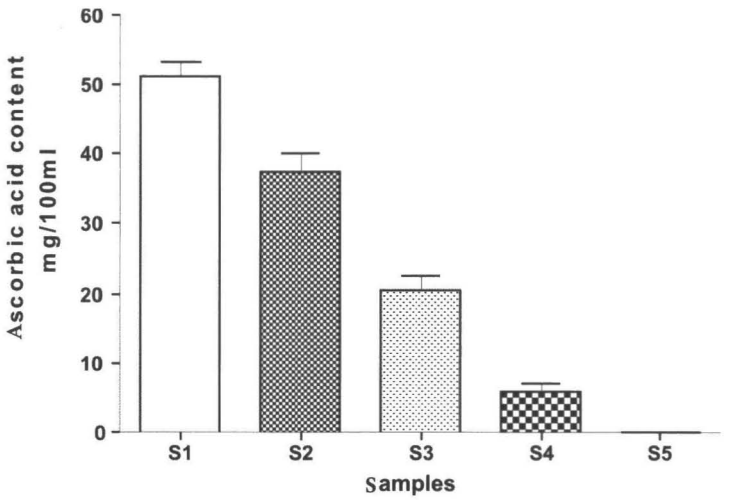

Fig 7. Ascorbic acid content of the prepared beverages, *Citrus sinensis* (**S1**), *Adansonia digitata* (**S2**), *Strychnos spinosa* (**S3**), *Parinari curatelifolia* (**S4**) and a commercial beverage, baobab nectar (**S5**) as determined by the DCPIP method

Ascorbic acid content of the fruits, *Parinari curatelifolia* and *Strychnos spinosa* have not been studied in depth there are only facts in literature that the two fruits contain ascorbic acid (vitamin C) with no supporting values. However, from this study, the ascorbic acid content of *Parinari curatelifolia* and *Strychnos spinosa* were 6.07 ± 2.19 and 20.65 ± 3.58 mg/100 mL respectively. Ascorbic acid is a well known reducing agent through its hydrogen donation abilities thus; it impacts this property on the beverages. This property of ascorbic acid makes it a good antioxidant component of the beverages since hydrogen atom is a major factor influencing radical scavenging activity as well as reducing power effects. In beverages derived from citrus fruits ascorbic acid have been fond to contribute about 65-100% of the antioxidant activity, but the case has been different for the fruits (Luximon-Ramma *et al.*, 2003). Total phenolic content by Folin-C is not very specific since not only phenolic compounds but also reducing compounds such as ascorbic acid are simultaneously extracted and determined (Dasgupta & De, 2004), thus ascorbic acid could have contributed to the antioxidant activities of the extracts.

Butanol-HCl Assay

Table 2 shows that the proanthocyanidin content for all samples was very low with the highest level being 1.07% leucocyanidin content in *Adansonia digitata.*

The assay is based on the oxidative depolymerisation of condensed tannins to yield red anthocyanidins (Schofield *et al.*, 2001). *Adansonia digitata* showed the highest anthocyanidin content as shown in Table 2 were it had a leucocyanidin content of 1.071 ± 0.021% whilst other beverages had leucocyanidin equivalence that was less than 1%. This means that *Adansonia digitata* contains more flavan-3-ols than any of the beverages studied. *Citrus sinensis* had the second highest levels of proanthocyanidins as it had leucocyanidin equivalence of 0.587 ± 0.025%. *Strychnos spinosa* and *Parinari curatelifolia* had proanthocyanidin content of 0.407 ± 0.035 and 0.357 ± 0.032% expressed as percentage leucocyanidin equivalence. Baobab nectar had the lowest anthocyanidin content as displayed by its low % leucocyanidin equivalence of 0.116 ± 0.021%. The low proanthocyanidin content of the baobab nectar can be attributed to small percentage of baobab added to the beverage. The low levels of condensed tannins as shown by results from the butanol-HCl assay are of benefit to the consumers since high levels of condensed tannins lead to precipitation of proteins since tannins are capable of forming insoluble complexes with proteins.

Table 2. Proanthocyanidin content of the beverages expressed as % leucocyanidin equivalence

Samples	% Leucocyanidin equivalence
Adansonia digitata	1.071 ± 0.021
Strychnos spinosa	0.407 ± 0.035
Parinari curatelifolia	0.357 ± 0.032
Citrus sinensis	0.587 ± 0.025
Baobab nectar	0.116 ± 0.021

Vanillin Assay

Flavanol content of the extracts of the beverages was relatively high although low levels were detected in *Citrus sinensis* and *Parinari curatelifolia*.

Adansonia digitata displayed high amounts of flavonols (124.2 ± 1.386 mg CAE/100 mL) as shown in Fig 8 which is about 10 times greater than that of *Citrus sinensis* extract. *Citrus sinensis* had a lower flavonol content mainly due to the fact that most flavonoids found in oranges are abundant in the skin and the mesorcarp especially hesperidins. The baobab nectar had almost the same levels of flavonols as *Strychnos spinosa* (55.6 ± 3.470 and 53.33 ± 9.46 mg CAE/100 mL, respectively). Flavonols detected in baobab nectar are probably due to the contribution of the baobab ingredient in the

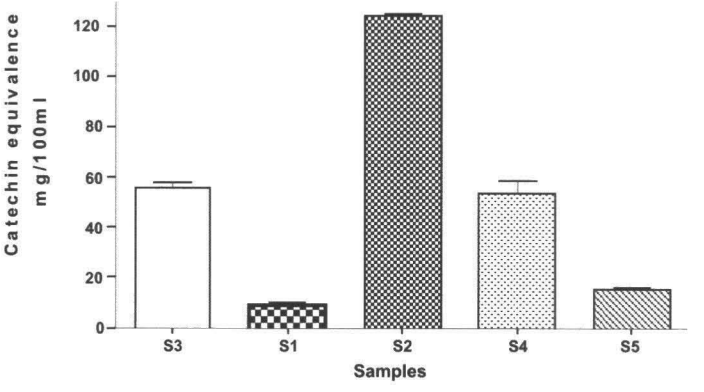

Fig 8. Anthocyanidin content of the beverages' extracts expressed as catechin equivalence (CAE) as determined by vanillin-HCl method for three beverages prepared from wild fruits *Adansonia digitata* **(S2)**, *Strychnos spinosa* **(S4)**, *Parinari curatelifolia* **(S5)** and two commercial beverages *Citrus sinensis* **(S1)**, Baobab nectar **(S3)**

preparation of the beverage. *Parinari curatelifolia* was observed to have flavonol content that is lower than that of the other wild fruit derived beverages. The vanillin method depends on the reaction of vanillin with flavan- 3-ol residues of condensed tannins forming colored complexes hence confirms the presence of flavonols in the sample.

CONCLUSIONS

Results indicate that methanolic extracts of the prepared beverages contain a number of antioxidant compounds which can effectively scavenge various free radical and reactive oxygen species under *in vitro* conditions. The extracts from the three wild fruit beverages had significant antioxidant activities although the antioxidant activity varied from sample to sample in the systems tested.

The antioxidant activities of the beverages' extracts can be presented in the following order: *Citrus sinensis* ≥ *Adansonia digitata* > *Strychnos spinosa* > *Parinari curatelifolia* > baobab nectar. Total phenolic content showed close correlation with the antioxidant activities of the samples. Phenolic content was highest in *Adansonia digitata* and *Citrus sinensis* and lowest in *Parinari curatelifolia.* The broad range of activity of the extracts suggests that multiple mechanisms are responsible for antioxidant activity of the extracts from the beverages.

The study shows the potential antioxidant properties of three commonly consumed wild fruit beverages, *Adansonia digitata*, *Strychnos spinosa* and *Parinari curatelifolia*. These fruit beverages can be used as supplement for antioxidants and ascorbic acid within existing nutritional programs which can prove to be a more effective and economical means of protecting the body against various oxidative stress than the supplementation with individual antioxidants such as vitamin C and E.

ACKNOWLEDGEMENTS

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4

Spontaneous Short-Term Fermentation of Garlic Potentiates its Anti-Oxidative Activities

YOSHIMI NIWANO[1,*], EMIKO SATO[1] AND MASAHIRO KOHNO[1]

ABSTRACT

Spontaneous fermentation of garlic for the relatively short period of time (40 days at 60–70°C, 85–95% relative humidity) potentiates its fundamental antioxidative properties. Scavenging activities against $O_2^{-\bullet}$ and H_2O_2 of 80% ethanol extract of the fermented garlic were increased 13-folds and more than 10-folds respectively, as compared with those of the control garlic extract. Polyphenol content of the extract were also increased about 7-fold in the fermented garlic. The results indicate that relatively short-term spontaneous fermentation potentiates anti-oxidative properties of garlic in fresh form, which is, at least in part, attributable to the increased level of polyphenols. Since $O_2^{-\bullet}$ is the primary upstream radical of the chain reaction with reactive oxygen species and H_2O_2 is generated from the scavenging reaction by superoxide dismutase, the fermented-garlic is suggested to possess desirable anti-oxidative properties. To further examine the mechanism by which H_2O_2 is scavenged, tetrahydro-β-carboline derivatives (THβCs), potent scavengers against H_2O_2, were quantitatively analyzed with liquid chromatography-mass spectrometry (LC-MS). (1R, 3S)-1-Methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTCC) and (1S, 3S)-MTCC were found in the fermented garlic extract whereas only trace levels of MTCCs were detected in the control garlic extract. Therefore, it is suggested that relatively short-term fermentation potentiates scavenging activity of garlic against H_2O_2 by forming THβCs, especially MTCCs.

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Key words : Anti-oxidative potency, garlic, polyphenols, radical scavenging, tetrahydro-β-carboline derivatives

INTRODUCTION

Foodstuffs possess two major functions. That is, the primary function is nutritional feature (life support), and the secondary function is gastational feature (taste, flavor, and texture). Recently, researchers have focused on balancing biodynamics, such as immunity, internal secretion, neurotic systems, and cardiovascular systems, as the tertiary function of foodstuffs. A typical example is French paradox. The French paradox refers to the fact that people in France suffer relatively low incidence of coronary heart disease (CHD), despite having a diet relatively rich in saturated fat (Renaud & Lorgeril, 1990). Incidence of atheroscleosis and CHD are associated with the elevated levels of low density lipoprotein (LDL) cholesterol in the blood. Frankel *et al.* (1993) have shown that the phenolic compounds in red wine exerts potent antioxidant activity, which results in inhibiting the oxidation of human LDL *in vitro* (Kanner *et al.*, 1994). Therefore, it has been postulated that the phenolic compounds in red wine may prevent the incidence of atherosclerosis. In addition, Kinsella *et al.* (1993) suggested that phenolic compounds in plant foods also be effective in preventing thrombosis, a fatal event in a large proportion of deaths from CHD and as reviewed by Wolframe (2008), green tea catechins can be regarded as food components useful for the maintenance of cardiovascular and metabolic health.

Garlic (*Allium sativum* L.) has been considered as a valuable healing agent by people of different cultures for thousands of year, and has long been used as a folk remedy for a variety of ailments. Even today, it is commonly used for its medicinal benefit through the world, especially Eastern Europe and Asia. Recently, it has also been suggested that garlic preparation including aged garlic prevents tumor promotion (Dorant *et al.*, 1993), cardiovascular disease (Kleijnen *et al.*, 1989), liver damage (Pal *et al.*, 2006) and aging (Moriguchi *et al.*, 1994) which are considered to be associated with reactive oxygen species (ROS) and lipid peroxidation. The intrinsic antioxidant activities of garlic (Rietz *et al.*, 1993), garlic extract (Numagami *et al.*, 1996) and some garlic constituents (Ide *et al.*, 1996) have been widely documented. Among the many commercial garlic products, aged garlic extract is known to contain unique and bioactive organic sulfur compounds such as S-allylcystein and S-allylmercaptocystein which show anti-oxidative effects (Ide *et al.*, 1999). In addition to organic sulfur compounds, it has been reported that aged garlic contains tetrahydro-β-carboline derivatives which possess potent H_2O_2

scavenging properties, and fermented garlic by a more than 10 months natural aging process have high antioxidant potency than non-fermented garlic (Ichikawa *et al.*, 2006).

We examined antioxidant properties of uniquely processed garlic, which was fermented for only 40 days without any additives, by using ESR-spin trapping method for $O_2^{-\bullet}$ determination and a spectrometric technique for H_2O_2 determination (Sato *et al.*, 2006a). Furthermore, 1,2,3,4-tetrahydro-β-carboline derivatives (THβCs), which have been reported to possess H_2O_2 scavenging activity, were quantitatively analyzed with liquid chromatography (LC)-mass spectrometry (MS) (Sato *et al.*, 2006b). In this review, the augmented antioxidant potency of this uniquely processed garlic has been described.

MATERIALS AND METHODS

Garlic in fresh form has been harvested in August from Aomori prefecture of Japan and was stored in dry and dark depots. The garlic's color was rendered black by spontaneous fermentation for 40 days at 60–70°C, 85–95% relative humidity without any additives (described as black garlic throughout the paper). Fig 1 shows representative examples of the black garlic. As a control, the garlic in fresh form without spontaneous fermentation was used (described as control garlic throughout the paper). Both of the control and black garlic were freeze-dried and pulverized in 80% ethanol solution followed by filtration through No. 2 filter paper. The obtained filtrate was used as garlic extract. For mass-spectrometry analyses, the obtained filtrate was further filtrated though a 0.2 μm PVDF filter.

Assay for superoxide dismutase (SOD)-equivalent activity was essentially identical to that described in our papers (Sato *et al.*, 2007; Niwano *et al.*, 2007). In brief, 50 μl of 2 mM hypoxanthine

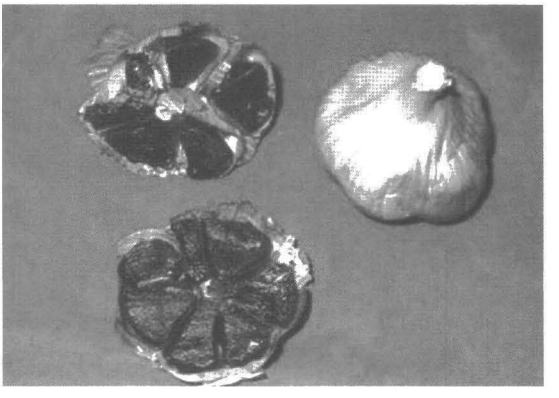

Fig 1. The representative examples of the black garlic

(HPX), 30 µl of dimethylsulfoxide (DMSO), 50 µl of the garlic extract dissolved in 80% EtOH, 20 µl of 4.5 M DMPO and 50 µl of 0.4 U/ml xanthine oxidase (XOD) were placed in a test tube and mixed. The mixture was transferred to the electron spin resonance (ESR) spectrometry cell, and the 5,5-dimethyl-1-pyrroline-N-oxide (DMPO)-OOH spin adduct was quantified 90 sec after the addition of XOD. Spin adduct formation of DMPO and typical reactive oxygen species is summarized in Fig 2. Signal intensities were evaluated from the peak height of the first signal of the DMPO-OOH spin adduct. To make the $O_2^{-\bullet}$ scavenging activities comparable to each other, the activity was converted to SOD-equivalent units using a calibration curve of the enzyme activity of authentic SOD.

Assay for •OH was essentially identical to that described in the previous paper (Sato *et al.*, 2008). A schematic drawing of ultrasound device operated at 1 MHz for hydroxyl radical generation is illustrated in Fig 3. In the assay, since ethanol is a potent scavenger against •OH (Zang *et al.*, 1995), 80% ethanol extract of the black garlic was mixed with the same volume of chloroform: pure water (1:1), and the water layer was recovered for the assay. A glass tube (15 × 85 mm) with the reaction mixture which consist of 880 µl of pure water, 100 µl of water extract of the black garlic and 20 µl of 111.25 mM DMPO dissolved in pure water was set in the device. Then the reaction mixture was exposed to sonication for 2 min. The reaction mixture obtained after the exposure was immediately transferred to the ESR spectrometry cell for the ESR analysis. Signal intensities were evaluated from the peak height of the second signal of the DMPO-OH spin adduct (Fig 2).

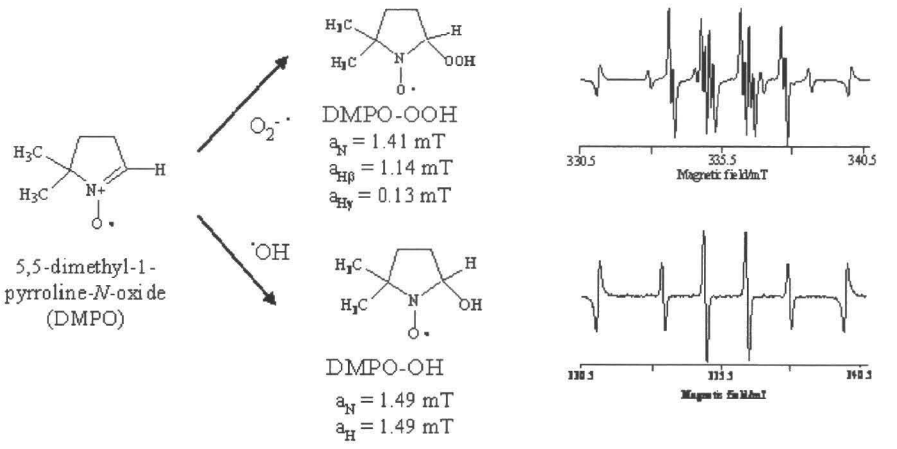

Fig 2. Spin adduct formation of 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) and typical reactive oxygen species ($O_2^{-\bullet}$ and •OH)

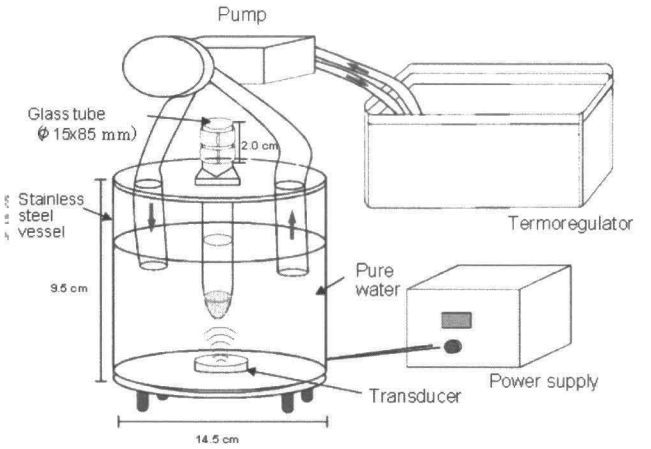

Fig 3. A schematic drawing of ultrasound device for •OH generation

The protocol used for H_2O_2 assay was the procedure using *N*-(carboxymethylaminocarbonyl)-4,4'-bis(dimethylamino)-diphenylamine sodium salt (DA-64) as a coloring agent provided by the manufacture (Wako Pure Chemical Industries, Osaka, Japan). In brief, 50 µl of each sample (garlic extract or 80% ethanol as a solvent) was added to 150 µl of H_2O_2 (final 21.3 µM) and mixed. Then, 100 µl of the mixture was added to 900 µl of reaction solution consisting of 0.1 mM DA-64, 0.1 M PIPES buffer (pH 7.0), 0.5% Triton X-100 and horse radish peroxidase (1 unit/ml), and the optical density at 727 nm was read 10 min after the onset of the reaction. The principle of the coloring reaction of DA-64 and H_2O_2 is illustrated in Fig 4 (Cheng *et al.*, 1982).

Total polyphenol content was determined by Folin-Denis method (Shanderl, 1970). In brief, 3.2 ml of pure water, 200 µl of each garlic extract, 200 µl of Folin & Ciocalteu's Phenol Reagent and 400 µl of saturated sodium carbonate solution were mixed. The optical density at 760 nm was read after standing for 30 min. A freshly prepared gallic acid was used as the standard.

Chemical structure of tetrahydro-β-carboline derivatives (THβCs) is shown in Fig 5. For the determination of (THβCs) that have been reported to be responsible for the scavenging activity against H_2O_2 in the fermented garlic (Ichikawa *et al.*, 2006), mass spectra were acquired by TOF mass spectrometer coupled to chromatographic separation at 35°C.

RESULTS AND DISCUSSION

Fig 6 shows the representative ESR spectra of DMPO-OOH obtained from the solvent control, the control garlic extract and the black

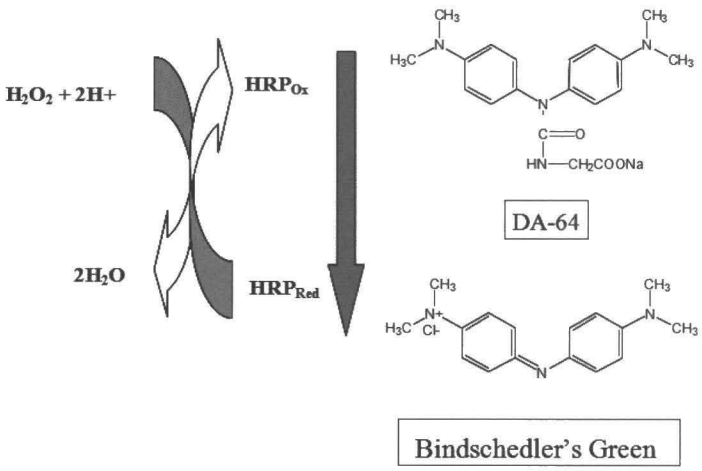

Fig 4. The schematic figure of coloring reaction by DA-64 and H_2O_2, HRP_{Ox} and HRP_{Red} indicate oxidized and reduced horse radish peroxidase, respectively

garlic extract in the HPX-XOD system as a $O_2^{-\bullet}$ generator. Since it has been reported that the addition of SOD (a scavenger for $O_2^{-\bullet}$) resulted in the disappearance of the ESR spectrum, DMPO-OOH was indicated to be derived from $O_2^{-\bullet}$ generated by the HPX-XOD reaction system. (Tanigawa *et al.*, 1994). SOD equivalent activity obtained from the control garlic is 29 U/g dry tissue, while the activity from the black garlic reached 368 U/g dry tissue, indicating that the relatively short-term fermentation can increase the $O_2^{-\bullet}$ scavenging activity of garlic by more than 10 times. Since scavenging activity of the plants for $O_2^{-\bullet}$ is mainly attributable to polyphenols as well as antioxidant vitamins (Saito *et al.*, 2008), the content of polyphenols were determined. The extract of the control garlic contained polyphenols as much as about 1,000 μg/g dry tissue, and the polyphenols in that of the black garlic were increased more than 7-fold as compared with those in the control garlic.

Fig 7 shows the percentages of H_2O_2 scavenged by the control garlic extract and the black garlic extract. The control garlic extract

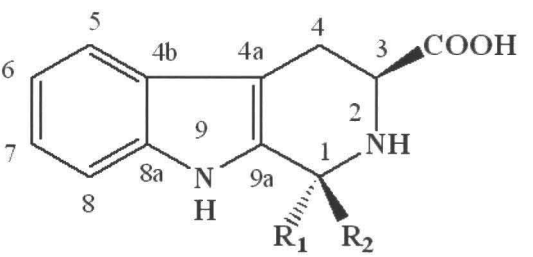

Fig 5. Chemical structure of the tetrahydro-β-carboline derivatives (THβCs)

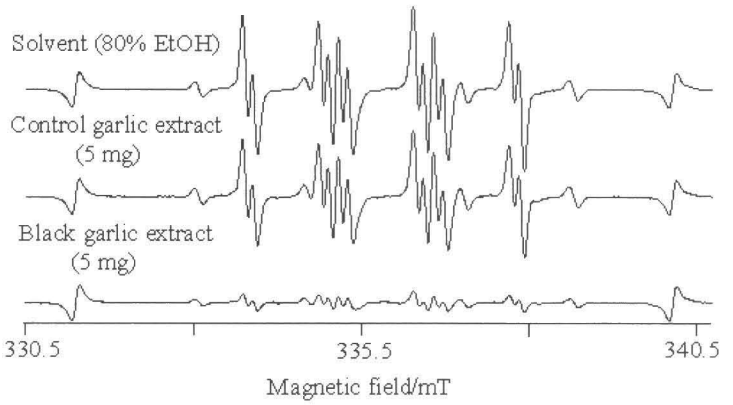

Fig 6. The representative ESR spectra of DMPO-OOH (for $O_2^{-\bullet}$ determination) from the solvent control (80% EtOH alone), the control garlic extract and the black garlic extract

reduced the amount of H_2O_2 by only 15%, whereas the black garlic extract completely scavenged H_2O_2. It means that the relatively short-term fermentation also extremely potentiates the scavenging activity of garlic for H_2O_2. The MS measurement of THβCs indicated that methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acids (MTCCs) were clearly increased in the black garlic, but not methyl-1,2,3,4-teterahydro-β-carboline-1,3-dicarboxylic acid (MTCdiCs).

Since polyphenols were remarkably increased in the black garlic, it is postulated that the black garlic possesses the ability to scavenge •OH. In our preliminary study, the water soluble fraction obtained from the 80% ethanol extract of the black garlic can scavenge •OH that were generated by an ultrasound device. A typical example is

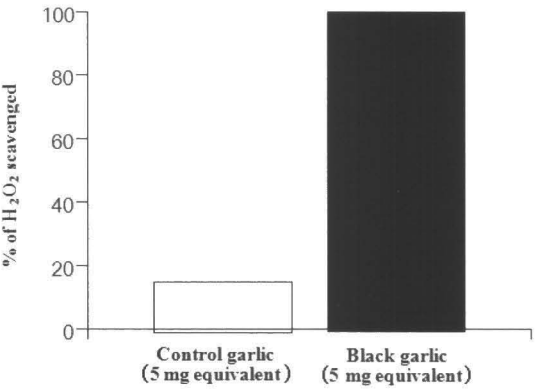

Fig 7. H_2O_2 scavenging activity of garlic extract. H_2O_2 scavenging activity is expressed as the % of reduced H_2O_2 concentration

shown in Fig 8 in which the spin adduct DMPO-OH was extremely reduced by the addition of the water soluble fraction.

Aged garlic extract manufactured by a more than 10 months natural aging process is well known to contain bioactive organic sulfur compounds such as S-allylcysteine and S-allylmercaptocysteine both of which show a variety of biological activities including anitoxidative properties (Ide *et al.*, 1999). In addition, tetrahydro-β-carboline derivatives which possess H_2O_2 scavenging activity have recently been identified in aged garlic extract (Ichikawa *et al.*, 2006). In the study with spontaneously fermented garlic for relatively short-term period, as is the case with the aged garlic, the $O_2^{-\bullet}$ scavenging activity and H_2O_2 scavenging activity are greatly increased (Figs 6 & 7). The increased $O_2^{-\bullet}$ scavenging activity is, at least in part, attributable to the increased amount of polyphenols. And the scavenging activity of the black garlic extract against H_2O_2 was at least 10 times more potent than that of the control garlic extract, indicating that the increased levels of THβCs seem to correlate with the increased activity. In other words, the potent scavenging activity against H_2O_2 by the black garlic was at least in part attributable to the increased levels of THβCs, especially MTCCs. Besides the scavenging activity against H_2O_2, it has been reported that THβCs inhibit platelet aggregation (Tsuchiya *et al.*, 1999) so that the preventative effect in thromboses is also expected.

In the case of SOD that is a specific and potent scavenger of $O_2^{-\bullet}$, SOD dismutates $O_2^{-\bullet}$ into H_2O_2 and O_2. H_2O_2 was then dismutated

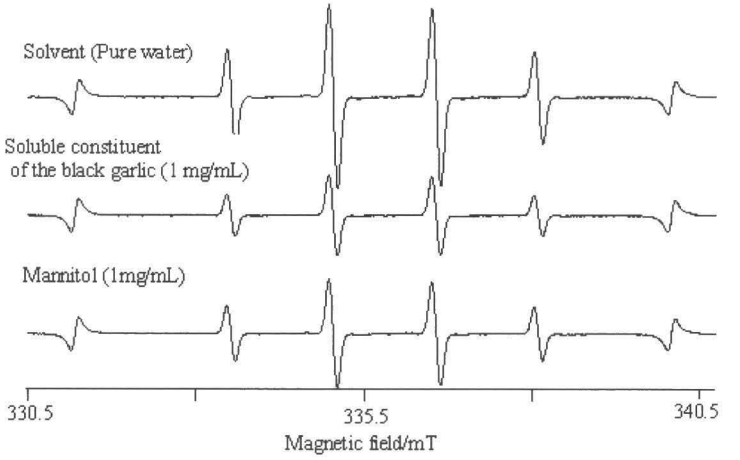

Fig 8. The representative ESR spectra if DMPO-OH (for •OH determination) from the solvent control (pure water), the black garlic extract, and mannitol as an authentic •OH scavenger

by catalase to H_2O and O_2. In other words, complete dismutation of $O_2^{-\bullet}$ into H_2O and O in the biological system requires both of SOD and catalase. In the case of the fermented garlic, not only $O_2^{-\bullet}$ scavenging activity but also H_2O_2 scavenging activities was increased, indicating that fermented garlic has an ability to completely dismutate $O_2^{-\bullet}$ into H_2O and O_2. H_2O_2 also gives rise to •OH formation through a Fenton-type reaction (Bannister *et al.*, 1987; Dunford, 1987).

The results clearly show that spontaneous fermentation of garlic for the relatively short period is enough to give it desirable anti-oxidative properties against ROS such as $O_2^{-\bullet}$, •OH and H_2O_2 (Fig 9).

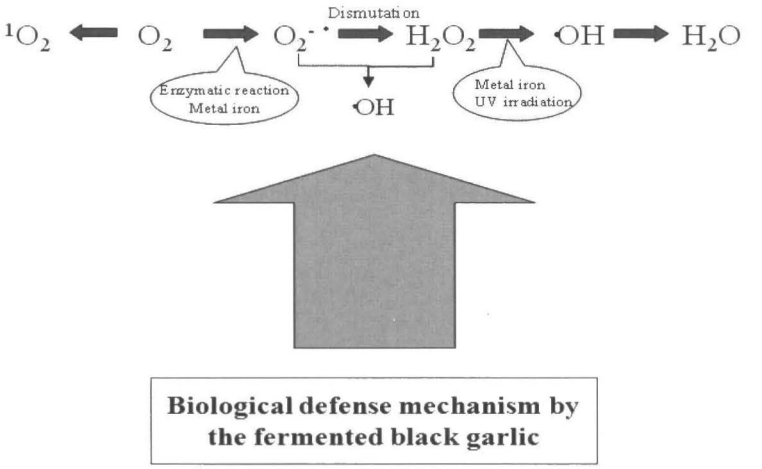

Fig 9. One of the proposed mechanisms by which the fermented black garlic augments the biological defense

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5

Natural Antioxidant Phytochemicals in Fruits, Berries and Vegetables and their Degradation Status During Processing

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ABSTRACT

Fruits, berries and vegetables are good sources of antioxidants, including carotenoids, ascorbic acid, tocopherols, flavonoids and phenolic acids. In comparison to fruits and berries, vegetables generally contain much lower amounts of antioxidant compounds. These phytochemicals have antioxidant activity, which are gaining a considerable amount of interest as bioactive components with beneficial health effects. As interest in functional foods and other products with possible health effects is escalating, a large number of industrial enterprises are now producing various 'antioxidant' concentrates. In addition, certain natural antioxidant phenols may act synergistically or even antagonistically, which further complicates predictions of antioxidant effective ness of mixed concentrates. Therefore, marketing of most natural antioxidant concentrates is based only on empirical knowledge from tests in model systems. The natural compounds in fruits and vegetables responsible for the antioxidant activity, their

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content levels and the compounds' fate during different methods of processing shows great variation. Food processing such as peeling, boiling or juicing may result in increased inhibition or decreased inhibition of oxidation depending on the changes in the antioxidant components. Antioxidant activity of fruits, berries and vegetables and their products therefore vary widely owing to differences in the raw materials as well as a result of different food processing methods that may induce changes in the antioxidant compounds. This chapter presents the antioxidative activity of different extracts obtained from the plant material, as well as of individual antioxidants isolated from them.

Key words : Antioxidant, fruits, berries, vegetables, processing

INTRODUCTION

Fruits, berries and vegetables contain different phytochemicals having antioxidant activity, which are gaining a considerable amount of interest as bioactive components with beneficial health effects (Ho, 1992). The physiological role of some of these antioxidants, such as vitamin E and vitamin C, is well established. The natural compounds in fruits and vegetables responsible for the antioxidant activity, their content levels and the compounds' fate during different methods of processing shows great variation. Food processing such as peeling, boiling or juicing may result in increased inhibition or decreased inhibition of oxidation depending on the changes in the antioxidant components (Spanos *et al.,* 1990). Transformation of antioxidants into more active compounds improves antioxidant activity, while destruction or loss of antioxidants generally decreases the antioxidant activity, but important exceptions exist. Antioxidant activity of fruits, berries and vegetables and their products therefore vary widely owing to differences in the raw materials as well as a result of different food processing methods that may induce changes in the antioxidant compounds (Hakkinen *et al.,* 2000). In addition, data on antioxidant activity of various fruits, berries and vegetables and their products can vary in response to differences in the preparation of samples for antioxidant testing. Finally, the use of different oxidation systems and methods to measure antioxidant activity affect the antioxidant results. This chapter presents the antioxidative activity of different extracts obtained from the plant material, as well as of individual antioxidants isolated from them.

Antioxidants from Fruits and Berries

It has been known for a long time that the phenolics, as well as some of the other antioxidant components, are closely associated

with the sensory attributes of fresh and processed fruits, berries and other plant foods (Table 1). Fruits and berries are good sources of antioxidants, including carotenoids, ascorbic acid, tocopherols, flavonoids and phenolic acids. Especially, the colour contribution by carotenoids (yellow to orange and red) and anthocyanins (red to purple and blue) is well known. Antioxidant activity of fruits and berries, their juices and wines vary widely partly due to the use of different oxidation systems and methods to analyse antioxidant compounds. By using the ORAC method, the extract of fresh strawberries had the highest total antioxidant capacity compared with the extracts of plum, orange, red grape, kiwi fruit, pink grapefruit, white grape, banana, apple, tomato, pear and honeydew melon. However, in lipid oxidation models (methyl linoleate, LDL) phenolic extracts from strawberries ranked among the least active antioxidants compared with the activities of other berries.

Beneficial biological functions of the traditional antioxidant vitamins, *i.e.* ascorbic acid, α-tocopherol and to a certain extent beta-carotene (provitamin A) have been intensively studied and the biological roles of these plant phenolics that exert antioxidant activity proved that phenolic phytochemicals also exert various protective effects in human beings. Because of the possible benefits of phenolic phytochemicals to human health, their quantitative occurrence and composition in various fruits and berries are recognized as recent field of investigation. It is now known that flavonoids and other phenolic compounds abundant in fruits and berries are generally recognized in relation to compilation of food compositional data, there are large variations in the levels of the constituents reported, depending on the species investigated, harvest time, fruit maturity stage, geographical origin etc. Differences in the methods employed for extraction and analyses also strongly affect the results. Some studies have evaluated the phenolic contents in fruits at more than one ripening stage. In the case of plums as well as with red grapes (intended for wine making) a marked increase in the content of phenolics of potential antioxidant activity was seen in the fully ripe stage in comparison with the less ripe stage (Meyer *et al.*, 1997; Thomas-Barberan *et al.*, 2001).

Antioxidant composition (anthocyanins, flavanols and proanthocyanidins, flavonols, hydroxycinnamates, carotenoids, vitamin C and vitamin E) of selected, commonly consumed fruits and berries is presented in Table 1. Large amounts of anthocyanins (up to 8100 $mgkg^{-1}$) are found in the strongly coloured fruits and berries including bilberries (wild clone of blueberries), blackcurrants, cherries, cranberries, red grapes and raspberries. The amount of flavanols is generally below 150 $mgkg^{-1}$with larger amounts found in

Table 1. Antioxidant compounds identified in different fruits and berries

Fruits and berries	Antioxidative compounds	Reference(s)
Apple juice	chlorogenic acid, phloretin glycosides	Miller & Rice-Evans, 1997; Miller *et al.*, 2000
Apple pomace	ascorbic acid, epicatechin, its dimer (procyanidin B2), trimer, tetramer, oligomer, quercetin glucosides, chlorogenic acid, phloridzin, 3-hydroxyphloridzin	Miller & Rice-Evans, 1997
Apple	chlorogenic acid	Lu & Foo, 2000
Grapefruit	naringin (naringenin 7-β-neohesperidoside)	Plumb *et al.*, 1996
Grapes	total phenolics, anthocyanins, flavonols, malvidin 3-*O*-(6-*O*-*p*-coumaroylglucosido)- 5-glucoside	Frankel & Meyer, 1998; Tamura & Yamagami, 1994
Wild grapes	malvidin-3,5-diglucoside	Igarashi *et al.*, 1989
Red grape juice	total phenolics, anthocyanins	Frankel *et al.*, 1998
White grape juice	hydroxycinnamates, flavan-3-ols	Frankel *et al.*, 1998
Grape seeds	procyanidin B2 3´-*O*-gallate	Da Silva *et al.*, 1991
Red wine	anthocyanins, catechin, gallic acid, resveratrol	Frankel *et al.*, 1995; Ghiselli *et al.*, 1998; Burns *et al.*, Unpublished
Peach	chlorogenic acid, neochlorogenic acid	Garcia *et al.*, Plumb *et al.*, 1996
Pear	chlorogenic acid	Plumb *et al.*, 1996
Orange juice	hesperidin, narirutin	Miller & Rice-Evans, 1997
Prunes, prune juice	chlorogenic acid, neochlorogenic acid	Donovan *et al.*, 1998
Tart cherries	cyanidin, 6, 7-dimethoxy-5,8,4´-trihydroxyflavone, genistein, chlorogenic acid, naringenin, genistin, 2-hydroxy-3-(*o* hydroxy-phenyl) propanoic acid, 1-(3´,4´-dihydroxycinnamoyl)-cyclopenta-2, 5-dio 1,1- (3´,4´-dihydroxy-cinnamoyl)-cyclopenta-2,3-diol	Haibo *et al.*, 1999a; Haibo *et al.*, 1999b; Haibo *et al.*, 1999c
Berries	anthocyanins, hydroxycinnamates, flavonols	Prior *et al.*, 1998; Costantino *et al.*, 1992; Abuja *et al.*, 1998; Kalt *et al.*, 1999

blackcurrants, cranberries, red wine grapes, peaches, plums and red raspberries. Apart from a few exceptions such as cranberries and red grapes, fruits and berries are generally also low in flavonols and high in phenolic acids such as hydroxycinnamates. Large amounts of hydroxycinnamates are present in cherries (300-1930 $mgkg^{-1}$), plums (121-896 $mgkg^{-1}$) and peaches (81-750 $mgkg^{-1}$). High molecular weight phenolics, tannins, are also found in fruits and berries with large amounts of ellagitannins in red raspberries (2200 $mgkg^{-1}$) and cloudberries (1800-2600 $mgkg^{-1}$) and moderate amounts in strawberries (90-200 $mgkg^{-1}$) (Rice-Evans *et al.,* 1996). The vitamin C content of fresh fruits and berries is generally high while that of provitamin A caro tenoids and vitamin E is low. Blackcurrants (1200-1500 $mgkg^{-1}$), cloudberries (1000 $mgkg^{-1}$), strawberries (550-1000 $mgkg^{-1}$)[4] and orange (510 $mgkg^{-1}$) are very rich in vitamin C. One exceptional berry is sea buckthorn berry with extremely large amounts of vitamin C (2000 $mgkg^{-1}$) as well as high amounts of beta-carotene (15 $mgkg^{-1}$) and vitamin E (32 $mgkg^{-1}$).

For antioxidant testing, either extracts or juices of fruits and berries have been used resulting in different antioxidant compositions owing to choice of extraction solvents (*e.g.* either water-soluble or lipid-soluble compounds extracted by one method) or use of fil tration (*e.g.* possible losses of antioxidant compounds). Many of the flavonoids and phenolic acids exert comparable or better radical scavenging activity than vitamin C and E in radical scavenging activity assays (Risch *et al.,* 1988).

Plums contain higher levels of epicatechin than catechin, with the total levels of these flavanol diastereoisomers being 5-50 mg kg^{-1} fresh weight of whole plums (Heinonen *et al.,* 1998). The antioxidant activities of phenolic extracts of berries against lecithin liposomes were significantly positively correlated to the content of hydroxycinnamates, but the amount of flavanols correlated to the antioxidant potency of extracts of berries in neither the *in vitro* LDL oxidation systems nor in the lecithin liposome assay (Table 2) (Kahkokonen *et al.,* 2001). Extracts of sweet cherries were found to be the best among a large number of other fruits in inhibiting oxidation *in vitro* while red grapes ranked second.

APPLE

Although the apple extracts tested were low in total phenolics as well as ascorbic acid (Rice-Evans *et al.,* 1996; Kahkonen *et al.,* 1999) but apple showed strong antioxidant activity towards oxidation of methyllinoleate. In apple juice, vitamin C activity represented a minor fraction of the total antioxidant activity, with chlorogenic acid and

phloretin glycosides as the major identifiable antioxidants (Miller & Rice-Evans, 1997; Tomas & Clifford, 2000). Dihydrochalcones such as phloretin glucosides and phloridzin amount to 5-223 $mgkg^{-1}$ in apple juice, this content being greater than that of fresh apples (Tomas & Clifford, 2000). According to Plumb *et al.* (1996) chi-orogenic acid contributes about 27% of the total activity of apple extract in scavenging hydroxyl radicals. During conventional apple juice production (straight pressing or pulp enzyming) more than 80% of the quercetin glycosides remained in the press cake and less than 10% was found in the raw juice. It was suggested that quercetin glycosides and antioxidant activity in apple juice could be increased over tenfold by extracting the pulp with an alcoholic solvent such as methanol or ethanol (Van Der Sluis *et al.,* 1999). Apple polyphenols isolated from gala apple pomace such as epicatechin (Choi *et al.,* 2000), its dimer (procyanidin B2) (Hayase & Kato, 1984), trimer, tetramer and oligomer, guercetin glucosides, chlorogenic acid, phloridzin (Rodriquez-Saona *et al.,* 1998) and 3-hydroxyphloridzin (Plumb *et al.,* 1997) showed strong antioxidant activities in β-carotene linoleic acid system and DPPH radical scavenging activities (Plumb *et al.,* 1996).

STONE FRUITS

Nectarines (*Prunus persica* var. nucipersica), peaches (*Prunus persica* L.), plums (*Prunus domestica),* sweet cherries (*Prunus avium* L.) and sour cherries (*Prunus cerasus* L.) comes in the category of stone fruits. In general, ascorbic acid is present in highest concentration in the fruit flesh, but the skin fraction also contains larger amounts of phenolics. Phenolic compounds in nectarines, peaches and plums, the anthocyanins and flavonols were found to be almost exclusively located in the peel tissues (Thomas-Barberan *et al.,* 2001). However, the flavanols, notably catechin, epicatechin, procyanidin, were also found in the fruit flesh with mean contents in the flesh of peaches and nectarines in the range 100-700 $mgkg^{-1}$ with plums were less potent. Neochlorogenic acid and chlorogenic acid, the two predominant phenolic compounds in prunes, were antioxidants toward oxidation of human LDL (Van Der Sluis *et al.,* 1999). According to the ORAC test prunes rank highest with more than twice the level of antioxidants than other high-scoring fruits such as raisins and blueberries. The inhibition of LDL oxidation by peach (*Prunus persica*) extracts, including raw and canned peaches, ranged between 56–87% with the antioxidant activity mainly attributed to the presence of hydroxycinnamic acids, chlorogenic and neochlorogenic acids, (Chang *et al.,* 2000) but not to carotenoids such as β-carotene and β-cryptoxanthin present (Garcia *et al.,* unpublished results).

Table 2. Radial scavenging and antioxidant activities in different test systems for ascorbic acid and selected phenolic antioxidants purified from fruits, berries and vegetables

Compound	Inhibition (%) of LDL oxidation at 5μm GAE★	Inhibition (%) of lecithin liposome oxidation at 10 μm GAE●	ORAC (μm trolox equivalents)■	TEAC (mm trolox equivalents)◆
Flavanones				
Naringenin			2.67	0.72
Hesperidin				1.37 (Risch *et al.*, 1988)
Flavonols				
Kaempferol			2.67	1.02
Quercetin	50.6		3.29	2.88
Rutin	67.6		0.56	2:4 (Risch *et al.*, 1988)
Myricetin	68.1		4.3	3.1 (Risch *et al.*, 1988)
Flavan-3-ols				
Catechin	87.8		2:49	2.4 (Abuja *et al.*, 1998)
Epicatechin	67.6		2.36	2.5 (Risch *et al.*, 1988)
Procyanidins				
Anthocyanins				
Cyanidin	79:4	pro-oxidant	2.2	2.38
Malvidin	59.3	23.9	2.0	1.80
Pelargonin	39.0	pro-oxidant	1.1	1.30 (Risch *et al.*, 1988)
Delphinidin	71.8	pro-oxidant	1.8	4.80
Hydroxycinnamates				
p-Coumaric	24.5		1.09	1.56
Ferulic	24.3		1.33	1.75
Caffeic	96.7		2.23	0.99
Chlorogenic	90.7			
Other				
Ascorbic acid	45.2 (at 10 μm)	2.5 (at 10 μm)	0.52	1.05
Gallic acid	63.3		1.74	3.0 (Lees & Francis, 1972
Ellagic acid	0-36			

★ (Kahkokonen *et al.*, 2001; Teissedre *et al.*, 1996; Heinonen *et al.*, 1998; Meyer *et al.*, 1998)
● (Heinonen *et al.*, 1998)
■ (Cao *et al.*, 1997; Wang *et al.*, 1997; Re *et al.*, 1999)
◆ (Guo *et al.*, 1997)

Total phenols as gallic acid equivalents, extracts of whole clingstone peach cultivars inhibited human LDL oxidation *in vitro* by 44-84% depending on the cultivar (Chang *et al.,* 2000). Extracts of peach peels contained more total phenols (910-1920 $mgkg^{-1}$ as gallic acid equivalents) than the extracts from flesh (430-770 $mgkg^{-1}$). Chang *et al.* (2000), found a stastically significant linear correlation between relative antioxidant activity and concentration of total phenols of peach extracts. Thus, the relative antioxidant activity of peel extracts was better than the extracts of whole peach and peach flesh extracts, even though the percentage inhibition at 10 µm was in a similar range for all types of peach extracts. The results signified that the antioxidant activity was widely distributed among the extracted peach phenolics. In peaches, the anthocyanins are mainly confined to the peel tissue (Thomas-Barberan *et al.,* 2001; Chang *et al.,* 2000). Strong correlation was found between the percentage relative inhibitory activity and redness of whole peach extracts when colour was measured on the Hunter scale (Chang *et al.,* 2000).

Plums contain high levels of hydroxycinnamic acids, notably neochlorogenic and chiorogenic acids, with neochlorogenic acid as the dominant compound with content levels in the range 500-770 $mgkg^{-1}$ fresh weight (Heinonen *et al.,* 1998). Individually, these compounds exert potent antioxidant activity on human LDL oxidation *in vitro* and have been shown to inhibit totally the LDL oxidation *in vitro.* Plum extracts tested *in vitro* were better inhibitors of lipid oxidation in human liver microsomes and phosphatidyl choline than peach, apple, grapefruit and pear extracts (Plumb *et al.,* 1996). Analyses of methanolic extracts of freshly harvested, unprocessed prune plums, cultivar Lapetited' Agen, showed the mean concentration of phenolics to be about 1100 $mgkg^{-1}$ fresh weight, where neochlorogenic acid constituted 73 wt% of the phenols (807 $mgkg^{-1}$) and chiorogenic acid was 13 wt% (144 $mgkg^{1}$); only low amounts of 3'-coumarylquinic acid (10 $mgkg^{-1}$) were detected (Donovan *et al.,* 1998). The level of anthocyanins in these plums were 76 $mgkg^{-1}$, while there was 54 mg kg^{-1} catechin and 27 $mgkg^{-1}$ of other flavonols, mainly rutin (Donovan *et al.,* 1998). In a study where five Californian plum cultivars were analyzed for their phenolic content, high levels of anthocyanins, dominated by cyanidin 3-glucoside (about 1040 $mgkg^{-1}$) and cyanidin-3-rutinoside (560 $mgkg^{-1}$) about 1600 $mgkg^{-1}$ fresh weight, were found in the skin of the blue plum cultivar 'Angeleno'. Other red and blue plum varieties also contained these two anthocyanin glucosides at lower levels, in their skin. In pitted prunes, anthocyanins and catechin were absent, and hydroxycinnamates dominated by neochlorogenic acid made up 98% by weight of the phenolic material, where the mean concentrations of phenols were 1840 $mgkg^{-1}$ (Donovan *et al.,*

1998). Extracts of prunes as well as of prune juice were shown to inhibit the copper catalysed oxidation of lipids in human LDL significantly with the prune extract exerting higher antioxidant activity than the prune juice (Thomas-Barberan *et al.*, 2001).

ORAC measurements evaluated on a per 100 gram weight basis ranked the 'antioxidant power' of dried plums, that is prunes, the highest among a range of other fruits, however, part of the increase could be due to the greater dry matter content in dried plums compared to fresh plums.

CITRUS FRUITS

Citrus fruits contain high levels of ascorbic acid and certain flavonoids. citrus peel, also contain the unique glucaric and galactaric acid conjugates of hydroxycinnamic acids, mainly as feruloyl and p-coumaroyl conjugates at levels of 170-250 $mgkg^{-1}$ in oranges and 3-10 times less in lemons and grapefruits (Risch *et al.*, 1987; Risch & Herrmann, 1987). Grapefruit (*Citrus paradisi*) extracts inhibited ascorbate/iron-induced lipid peroxidation of liver microsomes in a dose-dependent assay. Naringin (naringenin 7-b-neohesperidoside) a major component in grapefruit, is responsible for most of the hydroxyl radical scavenging activity of grapefruit (Plumb *et al.*, 1997). Grapefruit was also effective towards ascorbate/iron-induced lipid per-oxidation of P450-containing microsomes (Frankel & Meyer 1998). According to Wang *et al.* (1996) orange (*Citrus sinensis*) was more active than pink grapefruit in scavenging peroxyl radicals (ORAC assay) while grapefruit juice was more active than orange juice. Ascorbic acid is major nutrients in citrus fruits, owing to its activity as vitamin C, and it seems plausible that the presence of ascorbic acid may influence the antioxidant potency of citrus products. The ascorbic acid levels in various processed citrus juice products manufactured in Florida (orange juices, grape juices) range from 300 to 450 mgl^{-1} (Lee & Coates, 1997).

Flavonoids in the edible part of citrus fruits are dominated by hesperidin, which is a compound exhibiting only limited antioxidant and antiradical potency in various assay test systems (Risch *et al.*, 1988). Hesperidin concentrations in citrus are in the range 5400-5500 $mgkg^{-1}$ dry weight based on analyses of 66 different citrus species (Kawau *et al.*, 1999). When the $ABTS^{\cdot+}$ radical trapping efficiency of orange juice were evaluated in the TEAC assay, the antioxidant activity of orange juice was mainly due to the presence of hesperidin, naringin and narirutin. Citrus essential oils, which contain a large number of volatile components, notably high levels of limonone, exert radical scavenging effects against DPPH, where the essential oil of

the Korean lemon variety Chang lemon, Tahiti lime and Eureka lemon were found to be especially strong radical scavengers on DPPH *in vitro* (Choi *et al.,* 2000).

Extracts from citrus peel and seeds contain glycosylated flavanones and polymethoxylated flavones, especially of naringin, neohesperidin, hesperidin and narirutin, as well as hydroxycinnamates, with the flavanone content in the peels being higher than in the seeds (Choi *et al.,* 2000; Bocco *et al.,* 1998). In a model system using citronellal as the oxidising substrate, seed extracts of various citrus fruits exhibited greater antioxidant activity than the corresponding extracts of peels (Bocco *et al.,* 1998). Studied the antioxidant effect of by-products of the citrus juice industry and found that, in general, the seeds of lemon, bergamot, sour orange, sweet orange, mandarin, pummelo and lime possessed greater antioxidative activity than the peels. Thus, citrus products contain a range of very different types of antioxidant compounds, which are furthermore distributed differently in the separate fruit fractions.

BERRIES

Berries constitute a significant source of antioxidants, the most significant compounds being flavonoids, phenolic acids and to a minor extent ascorbic acid. The most potent berries are crowberry (*Empetrum nigrum*), cloudberry (*Rubus chamaemorus*), whortleberry (*Vaccinium uligonosum*), cranberry (*Vaccinium oxycoccus*) and rowanberry (*Sorbus aucuparia*), all being wild berries, while the cultivated berries such as strawberry (*Fragaria ananassa*), red currant (*Ribes rubrum*), blackcurrant (*Ribes nigrum*) and red raspberry (*Rubus idaeuss* exerted low antioxidant activity in inhibiting lipid oxidation (Kahkonen *et al.,* 1999). Carotenoids may also contribute to the antioxidant activity in, for example, carotenoid-rich sea buckthorn berry (*Hippophae rhamnoides* L. cv. Indian -Summer) had a high antioxidant activity in a beta-carotene bleaching method (Velioglu *et al.,* 1998). High antioxidant capacities is reported for strawberries by using radical model systems (Kalt *et al.,* 1999; Wang *et al.,* 1996; Garcia-Alonso *et al.,* 2001) while in lipid oxidation models (methyllinoleate, LDL) phenolic extracts from strawberries ranked among the least active antioxidants compared to the activities of other berries (Kahkokonen *et al.,* 2001; Kahkonen *et al.,* 1999).

Berry extracts inhibited LDL oxidation in the order: blackberries (*Rubus fructicosus*) > red raspberries > sweet cherries (*Prunus avium*) > blueberries (*Vaccinium corymbosum*) > strawberries (Haila, 1999). In the same study, sweet cherries were the most active towards oxidation of lecithin liposomes followed by blueberries, red raspberries,

blackberries and strawberries. Different blueberries and bilberries were reported to exhibit good antioxidant capacity in the ORAC assay (Prior *et al.,* 1998; Kalt *et al.,* 1999). The antioxidant capacity of blueberries was about three-fold higher than either strawberries or raspberries with only a small contribution of ascorbic acid (Costantino *et al.,* 1992) to the total antioxidant capacity compared to total phenolics and anthocyanins (Kalt *et al.,* 1999). Kahkonen *et al.* (2001), found a statistically significant correlation between the flavonol content and antioxidant activity of berries (R=0.78) and between hydroxycinnamic acid content and antioxidant activity (R=0.54). Blueberries and their wild clones, bilberries (*Vaccinium myrtillus*), have been shown to be very efficient antioxidants in many studies (Prior *et al.,* 1998; Smith *et al.,* 2000; Kahkonen *et al.,*1996; Satue-Gracia *et al.,*1997). One of the most potent antioxidant compounds in strongly coloured berries, such as blue-berries, are anthocyanins, although blueberries are also rich in hydroxycinna mates such as chiorogenic acid (Rice-Evans *et al.,*1996; Kalt *et al.,* 1999). Like several other flavonoids, anthocyanins are powerful free radical scavengers (Risch *et al.,* 1988; Wang *et al.,* 1996; Porter, 1993). They also show antioxidant activity in lipid environments such as emulsified methyl linoleate, liposome and human LDL (Satue-Gracia *et al.,* 1997; Abuja *et al.,* 1998). However, according to Costantino *et al.,* (1992) the activities of black raspberries, blackcurrants, highbush blueberries, blackberries, redcurrants and red raspberries toward chemically generated superoxide radicals were greater than those expected on the basis of anthocyanins and polyphenols present in the berries. It has been found also that the anthocyanins were able to reduce α-tocopheroxyl radical to α-tocopherol (Abuja *et al.,* 1998). Tart cherries (*Prunus cerasus*) were reported to exhibit antioxidant activity (Haibo *et al.,* 1999a; Haibo *et al.,* 1999b, 1999c). According to Haibo *et al.* (1999a) anthocyanidin and its aglycone, cyaniding, Yi *et al.,* (1997) isolated from tart cherries were responsible for the antioxidant action. Also spray-dried elderberry juice (*Sambucus nigra*), containing large amounts of anthocyanin glucosides, (Table 3) inhibited copper-induced oxidation of LDL (Vinson *et al.,* 1998). In this study, the anthocyanins were able to reduce alpha-tocopheroxyl radical to alpha-tocopherol. Kahkonen *et al.* (1996) isolated anthocyanins from blackcurrants, Blue berries and lingonberries (*Vaccinium vitis-idaea*) resulting in remarkable inhibition of the hydroperoxide formation of methyl linoleate and hexanal formation in LDL. Blackcurrant anthocyanins showed the highest radical scavenging potential against the DPPH radical, followed by bilberry and lingonberry. On the other hand, according to Costantino *et al.* (1992) the activities of black raspberries, blackcurrants, high bush blueberries, blackberries, red currants and red rasp-berries toward chemically generated superoxide radicals were

greater than those expected on the basis of anthocyanins and polyphenols present in the berries. It is possible that ascorbic acid contributes significantly to the antioxidant activity of berries and berry juices, as Miller and Rice-Evans (1997) have reported that blackcurrant juice has an ascorbate sparing effect.

GRAPES AND WINES

Grapes (*Vitis vinifera* and *Vitis lubruscana*), especially the dark red varieties, contain generous amounts of flavonoids and relatively high levels also of hydroxycinnamates that all exert potent anti-oxidant activities in various assay systems. However, several of the phenolics present in fresh grapes and grape juice are also potent antioxidants in various *in vitro* assays, including several containing biologically relevant lipid substrates, notably human LDL. In fresh grapes and

Table 3. Inhibition (%) of human low-density lipoprotein (LDL) oxidation *in vitro* in a copper-catalysed system of selected fruits, berries, their juices and wine tested at the level of 10 mm

Fruits, berries, their juices or wines	% Inhibition	Reference(s)
Red and blush table grapes	22–49	Plumb *et al.*, 1996
Red wine grapes	39–60	Plumb *et al.*, 1996
Red grape juice (Concord)	68–70	Frankel *et al.*, 1998
Red wine	37–65	Frankel *et al.*, 1995
White table grapes	30	Plumb *et al.*, 1996
White wine grapes	44–46	Plumb *et al.*, 1996
White grape juice	71–75	Frankel *et al.*, 1998
White wine	25–46	Frankel *et al.*, 1995
Peaches, fresh	64–87	Garcia *et al.*, unpublished results
Peaches, canned	56–85	Garcia *et al.*, unpublished results
Prunes	82	Donovan *et al.*, 1998
Prune juice	62	Donovan *et al.*, 1998
Blackberries	84	Haila, 1999
Sweet cherries	71	Haila, 1999
Blueberries	65	Haila, 1999
Red raspberries	79	Haila, 1999
Strawberries	54	Haila, 1999

grape juices the polyphenolic compounds are primarily present as glucosides, while the phenolics in wines are principally aglycones. Depending on the variety, red grapes may contain about 100-4000 $mgkg^{-1}$ of anthocyanins, 5–285 $mgkg^{-1}$ flavonols, mainly rutin, 0–25 $mgkg^{-1}$ flavanols, 2–25 $mgkg^{-1}$ hydroxycinnamates, and very low levels of hydroxybenzoic. The levels of phenolics in white grapes are about 20–25 times lower than in dark red grapes, and white grapes do not contain anthocyanins (Meyer *et al.,* 1997).

Both fresh grapes and commercial grape juices are a significant source of phenolic antioxidants (Frankel & Meyer, 1998). Extracts of fresh grapes inhibited human LDL oxidation from 22 to 60% and commercial grape juices from 68 to 75% (Plumb *et al.,* 1996; Frankel *et al.,* 1998). The antioxidant activities of grapes and grape juices were comparable to those found for wines (Frankel *et al.,* 1995). The LDL antioxidant activity correlated highly with the concentration of total phenolics for both grape extracts and commercial grape juices, with the level of anthocyanins and flavonols for grape extracts, with the levels of anthocyanins for Concord grape juices, and with the levels of hydroxycinnamates and flavan-3-ols with the white grape juice samples (Frankel *et al.,* 1998). Grape extracts were also shown to inhibit formation of both hydroperoxides and hexanal in lecithin liposomes (Velioglu *et al.,* 1998).

According to Wang *et al.* (1996) grapes and grape juices also had high ORAC activities. A major anthocyanin pigment, malvidin- 3, 5-diglucoside (Meyer *et al.,* 1998), with antioxidant activity, was isolated from wild grapes (*Vitis coignetiae*) (Igarashi *et al.,* 1989). Anthocyanins with malvidin nucleus, especially malvidin-*O*-(6-*O*-*p* coumaroylglucosido)-5-glucoside (Kahkonen *et al.,* 1999), isolated from Muscat Bailey. A grape proved to be more effective than (+) catechin and α-tocopherol (Lanningham-Foster *et al.,* 1995). According to Meyer *et al.* (1998) phenolic antioxidants that were released from grape pomace using enzymes significantly retarded human LDL oxidation. Oxygen radical scavenger ability of procyanidins for superoxide and hydroxyl radicals was evaluated by Da Silva *et al.* (1991). In this study, procyanidin B2 3′-*O*-gallate (Vinson & Hontz, 1995), isolated from grape seeds was found to exert maximum antioxidant activity. Red wines, extracts of different types of fresh grapes, 'grape skin extract', American Concord grape juice, as well as European red grape juices, strongly inhibit human LDL oxidation *in vitro* and this antioxidant activity is associated with the phenolic compounds (Meyer *et al.,* 1997; Frankel *et al.,* 1998; Landbo & Meyer, 2001; Yi *et al.,* 1997; Abu-Amsha *et al.,* 1996).

Thus, not only has the antioxidant activity of similarly diluted grape samples been shown to be proportional to concentration of

total phenols, but in certain cases, the antioxidant potency also correlates to the levels of different classes of compounds. Extracts of fresh grapes also inhibit both development of lipid hydroperoxides and their degradation to produce hexanal in lecithin in liposomes, and the relative antioxidant potency is statistically correlated with the total phenols (Pratt & Watts, 1964). Compared to the data obtained on human LDL oxidation *in vitro,* the grape extracts exhibiting highest anti-oxidant activity on lecithin liposomes were those of the red table varieties (Red Globe and Emperor) and the white wine grape varieties (Chardonnay and Sauvignon Blanc); (Pratt & Watts, 1964) these extracts had only low antioxidant potency on human LDL oxidation *in vitro* (Meyer *et al.,* 1997). The removal of phenolic compounds by polyvinyl-polypyrrolidone stripping abolishes the antioxidant activity of grape juices and a mixture of representative carboxylic acids of red wine do not exert antioxidant activity (Pratt, 1965).

In contrast, addition of ascorbic acid to European red grape juice samples significantly increased the antioxidant activities of the red grape juices on human LDL oxidation *in vitro* (Abu-Amsha, *et al.,* 1996). The phenolic profile of Concord grape juice is dominated by anthocyanins, levels range from about 300-450 mgL^{-1}, (Yi *et al.,* 1997) where the dominant compound, which is also the major contributor to the dark, purple-bluish colour, is delphinidin-*O*-3-monoglucoside. Concord grape juice exerted the highest antioxidant activity among com-mercial fruit juices followed by grapefruit, tomato, orange and apple juice in the ORAC antioxidant assay (Wang *et al.,* 1996).

ANTIOXIDANTS FROM VEGETABLES

The antioxidants present in commonly consumed vegetables include ascorbic acid, tocopherols, carotenoids and phenolic compounds such as flavonols and phenolic acids (Table 4). In comparison to fruits and berries, vegetables generally contain much lower amounts of antioxidant compounds. A large amount of vitamin C is found in sweet red pepper (1850 $mgkg^{-1}$) and significant amounts in Brussels sprouts (up to 900 $mgkg^{-1}$) and broccoli (750-830 $mgkg^{-1}$), while the amounts of vitamin E are generally below 10 $mgkg^{-1}$ in vegetables. Vegetables such as root and tuberous crops (carrots, potatoes, sweet potatoes, red beets etc.), cruciferous vegetables (cabbage, Brussels sprouts, broccoli etc.), green leafy vegetables (lettuce, spinach etc.), onions, tomatoes and other vegetables have been screened for antioxidant activity using different oxidation systems (Pratt & Watts, 1964; Pratt, 1965; Al-Saikhan *et al.,* 1995; Cao *et al.,* 1996; Ramarathnam *et al.,* 1997; Gazzani *et al.,* 1998; Beom *et al.,* 1998; Hollman & Arts, 2000; Plumb *et al.,* 1997). In early studies Pratt and

Watts (1964) and Pratt (1965) found that green onion tops were twice more potent as antioxidants than potato peel, green pepper and green onion and four times more potent than potatoes in inhibiting the coupled oxidation of β-carotene and linoleic acid. Using the same oxidation model Gazzani *et al.* (1998) reported that when prepared at 2°C, most vegetable juices showed initial pro-oxidant activity. This pro-oxidant activity was very high for eggplant, tomato, and yellow bell pepper.

In the cases of carrot, celery, garlic, mushroom, zucchini, tomato, and particularly eggplant juice, it was reported that the antioxidant activity of the vegetables was increased by boiling. This suggests that the pro-oxidant activity was due to peroxidases which were inactivated at high temperature. Kahkonen *et al.* (1999) showed that at the level of 5000 ppm on the basis of the plant dry weight the order of antioxidant activity was as follows: pea, legume (37% inhibition) > cucumber, leaf (35%) > pea (28%) > onion (11%) > carrot (10%). Compared to the poor activity of these vegetables, the peel extracts of beetroot, sugar beet, and potato showed remarkable antioxidant activity ranging from 86 to 99% inhibition.

By measuring the oxygen radical absorbance capacity (ORAC), Cao *et al.* (1996) reported that the antioxidant score decreased in the following order: kale > garlic > spinach > Brussels sprouts > alfalfa sprouts > broccoli flowers >beets > red bell pepper > onion > corn > eggplant > cauliflower > potato >sweet potato > cabbage > leaf lettuce > string bean > carrot > yellow squash > iceberg lettuce > celery > cucumber. Results on spiking plasma with vegetable extracts showed that beans, garlic, onions, asparagus, beet, potato and broccoli ranked highest in inhibiting the oxidation of low density lipoprotein (LDL) and very low density lipoprotein (VLDL) fractions (Frankel *et al.*, 1998). Table 4 illustrates the sparse literature on antioxidant compounds identified in different vegetables.

According to Hussein *et al.* (2000) although there was significant loss in vitamin C during storage of broccoli and green peppers, in most cases there was no difference in loss of vitamin C or beta-carotene between the processed and unprocessed vegetables, and the packaging systems. Carotenoids contribute to antioxidant activity, with beta-carotene (1644 $mgkg^{-1}$) and lutein (up to 203 $mgkg^{-1}$ in spinach) present in all vegetables and lycopene dominating in tomatoes (0.2–623 $mgkg^{-1}$) and tomato products. As a result of food processing involving 'heat treatment carotenoids undergo isomerisation 7°C which may decrease their antioxidant activity. On the other hand thermal processing is reported to increase carotenoid concentration, owing to greater extractability, enzymatic degradation and unaccounted losses of moisture and soluble solids (Rodriquez-Amaya, 1997).

In fresh vegetables only glycosylated flavonols and other flavonoids are present but aglycones may be found as a result of food processing (Matt *et al.,* 2000). Quercetin levels in vegetables are generally below 10 $mgkg^{-1}$, except for onions (340-347 $mgkg^{-1}$), kale (110-120 mg kg^{-1}) and broccoli (30-166 $mgkg^{-1}$), while present at 500-1200 mg kg^{-1} dry weight, and chlorogenic acid dominates (Clifford, 2000; Rodriquez-Amaya, 1997). The phenolics are concentrated in the potato skins; red skinned cultivars harbour up to 7 gkg^{-1} of p-coumaryl-anthocyanin conjugates in the peels and around only 25% of this level in the flesh (Clifford, 2000) and pelargonidin-3-rutinoside-5-glucoside appears to be the dominant anthocyanin compound in red-fleshed potatoes (Clifford, 2000). Concentrated aqueous extracts of red and brown potato skins, respectively, contained up to 12.5 gkg^{-1} of hydroxycinnamates, and chlorogenic acid accounted for 60-65 wt% of these, followed by caffeic acid (22-24 wt%). Ferulic acid and protocatechuic acid are also among the major phenolic acids in potato peels (Onyeneho & Hettiarachchy, 1993).

Root and Tuberous Vegetables

Carrot (*Daucus carota*) has been reported to exert low antioxidant activity compared to other vegetables (Meyer *et al.,* 1998; Vinson & Hontz, 1995; Ramarathnam *et al.,* 1997; Gazzani *et al.,* 1998; Beom *et al.,* 1998; Plumb *et al.,* 1997). Carrots are very rich in alpha- and beta-carotenes that range in content from 4000–8700 μg per 100 g (alpha) and 7000–16000 μg per 100 g (beta) in different orange carrot varieties (Alasalvar *et al.,* 2001; Bureau & Bushway, 1986; Hart & Scott, 1995; Sang *et al.,* 1997). The major phenolic compound in carrots is chloro genic acid, but dicaffeoylquinic acids, and several other hydroxycinnamic quinic kaempherol has only been detected in kale (21-70 $mgkg^{-1}$), endive (15-90 $mgkg^{-1}$), broccoli (60 $mgkg^{-1}$) and leek (10-60 $mgkg^{-1}$) (Matt *et al.,* 2000). The content of other flavonoids in vegetables is very low with some exceptions such as flavanones in celery leaves (apigenin, 750 $mgkg^{-1}$) (Matt *et al.,* 2000) or anthocyanins in purple sweet potatoes (Graf, 1992). Extracts of carrot leaves and peel showed antioxidant activity towards oxidation of pure methyl linoleate at 40°C while the carrot flesh was inactive (Vinson & Hontz, 1995). Boiling carrots for 30 min significantly improved their antioxidant activity towards coupled oxidation of β-carotene and linoleic acid (Hollman & Arts, 2000). In addition, the most polar fraction of carrots was found to be pro-oxidative. In general, flavonol levels in processed foods are lower than in fresh products (Hertog *et al.,* 1992). All vegetables contain phenolic acids such as hydroxycinnamates where either caffeic acid, ferulic acid, sinapic acid or coumaric acid has been conjugated with quinic acid and/or esterified with for example sugars (Lewis *et al.,* 1998; Graf, 1992;

Table 4. Antioxidant compounds in selected vegetables and their products, mg kg^{-1} fresh weight

Vegetable	Flavonols (quercetin)	Hydroxy-cinnamates	Carotenoids (beta-carotene)	Vitamin C	Vitamin E
Broccoli- boiled	15-65 (Plumb *et al.*, 1997; Matt *et al.*, 2000; Van *et al.*, 2000)	62-148 (Matt *et al.*, 2000)	4-27 (Van *et al.*, 2000; Heinonen *et al.*, 1989)	750-830 (Rastas *et al.*, 1997) 640 (Ewald *et al.*, 1998)	79 (Finnish Food composition table) 7 (Ewald *et al.*, 1998)
Brussels sprouts	0-6 (Matt *et al.*, 2000)	–	4.3 (Rastas *et al.*, 1997)	900 (Ewald *et al.*, 1998)	4 (Ewald *et al.*, 1998)
Carrots-boiled	–	–	11-770 (Rastas *et al.*, 1997) 101 (Clifford, 2000)	60 (Ewald *et al.*, 1998) 42 (Ewald *et al.*, 1998)	4 (Ewald *et al.*, 1998) 4 (Ewald *et al.*, 1998)
Onions-blanched-fried	340-420 (Matt *et al.*, 2000) 210-290 (Rodriquez-Amaya, 1997) 220-370 (Rodriquez-Amaya, 1997)	–	0.1 (Rastas *et al.*, 1997) 0.2	75 (Ewald *et al.*, 1998) 57 (Ewald *et al.*, 1998)	0.4 (Ewald *et al.*, 1998) 8 (Ewald *et al.*, 1998)
Pea-boiled-fried	1.4-1.6 (Rodriquez-Amaya, 1997) 0.8-1.0 (Rodriquez-Amaya, 1997) 1.3-2.0 (Rodriquez-Amaya, 1997)	–	3.6 (Rastas *et al.*, 1997) 3.6 (Ewald *et al.*, 1998)	200 (Ewald *et al.*, 1998)	2 (Ewald *et al.*, 1998)

Table 4. *Contd.*

Vegetable	Flavonols (quercetin)	Hydroxy-cinnamates	Carotenoids (beta-carotene)	Vitamin C	Vitamin E
Potatoes-boiled	–	140 (Lewis *et al.*, 1998)	0.1 (Rastas *et al.*, 1997)	100 (Ewald *et al.*, 1998) 100 (Ewald *et al.*, 1998)	1 (Ewald *et al.*, 1998) 1 (Ewald *et al.*, 1998)
Spinach	Tr (Matt *et al.*, 2000)	–	8-240 (Rastas *et al.*, 1997)	600 (Ewald *et al.*, 1998)	12 (Ewald *et al.*, 1998)
Tomatoes-juice-ketchup	2-14 (Matt *et al.*, 2000) 13 (Matt *et al.*, 2000)	–	0.2-623 (Rastas *et al.*, 1997) 99 (Rastas *et al.*, 1997)	140 (Ewald *et al.*, 1998) 140 (Ewald *et al.*, 1998) 80 (Ewald *et al.*, 1998)	7 (Ewald *et al.*, 1998) 7 (Ewald *et al.*, 1998) 23 (Ewald *et al.*, 1998)
Sweet red pepper	–	–	1.2-33 (Ewald *et al.*, 1998)	1850 (Ewald *et al.*, 1998)	22 (Ewald *et al.*, 1998)

Plumb *et al.,* 1997). According to Clifford (2000), commercial varieties of American potato may contain up to 1400 $mgkg^{-1}$ dry weight caffeoylquinic acids. In broccoli several hydroxycinnamic acid esters have been isolated in amounts of 62–148 $mgkg^{-1}$ (Plumb *et al.,* 1997).

Cao *et al.* (1996) reported that the antioxidant score of vegetables measured by ORAC assay decreased in the following order: kale > garlic > spinach > Brussels sprouts > alfalfa sprouts > broccoli flowers > beets > red bell pepper > onion > com > eggplant > cauli-flower > potato > sweet potato > cabbage > leaf lettuce > string bean > carrot > yellow squash > iceberg lettuce > celery > cucumber. Results on spiking plasma with vegetable extracts showed that beans, garlic, onions, asparagus, beet, potato and broccoli ranked highest in inhibiting the oxidation of the LDL and VLDL fractions (Vinson *et al.,* 1998). On oxidation of pure methyllinoleate at 40°C, the antioxidant activity was the following: pea, legume > cucumber, leaf > pea > onion > carrot (Wang *et al.,* 1996). Compared to the poor activity (10-37% inhibition) of these vegetables in inhibiting lipid oxidation, the peel extracts of beetroot, sugar beet and potato showed remarkable antioxidant activity ranging from 86 to 99% inhibition. By measuring the ORAC, Gazzani *et al.* (1998) reported that when prepared at 2°C, most vegetable juices showed initial pro-oxidant activity. This pro-oxidant activity was very high for eggplant, tomato and yellow bell pepper. In general the antioxidant activity increased after heat treatment suggesting that the pro-oxidant activity is due to peroxidases which are inactivated at high temperature during food processing.

Potatoes contain ascorbic acid and are characterized by high levels of conjugated hydroxycinnamates, acid conjugates are also present; in total the level of conjugated hydroxycinna mates is about 1.6 mg kg^{-1} and ascorbic acid contents are 30–50 $mgkg^{-1}$ fresh carrot weight (Wang *et al.,* 1996). Methanolic extracts of peels of sugar beet and red beetroot contain the same total level of phenolics (about 4.2 mg g^{-1} dry weight of starting material) and exhibited strong antioxidant activities in pure methyllinoleate at 40°C, almost blocking oxidation (Kahkonen *et al.,* 1999). Betacyanins, the major colour compounds in red beets, were shown to exert potential antioxidant activities in various model systems (Kanner *et al.,* 2001).

Potato (*Solanum tuberosum*) is considered a good source of antioxidants such as ascorbic acid, α-tocopherol and polyphenolic compounds (Table 5) (Kahkonen *et al.,* 1999; Ramarathnam *et al.,* 1997; Onyeneho & Hettiaranchchy, 1993; Lugasi *et al.,* 1997). Potato peelings especially also show high antioxidant activity (Al-Saikhan *et al.,* 1995; Cao *et al.,* 1996; Vinson & Hontz, 1995; Rodriquez *et al.,* 1994; Rodriquez *et al.,* 1994). The active compounds isolated from

potatoes (Onyeneho & Hettiaranchchy, 1993; Rodriquez *et al.*, 1994) especially potato peelings (Table 5), and other root crops such as the Japanese vegetable, burdock (*Arctium lappa* L), (Rice-Evans *et al.*, 1996) are derivatives of caffeic acid (Block *et al.*, 1992) such as chlorogenic acid (Verlangieri *et al.*, 1985) or caffeoylquinic acid derivatives with sugar moiety. According to Hayase and Kato (1984) these phenolic compounds are responsible for enzymatic browning and act as antioxidants in sweet potatoes (*Iopomea batatas*). Burdock (Beom *et al.*, 1998) and sweet potato (Meyer *et al.*, 1998; Beom *et al.*, 1998) extracts were also reported to be highly active towards lipid oxidation. Purple potatoes and peel have been shown to exhibit greater antioxidant activities than the white and yellow varieties (Kahkonen *et al.*, 1999; Vinson & Hontz, 1995). This difference in antioxidant activity may result partly from the presence of anthocyanins such as pelargonidin-3-rutinoside-5-glucoside 3 identified as the dominant anthocyanin in red-fleshed potato varieties (Rodriquez-Saona *et al.*, 1998). Also an anthocyanin, peonidin glycoside, isolated from purple sweet potatoes was reported to exhibit strong antioxidant activity (Sang *et al.*, 1997). According to Al-Saikhan *et al.* (1995) patatin, a water-soluble glycoprotein, appeared to be the major water-soluble compound that showed antioxidant activity of potatoes.

Similarly to carrot and potato peel, beetroot peel (*Beta vulgaris* L) and sugar beet peel (*Beta vulgaris esculenta*) showed remarkably high antioxidant activities (Vinson & Hontz, 1995). Beet ranked eighth

Table 5. Antioxidant compounds identified in different vegetables

Vegetables	Antioxidative compounds	Reference(s)
Bell peppers	Quercetin	Cao *et al.*, 1996; Ramarathnam *et al.*, 1997
Cruciferous vegetables	Phenolic compounds	Plumb *et al.*, 1996; Meyer *et al.*, 1998; Fenwick *et al.*, 1989
Onions	Quercetin, allicin	Al-Saikhan *et al.*, 1995; Cao *et al.*, 1996; Prasad *et al.*, 1995
Potato	Caffeic acid derivatives, patatin, chlorogenic acid	Ramarathnam *et al.*, 1997; Onyeneho & Hettiaranchchy, 1993; Rodriquez *et al.*, 1994; Block *et al.*, 1992
Purple sweet potatoes	Peonidin glycoside	Sang *et al.*, 1997
Spinach	Phenolic compounds	Meyer *et al.*, 1998; Castenmiller, 2000

among 23 vegetables assayed for inhibition of LDL oxidation (Meyer *et al.,* 1998). Homogenized potatoes and sweet potatoes only exhibited medium ORAC compared with, for example, kale, garlic, spinach and onions (Cao *et al.,* 1996). According to Lugasi *et al.* (1999) ethanolic extracts of whole potatoes have been demonstrated to reduce oxidizing DPPH radicals and to inhibit linoleic acid oxidation in suspension. More concentrated extracts of potato peels efficiently retarded carotene bleaching coupled to linoleic acid oxidation, (Onyeneho & Hettiarachchy, 1993) and slowed the oxidation of soybean oil (active oxygen method) (Zhan,1996). Most of antiradical scavenging effects and antioxidant activities exerted by potatoes and potato extracts is due to the presence of chlorogenic, protocatechuic and caffeic acid (Zhan, 1996; Lugasi *et al.,* 1997). Methanolic extracts of sweet potatoes also exhibit antioxidant activity to retard linoleate oxidation. The phenolics in a methanolic sweet potato extract were identified mainly as caffeoylquinic acids, notably chlorogenic acid, and various 'iso' chiorogenic acids, but the antioxidant activity of this sweet potato extract was not directly related to the phenolic profile, being ascribed as a result of a synergistic action of both phenolic compounds and amino acids. Peonidin glucoside, an anthocyanin purified from purple sweet potatoes, also exhibited antioxidant activity on linoleate oxidation.

CRUCIFEROUS VEGETABLES

Broccoli (*Brassica olearacea* L. cv *Italica* L.), Brussels sprouts *(B. olearacea* L. *Gemmifera*), red cabbage (*B. olearacea* L. cv *Rubra*), white cabbage (*B. olear acea* L. cv *Alba*) and cauliflower *(B. olearacea* L. cv *Botrytis*) have been reported to show significant antioxidant properties against lipid peroxidation (Ramanathnam *et al.,* 1997). Phenolic compounds such as flavonols and hydroxycinnamic acids in the cruciferous vegetables may be responsible for the antioxidant activity rather than the main bioactive compounds in crucifers, namely glucosinolates (Plumb *et al.,* 1996; Fenwick *et al.,* 1989). In contrast, cabbage, cauliflower and Brussels sprouts were pro-oxidants towards lipid peroxidation in microsomes containing specific cytochrome P450s (Van Der Sluis *et al.,* 1999).

Kale (*B olearacea L cv Acephala*), Brussels sprouts and broccoli were found to exert higher antioxidant activity than cauliflower and other vegetables (Meyer *et al.,* 1998; Ramarathnam *et al.,* 1997; Gazzani *et al.,* 1998; Beom *et al.,* 1998). White cabbages was reported to show more than 80% inhibition of coupled oxidation of β-carotene and linoleic acid (Hollman & Arts, 2000) and it was also an active hydroxyl radical scavenger (Van Der Sluis *et al.,* 1999). According to Plumb *et al.* (1996) purified glucosinolates, exhibited only weak antioxidant properties and thus are unlikely to account for the antioxidant effects

of extracts from cruciferous vegetables. Food processing involving heat treatment seems to have different effects on various cruciferous vegetables depending on the choice of the antioxidant activity measurement. Boiled (15 min) Brussels sprouts were found to promote peroxidation of human liver microsomes and of phospholipid liposomes, (Van Der Sluis *et al.,* 1999) while boiled (5 min) broccoli exhibited 96% inhibition of oxidation of β-carotene linoleic acid emulsion. Swede peel (*Brassica napus rapifera*) was inactive towards oxidation of methyl linoleate.

ONIONS

The antioxidant activity of onion (*Allium cepa*) and onion scales has been studied in lipid oxidation models (Meyer *et al.,* 1998; Al-Saikhan *et al.,* 1995; Cao *et al.,* 1996; Ramarathnam *et al.,* 1997; Beom *et al.,* 1998; Hollman & Arts, 2000) and in radical scavenging assays (Meyer *et al.,* 1998; Gazzani *et al.,* 1998). Both yellow and red onion were shown to be poor antioxidants towards oxidation of methyl linoleate, (Kahkonen *et al.,* 1999) moderately active towards coupled oxidation of beta-carotene and linoleic acid (Wegh & Luyten, 1998) and highly active towards oxidation of lower density lipoproteins (Onyeneho & Hettiaranchchy, 1993). Onion had also a poor antioxidant score in the ORAC activity test while garlic (*Allium sativum* L) gave a score that was four times higher (Gazzani *et al.,* 1998). Yin and Cheng (1998) reported that the presence of garlic bulb, garlic greens, Chinese leek, scallion, onion bulb, and shallot bulb significantly delayed lipid oxidation of phosphatidylcholine liposomes. While allicin (Cao *et al.,* 1996) is responsible for the antioxidant activity of garlic bulb (Haibo *et al.,* 1999c) compounds other than allicin are involved in determining the antioxidant effect of other *Allium* members. According to Velioglu *et al.* (1998) anthocyanin-rich vegetables including red onion scales generally showed very strong activities towards oxidation of β-carotene linoleic acid model system. Similarly, green onion tops were reported to be twice as active as green onions with quercetin (Gazzani *et al.,* 1998) included in the antioxidant substances (Pratt, 1965; Cao *et al.,* 1996; Ramarathnam *et al.,* 1997).

TOMATO

The interest in tomato (*Lycopersicon esculentum*) is due to its high concentration of lycopene (Gazzani *et al.,* 1998) as well as phenolic compounds present. Tomato was reported to exert antioxidant activity in some studies (Vinson & Hontz, 1995; Meyer *et al.,* 1998) while in other experiments it acted as pro-oxidant (Gazzani *et al.,* 1998; Hollman & Arts, 2000). Among commercial juices tested, tomato juice has a higher oxygen radical absorbance capacity than orange and

apple juices (Wang *et al.,* 1996). In this study, tomato juice had much higher ORAC than the acetone extract of fresh tomatoes, which may be due to differences in the varieties of tomatoes used. In addition, it was not clear whether vitamin C was added to the commercial tomato juice. Antioxidant activity of tomato juice decreased after initial 2–5 h of heating but was restored after prolonged heating (Anese *et al.,* 1999).

In beef homogenates, tomato significantly inhibited lipid peroxidation (Plumb *et al.,* 1997). The antioxidant effect of tomato is most probably due to synergism between several compounds and it is not due to lycopene content alone as pure lycopene and several other carotenoids act as prooxidants in a lipid environment (Ramarathnam *et al.,* 1997; Haila *et al.,* 1996). Bell peppers have been shown to exert low antioxidant activity (Meyer *et al.,* 1998; Ramarathnam *et al.,* 1997; Gazzani *et al.,* 1998) or pro-oxidant activity (Hollman & Arts, 2000).

Other vegetables investigated for antioxidant activity include asparagus (Meyer *et al.,* 1998) celery (Meyer *et al.,* 1998; Gazzani *et al.,* 1998; Hollman & Arts, 2000), corn (Meyer *et al.,* 1998; Gazzani *et al.,* 1998) , cucumber (Meyer *et al.,* 1998; Vinson & Hontz, 1995; Hollman & Arts, 2000) eggplant, (Gazzani *et al.,* 1998; Hollman & Arts, 2000), pea (Vinson & Hontz, 1995) and zucchini (Hollman & Arts, 2000). In a study by Wenli *et al.* (2001) lycopene concentrate extracted from tomato paste containing 50% lycopene and 50% other lipid-soluble substances (probably including tocopherols) was shown to scavenge oxygen radicals effectively and to inhibit lipid peroxidation.

Lycopene in tomatoes seems to be more stable compared to other carotenoids to changes during peeling and juicing of vegetables. According to Anese *et al.* (1999) antioxidant activity of tomato juice decreased after an initial 2-5 h of heating but was restored after prolonged heating. Gazzani *et al.* (1998) report that while boiled vegetable juices were generally found to exert antioxidant activity, tomato juice was pro-oxidant. Apart from lycopene, another interesting antioxidant compound, naringenin chalcone, is present in tomato skin (64 $mgkg^{-1}$) and may be present in juice, paste and ketchup (Plumb *et al.,* 1997). In tomato processing to ketchup, naringenin chalcone is transformed to naringenin and wine (Rice-Evans *et al.,* 1996; Kahkokonen *et al.,* 2001; Yanishlieva-Maslarova *et al.,* 2001; Beom *et al.,* 1998). Also the antioxidant activity in tomato juice was comparable to that of fresh vegetables in most studies.

GREEN LEAFY VEGETABLES

The antioxidant activity of green leafy vegetables has been reported to be low: spinach (*Spinacia olearacea* L) ranked 18th and lettuce

(head) (*Lactuca sativa* L cv *Capita*) 22nd among 23 vegetables assayed for inhibition of LDL (Vinson *et al.,* 1998). On the other hand spinach expressed a very high ORAC activity while that of leaf lettuce and iceberg lettuce was poor (Gazzani *et al.,* 1998). Yet, according to Vinson *et al.* (1998) the phenols in spinach were able to enrich the lipoproteins by binding with them and subsequently protect them from oxidation. Moderate antioxidant activity of spinach was reported towards oxidation of linoleic acid (Beom *et al.,* 1998). Differently processed spinach samples were also found to inhibit formation of lipid hydroperoxides but to act as pro-oxidants in cooked meat (Castenmiller, 2000). Blends of two to four vegetables including spinach increased the inhibitory effect on lipid peroxidation, mainly due to the high levels of antioxidants in spinach. According to Beom *et al.* (1998) blending spinach with other vegetables resulted in increased antioxidant activity in iron-catalysed model systems. The antioxidant activity' of spinach decreased during storage after modified atmosphere packaging (MAP) which could be due to decrease in the ascorbic acid content (Castenmiller, 2000). Differently processed, that is, minced or enzymatically juiced spinach samples, were found to inhibit formation of lipid hydroperoxides but to act as pro-oxidants in cooked meat (Gil *et al.,* 1999).

The authors also reported a 50% loss of total flavonoids and 60% loss of vitamin C in the cooking water while boiling spinach. However, the vitamin C content of the cooked tissue was higher than in spinach stored in MAP.

Effect of Different Processing Technologies on Antioxidant Activity

As interest in functional foods and other products with possible health effects is escalating, a large number of industrial enterprises are now producing various 'antioxidant' concentrates. Industrial enterprises range from the traditional juice producers and large companies specializing in natural flavours and colours to new companies specializing in health promoting supplements. There is a scarcity of published knowledge available on the molecular composition and the proven health effects of most of these antioxidant concentrates, but many of them are nevertheless claimed and marketed as having potential physiological benefits, or at least to 'supply high amounts of antioxidants'. In addition, certain natural antioxidant phenols may act synergistically or even antagonistically, which further complicates predictions of antioxidant effectiveness of mixed concentrates. Therefore, marketing of most natural antioxidant concentrates is based only on empirical knowledge from tests in model systems.

Food processing involves changes in structural integrity of the plant material and this produces both negative and positive effects. When the negative and positive effects counterbalance each other, no change in the antioxidant activity occurs. The antioxidant activity is diminished owing to inactivation of antioxidant compounds caused by oxidation, for example, by enzymes (polyphenoloxidase and others) or leaching into the cooking water. Both negative changes have a greater impact on the water-soluble antioxidants, vitamin C, flavonoids and phenolic acids, than on the lipid-soluble antioxidants, carotenoids and tocopherols. The positive effects of food processing include transformation of antioxidants into more active compounds, such as the deglycosylation of onion quercetin, (Yanishlieva-Maslarova *et al.,* 2001) as well as an increase in the antioxidant activity owing to inhibition of enzymes (Onyeneho & Hettiaranchchy, 1993). Peeling and juicing result in substantial losses of carotenoids, anthocyanins, hydroxycinnamates and flavanols as the fruit and berry skins and vegetable peels are very rich in these antioxidant compounds. However, the antioxidant activity of fresh fruits and berries is comparable to that of their processed products such as juices, jam, jelly etc.

Food processing of fruits and berries into juices and jams, and drying of fruits generally result in lower amounts of antioxidant compounds. For example, losses of anthocyanins in juices and purees of strawberries, strawberry and blackcurrant syrups, cranberry juice, raspberry juice and wine have been reported by Miller *et al.* (2000); Lu & Foo (2000); Plumb *et al.* (1996); Frankel & Meyer (1998); Tamura & Yamagami 1994 as well as phenolic degradation during processing of apple juice. In domestic berry processing practices, a quercetin loss of 15% was observed in strawberry jam, 85% loss in blackcurrant juice, 40% loss in bilberry soup and 85% loss in lingo berry juice in their making procedures. Flavanols are effectively extracted into apple cider, blackcurrant juice and red wine, the amounts being higher than those of the raw materials. An increase in ellagic acid in raspberry jams was reported to occur; most likely owing to release of ellagic acid from ellagitannins with the thermal treatment, although according to Gil *et al.* (1999) ellagic acid content in strawberry jam was 80% that of unprocessed strawberries. As for other antioxidant compounds, peeling and juicing result in substantial losses of provitamin A carotenoids, often surpassing those associated with heat treatment (Spanos *et al.,* 1990).

Moreover, the stability of carotenoids differs in different foods even when the same processing conditions are used. Ascorbic acid of fruit juices such as orange, peach, grapefruit, pineapple, apple and mango juice is readily oxidized and lost during staying of the juices

with losses ranging from 29 to 41% when stored at room temperature for four months found a marked difference in the stability of ascorbate in green leafy vegetables when compared with fruits. For example, in spinach more than 90% of the ascorbate was lost within three days after harvest when stored at ambient temperature while losses in ascorbate during storage of blueberries, raspberries and strawberries were minimal.

Food processing such as juicing, involving juice extraction, heating steps and juice clarification treatment has an impact on the putative antioxidant composition as well as the antioxidant activity of berries. For instance, industrial clarification treatment of blackcurrant juice to remove cloud and sediments decrease the contents of the four major anthocyanins by 19-29%. Also the level of ascorbic acid and flavonols decreases, but the flavonols apparently relatively less than the other compounds (Kabasakalis *et al.,* 2000). When tested at equimolar doses of total phenols, the antioxidant activity on human LDL oxidation *in vitro* was improved after clarification treatment. This suggests that the overall composition of putative antioxidants in the blackcurrant juice improved, even though the total level of antioxidants decreased (Kabasakalis *et al.,* 2000).

Thus, to obtain a more comprehensive understanding of the effects of processing, it appears important to accompany antioxidant evaluations with detailed compositional studies of the putative antioxidants. Meyer *et al.* (1999); Makris and Rossiterl (2001) assessed the impact of domestic processing, including chopping, maceration and boiling on onion bulbs. While quercetin 3,4'- diglucoside and quercetin-4'-monoglucoside were virtually unaffected by chopping, boiling for 60 min caused overall flavonol losses of 20.6% in the onions. In contrast, Ewald *et al.* (1998) reported that the greatest loss of quercetin and kaempherol in onion took place during the peeling, trimming and chopping before blanching. Further processing by cooking, frying and warm-holding of blanched onion had only a small effect on flavonoid content. Chopping did not consider ably influence the antioxidant capacity of onion bulbs, but boiling did provoke notable changes measured by the coupled oxidation system of beta-carotene and linoleic acid (Makris & Rossiter, 2001). Boiling of juiced onion for 10 min resulted in pro-oxidant activity that was reversed into antioxidant activity with prolonged heat treatment (Gazzani *et al.,* 1998). On the other hand, incubation of pulped onion at 37°C resulted in improved antioxidant activity partly caused by the enzymatic (endogenous glycosidases and glycosyltransferases) conversion of quercetin diglucosides into the monoglucoside and aglycone forms (Wegh & Luyten, 1998). It was suggested that the increment of anti-oxidant activity through enzymes naturally present in vegetables could be used to replace food antioxidants.

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6

The Grape Fruit Flavanone, Naringin Reduces Ferric Ion Induced Oxidative Stress *In vitro*

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ABSTRACT

The effect of naringin, a grape fruit flavanone was studied on the ferric ion-induced oxidative stress in the mitochondrial fraction of mouse liver and $HepG_2$ cells in vitro. The iron was found to increase the oxidative burden in both the mitochondrial fraction and $HepG_2$ cells in vitro as evident by a rise in the lipid peroxidation, protein oxidation, DNA damage and depletion in glutathione concentration. Iron-treatment negatively altered various antioxidant enzymes including glutathione-S-transferase (GST), glutathione peroxidase (GSHpx), catalase and superoxide dismutase (SOD), whereas naringin supplementation led to an increase in the superoxide dismutase and catalase and reduction in DNA strand breaks, followed by enhanced DNA repair capacity in $HepG_2$ cells. Pretreatment of mitochondrial fraction and $HepG_2$ cells with naringin significantly reduced the iron-induced lipid peroxidation, protein oxidation, DNA damage followed by inhibition of the iron-induced depletion of GSH, GST, GSHpx, catalase and SOD. Naringin did not exhibit pro-oxidant activity as revealed by the ferric iron reduction assay. Iron free coordination site assay indicated that naringin was unable to occupy all the active sites of iron. These results suggest that the observed protective effect of naringin was because of the antioxidant nature of naringin. Naringin was able to share the burden of endogenous oxidant system, as it inhibited the iron-induced depletion of all important antioxidant enzymes as well as GSH.

Key words : Naringin, ferric-ion, protein oxidation, DNA oxidation, lipid peroxidation, glutathione

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INTRODUCTION

Iron is essential component of all cells, as it plays an important role in numerous redox reactions. It is critically involved in oxygen transport and in a variety of cellular processes ranging from respiration to DNA synthesis. The central position of iron in life processes is due to its flexible coordination chemistry and redox potential, which is fine tuned by coordinating ligands. However, these physical properties enable iron to be an essential factor for a wide range of proteins involved in controlled redox reactions and allow iron to be toxic when not carefully handled by proteins and shielded from surrounding media (Ryan & Aust, 1992). The toxicity produced by iron in biological systems generally is ascribed to the enhanced production of powerful oxidants and can cause severe damage to biological molecules (Halliwell & Gutteridge, 1984). In the presence of physiological reductants, iron can redox cycle between the two oxidation states, thereby generating the production of highly reactive oxygen species continuously (Halliwell & Gutteridge, 1985). Triplet dioxygen cannot directly react with biomolecules in the ground state; iron as well as other transition metals, can relieve the spin restriction of oxygen and dramatically enhance the oxidation rate (Miller *et al.*, 1990). To avoid excess oxidation, the cells maintain the concentration of free iron as low as possible. Under normal conditions iron levels are tightly controlled and iron-catalyzed free radical reactions are kept minimal. However, in some situations the iron balance may be disturbed either locally or systemically resulting its participation in Fenton chemistry (Hider & Singh, 1992; Reif, 1992; Nathan, 1995).

Free radicals have been implicated in a variety of diseases like cancer, atherosclerosis, acute hypertension, inflammatory diseases, transplantation injury and ageing. Small amounts of potentially toxic reactive oxygen species can be generated in eukaryotic cells by normal oxidase action and during the course of electron transport in mitochondria or microsomes (Halliwell & Gutteridge, 1990; Forman & Boveris, 1992). During electron transport to molecular oxygen, as well as in various hydroxylation reactions, toxic partial reduction products of oxygen may be formed. The most important are the superoxide anion and hydrogen peroxide, which are extremely reactive and capable of irreversible damage to various biomolecules in the presence of excess iron. Both superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) have their inherent toxicity, but cell damage ensues more rapidly when they react with iron (Ryan & Aust, 1992). Iron-mediated reduction of H_2O_2 by $O_2^{\bullet-}$ (reduction of Fe^{3+} by $O_2^{\bullet-}$ coupled with Fenton-type reduction of H_2O_2 by Fe^{2+}), gives rise to hydroxyl radical ($HO^{\bullet}$), an exceedingly strong and indiscriminate oxidant that can abstract allylic hydrogens, add (hydroxylate), or accept electrons,

depending on the target molecule (Halliwell & Gutteridge, 1984). Hydroxyl free radicals ($HO^{\bullet}$) generated by Fenton chemistry (H_2O_2/ iron) give rise to primary stage LOOHs. These LOOHs may undergo iron-mediated one-electron reduction and oxygenation to give epoxyallylic peroxyl radicals ($OLOO^{\bullet}$), which trigger exacerbating rounds of free radical-mediated chain reaction. The chain reaction is thought to contribute to lipid peroxidation, DNA damage and protein oxidation (Niedernhofer *et al.*, 2005).

Eukaryotic cells are equipped with a repertoire of primary and secondary defenses against lipid peroxidation and other deleterious effects of oxidative stress. Potentially lethal injury can occur if these defenses are overwhelmed (Jagetia *et al.*, 2003). Primary defenses are mainly preventative, whereas secondary defenses have a "back-up" protective role, which might typically involve excision/repair of any lesions that do develop. Primary cytoprotection relies on the scavenging/inactivation of reactive oxygen species or redox metal ions before lipid peroxidation takes place. Enzymes involved in primary cytoprotection include SOD, GSHpx, which scavenges H_2O_2 efficiently at relatively low concentrations and catalase, which scavenges H_2O_2 efficiently at relatively high concentrations.

Flavonoids occur ubiquitously in the plant kingdom and are common components of human diet (Graziani *et al.*, 1983). Flavonoids have been shown to exert structurally dependent, highly specific effects on a variety of enzymes and are able to interfere with numerous cellular processes, including growth and differentiation (Swiader & Zarawska, 1996). The diverse effects of flavonoid may relate to their structural similarity to ATP and hence to their ability to compete with ATP for binding to various enzymatic sites (Lin, 1996). Naringin or 4',5,7-trihydroxyflavanone 7-rhamnoglucoside, a glycoside is the predominant flavanone found in the grapefruit and related citrus species. Like most flavonoids, naringin has metal chelating, antioxidant, and free radical scavenging properties (Jung *et al.*, 1983; Kroyer, 1986; Chen *et al.*, 1990) and has been reported to offer protection against mutagenesis (Francis *et al.*, 1989) and lipid peroxidation (Maridonneau-Parini, 1986; Jagetia & Reddy, 2005). Naringenin, the aglycone is readily formed from naringin in humans. The ability of naringin and naringenin to inhibit certain isoforms of cytochrome P450 may account for its effects on procarcinogen activation and drug metabolism (Guengerich & Kim, 1990). Naringin has been reported to reduce radiation-induced chromosome damage (Jagetia & Reddy, 2002; Jagetia *et al.*, 2003) and inhibit benzo-a-pyrene-induced carcinogenesis in mice (Jagetia & Reddy, 2004). The present study was undertaken to investigate the effect of naringin in the ferric ion-induced oxidative stress in the liver mitochondrial fraction of mice and $HepG_2$ cells *in vitro*.

MATERIALS AND METHODS

Chemicals

Naringin and TCA were procured from Acros Organics Ltd, Belgium, while glutathione, 2-thiobarbituric acid, 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB), diethylenetriamine pentaacetic acid (DTPA), butylated hydroxytolune, cumene hydroperoxide, EDTA, 1-chloro-2, 4-dinitrobenzene, ethidium bromide, 2,4-dinitrophenyl hydrazine, guanidine hydrochloride and tetraethoxypropane were purchased from Sigma Chemicals Co. St. Louis, USA.

Naringin or 4',5,7-trihydroxyflavanone 7-rhamnoglucoside is one of the bitter principles present in the grape fruit (Fig 1).

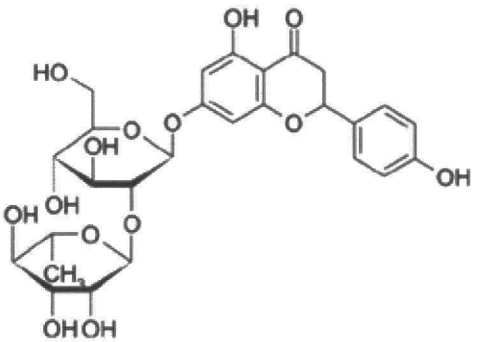

Fig 1. Chemical structure of naringin, 4',5,7-trihydroxyflavanone 7-rhamnoglucoside

Experimental

Evaluation of the Antioxidant Activity in Cell Free System

The antioxidant activity of naringin was evaluated in mitochondrial fraction isolated from mice.

Preparation of Mitochondria

The mice were euthanized and the livers were perfused with isotonic cold saline. The liver was homogenized in 0.25 M sucrose containing 1 mM EDTA. The homogenate was spun at 3000 g for 10 min to remove cell debris and nuclei. The supernatant was centrifuged at 10000 g using a Sorvall RC5C centrifuge to obtain mitochondria and washed three times with 0.05 M sodium phosphate buffer (pH 7.4) so as to remove last traces of sucrose. The final pellet was resuspended in phosphate buffer (Kamat *et al.*, 2000). The protein contents were

estimated by the modified method of (Lowry *et al.*, 1951). The mitochondrial fraction was grouped into Control group- without any treatment, Iron group – loaded with iron and Naringin iron group - treated with naringin before iron overload.

A pilot experiment was carried out with various concentrations of naringin using lipid peroxidation as the end point and 50 nm concentration provided the maximum reduction in iron-induced lipid peroxidation (data not shown) hence further studies were carried out using 50 nM naringin.

Generation of Iron-induced Free Radicals

Free radicals were generated using Fe^{3+} ions (Sreejayan & Rao, 1993). Briefly, 0.15 M KCl was added to liver microsomal fraction (0.5 mL), followed by the addition of 0 or 50 nm naringin. The peroxidation was initiated by adding 50 µM ferric chloride making the final volume up to 1.5 mL. Thereafter, the mixture was incubated for 0, 10, 20, and 30 min at 37°C. The samples were incubated and immediately processed for the following biochemical estimations:

Lipid Peroxidation

TBARS assay was performed according to the standard protocol (Gelvan & Saltman, 1990). Briefly, the samples were incubated with a mixture of trichloroacetic acid (15%), thiobarbituric acid (0.375%), and butylated hydroxytoluene (0.01%) in 0.25 N HCl at 95°C for 25 min. The reaction mixture was allowed to cool to room temperature and was centrifuged at 8,000 g. The supernatant was collected and the absorbance was recorded against the blank using a double beam UV-VIS spectrophotometer (Shimadzu Corporation, Japan). The lipid peroxidation has been expressed as TBARS that were determined against a standard curve prepared with tetraethoxypropane.

Protein Carbonyl Content

Protein carbonyl contents were estimated by the method of Palamanda and Kehrere (1992). Briefly, homogenate was incubated with dinitrophenyl hydrazine at room temperature for 30 min. After addition of 20% cold TCA, mixture was centrifuged and the pellet was mixed with guanidium hydrochloride. The OD was read at 280 nm and 370 nm.

DNA Oxidation

DNA oxidation was estimated by the method of Borkitt (1994). Briefly, samples were incubated with phosphate buffer and ethidium bromide.

The samples were analysed using a spectrofluorometer at an excitation of 510 nm and emission of 590 nm.

Glutathione

GSH content was measured by the method of Moron *et al.* (1979). Briefly, proteins were precipitated by 25% TCA, centrifuged and the supernatant was collected. The supernatant was mixed with 0.2 M sodium phosphate buffer pH 8.0 and 0.06 mM DTNB and incubated for 10 min at room temperature. The absorbance of the sample's was read against the blank at 412 nm and the GSH concentration was calculated from the standard curve.

Glutathione Peroxidase

Glutathione peroxidase was assayed by the modified method of Tappel (1978). Briefly, the mitochondrial samples were mixed with stock solution containing glutathione reductase, GSH, NADPH and incubated at 37°C for 5 min, followed by the addition of cumene hydroperoxide. The absorbance was recorded against the blank at 340 nm.

Glutathione-S-Transferase

Glutathione-S-transferase was assayed by the method of Habig *et al.* (1974). Briefly, the incubation mixture contained 0.1 M potassium phosphate buffer, 1 mM EDTA, glutathione reductase, 10 mM GSH, 12 mM *tert*-butyl-hydroperoxide and the samples were incubated for 10 min at 37°C. The absorbance was read against the blank at 340 nm.

Catalase

The catalase activity was estimated by catalytic reduction of hydrogen peroxide using the method of Abei (1984). Briefly, cumene hydroperoxide was added to the sample and was incubated at 37°C. The decomposition of hydrogen peroxide was monitored by recording the absorbance against the blank at 240 nm.

Superoxide Dismutase

Total SOD activity, was determined by the pyrogallol autooxidation method (Marklund & Marklund, 1974). Briefly, the mitochondrial sample was added to 62.5 mM Tris-cacodylic acid buffer, containing 1 mM diethylenetriaminepentaacetic acid (DTPA), followed by the addition of 4 mM pyrogallol. The autooxidation of pyrogallol was monitored against the blank at 420 nm.

Reduction of Ferric Ions

Reduction of Fe^{3+} to Fe^{2+} was measured by *O*-phenanthroline complex method (Rajkumar & Rao, 1993). Briefly, 50 µm *O*-phenanthroline, ferric chloride (1 mM), and various concentrations of naringin (5-1000 nM) was incubated for 10 min at room temperature. The absorbance of the resultant mixture was measured at 510 nM. The control consisted of 5 mM ascorbic acid instead of naringin and the absorbance obtained was considered as equivalent to 100% reduction of all the ferric ions present.

Presence of Free Coordination Site

Reaction mixture containing Fe^{3+} (0.6 mM), naringin (50-1000 nM), Tris HCl buffer pH 7.4 (50 mM) and sodium azide (50 mM) was scanned in the region of 350 – 500 nm (Graf & Bryant, 1984). A corresponding blank (without azide) and a control, where naringin was replaced by EDTA (5 mM) were scanned in the region of 350 – 500 nm.

Antioxidant Activity in Hepg2 Cells In vitro

Cell Line and Culture

$HepG_2$ cells obtained from National Centre for Cell Sciences (Pune, India) were used throughout this study. Cells were grown in Eagle's minimum essential medium (MEM) supplimented with 10% fetal calf serum, 1% L-glutamine and 50 µg/mL gentamicine sulfate. Cells were routinely grown in 25 cm^2 flasks with loosened caps in a humidified atmosphere of 5% CO_2 in air at 37°C in a CO_2 incubator. The cell cultures were grouped into Control group -without any treatment, Iron group –loaded with iron and Naringin+iron group – treated with naringin before iron overloading.

Iron Loading of $HepG_2$ Cells

Iron loading of $HepG_2$ cells using ferric citrate was performed as described previously (Cragg *et al.*, 1998). Ferric citrate solution was freshly prepared for each experiment. Cells were exposed to 0, 0.5, 1, 2.5, 5 and 10 mM naringin followed by the addition of 1 mm ferric citrate in media for 20 h. After exposure to ferric citrate, cells were washed twice with iron-free phosphate buffered saline (PBS) and exposed to 50 µM hydrogen peroxide in an iron-free media for 30 min at 37°C. After hydrogen peroxide exposure, cells were washed twice and harvested by trypsinization. Cells were homogenized in phosphate buffer. Lipid peroxidation, protein oxidation, DNA oxidation, GSH and antioxidant enzymes were estimated.

Effect of Iron and Naringin on DNA Strand Breaks and Repair

DNA strand breaks and repair was determined by the fluorometric analysis of DNA unwinding (FADU) method as described by Birnbiom and Jevcak (1981). Briefly, cells were exposed to naringin and iron. The DNA strand break and repair was evaluated at different time intervals where the cells were trypsinised and incubated with phosphate buffer containing myo-inositol, urea SDS and NaOH. Cells were sonicated for few seconds followed by the addition of ethidium bromide. The fluorescence was read using a spectrofluorometer (λ_{ex} 520 nm, λ_{em} 590 nm) at room temperature.

RESULTS

The lipid peroxidation, protein oxidation, DNA oxidation, glutathione and activities of enzymes like GSHpx, GST, catalase and SOD are shown as mean ± SEM (standard error of the mean) in Tables 1–7 and Figs 2-4.

Lipid Peroxidation

Treatment of mitochondrial fraction with ferric iron resulted in a time dependent elevation in the lipid peroxidation up to 30 min post-treatment (Table 1), whereas pre-treatment of mitochondrial fraction with 50 nM naringin reduced the ferric ion-induced lipid peroxidation significantly (Table 1). Ferric-iron elevated the lipid peroxidation in $HepG_2$ cells, whereas naringin (1 mM) pre-treatment significantly inhibited the iron induced lipid peroxidation significantly (Table 6).

Protein Carbonyl Content

Treatment of mitochondrial fraction with ferric iron resulted in a time dependent elevation in the protein carbonyl levels up to 30 min post-treatment (Table 2). The pre-treatment of mitochondrial fraction with 50 nM naringin reduced the ferric ion-induced protein oxidation. Ferric-iron elevated the protein oxidation in $HepG_2$ cells and 1 mM naringin significantly inhibited the iron induced protein oxidation (Table 6).

DNA Oxidation

Treatment of mitochondrial fraction with ferric ions resulted in a time dependent elevation in the DNA oxidation up to 30 min post-treatment (Table 3). Treatment of mitochondrial fraction with 50 nm naringin reduced the ferric ion-induced DNA oxidation. Ferric-iron elevated the DNA oxidation in $HepG_2$ cells while treatment of cells with 1 nM naringin significantly inhibited the iron induced DNA oxidation (Table 6).

Table 1. Effect of naringin on the iron-induced lipid peroxidation in mouse liver mitochondria *in vitro*

Treatment	Lipid peroxidation nM/mg protein ± SEM			
	Post treatment time periods (min)			
	0	10	20	30
Control	0.69 ± 0.03	0.76 ± 0.09	0.83 ± 0.11	0.75 ± 0.07
Fe control	0.73 ± 0.07	2.21 ± 0.13	2.89 ± 0.11	3.1 ± 0.6
NIN 50 nM	0.70 ± 0.09	0.81 ± 0.05	0.80 ± 0.07	0.73 ± 0.2
Fe+ NIN 50 nM	0.71 ± 0.13[a]	1.89 ± 0.09**	2.26 ± 0.19**	2.52 ± 0.3*

Values are mean ± Standard error of the mean (SEM) of four experiments. [a]$p>0.05$ non significant; *$p<0.01$; **$p<0.01$ compared with respective iron treated group.

Glutathione

The introduction of iron to mitochondrial fraction caused a time dependent depletion in the GSH concentration that reached a nadir at 30 min post-treatment. However, 50 nM naringin arrested this decline significantly, when compared with the non-drug treated group (Table 4). In $HepG_2$ cells the iron overload depleted the GSH concentration significantly. The pre-treatment of $HepG_2$ cells with 1 mm naringin significantly elevated the cellular glutathione levels when compared with iron treated group (Table 6).

Glutathione Peroxidase

The presence of iron drastically reduced glutathione peroxidase, this reduction was approximately 2 fold when compared to non-iron treated

Table 2. Effect of naringin on the iron-induced protein carbonyl levels in mouse liver mitochondria *in vitro*

Treatment	Protein oxidation nM/mg protein ± SEM			
	Post treatment time periods (min)			
	0	10	20	30
Control	0.99 ± 0.06	1.01 ± 0.21	0.98 ± 0.09	0.96 ± 0.10
Fe control	1.01 ± 0.10	1.19 ± 0.08	1.42 ± 0.15	1.76 ± 0.16
NIN 50 nM	0.98 ± 0.21	0.97 ± 0.1	1.01 ± 0.12	0.98 ± 0.08
Fe+ NIN 50 nM	1.07 ± 0.31[a]	1.18 ± 0.12[a]	1.40 ± 0.11[a]	1.66 ± 0.09[a]

Values are mean ± Standard error of the mean (SEM) of four experiments. [a]$p>0.05$ non significant compared with respective iron treated group.

Table 3. Effect of naringin on the iron-induced DNA oxidation levels in mouse liver mitochondria *in vitro*

Treatment	% Undamaged double stranded DNA ± SEM			
	Post treatment time periods (min)			
	0	10	20	30
Control	99 ± 0.001	99 ± 0.004	99 ± 0.002	99 ± 0.01
Fe control	99 ± 0.005	92 ± 0.02	80 ± 0.03	76 ± 0.005
NIN 50 nM	98 ± 0.001	99 ± 0.001	99 ± 0.004	99 ± 0.001
Fe^{+} NIN 50 nM	99 ± 0.009[a]	93 ± 0.009*	84 ± 0.04*	82 ± 0.01*

Values are mean ± Standard error of the mean (SEM) of four experiments.
[a]$p>0.05$ non significant; *$p<0.001$ compared with respective iron treated group.

fraction. The naringin treatment inhibited this inactivation of the enzyme thus increasing the availability of reactive enzyme species (Table 5). Iron overload significantly reduced the GSHpx levels in $HepG_2$ cells when compared with control group. Naringin did not alter the GSHpx levels in $HepG_2$ cells when compared with control group. Treatment with 1 mM naringin before iron overload significantly increased the GSHpx levels when compared with iron treated group (Table 6).

Glutathione-S-Transferase

The presence of iron in the mitochondrial fraction reduced the activity of GST by 2.7 fold when compared with the non-iron treated fraction. Addition of 50 nM naringin to mitochondrial fraction protected against

Table 4. Effect of naringin on the iron-induced glutathione levels in mouse liver mitochondria *in vitro*

Treatment	Glutathione nM/mg protein ± SEM			
	Post treatment time periods (min)			
	0	10	20	30
Control	14.9 ± 1.1	14.1 ± 0.9	14.5 ± 0.5	14.1 ± 0.9
Fe control	13.2 ± 1.9	9.13 ± 1.8	7.12 ± 1.0	6.93 ± 1.7
NIN 50 nM	14.5 ± 0.98	13.9 ± 1.21	14.54 ± 0.39	14.30 ± 0.9
Fe^{+} NIN 50 nM	13.61 ± 1.7[a]	13.1 ± 0.82*	12.2 ± 0.9**	11.3 ± 1.23*

Values are mean ± Standard error of the mean (SEM) of four experiments.
[a]$p>0.05$ non significant; *$p<0.05$ **$p<0.001$ compared with respective iron treated group.

Table 5. Effect of naringin on the iron-induced depletion of antioxidant enzymes in mouse liver mitochondria *in vitro*

Treatment	GSHpx	GST	Catalase	SOD
Control	176.3 ± 2.11	4.98 ± 0.48	82.31 ± 2.87	4.98 ± 0.65
Fe control	91.25 ± 3.12	1.83 ± 0.75	35.65 ± 3.14	1.53 ± 0.87
NIN 50 nM	168.24 ± 0.98	4.87 ± 1.15	80.12 ± 2.11	4.78 ± 1.12
Fe+ NIN 50 nM	125.7 ± 4.50*	2.68 ± 1.26[a]	52.43 ± 2.11*	2.90 ± 0.74[a]

Values are mean ± Standard error of the mean (SEM) of four experiments.
[a]p>0.05; *p<0.001 compared with respective iron treated group.
GSHpx - nm of GSH utilized/min/mg protein, GST-µm CDNB formed/min/mg protein, catalase -µm H_2O_2 ecomposed/min/mgprotein & SOD-Units/mg protein.

the iron-induced inhibition in the GST activity The presence of naringin did not allow the loss of GST activity, which was 2 folds greater than the iron-treated group (Table 5). Iron overload significantly reduced the GST levels in $HepG_2$ cells when compared with control group, whereas naringin alone did not alter the GST levels when compared with control group. Treatment of $HepG_2$ cells with 1 mM naringin before iron treatment significantly elevated the cellular GST levels when compared with iron treated group (Table 7).

Catalase

The activity of catalase was found to be inhibited by iron overload, where the activity of catalase was reduced to less than half of the control value (Table 5). The introduction of naringin arrested the iron-induced inactivation of catalase. Treatment of $HepG_2$ cells with naringin elevated catalase levels significantly in non-iron treated

Table 6. Effect of naringin on the iron-induced alteration in biomolecules and glutathione levels in $HePG_2$ cells *in vitro*

Treatment	TBARS	Carbonyls	DNA	GSH
Control	3.56 ± 0.4	1.03 ± 0.20	96 ± 0.01	52.12 ± 4.2
Fe control	10.35 ± 2.13	2.31 ± 0.32	59 ± 0.03	26.14 ± 1.89
NIN 1 mM	3.24 ± 1.19	0.91 ± 0.11	98 ± 0.01	50.01 ± 3.20
Fe+ NIN 1 mM	5.46 ± 2.74[a]	1.56 ± 0.06*	76 ± 0.09**	37.81 ± 2.99*

Values are mean ± Standard error of the mean (SEM) of four experiments.
[a]p>0.05 non significant; **p<0.001 *p<0.05 compared with respective iron treated group.
TBARS-(nmols/mg protein), carbonyls-(nmol/mg protein), % undamaged double stranded DNA & GSH-(nmol/ mg protein).

group while iron overload significantly reduced the catalase activity in $HepG_2$ cells when compared with control group. The naringin (1 nM) treatment significantly elevated the cellular catalase levels when compared with iron treated group (Table 7).

Superoxide Dismutase

Addition of iron to mitochondrial fraction induced significant decline in the SOD activity and this decline was 3.25 folds, when compared to the untreated fraction. Treatment of mitochondrial fraction with naringin *in vitro* arrested the iron-induced decline in SOD activity (Table 5). Naringin itself increased the SOD activity in non-iron treated $HepG_2$ cells significantly when compared with control group. Iron overload significantly reduced the SOD activity in $HepG_2$ cells when compared with control group, whereas treatment of $HepG_2$ cells with naringin before iron overload significantly elevated the cellular SOD levels when compared with the iron treated group (Table 7).

Reduction of Ferric Ions

The evaluation of the reduction of ferric iron to ferrous iron showed that naringin was unable to reduce ferric iron to ferrous iron (Fig 2).

Iron Free Coordination Site

Spectroscopic determination of azide binding is a simple and reliable method for predicting the oxidative reactivity of Fe^{3+} (Graf & Bryant, 1984). Naringin complexed with Fe^{3+} and the spectra of iron-naringin complex showed a shift, which was mainly due to displacement of

Table 7. Effect of naringin on the iron-induced changes in antioxidant enzymes in $HePG_2$ cells *in vitro*

Treatment	GSHpx	GST	Catalase	SOD
Control	256.8 ± 6.81	7.23 ± 0.98	138.1 ± 3.24	5.38 ± 1.10
Fe control	172.5 ± 4.12	5.32 ± 1.14	72.61 ± 4.11	2.34 ± 1.71
NIN 1 mM	260.2 ± 6.41	7.50 ± 0.54	152.3 ± 2.61	7.11 ± 0.90
Fe+NIN 1 mM	170.2 ± 3.19[a]	5.9 ± 0.90[a]	109 ± 3.59**	3.21 ± 0.98*

Values are mean ± Standard error of the mean (SEM) of four experiments. $p>0.05$ non significant; $^{**}p<0.001$ $^{*}p<0.05$ compared with respective iron treated group.
GSHpx - nm of GSH utilized/min/mg protein, GST-µm CDNB formed/min/mg protein, catalase -µm H_2O_2 ecomposed/min/mg protein & SOD-Units/mg protein.

water molecule from the coordination site by azide. Such a shift was observed with ligands such as EDTA and EGTA which have free coordination site. The naringin has similar effect on iron-coordination site as was observed for EDTA and EGTA (Fig 3).

DNA Strand Breaks and Repair

The DNA strand breaks induction was expressed as percent remaining double stranded DNA (Fig 4). Ferric iron induced DNA strand breaks in a time dependent manner and a maximum number of DNA strand breaks were observed at 24 h in $HepG_2$ cells treated with ferric iron. Thereafter, DNA strand breaks showed reparation of iron-induced damage that progressed steadily up to 72 h the last time period evaluated, where the numbers of breaks were few (Fig 4). The pattern of DNA strand breakage was similar in naringin pretreated group except that naringin treatment significantly reduced the iron-induced DNA breaks in $HepG_2$ cells and the repair was also higher when compared with the iron treatment (Fig 4).

DISCUSSION

Free radicals are important intermediates in natural processes, which are involved in progression of several diseases including aging, cytotoxicity, mutagenesis and carcinogenesis (Gutteridge & Halliwell, 2000). The intracellular levels of iron, is critical in defining the extent of hydroxy radical production from superoxide and hydrogen

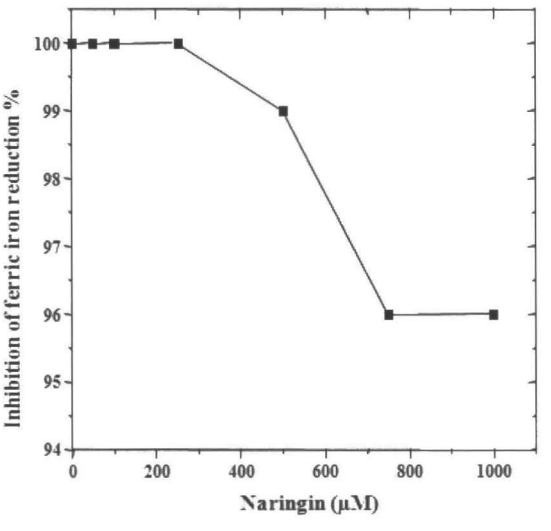

Fig 2. Effect of various concentrations of naringin on the reduction of Fe^{3+} to Fe^{2+} in cell free system

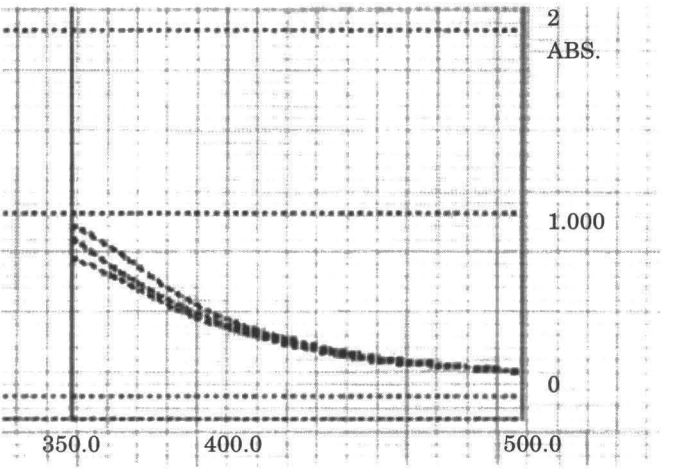

Fig 3. Effect of naringin on the spectrophotometric determination of iron free coordination sites *in vitro*. Upper curve-EGTA, middle curve-naringin and lower curve-EDTA

peroxide. Iron-induced oxidative stress is involved in aging (Herman, 2001), heart and cardiovascular diseases (Stevens *et al.*, 2002), gastrointestinal tract disorders, diabetes, cataractogenesis, degenerative retinal damage, autoimmune nephrotic syndromes, heavy metal nephrotoxicity, Parkinson's and Alzheimer's diseases

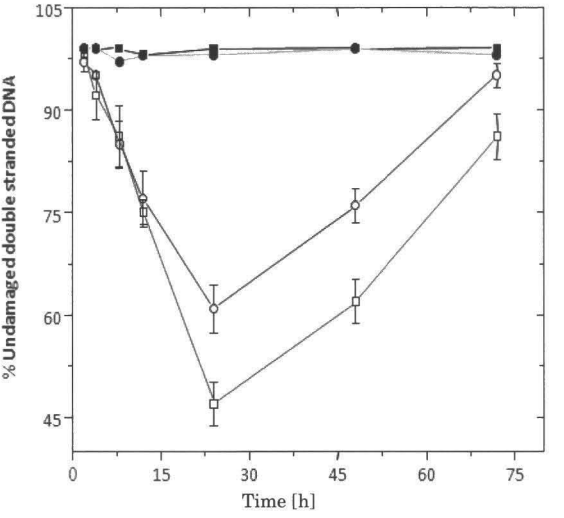

Fig 4. Alteration in the iron-induced DNA strand break and repair by naringin. Solid suqares(■) non-drug treated control; Open squares(□) iron Control; Solid circles(●) Naringin alone and Open circles(o) Naringin^{+} iron

(Halliwell, 2001), bronchopulmonary dysphasia (Repine *et al.*, 1997), and ischemia reflow states (Young & Woodside, 2001). Cells are equipped with a repertoire of endogenous antioxidant defense machinery to protect themselves from metabolic calamities (Beckman & Ames, 1998). These include hydrophilic radical scavengers such as GSH, urate and ascorbate, enzymatic scavengers such as SOD, catalase and GSHpx. In several circumstances like carcinogenesis, radiation exposure, diabetes and many other pathological conditions, they all are burdened with excess oxidative stress that forces the cells to undergo oxidative cellular damage. In such situations cells may require exogenous supply of antioxidants to reinforce their antioxidant mechanism as endogenous supply may not be enough to combat sudden oxidative stress. The natural products and certain dietary ingredients may be helpful to relieve the oxidative stress of the cells. It is essential to screen the antioxidant properties of certain natural products and/or dietary ingredients, which can share the burden of intracellular antioxidant machinery. Therefore, any drug which can reduce the iron-induced damage may be of potential value in one or more of these disorders. Naringin, a citrus flavanone has been evaluated for its ability to reduce the iron-induced oxidative damage *in vitro*.

Mitochondria plays a central role in energy metabolism within the cell, and mitochondrial dysfunction leads to various neurodegenerative disorders and to the so-called "mitochondrial diseases". A vast amount of evidence points to the implication of mitochondria in such complex processes as apoptosis and cardioprotection. The identification of mitochondria as primary or secondary targets of a drug may help to better understand the drug's mechanism of action and open new perspectives for its application. Oxygen radicals are destructive to a variety of cell components including lipid membranes and produce peroxidation of lipids. Therefore, lipid peroxidation has been used as an indirect measure of oxidative stress. The end products of stable aldehydes reacts with thiobarbituric acid (TBA) to form thiobarbituric acid-malondialdehyde adduct (Girotti, 1990). Naringin significantly inhibited the ferric ion-induced lipid peroxidation in mitochondrial fraction as well as $HepG_2$ cells. Compounds that are able to scavenge free radicals and/or chelate iron can protect the cells from reactive oxygen species-induced lipid peroxidation (Halliwell *et al.*, 1987). Our study has revealed that inhibition of lipid peroxidation by naringin is mainly by free radical scavenging and antioxidant activity but not through iron chelating activity. Naringin has been reported to inhibit lipid peroxidation in brain and kidney and irradiated liver (Ng *et al.*, 2001; Jagetia & Reddy, 2005). Similarly, naringin has been reported to inhibit the H_2O_2 induced lipid peroxidation (Kanno *et al.*, 2003). The flavonoids

including rutin and quercetin have been reported to inhibit the iron-induced lipid peroxidation by chelating iron ions (Afanas'ev *et al.*, 1989). The other plant flavonoids, like *baicilein, luteolin*, naringenin, and quercetin have also been found to suppress the Fenton reaction characteristic of the iron-ATP complex (Cheng & Breen, 2000).

Byproducts of lipid peroxidation and free radicals may cause further damage to important biomolecules like proteins and DNA. Therefore, we have further evaluated the oxidative damage in proteins and DNA. Although it is generally far less monitored than lipid peroxidation as a marker of oxidative damage, oxidative damage to proteins and DNA is also very critical. Both can nonspecifically bind iron either as a ferrous or as a ferric form and therefore undergo site-specific damage. Radical mediated protein oxidation was measured by the estimation of generic marker of protein oxidation *i.e.* carbonyl contents. Hydrogen abstraction at the α-amino carbon occurs with a subsequent single electron transfer from cation radical to ferric iron, leading to an imminium cation and ferrous iron leading to the formation of aldehyde derivative due to spontaneous hydrolysis. Ferrous iron is bound to a target molecule, and hydroxyl radicals produced by a reaction with hydrogen peroxide reacts very closely to the metal binding sites according to so called site specific Fenton reaction. This type of damage is insensitive to inhibition by hydroxyl radical scavengers. It must be pointed out that such a mechanism is catalytic and thus can be repeated on other target molecules or on other sites of the same macromolecule. Ferric iron- induced free radicals caused protein oxidation in both mitochondrial fraction and $HepG_2$ cells. Protein oxidation is known to give rise to alterations to both the backbone and side chains of the molecule; which leads to the denaturation and loss of biological activities of various important proteins, leading to cell death. Naringin reduced the protein oxidation in both mitochondrial and $HepG_2$ cells. This could be one of the reasons of observed inhibitory activity of naringin against the oxidative stress induced by ferric iron.

Oxidative DNA damage refers to the functional or structural alterations of DNA resulting from the insults of ROS. Because of its polyanionic nature, DNA is known to bind various metal ions and is therefore especially prone to iron-dependent site-specific oxidative damage. Oxygen free radicals induce a variety of lesions in DNA, including oxidized bases, abasic sites and DNA strand breaks. Naringin significantly reduced the oxidative DNA damage induced by ferric iron. This DNA oxidation assay is based on the fact that a highly fluorescent complex is formed between native DNA and the intercalating agent ethidium bromide. When DNA is modified following exposure to free radicals, the intercalation by ethidium bromide is

disrupted and the fluorescence of the ethidium bromide-DNA complex is compromised. Several forms of DNA lesions including strand scission, base oxidation and base liberation are believed to contribute to the loss of fluorescence. Hence the assay is not specific for any single lesion. Therefore, the DNA oxidation study was further confirmed by FADU assay. The alkaline unwinding assay is closely related to the comet assay and can measure the induction and rejoining of DNA strand breaks (Birnboim & Jevcak, 1981). The naringin pretreatment reduced the ferric iron-induced DNA strand breaks significantly. This reduction in DNA strand breaks by naringin may be due to its ability to scavenge free radicals and also its antioxidant activity. Naringin has been reported to inhibit the H_2O_2 induced DNA damage (Kanno *et al.*, 2003). The naringin treatment has also been reported to reduce the radiation-induced chromosome damage in mice bone marrow and scavenge free radicals *in vitro* (Jagetia & Reddy, 2002; Jagetia *et al*, 2003).

The mechanism of inhibition of iron-induced damage by naringin was further evaluated by estimating antioxidant status, iron free coordination site and reduction of ferric iron. Glutathione is an abundant and ubiquitous antioxidant, a tripeptide and essential biofactor synthesized in all living cells. It functions mainly as an effective intracellular reductant (Rahman & MacNee, 1999). It protects cells from free radical mediated damage caused by drugs and ionizing radiation. It forms an important substrate for GSHpx, GST and several other enzymes, which are involved in free radical scavenging (Meister & Anderson, 1983; Brigelius-Fhole, 1999). Naringin inhibited the iron-induced decline in glutathione in the mitochondrial fraction as well as $HepG_2$ cells. A similar effect has been observed earlier in irradiated liver (Jagetia & Reddy, 2005). Similarly, caffeine another natural product has been reported to protect the mitochondrial fraction against the radiation induced GSH decline *in vitro* (Kamat *et al.*, 2000). Certain pro-glutathione agents like alpha-lipoic acid and N-acetyl cysteine have been found to possess sparing effect on GSH levels and protect cells from glutamate insult (Kobyashi *et al.*, 2000). Treatment of rats with a lignan-enriched extract of the fruit of *Schizandra chinensis* enhanced the hepatic antioxidant/detoxification system, as indicated by increase in the hepatic reduced glutathione (GSH) level as well as hepatic glutathione reductase and glutathione-S-transferase activities (Ip *et al.*, 1996). GSH participates non-enzymatically and enzymatically (GST) in the protection against toxic compounds. The presence of naringin would have taken the burden upon itself thereby sparing the GSH depletion. This helped the mitochondria and $HepG_2$ cells to overcome the iron-induced oxidative stress. An earlier study has reported that the antioxidant activity of naringin is similar to that of GSH (Cheng & Breen, 2000).

Abstraction of hydrogen from methylic group results in the formation of a carbon centered free radical, which reacts rapidly with molecular oxygen to generate peroxyl radical. Peroxyl lipid can initiate a chain reaction of lipid peroxidation leading to the formation of peroxides. These destructive membrane lipid byproducts may cause further damage to important biomolecules including proteins and DNA (Lim *et al.*, 2004). The GST acts like a peroxidase and remove the stable peroxides from the system resulting in the reduction in the peroxide induced damage (Jeon *et al.*, 2001). Iron compounds can increase the rate of lipid peroxidation cycle. Naringin has arrested the iron-induced decline in GST and thus reducing the iron-induced damage. An identical effect has been observed earlier, where naringin arrested radiation-induced decline in GST activity in mouse liver (Jagetia & Reddy, 2005). Superoxide and hydrogen peroxide are important byproducts in usual cellular energy metabolism. As such they are not highly toxic but uncompartmentalized excess iron can initiate the formation of $HO^{\bullet}$ radical and can influence lipid peroxidation via Fenton/Haber-Weiss reaction (Agarwal & Kale, 2001). Cells are equipped with an impressive repertoire of antioxidant enzymes, such as superoxide dismutase, which hastens the dismutation of $O_2^{\bullet-}$ to H_2O_2 and catalase and glutathione peroxidase, which convert H_2O_2 into water. SOD brings first line of defense against free radicals by dismutating toxic superoxide into less toxic hydrogen peroxide. SOD works in concert with other H_2O_2 removing enzymes (Cohen, 1986).

SOD is also required for the growth of aerobes without excessive DNA damage in the presence of superoxide. Selenium containing GSHpx decomposes H_2O_2 and other peroxides which initiate free radical chain reaction. Catalase heme enzyme, brings the decomposition of high amounts of H_2O_2 and other peroxides. SOD, GSHpx and catalase in concerted action protect the oxidative attack of superoxide and hydrogen peroxide in the cells. Naringin has arrested the iron-induced depletion of SOD, GSHpx, and catalase in mitochondrial fraction and $HepG_2$ cells (Table 7). Earlier studies from this laboratory have shown a similar effect in irradiated liver of mouse (Jagetia & Reddy, 2005). Naringin, has been reported to play an important role in regulating antioxidative capacities by increasing the SOD, GSHpx and catalase activities by up-regulating the gene expressions of SOD, catalase, and GSHpx and protecting the plasma vitamin E (Jeon *et al.*, 2001, 2002). Naringin has been reported to prevent H_2O_2 induced cytotoxicity, apoptosis and genotoxicity (Cheng & Breen, 2003). It has also been found to scavenge superoxide anions generated by phenazin methosulphate-NADH system (Chen *et al.*, 1990). Naringin has been reported to scavenge free radicals *in vitro* (Jagetia *et al.*, 2003).

Certain plant based flavonoids have been reported for their pro-oxidant nature, when flavonoids reduce transition metal ions, it is assumed that these flavonoids can exert pro-oxidant effects by promoting Fenton or Haber-Weiss reactions. Therefore, an attempt was made to reveal the pro-oxidant nature of naringin if any. The ability of naringin to reduce ferric to ferrous ions was tested, and it was observed that naringin could not reduce ferric ions to ferrous ions indicating that it does not exert pro-oxidant effect. Flavonoids including myricetin and quercetin possess a high Fe^{3+} reducing activity, providing evidence for the importance of the simultaneous presence of both the catechol group in the B-ring and the 3-hydroxyl group in C-ring. The presence of the 2,3-double bond in conjugation with the 4-oxo group in the C-ring is also particularly important for Fe^{3+} reducing activity. Naringin lacks the catechol group in the B-ring, 3-hydroxyl group in C-ring and the presence of 2,3-double bond (Chen & Breen, 2000). Therefore, it is concluded that naringin as such does not have prooxidant activity. Naringin has been unable to completely inhibit the generation of ferric iron-induced free radical damage. This may be due to the incomplete binding of naringin with iron. To prove our point further we have evaluated the iron free coordination site. By definition chelation requires the presence of two or more atoms on the same molecule capable of metal binding, forming a coordinate bond, the interaction between an electron donor and an electron acceptor. Depending on the number of covalently linked donor groups associated with the chelating agent, varying stoichiometry of metal-ligand can be found in order to satisfy the coordination requirement of the metal ion. Both ferrous and ferric ions have a coordination number of six, *i.e.* most of complexes are octahedral. Bidentate ligands therefore form 3:1 complexes, whereas hexadentate ligands form 1:1 complexes. However, some ligands may also form polynuclear chelates. The stability of metal chelate in solution is influenced by the number of donor groups present on the same molecule according to the so-called chelate effect *i.e.* hexadentate ligands form more stable complexes than the corresponding bidentate or tridentate ligands. Spectroscopic determination of azide binding studies revealed that naringin forms a complex with iron and even in this complexed form, iron still retains its catalytic properties, because of the presence of free coordination sites. Many chelating agents such as EDTA, EGTA, nitrolotriacetic acid etc., behave in a similar manner. However chelating agents like desferrioxamine and phytic acid etc., will completely block the iron free coordination sites and they will inhibit the generation of iron-catalysed free radicals. The metal chelating sites with in a flavonoid molecule containing hydroxyl groups at 3, 5, 3', and 4', positions. The 2,3-double bond increases the planarity of the molecule and confers higher rigidity to

the C-ring and holds the A and C rings in a more coplanar position allowing the 3-hydroxyl/4-oxo groups and 5-hydroxyl/4-oxo groups to be closer. Lack of 2,3 bond in C-ring and hydroxyl groups in naringin could not arrest the generation of iron catalysed free radicals because of the functionally active free coordination sites.

In conclusion, naringin can inhibit the adverse effects of ferric ion induced oxidative stress, protein and DNA damage and may protect the cellular environments form free radical damage. Therefore, naringin, a grapefruit flavanone present in citrus fruits, may be of a great potential for use in inhibiting the oxidative stress in man.

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7

Scavenging Capacity of *Allium* Species

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ABSTRACT

Allium species were investigated in order to evaluate their free radical scavenging capacities. The antioxidative enzymes and scavenger activities were determined by ESR and DPPH methods. Beside the quantities of non-enzymic antioxidants, malonyl-dialdehyde and HO• radical were investigated. Total antioxidant activities of selected Allium extracts were determined by FRAP method. Obtained results suggested that bulbs and leaves of cultivated A. nutans L. could be the promising sources for further investigation as raw materials for producing non-toxic natural antioxidants for food, pharmaceutical and cosmetic industries. Cultivation of some wild varieties such as A. flavum L. could be future task in order to produce Allium sorts with strong antioxidant capacity.

Key words : *Alliums*, antioxidants, ESR, DPPH, scavenging capacity

INTRODUCTION

Alliums health benefits especially of garlic (*Allium sativum*) and onion (*Allium cepa*) are known for thousands of years, but recently the interest in research of other *Allium* species was also observed (Reuter, 1995). Alliums have been used as food and medicinal agents for more than 4,000 years. Reference to their medical use appears in the Codex Ebes in Egypt about 1500 BC, as well in the writings of Hippocrates, Herodotus, Pasteur, and Albert Schweitzer, among

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others. Folklore claimed that garlic would keep vampires and other bloodsuckers away.

Today garlic and onion are used for their flavor, aroma and taste, being prepared domestically or forming basic materials for a variety of food manufacturing processes (dehydradation freezing, canning and pickling). Onions were among the earliest vegetables to be processed, canned, dried and frozen (Brewster & Rabinowitch, 1990). People use garlic and onion to help with several different types of ailments *viz.* high cholesterol, high blood pressure, excess blood clotting and coagulation, atherosclerosis, inflammation, bacterial infections, fungal infections, diabetes and cancer (Koch & Lawson, 1996). Much of the data about human use came from reports of lowered rates and risks of disease (such as cancer) in people with relatively high levels of garlic or other *Alliums* consumption.

More than 200 components of garlic and onion have been identified, including vitamins (Brewster & Rabinowitch, 1990), sulphur containing compounds, amino acids, proteins (Block *et al.*, 1996), lipids, and trace elements as Se (Ip & Lisk, 1996), flavorous (Hollman & van der Gaag, 1998), enzymes (Roberts & Tyler, 1999) and different antioxidants (Ide & Itakura, 1996).

Our previous study concerning antioxidant ability of different *Allium* species (Štajner *et al.*, 1998, 1999, 2002) showed that all plant parts posses antioxidant abilities, especially leaves, but in human diet and also in industries bulbs are mainly used. Therefore, the aim of this study was to explore leaves and bulbs for antioxidant activities of some wild and cultivated *Alliums* in order to point to species which could be the sources of non-toxic natural antioxidants for food, pharmaceutical and cosmetic industries.

MATERIALS AND METHODS

Plant Material

Allium plants were collected in the flowering phase. For the experiment, fresh leaves and bulbs of both cultivated (*Allium nutans* L., *Allium pskemenese* B. Fedtch. L., *Allium fistulosum* L. and *Allium sativum* L.) and wild (*Allium flavum* L., *Allium roseum* L. and *Allium subhirsutum* L.) species were used (Figs 1-7). Voucher herbarium specimens are deposited at the Institute of Biology, University of Novi Sad.

Methods

One g of fresh plant material was grounded with quartz sand in a cold mortar. The ground material was suspended in 5 mL 1 mol/L

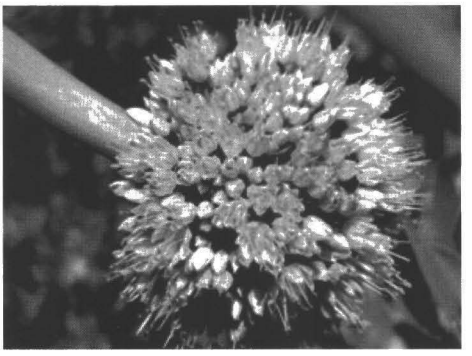

Fig 1. *Allium fistulosum* in blossoming phase (www.hear.org/starr/plants/images/image/?q=070714-7581)

K_2HPO_4 at pH 7.0. Centrifugation for 10 min at 4°C and 15,000 g, the aliquots of the supernatant were used for superoxide dismutase (SOD) activity measurements. 20 ml of Tsuchiachi solution (chloroform/ethanol 3/5) was added to the supernatant prior to measurement of the enzyme activity. The SOD activity was determined in aliquots by the method of Misra & Fridovics (1972), based on the inhibition of transformation of adrenaline to adrenochrome at pH 10.2 (Matkovics *et al.*, 1977).

Carotenoids were extracted with acetone and determined spectrophotometrically using molar extinction coefficients according to Wettstein (1957). The amount of reduced glutathione (GSH) was determined with Ellman reagent at 412 nm (Sedlak & Lindsay, 1968).

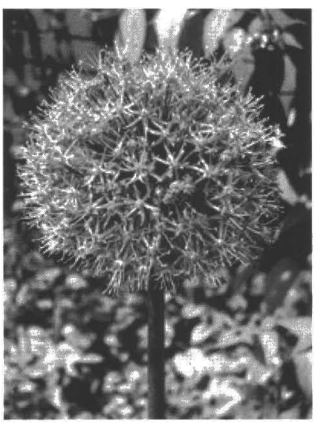

Fig 2. *Allium sativum* in blossoming phase (http://farm1.static.flickr.com/223/468307008_cc87abd16f.jpg?v=0)

Fig 3. *Allium flavum* in blossoming phase (www.robsplants.com/plants/AlliuFlavu.php)

Fig 4. *Allium nutans* in blossoming phase (http://1000naturephotos.1000wallpapers.com/photos/1/Flore/Par+ordre+alphab%E9tique/Alliums_Alliums/Allium+nutans_Allium+nutans.jpg)

Lipid peroxidation (LP) was determined by the thiobarbituric acid (TBA) method; values were given as equivalent amounts of malonyldialdehyde (MDA); the calibration curve was prepared with malonyldialdehyde bis-diacetal (Placer & Hohnson, 1968). Hydroxyl radical was determinated by the inhibition of deoxyribose degradation (Cheesman & Esterbaurer, 1988).

The influence of the phosphate buffer (pH 7) *Allium* extract on hydroxyl radical ($HO^{\bullet}$) formation was studied by electron spin resonance (ESR) using a spin trapping method (Hiramoto & Kikugawa, 1996). The scavenging activity of the extract was estimated by the

Fig 5. *Allium psekemense* in blossoming phase (www.en.wikipedia.org/wiki/File: Allium_pskemense.jpg)

Fig 6. *Allium roseum* in blossoming phase (www.dutchbulbs.com/images/products/small/1939.jpg)

Fig 7. *Alliumsu subhirsutum* in blossoming phase (www.maltawildplants.com/LILI/Pics/ALLSH/ALLSH-Allium_subhirsutum_t.jpg)

percentage decrease of the relative intensity of the signal of DMPO-OH radical adduct with reference to the control without extract.

Radical scavenging capacity was determined using 1, 1-diphenyl-2-pycril-hydrasil radical (DPPH). Reduction of DPPH radical was determined measuring disappearance of DPPH at 515 nm. RSC is expressed by percents compared to the control (Abe & Hirota, 1998). The percent inhibition of the DPPH radical (RSC) by the samples was calculated using the formula:

$$RSC = \frac{Ac - Ax}{Ac} \times 100\ \%$$

Where Ac is absorbance of the control and Ax is absorbance of the sample after 30 min of incubation.

Total antioxidant capacity was estimated according to the FRAP (Ferric Reducing Antioxidant Power) assay (Benzie & Strain, 1999). Total reducing power is expressed as FRAP units. FRAP unit is equal with 100 µmol/dm3 Fe^{2+}. FRAP value was calculated using formula:

$$\text{FRAP value} = \frac{\Delta\ A\ \text{sample}}{\Delta\ A\ \text{standard}}$$

All the experiments were repeated three times.

RESULTS AND DISSCUSSION

In Table 1, SOD activities and GSH quantities in leaves and bulbs and carotenoid content in leaves of different *Alliums* are presented.

All investigated leaves and bulbs exhibited SOD activity. In leaves, SOD activity ranged from 4.08 U/mg protein in *A. pskemenese* to 62.20 U/mg protein in *A. fistulosum* and in bulbs from 1.24 U/mg protein in *A. flavum* to 127.11 U/mg protein in *A. sativum*. SOD present in leaves and bulbs remove $O_2^{\bullet -}$ from the compartments where radicals are formed including chloroplast and mitochondria, controlling oxidative stress in plants (Alsher & Heath, 2002). GSH quantity in leaves (Table 1) ranged from 0.125 nmol/mg protein (*A. nutans*) to 0.646 nmol/mg (*A. roseum*) and in bulbs from 0.072 nmol/mg in *A. nutans* to 0.646 nmol/mg in *A. subhirsutum*. The quantity of GSH, powerful nonenzymic antioxidant, mainly, was the highest in leaves and lower in bulbs of examined *Alliums* what indicated high antioxidant capacity of leaves. High GSH quantity is also beneficial for lipid peroxide balance in tissue (Filomeni *et al.*, 2002).

Carotenoids content (Table 1) was detected in leaves of all investigated *Alliums*, but there is no evidence of it's presence in bulbs. The highest carotenoid content (2.87 mg/g) was observed in leaves of *A. fistulosum* and the lowest in *A. pskemenese*, 0.66 mg/g. It is well known that carotenoids, flavonoids and other polyphenol compounds scavenge lipid peroxyl radicals resulting in formation relatively stable antioxidant radical which react slowly with substrate (LOOH) and so reduce LP (Štajner *et al.*, 1999). It is well known that they also act as powerful scavenger of activated oxygen (Halliwell & Guteridge, 1989).

The accumulation of $HO^{\bullet}$ radicals and MDA, the most toxic oxygen species and main products of lipid membrane peroxidation, is presented in Table 2. The lowest $HO^{\bullet}$ quantities were observed in leaves of *A. pskemenese* (0.07 nmol/mg) and bulbs of *A. nutans* (1.16 nmol/mg) and the highest in leaves of *A. subhirsutum* (39.27 nmol/mg) and bulbs of *A. roseum* (96.23 nmol/mg). The lowest MDA quantities were observed in leaves of *A. fistulosum* (4.98 nmol/mg) and bulbs of *A. nutans* (14.28 nmol/mg) and the highest in leaves of *A. subhirsutum* (37.12 nmol/mg) and in bulbs of *A. pskemenese* (113.29 nmol/mg).

According to our results MDA accumulation could be correlated with the high quantity of toxic $HO^{\bullet}$ what provoke membranes deterioration. The leaves exhibited the lowest lipid peroxidation due the accumulation of relatively low $HO^{\bullet}$ quantities (Halliwell & Guteridge, 1989) and also presence of carotenoids.

Scavenging activities and total antioxidant capacities measured by FRAP method in leaves of different *Alliums* are presented in Table 3. All investigated *Alliums* showed huge scavenging ability. ESR data demonstrate that phosphate buffer extracts possess similar $HO^{\bullet}$

Table 1. SOD activities and GSH quantities in leaves and bulbs and carotenoid content in leaves of different *Alliums*

***Allium* sort**	**SODU/mg protein (leaves)**	**SODU/mg protein (bulbs)**	**GSHμmol/mg protein (leaves)**	**GSHμmol/mg protein (bulbs)**	**Carotenoids mg/g (leaves)**
A. sativum L.	52.47 ± 7.11	127.11 ± 12.62	0.215 ± 0.009	0.184 ± 0.002	2.57 ± 0.00
A. pskemenese L.	4.08 ± 0.84	16.18 ± 6.26	0.177 ± 0.004	0.336 ± 0.011	0.66 ± 0.01
A. fistulosum L.	62.20 ± 3.85	43.03 ± 4.93	0.497 ± 0.008	0.219 ± 0.006	2.87 ± 0.03
A. nutans L.	20.27 ± 3.27	3.73 ± 1.57	0.125 ± 0.004	0.072 ± 0.002	2.24 ± 0.01
A. flavum L.	10.62 ± 2.02	1.24 ± 0.9	0.146 ± 0.002	0.112 ± 0.002	1.10 ± 0.01
A. roseum L.	25.91 ± 3.36	43.36 ± 5.23	0.646 ± 0.08	0.279 ± 0.05	0.874 ± 0.02
A. subhirsutum L.	18.45 ± 2.18	14.75 ± 1.05	0.525 ± 0.13	0.646 ± 0.08	1.428 ± 0.03

Table 2. Quantities of $HO^{\bullet}$ and MDA in leaves and bulbs of different *Alliums*

***Allium* sort**	**$HO^{\bullet}$ nmol/mg protein (leaves)**	**$HO^{\bullet}$ nmol/mg protein(bulbs)**	**MDA nmol/mg protein(leaves)**	**MDA nmol/mg protein(bulbs)**
A. sativum L.	2.10 ± 0.29	3.73 ± 0.05	24.55 ± 1.02	42.73 ± 0.92
A. pskemenese L.	0.07 ± 0.02	17.52 ± 0.34	7.12 ± 0.77	113.29 ± 3.84
A. fistulosum L.	0.20 ± 0.02	2.86 ± 0.32	4.98 ± 0.34	26.38 ± 0.39
A. nutans L.	0.30 ± 0.04	1.16 ± 0.07	12.40 ± 0.16	14.28 ± 0.23
A. flavum L.	0.43 ± 0.13	9.53 ± 0.02	7.74 ± 0.40	89.51 ± 4.10
A. roseum L.	24.49 ± 10.52	96.23 ± 21.11	35.70 ± 7.41	92.82 ± 18.82
A. subhirsutum L.	39.27 ± 7.13	65.21 ± 8.03	37.12 ± 12.03	73.69 ± 19.52

Table 3. Scavenging activity and total antioxidant capacity measured by FRAP in leaves of different *Alliums*

***Allium* sort**	**Scavenger activity (%)**		**FRAP (mM Fe^{2+})**
	DPPH	**ESR**	
A. sativum L.	80.14 ± 5.12	90.36	16.02 ± 1.05
A. flavum L.	88.20 ± 7.01	94.30	15.32 ± 0.88
A. psekemense B. Fedtsch	45.10 ± 2.43	50.22	3.40 ± 0.23
A. nutans L.	84.17 ± 3.75	70.97	7.66 ± 0.70
A. fistulosum L.	85.11 ± 6.18	87.09	14.75 ± 0.12
A. roseum L.	63.38 ± 4.18	84.61	6.47 ± 0.72
A. subhirsutum L.	46.40 ± 10.11	81.92	4.17 ± 0.49

scavenging activities which is crucial because hydroxyl radicals are the major active oxygen species causing lipid oxidation and enormous biological damage (Ide & Itakura, 1996).

The highest HO• and DPPH scavenger activities were observed in wild *A. flavum* extract (94.3% and 88.20%). Other results concerning *A. flavum* support this assessment because quantities of HO• and MDA were low (Table 2). Scavenging activity of *A. sativum* extract was also high (90.36%) which was is in agreement with results of number of authors (Helen *et al.*, 2000; Briggs *et al.*, 2001; O'Reilly *et al.*, 2001) who referred about garlic's and onions antioxidant and pharmacological activities. Total antioxidant capacities measured by FRAP method were also high especially in *A. sativum* and *A. flavum* leaf extracts. Our results confirmed that antioxidant and scavenger activities influence the pharmacological activity of garlic and also other *Allium* species possess huge antioxidant capacity.

Our results suggested that bulbs and leaves of cultivated *A. nutans* L. due to high antioxidant and scavenger capacities could be the promising source for further investigation in order to produce non-toxic natural antioxidants which could be used in food, pharmaceutical and cosmetic industries. However, cultivation of some wild varieties such as *A. flavum,* could be future task in order to produce *Allium* sorts with strong antioxidant abilities. Healthy and safe natural antioxidants that provide good protection against oxidative damage which occurs both in the body and our daily foods, medicaments, and cosmetics could replace artificial toxic antioxidants. Therefore new plant species, as natural sources, such as leaves and bulbs of investigated *Allium* plants due its antioxidant abilities, could be introduced for this purpose.

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8

Effect of *Aquilegia vulgaris* on Liver Antioxidant Status in Rats Treated with Diethylmaleate

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ABSTRACT

The aim of the study was to investigate the potential protective action of ethanol and ethyl acetate extracts and isocytisoside from Aquilegia vulgaris (L.) (Ranunculaceae) in the model of oxidative stress evoked by diethylmaleate (DEM), a known GSH-depleting agent. Rats pretreated with DEM (18 mmol/kg b.w.) were given per os extracts as well as isocytisoside (100 mg/kg b.w.) obtained from A. vulgaris. Hepatic glutathione level depleted by DEM to 50% of the control level was further decreased after the substances tested administration. The substances tested caused significant reduction of uninduced and enzymatically-driven microsomal lipid peroxidation in the liver of rats treated with DEM by 30%-54%. Activity of antioxidant enzymes inhibited by DEM was significantly restored after administration of the substances tested, in particular of GR and CAT activity. Reduced activity of GPx was raised only by ethanol extract.

Key words : *Aquilegia vulgaris,* lipid peroxidation, glutathione, diethyl malete, liver

INTRODUCTION

There is a strong demand for the development of therapeutic and chemopreventive antioxidant agents with limited cytotoxicity to

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enhance the antioxidant capacity of the body and to attenuate the damages induced by ROS (Dhiman & Chawla, 2005).

Aquilegia vulgaris (L.)(Ranunculaceae) is a perennial herb indigenous in central and southern Europe. Leaves and stems of *A. vulgaris* have been used in folk medicine against liver and bile duct disorders especially for the treatment of jaundice. The herb is a component of immunostimulating preparation Padma 28 and homeopathic drugs (2000). In our previous research we have isolated and identified several flavonoids (Bylka, 2001; Bylka *et al.*, 2002; Bylka & Matlawska, 1997a; Bylka & Matlawska, 1997b) and phenolic acids (Drost-Karbowska *et al.*, 1996) in aerial parts of the plant as well as alkaloids in roots (Szaufer-Hajdrych *et al.*, 1998). The predominant compound was 4'-methoxy-5,7-dihydroxyflavone 6-*C*-glucopyranoside (isocytisoside) (Bylka & Matlawska, 1997a). We have also found that ethanol extract of *A. vulgaris* and isocytisoside could protect against hepatotoxicity induced by carbon tetrachloride in rats as assessed by inhibition of transaminases and sorbitol dehydrogenase leakage to serum and by histopathological examination (Adamska *et al.*, 2003).

Glutathione is an important intracellular peptide with multiple functions including detoxifying electrophiles, maintaining the essential thiol status of protein, scavenging free radicals, providing a reservoir for cysteine, modulating critical cellular processes such as DNA synthesis (Lu, 1999). Several studies have shown that the rapid depletion of reduced glutathione in the liver is associated with lipid peroxidation and cell death. It has been postulated that the loss of GSH may compromise cellular antioxidant defenses and lead to the accumulation of ROS that are generated as by-products of normal cellular function (Tirmenstein *et al.*, 2000). Therefore, cell death is linked to the oxidative damage since antioxidants and ferric ion chelators prevented both the lipid peroxidation and the cell killing without any effect on the extent of GSH depletion (Miccadei *et al.*, 1988).

Extracts from *A. vulgaris* and isocytisoside were shown to inhibit microsomal lipid peroxidation, scavenge superoxide radical and chelate Fe^{2+} *in vitro*. Hence, we aimed to investigate the potential protective action of these substances in the model of oxidative stress evoked by GSH depletion. We have used diethylmaleate (DEM), a known GSH-depleting agent which is mainly conjugated with GSH by glutathione S-transferase without prior metabolism (Boyland & Chasseaud, 1967).

The study was designed (i) to determine the changes in the concentration of hepatic GSH, the level of lipid peroxidation in the

liver and the activities of hepatic antioxidant enzymes in rats treated with DEM, (ii) to evaluate the protective efficiency of isocytisoside and two extracts from *A. vulgaris* against DEM-induced changes in the mentioned parameters.

MATERIALS AND METHODS

Chemicals and Plant Material

The chemicals used were purchased from Sigma Chemical Co. *Aquilegia vulgaris* stems and leaves were collected in the Botanical Garden of A. Mickiewicz University, Poznañ, Poland in June 1999. A voucher specimen is deposited in the authors' laboratory (No. KF 1261999).

Ethanol and ethyl acetate extracts were prepared as described elsewhere (Adamska *et al.*, 2003; Jodynis-Liebert *et al.*, 2005). Isocytisoside, 4'-methoxy-5,7-dihydroxyflavone-6-C-glucopyranoside was isolated from methanol extract by column chromatography and identified by UV and NMR analysis (Bylka & Matlawska, 1997b).

The extracts were analysed by TLC as described previously (Bylka, 2001; Bylka & Matlawska, 1997a; Bylka & Matlawska, 1997b). Isocytisoside predominated in both extracts. Besides, the extracts contained: isocytisoside 7-*O*-glucoside, isoorientin, orientin, isovitexin 4'-*O*-glucoside, apigenin 7-*O*-rutinoside, apigenin 7-*O*-glucoside and apigenin. Additionally the ethanol extract contained phenolic acids: caffeic, ferulic, p-coumaric, resorcylic, p-hydroxybenzoic, vanilic, sinapic and chlorogenic (Drost-Karbowska *et al.*, 1996).

Phytochemical Analysis

Quantitative analysis of isocytisoside was performed by HPLC method. Lachrom-Merck chromatograph equipped with DAD detector and Zorbax SB-C18 column (250 × 4.6 mm; 5 μm) was used. The mobile phase was methanol-water-formic acid (40:60:1) at a flow rate 1 ml/min. The standard curve was made in the range 2-12 μg. The content of isocytisoside in ethyl acetate subextract was 5% and in ethanol extract 1.5%.

Experimental Design

Forty eight male Wistar rats (230 ± 10 g) were randomly assigned to 6 groups. The rats were housed in an animal facility at 22 ± 1°C with 12 h light-dark cycle, controlled humidity and circulation of air. The rats were fed commercial diet (ISO 9001 certified Labofeed H).

Groups II-VI were given intragastrically diethylmaleate at a dose 18 mmol/kg b.w. After 4 h the animals were treated as follows: group

II was given vehiculum; group III - isocytisoside; group IV – ethyl acetate extract; group V - ethanol extract, group VI - α-tocopherol. The substances tested were administered intragastrically at a dose 100 mg/kg b.w. α-Tocopherol at the same dose was used as a positive control. Group I (controls) was given vehiculum only - the mixture of water and olive oil (1:1 v/v) with a drop of Tween 20.

19 h after the first treatment animals were sacrificed by decapitation. The livers were removed, perfused with ice-cold 1,15% KCl and homogenised in buffered sucrose solution (TRIS, pH=7.55). Microsomal and cytosol fractions were prepared by differential centrifugation according to the standard pro-cedure. Protein concentration in the fractions was determined using Folin-Ciocalteu reagent. Liver homogenate for glutathione determination was prepared in phosphate buffer, pH 7.4.

The experiment was performed according to the Local Animal Ethics Committee guidelines for animal experimentation.

Biochemical Assays

Microsomal lipid peroxidation in the liver was assayed in three different experimental systems: i) Fe^{3+}/ADP/NADPH–stimulated peroxidation – enzymatic, ii) Fe^{2+}/ascorbate-stimulated peroxidation – non-enzymatic, iii) – uninduced peroxidation. Lipid peroxidation was evaluated by measuring thiobarbituric acid reactive substances (TBARS). The results were expressed in nmol malondialdehyde per mg protein (Sanz *et al.*, 1994).

GSH level was assayed in the liver homogenate prepared in phosphate buffer (pH 7,4) by the method of Sedlak and Lindsay (1968) with Ellman's reagent.

Glutathione peroxidase (GPx) activity was determined according to Mohandas *et al.* (1984). Hydrogen peroxide was used as a substrate.

Glutathione reductase (GR) was assayed by measuring NADPH oxidation at 340 nm using oxidized glutathione as a substrate (Mohandas *et al.*, 1984).

Catalase (CAT) activity was determined according to Beer and Sizer (1952). The rate of H_2O_2 reduction was a measure of CAT activity.

Superoxide dismutase (SOD) activity was determined by the method of Sun and Zigman (1978). Inhibition of spontaneous epinephrine oxidation was a measure of SOD activity.

Glutathione S-transferase (GST) activity measurement was based on the spectrophotometric determination of 1-chloro-2,4- dinitrobenzene

(CDNB) conjugate formed in a GSH coupled reaction (Mohandas *et al.*, 1984).

Statistical Analysis

The data were expressed as mean ± SD. One way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test for multiple comparisons were used.

RESULTS

Diethylmaleate treatment caused the significant depletion of hepatic glutathione almost to 50% of the control level. Treatment with all substances tested, including -tocopherol, resulted in the further decrease in GSH level by 25%–50% as compared to that in DEM treated rats (Table 1).

Microsomal lipid peroxidation (LPO) in the liver was assessed

Table 1. Effect of *Aquilegia vulgaris* extracts and isocytisoside on hepatic microsomal lipid peroxidation and reduced glutathione in diethylmaleate-treated rats

Treatment	Lipid peroxidation (nmol TBARS mg^{-1} protein)			GSH (μmol g^{-1} tissue)
	Uninduced	Fe^{2+}/ ascorbate	Fe^{3+}/ADP/ NADPH	
Control	2.61 ± 0.47	43.3 ± 6.0	61.0 ± 9.9	4.77 ± 1.14
DEM	3.49 ± 0.42[a] 34%	63.3 ± 8.3[a] 46%	90.8 ± 10.7[a] 49%	3.09 ± 0.53[a] 45%
DEM + IST	2.32 ± 0.19[b] 34%	61.9 ± 9.2	41.9 ± 5.2[b] 54%	1.93 ± 0.39[b] 38%
DEM + EAE	2.06 ± 0.28[b] 41%	36.1 ± 4.5[b] 43%	53.9 ± 10.1[b] 41%	2.34 ± 0.41[b] 24%
DEM + EE	2.44 ± 0.35[b] 30%	60.2 ± 5.0	57.4 ± 9.5[b] 37%	1.53 ± 0.14[b] 50%
DEM + -toc	1.89 ± 0.25[b] 46%	53.1 ± 5.5[b] 16%	44.7 ± 8.1[b] 51%	2.36 ± 0.24[b] 24%

Results are mean ± SD, n=8. Control rats were administered vehicle only
DEM - diethylmaleate, IST - isocytisoside, EE - ethanol extract, EAE - ethyl acetate extract,
-toc - -tocopherol
a) significantly different from control, P≤"0.05
b) significantly different from DEM-treated rats, P≤"0.05.

Table 2. Effect of *Aquilegia vulgaris* extracts and isocytisoside on antioxidant enzymes in the liver of diethylmaleate-treated rats

Treatment	GPx nmol NADPHx $min^{-1} \times mg^{-1}$ protein	GR nmol NADPHx $min^{-1} \times mg^{-1}$ protein	GST nmol CDNBx $min^{-1} \times mg^{-1}$ protein	SOD Ux mg^{-1} protein	CAT Ux mg^{-1} protein
Control	63.6 ± 8.4	19.8 ± 1.9	284.5 ± 28.7	4.66 ± 0.29	56.1 ± 6.2
DEM	50.5 ± 9.6[a] ↓ 23%	24.9 ± 2.4 ↑ 26%	293.0 ± 24.4	3.80 ± 0.53[a] ↓ 19%	73.3 ± 9.0[a] ↑ 31%
DEM + IST	51.8 ± 8.2	18.8 ± 1.9 ↓ 24%	269.7 ± 26.1	3.37 ± 0.46[b] ↓ 11%	59.6 ± 5.8[b] ↓ 19%
DEM + EAE	49.6 ± 8.0	17.5 ± 2.5 ↓ 30%	258.2 ± 31.5	5.83 ± 0.73[b] ↓ 53%	49.0 ± 5.6[b] ↓ 33%
DEM + EE	63.7 ± 10.2[b] ↑ 26%	18.3 ± 1.9 ↓ 27%	280.1 ± 27.0	5.46 ± 0.62[b] ↓ 44%	64.6 ± 8.4[b] ↓ 12%
DEM + α-toc	56.0 ± 7.6	24.2 ± 1.5	309.9 ± 30.2	5.74 ± 0.92[b] ↓ 51%	68.5 ± 2.6

Results are mean ± SD, n=8. Control rats were administered vehicle only
DEM - diethylmaleate, IST - isocytisoside, EE - ethanol extract, EAE - ethyl acetate extract,
α-toc - α-tocopherol
a) significantly different from control, $p \leq 0.05$
b) significantly different from DEM-treated rats, $p \leq 0.05$.

using three assays: LPO stimulated with Fe^{2+}/ascorbate (non-enzymatic), with Fe^{3+}/ADP/NADPH (enzymatic) and uninduced LPO. A significant increase in the level of TBARS was observed in rats treated with DEM, by 34%-49%, as compared to that in controls. All substances tested caused significant reduction of uninduced and enzymatically-driven LPO, by 30%-54% as compared to those in DEM-treated rats. As a result the level of TBARS in these groups was even lower than that in controls. The degree of LPO reduction by isocytisoside and both extracts was comparable to that caused by α-tocopherol. Isocytisoside and ethanol extract did not affect Fe^{2+}/ascorbate-stimulated lipid peroxidation in DEM-treated animals, only ethyl acetate extract inhibited non-enzymatic lipid peroxidation by 43%. Its efficiency was greater than that of α-tocopherol which reduced non-enzymatic LPO only by 16% (Table 1).

Effect of the substances tested on hepatic antioxidant enzymes was shown in Table 2. The response of these enzymes to DEM and test substances was differentiated. DEM treatment produced a rise in glutathione reductase (GR) and catalase (CAT) activities by approximately 30%, and a decrease in glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities by about 20%. Glutathione S-transferase (GST) was not affected by DEM. Administration of isocytisoside and the extracts caused the restoration of GR and CAT activity, ethyl acetate extract being the most active (Table 2). α-Tocopherol did not change the elevated activity of these enzymes in DEM-treated rats. Reduced activity of GPx was raised to the level of that in control group only by ethanol extract. Activity of SOD in DEM-treated rats was further reduced by isocytisoside administration while both extracts raised the activity of SOD up to the level exceeding the control value. Similar effect of α-tocopherol was observed (Table 2).

DISCUSSION

It is generally accepted that the decrease in cellular GSH level may disturb antioxidant defense system and cause the accumulation of reactive oxygen species which are normally produced in cells. When GSH depletion reaches certain threshold values, lipid peroxidation develops leading to cell death (Tirmenstein *et al.*, 2000). As might be expected, in our experiment administration of diethylmaleate to rats caused GSH depletion and enhanced lipid peroxidation in the liver. Substances tested appeared to be efficient in attenuating microsomal lipid peroxidation. TBARS level was reduced, especially in the assays of uninduced and enzymatically-driven system. The enzymatic NADP-dependent lipid peroxidation is catalyzed by the NADPH-cytochrome P450 reductase and propagated by cytochrome P450 with the

generation of free radicals, $O_2^{\bullet}$ and ROO$^{\bullet}$ (Sevanian *et al.*, 1990). Both extracts tested and isocytisoside were previously demonstrated to scavenge superoxide anion and showed iron chelating ability (Murias *et al.*, 2005). Therefore, the mechanism of inhibition of lipid peroxidation observed in the present study might involve the formation of complexes between iron and components of the extract. This would inhibit free radicals generation and terminate lipid peroxidation.

Neither the extracts tested nor isocytisoside reversed the GSH depletion caused by DEM treatment. GSH level was even reduced in comparison with that in DEM-treated rats. α-Tocopherol, the model antioxidant caused a similar effect. The reason for the further reduction of GSH level in DEM-treated rats by the substances tested is not clear. It could be related to the specific mechanism of DEM action since in our previous studies the extracts tested decreased the level of GSH in rats pretreated neither with APAP (Jodynis-Liebert *et al.*, 2005) nor carbon tetrachloride (unpublished data). Other authors' findings confirmed our observations. Miccadei *et al.* (1988) exposed cultured hepatocytes to diethylmaleate to deplete cellular GSH. Pretreatment with a ferric ion chelator and LPO inhibitor, deferoxamine, or the addition of an antioxidant, N, N'-diphenyl-p-phenylenediamine to the culture medium prevented both the lipid peroxidation and the cell death produced by DEM. However, neither of these two compounds prevented GSH depletion. Similar results were obtained when deferoxamine was used in experiments with two others GSH-depleting agents, bromobenzene (Casini *et al.*, 1987) and allyl alcohol (Pompella *et al.*, 1991). Studies by Comporti *et al.* (1991) supported our findings that model antioxidant, α-tocopherol, did not prevent GSH depletion. It should be emphasized that similarly to the effect demonstrated by mentioned above antioxidants, the substances tested in our experiment protected against lipid peroxidation despite of the lack of their activity against GSH depletion.

The response of hepatic antioxidant enzymes to DEM was not consistent. Usually treatment with prooxidant such as carbon tetrachloride or bromobenzene results in an inhibition of antioxidant enzymes. It is due to the inactivation of the enzymes by lipid peroxides and ROS (Halliwell & Gutteridge, 1984). On the other hand it is known that moderate cellular damage can induce the transcriptional activity of antioxidant enzymes (Toyokuni *et al.*, 2003). Our results are in accordance with these findings. We observed moderate but significant increase in hepatic CAT activity in rats treated with DEM. When cellular GSH is depleted glutathione peroxidase cannot remove endogenous hydrogen peroxide. Its accumulation leads to lipid

peroxidation and induces catalase activity. Increased activity of glutathione reductase in response to oxidative stress evoked by DEM could be also considered a part of adoptive mechanism. The role of GR is to regenerate oxidized GSH. In oxidative stress the requirement for reduced GSH is enhanced, thus the activity of GR may be stimulated.

All antioxidant enzymes act against ROS but the mechanism responsible for the regulation of their expression may be different. In our experiment the activity of two enzymes, GPx and SOD were reduced after DEM treatment while GST activity remained unchanged. It can be suggested that SOD and GPx are especially susceptible to oxidative stress evoked by DEM. On the other hand the response of antioxidant enzymes to DEM can depend on the experimental model. In the brain of rats treated with DEM the increased activity of CAT and GST as well as the reduced activity of SOD was found. Se-dependent GPx activity was not changed (Gupta *et al.*, 2000). In isolated rat hepatocytes DEM caused the inhibition of CAT activity and did not change SOD and GPx activity (Haidara *et al.*, 1999). It is known that cellular GSH depletion leads to the accumulation of ROS species including H_2O_2. Pigeolet *et al.* (1990) reported the inhibition of SOD and GPx activity by H_2O_2 *in vitro*. This is consistent with the decrease in GPx and SOD activity following GSH depletion in DEM-treated rats.

GST activity was not changed in DEM-treated rats. The GSTs are known to play an important role in the protection of cellular macromolecules from attack by reactive electrophiles. DEM, an electrophilic substrate of GST is not considered to affect the activity of the enzyme although it is known that a lot of xenobiotics evoking oxidative stress are concomitantly inducers of GST (Daniel, 1993).

The activity of CAT and GR enhanced by DEM was reduced to the level of the control group after substances tested administration. The decreased activity of SOD was raised by two extracts to exceed the level observed in the controls. Only ethanol extract was efficient in restoring GPx activity decreased by DEM pretreatment. α-Tocopherol administration did not change the activity of antioxidant enzymes modulated by DEM except for SOD. This may suggest that the protective action of the substances tested might not be directly related to their antioxidant activity. Assuming that the change in the activity of antioxidant enzymes in DEM-treated rats is the response to oxidative stress, it could be postulated that the substances tested exerted their protective action by restoring the activity of antioxidant enzymes counteracting changes evoked by oxidative insult. In summary, a single dose of DEM led to the depletion of hepatic GSH, enhanced hepatic microsomal lipid peroxidation and affected the

antioxidant enzymes activity. The administration of isocytisoside and other two extracts from *A. vulgaris* restored the antioxidant enzymes activity and decreased the level of hepatic lipid peroxidation.

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9

Antioxidant Activity, Medicinal Plants and Nervous Disorders: A Review

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ABSTRACT

Oxidative stress arise when the antioxidant defense system of the human body is not entirely efficient. Several chemical moiety have been identified as causative agents for oxidative stress which includes ROS, RNS and free radicals. In response to mild oxidative stress body increase its antioxidant defence, but severe oxidative stress increase free radicals which can lead to cell injury or cell death. Evidence suggest that oxidative stress induced free radicals contribute to various diseases including neurodegenerative diseases, chronic inflammatory diseases, cancer and cardiovascular disease. Evidence suggests the occurrence of oxidative damage in Alzheimer's and in Parkinson's brain. Oxidative stress in brain cause neuronal lipid peroxidation, protein oxidation and DNA oxidation by free radical mechanisms that can be inhibited by antioxidants. Many medicinal herbs are being used since ancient time in Indian traditional system for treatment of various diseases including neurodegenerative diseases. Recent investigations have shown antioxidant properties in various plants/products and their efficacy in quenching free radicals. In the present chapter we have reviewed the role of various medicinal herbs for their antioxidative effects against neuro-degenerative diseases.

Key words : Antioxidants, medicinal plants, central nervous system, neurodegenerative diseases

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INTRODUCTION

Oxidative stress arise when the antioxidant defense system of the human body is not entirely efficient. In response to mild oxidative stress the body can increase its antioxidant defense but unfortunately severe oxidative stress increase free radicals which can lead to cell injury or cell death. Evidence suggest that oxidative stress induced free radicals contribute to various diseases including neurodegenerative diseases, chronic inflammatory diseases, cancer and cardiovascular disease (Halliwell, 2001). Evidence also suggests the occurrence of oxidative damage in Alzheimer's disease brain and a central role for amyloid-β-peptide (Butterfield *et al.,* 2001). It has been postulated that amyloid-β-peptide induces neuronal lipid peroxidation, protein oxidation and DNA oxidation by free radical mechanisms that can be inhibited by antioxidants (Butterfield *et al.,* 2001).

The nervous system – the brain, spinal cord, and peripheral nerves are rich in both unsaturated fatty acids and iron. The high lipid content of nervous tissue, coupled with its high aerobic metabolic activity, makes it particularly susceptible to oxidative damage. The high level of iron may be essential, particularly during brain development, but its presence also means that injury to brain cells via the iron-catalysed formation of ROS (Bauer & Bauer, 1999; Andorn *et al.,* 1990). Those brain regions that are rich in catecholamines are exceptionally vulnerable to free radical generation, because adrenaline, noradrenaline, and dopamine can spontaneously break down (auto-oxidise) to free radicals, or can be metabolized to free radicals by the endogenous enzymes such as MAO (monoamine oxidases). One such region of the brain is the substantia nigra (SN), where a connection has been established between antioxidant depletion (including GSH) and cell degeneration (Perry *et al.,* 2002). A number of *in vitro* studies have shown that antioxidants both endogenous and dietary can protect nervous tissue from such damages by oxidative stress (Contestabile, 2001). It was shown earlier that vitamin-E prevent neuronal damage from reactive nitrogen species. Both vitamin-E and beta-carotene were found to protect rat neurons against oxidative stress from exposure to ethanol (Copp *et al.,* 1999). Most *in vivo* and clinical studies in neurological diseases have focused on vitamin-E. It was found that the risk for Parkinson's disease was lower for subjects who had higher dietary intakes of antioxidants, particularly vitamin-E. Low dietary intakes of beta-carotene was associated with impaired cognitive function in group of persons aged 55-95 (Hellenbrand *et al.,* 1996). Those patients suffering from Parkinson's disease had consumed less beta-carotene and vitamin-C than those who did non-suffer of the disease, implying that dietary

antioxidants do play a protective role in Parkinson's disease (Fahn, 1991). About 20% of familial ALS (FALS) cases are associated with a mutation in the gene for copper/zinc superoxide dismutase, an important antioxidant enzyme, and *in vitro* experiments demonstrated that expression of the mutant enzyme in neuronal cells cause cell death, which could be prevented by small antioxidant molecules such as glutathione and vitamin-E (Ferrante *et al.,* 1997). Thus there are substantial evidences that oxidative stress is a causative or at least ancillary factor in the pathogenesis of major neurodegenerative diseases, including Parkinson's, Alzheimer's disease and amyotrophic lateral sclerosis (ALS, "Lou Gehrig's disease") as well as in cases of stroke, trauma, and seizures (Ghadge *et al.,* 1997). Evidence of increase in lipid peroxidation and oxidation of DNA and proteins has indeed been seen in the substantia nigra (SNc) of patients affected with Parkinson's disease. Similar increase in markers of oxidative stress have also been seen in Alzheimer's disease, Huntington's disease and in both familial ALS and sporadic ALS (SALS) patients (Saggu *et al.,* 1989). Schizophrenia (SCZ) is also believed to have a component of free-radical overload. Lipid peroxides have been found elevated in their blood and increased pentane gas, a marker for lipid peroxidation in the breath of schizophrenics as compared with normal volunteers and with patients having other psychiatric illness (Phillips *et al.,* 1993).

THE BRAIN IS HIGHLY VULNERABLE TO OXIDATIVE STRES

The human brain uses more oxygen and produces more energy per unit mass than any other organ. Both features of brain metabolism translate in to extremely high oxidative phosphorylation, accompanied by correspondingly high electron leakage. The brain has high iron content that can catalyze oxidation, also particularly loaded with unsaturated fatty acids in the myelin sheath, and long chain fatty acids in the cell membranes are highly susceptible to peroxidation, make this organ exceptionally vulnerable to oxidative degeneration (Floyd & Hensely, 2002). Anatomical and histological studies have established the existence of selective regional vulnerability to oxidative stress. The dopaminergic neurons in the SNc are selectively injured in Parkinson's disease, whereas motor neurons in the spinal cord are selectively lost in ALS (amyotrophic lateral sclerosis). Loss of cholinergic neurons occurs frequently in the forebrain of Alzheimer's. Despite this regional sensitivity, oxidative processes may represent a specific and selective unifying mechanism for neurodegeneration.

Evidence is now mounting that the mitochondria are the most vulnerable functional subset of brain tissue, as they have antioxidant

defenses inferior to the greater cell. Thus mitochondrial DNA 10-100 times more likely to become damaged than nuclear DNA (Floyd & Hensely, 2002). Neurons also have constant calcium flux, and the mitochondria provide backup for calcium homeostasis. Thus, mitochondrial insufficiency could tip the delicate intracellular calcium balance toward cell death. The flood of ROS generated in the neuronal and glial mitochondria during hypoxic-hyperoxic ischemic insult can be acutely devastating to brain tissue (Aliev *et al.,* 2002).

Sources of Oxidative Stress in Brain

The brain utilize about 25% of respired oxygen *i.e.* 3.5 mL oxygen/ 100 g of brain tissue/minute (Kish *et al.,* 1992). About 2% of this oxygen consumed becomes reactive oxygen species (ROS) (Boveris & Chance, 1973). Free radicals are generated in the brain during the normal intake of oxygen, normal aerobic respiration, normal oxidative metabolism of certain substrates and also during infection. Mitochondria of one rat neuron cell will process about 10^{12} oxygen molecules and reduce them to water. During this process, superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl ions ($OH^{\bullet}$) are produced. Partially reduced oxygen, which represents about 2% of consumed oxygen, leaks out from the mitochondria and generates about 20 billion molecules of O_2^- and H_2O_2 per cell per day (Boveris & Chance, 1973; Ames *et al.*, 1993). During bacterial or viral infection, phagocytic cells generate high levels of NO^- (nitric oxide), O_2^- and H_2O_2 in order to kill infective agents; however, these radicals can also damage normal cells (Ames *et al.*, 1993). During degradation of fatty acids and other molecules by peroxisomes, H_2O_2 is produced as a by product. During oxidative metabolism of ingested toxins, free radicals are also generated.

Some brain enzymes such as monamine oxidase (MAO), tyrosine hydroxylase and L-amino acid oxidase also produce H_2O_2 as a normal by product of their activity (Coyle & Puttfarcken, 1993). Auto-oxidation of ascorbate and catecholamine generates H_2O_2 (Graham, 1978). Oxidative stress can also be generated by Ca^{2+} mediated activation of glutamate receptors. The Ca^{2+}-dependent activation of phospholipase A_2 by N-methyl-D-aspartate (NMDA) release araechidonic acid, which then liberates $O_2^{-\bullet}$ during the biosynthesis of eicosanoid (Chan & Fishman, 1980). Another radical, $No^{\cdot}$, is formed by nitric oxide synthase stimulated by Ca^{2+}. NO^- can react with O_2^- to form peroxynitrite anions that can form $OH^{\cdot}$, the highly reactive hydroxyl radical. NMDA receptor stimulation produces marked elevations in O_2^- and $OH^{\bullet}$ levels in brain (Lafon-Cazal *et al.,* 1993). Some enzymes such as xanthine oxidase and flavoprotein oxidase (*e.g.* aldehyde oxidase) also form superoxide anions during metabolism

of their respective substrates. Oxidation of hydroquinone and thiol and synthesis of uric acid from purines form superoxide anions. Certain external agents can increase oxidative stress. For example cigarette smoking increase the level of NO by about 1000 ppm (Kiyosawa *et al.*, 1990; Reznick *et al.*, 1992) and depletes antioxidant levels (Scheltman *et al.*, 1991; Duthie *et al.*, 1991). Free iron and copper can increase the level of free radicals (Winterbourn, 1995). Some plants/products ingested as food contain large amounts of phenolic compounds such as chlorogenic and caffeic acid which can be oxidized to form radicals (Ames *et al.*, 1990; Gold *et al.*, 1992). Thus several different types of radicals are constantly formed in the brain. Their levels can be increased by enhanced turnover of catecholamines, increased levels of free iron, impaired mitochondrial functions, decreased glutathione levels, etc.

Antioxidants

Anti-oxidants are substances that prevent or repair the oxidative damage to cells and its constituents. They are effective in preventing damage to lipids, proteins and DNA in neurons as well as in other cells. In generous words, antioxidants include endogenous, enzymatic and non-enzymatic defense systems such as Superoxide dismutase, Glutathione peroxidase, Catalase, Glutathione reductase, reduced Glutathione, Vitamins-C, E, Carotenoids, Manganese, reduced selenium, Alphalipote acid etc. (Ames *et al.*, 1993; Smith *et al.*, 1999). This defense system generally reduces with age and is vulnerable to various environmental or external factors, which include pollution, drugs, radiation, physical exercise, and stress. The different antioxidants act to diminish oxidative damage *in vivo* and their mechanism of action are highly varied (Table 1).

Antioxidant enzymes (Table 1), which can protect cells against the damaging effects of these free radicals include catalase, superoxide dismutase and glutathione peroxidase. Therefore, decreased levels of catalase, glutathione peroxidase or superoxide dismutase can also enhance the amounts of free radicals. Natural dietary antioxidants include vitamins A, C and E, carotenoids, flavanoids and polyphenols. Some biosynthetic antioxidants include co-enzyme Q_{10}, a-lipoic acid, glutathione, NADH and urates. Consumption of a diet low in antioxidants may also increase the levels of free radicals. Thus, maintenance of a balance in the favor of antioxidant is essential for the protection of brain function. When this balance is shifted in favor of oxidants, the epigenetic components of neurons suffer damage, accumulation of which may initiate degeneration and eventually cause death of neurons (Prasad *et al.*, 2002).

Table 1. Antioxidants and their mechanism of action

Antioxidants	Mechanism of action
(A) Enzymatic antioxidants - free radical deactivating enzymes	
Superoxide dismutase (SOD)	Catalyses dismutation of superoxide radical to hydrogen peroxide and water
Catalase (CAT)	Reduce hydrogen peroxide to water
Glutathione peroxidase (GP_x)	Reduce hydrogen peroxide to water
(B) Non-enzymatic antioxidants - free radical scavengers	
Vitamin-C (Ascorbic acid)	Assists alpha tocopherol in inhibition of lipid peroxidation
Vitamin-A (Retinol)	Scavenges the lipoperoxyl radical
Vitamin-E (α-tocopherol)	Lipid soluble scavenger captures and extract electron
Iron chelators	Prevent iron ions from participating in reactions as OH production and lipid peroxidatoin
Selenium	Co-factor for glutathione peroxidase
(C) Plant derived antioxidants - phytonutrients	
Flavonoids	Inhibit lipid peroxidation; decrease LDL oxidation
Lycopene	Non-provitamin A carotenoid; scavenges peroxyl radical
Terpenoids	Inhibit iron - induced mitochondrial lipid peroxidation

ANTIOXIDANT DEFENCE SYSTEM

Primary Defence System/Enzymatic Defence System

The enzymatic defence system includes antioxidant enzymes *viz.* superoxide dismutase (SOD), catalase (CAT); and glutathione peroxide which catalyze and reduce oxidants primarily in the body and constitute primary antioxidant defense system.

Superoxide Dismutase (SOD)

SOD catalyses the destruction of the O_2^- free radical. It protects oxygen-metabolizing cells against harmful effects of superoxide free radicals (Fridovich, 1972, 1973; Lavelle *et al.,* 1973; Paschen & Weser, 1973; Petkau *et al.,* 1975). The enzyme catalyses the dismutation of superoxide into oxygen and hydrogen peroxide. It is an important

antioxidant defense in nearly all cells exposed to oxygen. The cytosols of virtually all eukaryotic cells contain an SOD enzyme with Cu-Zn-SOD. The Cu-Zn enzyme is a homodimer of molecular weight 32,500 Daltons. In human, three forms of superoxide dismutase are present. SOD_1 is located in the cytoplasm, SOD_2 in the mitochondria and SOD_3 is extra cellular. The genes are located on chromosomes 21, 6 and 4, respectively.

Catalase (CAT)

CAT present in the peroxisomes of nearly all aerobic cells, serves to protect the cell from the toxic effects of hydrogen peroxide by catalyzing its decomposition in to molecular oxygen and water without the production of free radicals. The enzyme exists as a dumb bell-shaped tetramer of four identical subunits (22,000 to 350,000 KD). Each monomer contains a home prosthetic group at the catalytic center. CAT can also oxidize different toxins, such as formaldehyde, formic acid and alcohols. Any heavy metal ion (such as copper cations in copper (II) sulfate) will act as a noncompetitive inhibitor on CAT. The poison cyanide is a competitive inhibitor of CAT. Enzyme also exhibits peroxidase actions and catalyses the oxidation of various hydrogen donors in the presence of relatively lower concentration of hydrogen peroxide (Oshimo *et al.,* 1973). Though CAT is present in the brain in low concentration. *In vitro* studies in a rat neuronal cell line have indicated that CAT activity can be induced by nerve growth factors (Jackson *et al.,* 1990).

Glutathione Peroxidase (Gpx)

Gpx is the general name of an enzyme family with peroxidase activity whose main biological role is to protect the organism from oxidative damage. The biochemical function of Gpx is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water. There are several iso–enzymes encoded by different genes, which vary in cellular location and substrate specificity. Gpx-I is the most abundant version found in the cytoplasm of all mammalian cells, whose preferred substrate is hydrogen peroxide-

$$2\ GSH + H_2O_2 \longrightarrow GSSG + 2H_2O$$

where, GSH represents reduced monomeric glutathione, and GSSG represents glutathione disulphide. Glutathione reductase reduces the oxidized glutathione to complete the cycle.

$$GSSG + NADPH + H^+ \longrightarrow 2GSH + NADP^+$$

Glutathione peroxidase is a selenium-containing tetrameric glycoprotein. Mice genetically engineered to lack glutathione peroxidase-I are phenotypically normal, indicating that this enzyme is not critical for life. However, glutathione peroxidase-4 knockout mice die during early embryonic development.

Glutathione (GSh)

GSh is a ubiquitous tripeptide, γ-glutamyl steinyl glycine, found in plants, microorganisms, and all mammalian tissues. Glutathione exists in the reduced (Huang *et al.,* 1992) and disulfide oxidized (GSSG) forms (De Leve & Kaplowitz, 1991). Eukaryotic cells have three major reservoirs of GSH. Almost 90% of cellular GSH is in the cytosol, 10% in the mitochondria, and a small percentage in the endoplasmic reticulum (Huang *et al.,* 1992; Meredth & Reed, 1982; Suthanthiran *et al.,* 1990). GSH serves several vital functions, including detoxifying electrophiles; maintaining the essential thiol status of proteins by preventing oxidation of –SH groups or by reducing disulfide bonds induced by oxidant stress, scavenging free radicals, critical cellular processes such as DNA synthesis, microtubular - related processes and immune function. The brain maintains a high concentration of GSH for antioxidant defense. Depletion of total glutathione is a marker for oxidative stress in ischemic insult (Park *et al.,* 2000; Namba *et al.,* 2001). Ischemic effects are worsened by pharmacological depletion of glutathione, but improved by administration of a glutathione mimetic, glutathione mono isopropyl ester, N-acetyl cysteine, a glutathione precursor. GSH plays multiple roles in cells during DNA synthesis and repair, protein synthesis, enzymatic activation, and as a free radical scavenger (Meister & Anderson, 1983).

Non-enzymatic Free Radical Scavengers

Ascorbic Acid/Vitamin-C

Vitamin-C is a water soluble antioxidant and able to react with aqueous free radicals and reactive oxygen species. Vitamin C prevents the conversion of nitrates (from tobacco smoke, smog, bacon, and lunch meats) into cancer-causing substances. It also aids in the metabolization of folic acid, regulation of the uptake of iron, and is required for the conversion of the amino acids L-grosine and L-phenylalanine into noradrenaline. The converstion of tryptophan into seratonin, the neurohormone responsible for sleep, pain control and well being, also requires adequate supplies of vitamin C. A deficiency of ascorbic acid can impair the production of collagen which leads to joint pain, anemia, nervousness, retarded growth, reduced immune response, and increase susceptibility to infections.

Vitamin-E

Vitamin E is a collection of eight fat soluble compounds, tocopherols (methyl derivatives of tocol) and tocotrienols:- alpha -Tocopherol; the most common and biologically active (5, 7, 8-trimethyltocol), beta-Tocopherol (5, 8-trimethyltocol), gamma-Tocopherol (7, 8–trimethyltocol), delta-Tocopherol (8-trimethyltocal), alpha–Tocotrienol, beta–Tocotrienol, gamma–Tocotrienol, delta–Tocotrienol. The most important sources of vitamin E are vegetable oils; including soya, palm, corn, soft lower, sunflower, wheat germ, nut oils and likewise other sources are nuts, seeds, whole grains, leafy green vegetables, chick peas, avocados, sweet potatoes, sweet corn, red peppers, carrots, parsnips, milk, eggs, and cheese. The principal role of vitamin E is as a powerful antioxidant, protecting body cells from the detrimental effects of free radicals and protecting unsaturated lipids against oxidation. Together with vitamin A and vitamin E, it forms the trio of antioxidant vitamins which are thought to help prevent cancer and cardiovascular disease.

OXIDATIVE STRESS, AGEING AND RELATED NERVOUS DISORDERS

Most diseases associated with the human ageing process are known to have a strong oxidative stress component (Tandon & Vohra, 2006). The accumulation of net damage due to oxidative stress over a period of time is considered responsible for the age related disorders, etc. Alzheimer's, Parkinson's, rheumatoid arthritis, cancers, cardiovascular disorders, etc. leading to death. Thus, pharmaceutical drugs and other therapies that act to lower oxidative stress, represent a major approach in treating these diseases as well as intervening with the ageing process itself. In brief, longer-lived species generally show higher cellular oxidative stress resistance and lower level of mitochondrial ROS production than shorter-lived species.

Neurodegenerative disorders (Table 2) are a heterogeneous group of diseases of the nervous system, including the brain, spinal cord, and peripheral nerves, that have different etiologies. Many are hereditary, some are secondary to toxic or metabolic processes, and others result from infections. Due to the prevalence, morbidity, and mortality of the neurodegenerative diseases, they represent significant medical, social and financial burden on the society. Neuropathologically, these are characterized by abnormalities of relatively specific regions of the brain and specific populations of neurons. The degenerating neuron clusters in the different diseases determine the clinical phenotype of that particular illness. Recent investigations in medical biotechnology and genetics have identified

Table 2. Characteristic features of a few neurodegenerative diseases

NDG diseases	Clinical features	Neuropathology
Alzheimer's disease (AD)	Dementia, progressive deterioration of thought, judgment, language skills, visual-spatial perception and mood	Generalised atrophy with shrinkage of the hippocampus and amygdala. Selective death of cells in neocortex, hippocampus, amygdala, basal forebrain, and brainstem. The senile plaque, an extracellular deposit of amyloid, composed of Ab peptide derived from amyloid precursor protein.
Amytrophic lateral sclerosis (ALS)	Progressive weakness, atrophy of skeletal muscles weakness of chest muscles dysfunction in the larynx and pharynx.	Loss of primary motor neurons in the neocortex. Presence of abnormal phosphorylated neurofilaments.
Huntington's disease (HD)	Cognitive impairments (dementia), personality change, psychological symptoms including irritability and depression	Marked atrophy of striatum (caudate and putamen) Generalized cortical atrophy with decreased brain weight. Intranuclear inclusions with cleaved fragments.
Parkinson's disease (PD)	Slowness of voluntary movement (bradykinesia), rigidity, tremor. Cognitive deficits (dementia).	Loss of the pigmented neurons in the SNc (pars compacta).

specific genes for various neurodegenerative disorders. Specially bred animal models have been developed to be used in the study of the etiological factors and underlying pathogenic mechanisms of these diseases (Singh *et al.*, 2004).

ANTIOXIDANT ACTIVITY AND MEDICINAL PLANTS

Plants play a significant role in maintaining human health and improving the quality of human life since ancient times. Herbs have been used in many domains including medicine, nutritive agents, flavor, beverages, food dye, repellents, fragrances, cosmetics, smoking purposes. Recently, interest has increased considerably in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants, which needs to be restricted due to their adverse effects. Many medicinal plants contain large amounts of antioxidants such as polyphenols, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. Various epidemiological studies have demonstrated beneficial effects of high intake of fruits and vegetables and herbal preparations in age-related diseases. Antioxidant principles from herbal resources are multifaceted in their effects and provide enormous scope in correcting the imbalance through regular intake of a proper amount of such herbs in diet. It has been assumed that nutritional intervention to increase intake of phyto-antioxidants may reduce threat of free radicals and many chronic diseases. Selected Indian medicinal plants with antioxidant activity are included in Table 3.

Currently, much of the worlds attention has been focused on Indian medicinal plants commonly used in traditional Indian medicinal system that could arrest/delay ageing and rejuvenate whole functional dynamics of the body system. This revitalization and rejuvenation is known as "Rasayan Chikitsa" in "Ayurvedic" system of medicine. Rasayana drugs are used against a large number of disorders with no pathophysiologic connections according to modern medicine. This group of plants generally possess strong antioxidant activity. Rasayana is a unique concept of Ayurveda which means vital nourishment (Rasa + Ayana) representing a holistic approach responsible for preventive aspect against ageing as well as curative aspect against diseases. Sharangdhar in 16^{th} century AD described Rasayanas as 'Jaravyadhi Vinasanam' which literal means checking the advancement of age (Jara) as well as destroyer of disease (Vyadhi). Most of these drugs are strength-giving, besides controlling vitiation of *vata* and *pitta* as a result of any disease condition all the Vayahsthapana (anti-ageing) drugs (Table 4) are Tridosh-Shamak, thereby, keeping the body disease free, except Shatavar and Jeevanti which are also strength giving (Kapha vardhak).

Table 3. Selected Indian medicinal plants with antioxidant property

Acacia catechu	*Chenopodium album*
Aegle marmelos	*Cinnamomum verom*
Aloe vera	*Cissus quadrangularis*
Alstonia scholaris	*Coccinia grandis*
Anacardium occidentale	*Commiphora wightii*
Andrographis paniculata	*Coriandrum sativum*
Asparagus racemosus	*Cucumis melo*
Azadirachta indica	*Cucurbita pepo*
Bacopa monnieri	*Curcuma longa*
Bauhinia racemosa	*Cynodon dactylon*
Benincasa hispida	*Desmodium gangeticum*
Brassica oleracea	*Emblica officinalis*
Butea monosperma	*Eugenia jambolana*
Caesalpinia bonduc	*Evolvus alsinoides*
Cammellia sinensis	*Foeniculum vulgare*
Cannabis sativa	*Garcinia pedunculata*
Capsicum annum	*Glycyrrhiza glabra*
Corica papaya	*Hedychium spicatum*
Cassia fistula	*Hernidesmus indicus*
Cassia fora	*Ipomea reptans*
Cedrus deodara	*Leucas aspera*
Celastrus paniculatus	*Litsea glutinosa*
Centella asiatica	*Terminalia arjuna*
Chenopodium quinoa	*Terminalia bellirica*
Mangifera indica	*Terminalia chebula*
Mentha spicata	*Tinospora cordifolia*
Momordica charantia	*Trigonella foenum-graecum*
Morinda citrifolia	*Tylophora indica*
Moringa olifera	*Vigna radiata*
Nelumbo nucifera	*Vitex negundo*
Nigella sativa	*Vitis vinifera*
Nyctanthes arbortristis	*Withania somnifera*
Ocimum sanctum	

Table 3. *Contd.*

Zingiber officinale	*Rubia cordifolia*
Paederia foefida	*Santalum alben*
Pedilanthus tithymaloides	*Semecarpus anacardium*
Picrorhiza kurroa	*Sida cordifolia*
Piper betel	*Sorghum bicolor*
Psoralea corylifolia	*Swertia chirata*
Punica granatum	*Tamarindus indica*
Rheum officinale	*Tectonia grandis*

The modern scientific evaluation (Table 5) of some of the major Vayahsthapan Rasayanas (anti-ageing) and Jeevaniya (life-promoting) drugs shows that most of the vayahsthagana drugs have anti-oxidant property, besides several other pharmacological actions (Tandon & Vohra, 2006).

Dietry Antioxidants

A number of dietary antioxidants (Table 6), exist that are collectively known as phytonutrients or phytochemicals having antioxidant activity, example flavonoids which are group of polyphenolic compounds. These are widely distributed in plants as glucosylated derivatives, responsible for the different brilliant shades such as blue, scarlet, and orange. They are found in leaves, flowers, fruits,

Table 4. Some anti-ageing (Vayahsthapana) drugs in Charak samhita

	Name	
	Botanical	**Sanskrit**
1.	*Asparagus racemosus*	Shatavar
2.	*Boerhaavia diffusa*	Punarnava
3.	*Centella asiatica*	Mandook parni
4.	*Clitoric ternatea*	Aparajita
5.	*Desmodium gangeticum*	Shalparni
6.	*Emblica officinalis*	Amlaki
7.	*Leptadinea reticulate*	Jeevanti
8.	*Pluchea lanceolata*	Raasina
9.	*Terminalia chebula*	Abhaya
10.	*Tinospora cordifolia*	See net

Table 5. Modern Evaluation of some Vayahsthapana Drugs

S. No.	Name of plant	Activities reported
1.	*Asparagus racemosus*	Immunostimulant, adaptogenic, anti-oxidant, galactogogue, anti-abortive, anti-ulcer, anticancer.
2.	*Boerhaavia diffusa*	Diuretic, anti-inflammatory, analgesic, anti-fibrinolytic, hepatoprotective, cholerectic; cardiotonic, Ca-channel antagonist, immunosuppressant, anti-cancer, anti-oxidant.
3.	*Centella asiatica*	Improves cognitive functions and deficits, anti-epileptic, sedative, anti-anxiety, vascular diseases, wound healing, and anti-oedens; anti-eczema, anti-psoriasis and anti-leproitic, anti-ulcer, anabolic; radio protective, anti-oxidant; antiviral and anti-tumor.
4.	*Desmodium gongeticum*	Anti-inflammatory, analgesic, anti-cancer, anti-cholinesterase (helps dementia), anti-oxidant.
5.	*Emblica officinalis*	Cardio and hepatoprotective, antidiabetic, anti-ulcer, and anti-pancreatitis; immunomodulatory, adaptogenic, hypolipidaemic, anti-tussive, anti-inflammatory, anti-pyretic, anti-bacterial, anti-cancer and anti-HIV and anti-oxidant.
6.	*Leptadenia reticulata*	Promotes eyesight, good in chest congestion, galactogogue, antibacterial, hypotensive, anticancer and anti-oxidant.
7.	*Terminalia chebula*	Antibacterial, anti-ulcer, anti-fungal, antiviral; purgative, feeding behaviour regulator; hypoglycaemic, hypocholesterolaemic and anti-atherosclerotic, cardio protective, cardiotonic, hypotensive; anti-inflammatory, anti-anaphylactic, immunosuppressant, anti-cancer; anti-oxidant.
8.	*Tinospora cordifolia*	Immunomodulatory, adaptogenic and anti-oxidant; anti-inflammatory, analgesic, anti-pyretic and anti-arthritic, anti-asthma; photo-protective, radio-protective and hepatoprotective, hypoglycemic, anticancer.
9.	*Clitorea fernatia*	Nootropic, memory enhancer, anxiolytic, antidepressant, anticonvulsant, insecticidal, anti-pyretic, anti-inflammatory, anti oxidant.

seeds, nuts, grains, spices, roots etc. (Pietta, 2000; Weisburger, 1997). Many of the biological activities of flavonoids are attributed to their antioxidant properties and free radical scavenging capabilities (Table 7). A number of flavonoids efficiently chelate trace metals, which play an important role in oxygen metabolism. Free iron is a potential enhancer of ROS formation as it leads to reduction of H_2O_2 and generation of the highly aggressive hydroxyl radical. Free copper mediates LDL oxidation and contributes to oxidative damage due to lipid peroxidation (Shu, 1998). Due to the inefficiency of endogenous defense system in some physiopathological situations, increasing amounts of dietary antioxidants will be useful to diminish the cumulative effects of oxidative damage (Sun *et al.,* 2002; Smith *et al.,* 1999). Isoflavonoids include the isoflavones genistein and daidzein, which occur mainly as the glycosides genistan and daidzin, found respectively in soybeans and in other legumes (Wiseman, 2000). Soy foods are made from Soya beans and include both fermented and non-fermented foods. Non-fermented soy foods contain isoflavones mostly present as β-glucosides, some of which are esterified with malonic acid or acetic acid. Fermented soy foods such as miso or tempeh contain mostly unconjugated isoflavons. Some alcoholic beverages such as beer contain significant amounts of isoflavonoids. Several herbs and spices have also been reported to exhibit antioxidant activity, including rosemary, sage, thyme, nutmeg, turmeric, white pepper, chilli-pepper, ginger and several Chinese medicinal plants extracts (Lee *et al.,* 2003).

The majority of the active antioxidant compounds are flavonoids, isoflavones, flavones, anthocyanins, coumarins, lignans, catechins and isocatechins. In addition to the above compounds found in natural foods, vitamin-C and E, β-carotene, and α-tocopherol are known to possess antioxidant potential (Cai *et al.,* 2004; Tsao & Akhtar, 2005). Table 6 shows list of neutralizing antioxidants against ROS and additional physiological antioxidants (Sies & Staul, 1995; Anderson, 1996).

MEDICINAL PLANTS NATIVE TO INDIA WITH ANTIOXIDANT AND CNS ACTIVITY

Withania somnifera (Dunal)

W. somnifera is also called aswagandha, asgandhi, asoda or amukkira. The shrub grows wild in India (Handa & Kapoor, 2003). It is wildly distributed from southern Europe to India and Africa.

Ashwagandha (Fig 1) consists of dried roots and stem bases of the plant. The drug occurs in unbranched, straight cylindrical buff colored pieces of variable length. The outer surface shows longitudinal

Table 6. Foodstuffs Containing Antioxidant Constituents (Singh *et al.*, 2004)

Food stuffs	Constituents act as antioxidant	Food stuffs	Constituents act as antioxidant
Citrus fruits/black tea	Quercetin, rutin, hesperetin, naringin	Cloves	Eugenol, cryophyllene
Tomato juice/green tea	Kaempferol	Fenugreek seeds	Diosgenin, sapogenin
Tomato juice/vegetables	Fisetin	Rosemary	Camosol
Tomato juice/vegetables	Myricetin	Mint (pudina)	Menthofuran, menthol
Popolis/fruits	Galangin	Garlic	Allyl sulfide
Soyabean/soy foods	Daidzein, daidzin	Bel	Umbelliferone, marmalosin
Red clover	Biochanin A, formononetin	Cereal products	Apigenin, luteolin
Fruits/vegetables	Cyanidin, cyanin	Milk products	Casein, vitamin - D
Fruits/vegetables	Chrysin	Fish	Cord oil (Vitamin-A)
Soyabeans	Gemistein, genistin	Eggs	Vitamin - A
Tomato	Lycopene	Black pepper	Piperine, piperidine, piperatine
Green & red chilli peppers	Capciasin	Cinnamon	Eugenol, phellandrene
Cruciferous vegetables	Isothiocyanate, erucic acid	Green & black tea	(+)-Catechin, (-) Epicatechin, (-)-Epigallocatechin

Table 6. *Contd.*

Food stuffs	Constituents act as antioxidant	Food stuffs	Constituents act as antioxidant
Ginkgo biloba	Bilobalide, ginkgolides	Saffron	Crocetin
Virgin olive oils	3,4-dihydroxyphenylethanol (DOPET)	Ginger	Gingerol
Tulsi (basil)	Eugenol, nerol, camphene	Karela	Vicine, nomoridicine
Ginseng	Ginsenosides	Amla	Corilagin, ellagic acid, gallotanins
Walnuts, almonds	Oleic acid, alpha-linolenic acid, vitamin-E, minerals	Saunf	Anethole
Turmeric	Curcumin	Shah root	Betulinic acid

Table 7. Reactive oxygen species and their corresponding neutralizing antioxidants and also additional antioxidants

ROS	Endogenous antioxidants		Exogenous antioxidants
	Direct role	Indirect role	
Hydroxyl radical	GPx (Cofactor selenium)	–	Vitamin C, lipoic acid
Lipid peroxide	GPx (Cofactor selenium)	–	Vitamin E, beta-carotene
Superoxide radical	SOD (Cofactor/ Cu/Zn/Mn)	Ceruloplasmin (Cu) Metallothionin (Cu) Albumin (UU)	Vitamin C
Hydrogen peroxide	CAT (Cofactor iron)	Transferin (iron) Ferritin (iron) Myoglobin (iron)	Vitamin C, beta-carotene, lipoic acid
Prooxidant/anti oxidant equilibrium	GSH, lipoic acid, N-acetyl cysteine NAdPH and NADH Ubiquinone	Bilirobin uric acid	Flavonoids

wrinkles. The odour is not characteristic but the taste is bitter (Handa & Kapoor, 2003). *W. somnifera* is widely used in ayurvedic medicine, the traditional medical system of India. It is an ingredient in many formulations prescribed for a variety of musculoskeletal conditions (*e.g.* arthritis, rheumatism), and as a general tonic to increase energy, improve overall health and longevity, and prevent disease in athletes, the elderly, and during pregnancy (Chatterjee & Prakrashi, 1995; Bone, 1996). Many studies have shown that *W. somnifera* also have anti-inflammatory properties (Anbalagan & Saddique, 1981, 1984; Somasundaram *et al.*, 1983). Animal stress studies have been performed to investigate its use as an antistress agent (Dadkar *et al.*, 1987; Archana & Namasivagan, 1999; Dhuley, 1998) and antioxidant property (Ames *et al.*, 1993; Panda & Kar, 1997; Shukla, 2000). In a study stress produced depression anxiety and retention deficit in young and old rats; administration of *W. somnifera* methanolic extract 250 mg/kg during shock period in young and old rats attenuated the stress induced depression and enhanced memory (Ramanathan *et al.*, 2003). A significant decrease in lipid-peroxidation occurred in *W. somnifera* administered hyper cholesteremic animals when compared to their normal counterparts (Visavadiya & Narsimhacharya, 2006). The chemistry of *W. somnifera* has been extensively studied and over 35 chemical constituents have been identified, extracted, and

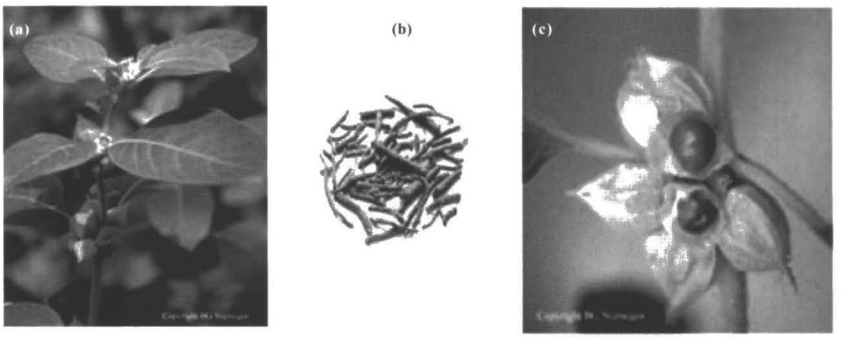


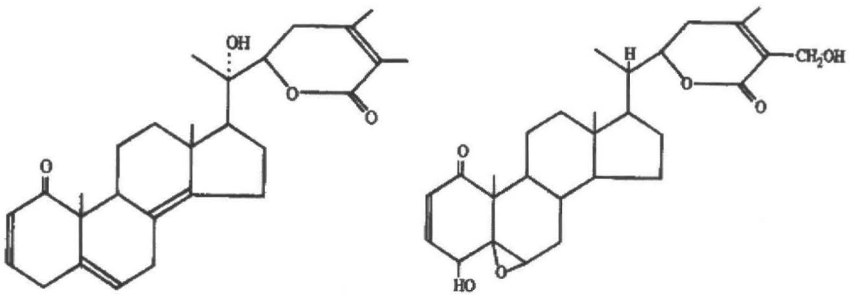
Fig 1. *Withania somnifera*; a. plant, b. dried roots, c. fruit

isolated (Rastogi & Malhotra, 1998). The biologically active chemical constituents are alkaloids (isopelletierine, anaferine), steroidal lactones (Withanolides, Withaferins), saponins containing an addtional acyl group (Sitoindoside VII and VIII), and wthanolides with a glucose at carbon 27 (Sitoindoside IX and X) *Withania somnifera* is also rich in iron (Fig 2).

Since traditional Ayurvedic use of *W. somnifera* has included many diseases associated with free radical oxidative damage, it has been considered likely that the effects may be due to a certain degree of antioxidant activity. The active principles of WS- Sitoindosides VII-X and Withaferin A (glycowithanolides), have been tested for antioxidant activity using the major free-radical scavenging enzymes, SOD, CAT and GPX levels in the rat brain frontal cortex and striatum. Decreased activity of these enzymes leads to accumulation of toxic oxidative free radicals and resulting degenerative effects (Vimal, 2007; Meena, 2008). An increase in these enzymes would represent increased antioxidant activity and a protective effect on neuronal tissue. Active glycowithanolides of *W. somnifera* (10 or 20 mg/kg intraperitoneally) given once daily for 21 days; showed dose related increase in all enzymes; the increases comparable to those seen with deprenyl (a known antioxidant) administration (2 g/kg/day intraperitonelly. This implies that *W. somnifera* does have an antioxidant effect in the brain which may be responsible for its diverse pharmacological properties (Bhattacharya *et al.*, 1997; Meena, 2008).

Nervous System Effects of *W. somnifera*

Total alkaloid extract (ashwagandholine, AG) of WS roots has been studied for its effects on the central nervous system (Malhotra *et al.*, 1965). AG exhibited a taming and a mild depressant (tranquilizer) effect on the central nervous system in monkeys, cats, dogs, albino rats and mice. AG had no analgesic activity in rats but increased



Withanolide Withaferine A

Fig 2. Structures of chemical constituents of *Withania somnifera*. Chemical structure of withanolides (i) and withaferin A (ii)

metrazol toxicity in rats and mice, amphetamine toxicity in mice, and produced hypothermia in mice. It also potentiated barbiturate, ethanol and urethane-induced hypnosis in mice. Effects of sitoindosides VII-X and withaferin on brain cholinergic, glutamatergic and GABAergic receptors in male wistar rats, (Schliebs *et al.*, 1997) have shown that they slightly enhanced acetylcholinesterase (AchE) activity in the lateral septum and globus pallidus, and decreased in the vertical diagonal band, accompanied by enhanced M_1 - muscarinic-cholinergic receptor-binding in lateral and medial septum and in frontal cortices, whereas the M_2 - muscarinic receptor-binding sites in a number of cortical regions including cingulate, frontal, piriform, parietal, and retrospinal cortex. Sitoindosides VII-X and withaferin affect the cortical and basal forebrain cholinergic-signal-transduction cascade. The drug-induced increase in cortical muscarinic acetylcholine receptor capacity might partly explain the cognition enhancing and memory-improving effects of *W. somnifera* extracts in animals and in humans. Ashwagandholine, total alkaloids extracted from extracts of WS roots, caused relaxant and antispasmodic effect against various agents that produce smooth muscle contractions in intestine, uterine, tracheal, and vascular muscles (Malhotra *et al.*, 1965).

Contraindications and Toxicity

Large doses of ashwagandha may possess abortifacient properties so that drug should not be prescribed during pregnancy. It is also contraindicated in conjunction with sedatives or anxiolytics. Drug should not be taken with positive inflammatory conditions or advance arterial blockade. In such conditions it must be taken with Yogaraj Guggal. Herb is relatively safe, if taken in suggested dosage. Drug also should not be taken with barbiturates as it potentiates their

effects. Ashvagandha is traditionally avoided in lymphatic congestion, during colds and flu (Frawley & Lad, 1986).

Centella asiatica (Linn.) Urban

Also called Brahmi, Manduka parani, Mandooki, Divya, Bhekaparni and Indian pennywort. It is a small creeping herbaceous plant found in damp, shady places throughout the tropical regions of the world. In India, it is very common throughout the peninsula from the Himalayas to Tamil Nadu at altitudes up to 600 m above the sea level. *C. asiatica* (Fig 3) of the family Apiaceae is a creeping, perennial, aromatic herb inhabit tropical and sub tropical regions of the world. The plants finds mention in *Shsruta samhita* and is an important component of the Indian pharmacopoeia. The plant has been widely recommended for use in wound healing, treatment of skin lesions, leprosy, eczema and psoriasis. It is used as a nervine tonic for improving memory and in insanity. It is also effective as a diuretic, alternative and tonic, having cooling and antipyretic properties. It is also used in leucoderma, inflammation, anemia, blood disease, syphilis and tuberculosis.

Chemical constituents

Plant contains aminoacids, aspartic acid, glycine, glutanic acid, α-alanine and phenyl alanine, β-sitosterol, palmitic acid and stearic acid, two saponins (brahmoside and brahminoside) three terpene acids–(brahmic acid, isobrahnic acid and betulic acid); mesoinositol, an oligosaccharide centellose and asiatic acid. Thankuniside and asiaticoside are other glycosides reported from the plant. Plant also contains stigmasteral and indocentoic acid. A bitter principle, vellarine, peptic acid and a resin are present in the leaves and roots. Alcoholic extract of the herb yields an essential oil, green in colour and possess strong odour of the original herb, a fatty oil, sitosterol, tannin and resinous substance. The triterpenoid compounds are the chief pharmacologically active substances in *C. asiatica*. The glycosides

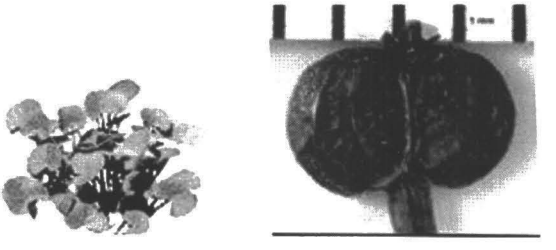


Fig 3. *Centella asiatica* – a) plant, b) dried leaf

such as asiaticoside, madecassoside, centelloside, branmoside, brahminoside, thankuniside, isothankuniside and asiaticoside A and B have been also reported. Asiaticoside ($C_{48}H_{78}O_{79}$) and ester of asiatic acid with two molecules of glucose and one molecule of rhamnose has proved effective in healing leprosy, tuberculosis and other skin diseases. Centelloside that is an ester of centellic acid ($C_{30}H_{43}O_6$) and glucose and fructose. Brahmoside ($C_{47}H_{78}O_{19}$) and brahminoside ($C_{53}H_{88}O_{24}$) are tri and tetra-glycoside of brahmic and with glucose, Rhamnose are also reported (in plants from India). The plant also possesses stigmasterol, stigmastside. A fatty oil consisting of the glucosides of olive, unoleic, linolenic, lignoceric, palmitic and stearic acids is obtained from this plant. Amino acids such as aspartic acid, glutamic acid, glycine, alanine, phenylalanine, serine, threonine, histidine and lysine have been reported from different parts of this plant.

Medicinal Uses

Plant parts are useful in diseases of the skin, nervous system and blood. The plant bears many synonyms in *Nighantus* where it is described as cold, moist, sweet, light and alterative ; It is said to improve the memory and understanding and to cure, anxiety, poor memory, ADD/ADHO, senility, Alzheimer's disease, epilepsy, chronic fatigue, premature aging, alopecia, gastrointestinal ulcers, eczema, psoriasis, leprous ulcers, venereal diseases, burns, jaundice, hepatitis, hypertension, anemia, diabetes, bronchitis, edema, varicosities, phlebitis venous insufficiency, fever, immunodeficiency, autoimmune disorders, cancer leprosy, jaundice, gonorrhea and fever (Nadkarni, 1908, 1976; Dash & Junius, 1983; Frawley & Lad, 1986; Varier, 1994). The leaves are only recognized in the *Pharmacopeia of India*, but many investigators have advocated the use of entire plant, root, twigs, leaves and seeds in medicine.

Brahmi (*C. asiatica*) is one of the recognized drugs used for *rasayana* (rejuvenation) purpose. Two common forms in which the drug is used are as a *Swarasam* given as it is, and in prepared form as a *Ghritam*. These will improve the colour of the body, youth, memory and give long life. The administration of asiaticoside, an isolated constituent of *C. asiatica*, significantly increased the levels of SOD, CAT, GPx, vitamin E and ascorbic acid in rats. The level of antioxidant activity was highest during the initial stages of treatment. Asiaticoside derivatives were found to inhibit or reduce H_2O_2 induced cell death and lower intra-cellular free radical concentration, protecting against the effects of beta-amyloid neurotoxicity (Mook-Jung *et al.*, 1999). A two compartment passive avoidance task test (with rats) showed an improvement in 24 h retention. Assessment of

turnover of biogenic amines (norepinephrine, dopamine and serotinin) showed significant reductions of these amines and their metabolites in the brain following oral administration of a fresh juice (1 mL = 0.38 g fresh leaves), at a dose of 0.18 g/kg for 15 days. The decrease of amine levels was correlated to improved learning and memory in rats (Leung & Foster, 1996). A water-soluble fraction of *C. asiatica* was found to have an anxiolytic effect in animals comparable to diazepam (Leung & Foster, 1996). An extract of *C. asiatica* was found to increase brain GABA levels (Chatterjee *et al.*, 1992). Effects of *Centella asiatica* in mentally challenged children (half were given 500 mg tablets of dried whole plant, and half placebo and intelligence quotient tests was conducted. at the outset of the study, and again at interval of 3 months. Results indicated that children who took the *C. asiatica* tablet showed significant improvements in co-operation, memory, concentration, attention, vocabulary and social adjustment. Intraperitoneal injections of brahmoside and brahminoside were found to have a CNS – depressant effect in mice and rats (Ramaswamy *et al.*, 1970). Six week treatment in patients of anxiety necrosis reduced anxiety levels and showed improvement in the mental fatigue rate and immediate memory.

Contraindications and Toxicity

A water-soluble fraction of *Centella asiatica* was reported to inhibit hepatic enzymes responsible for barbiturate metabolism (Leung & Foster, 1996). *Centella asiatica* has also been found to have a GABA nergic activity (Chatterjeee *et al.*, 1992). The triterpene constituents have shown to lack any kind or teratogenic effects (Bosse *et al.*, 1979), relaxation of the rat uterus has been documented for brahmoside and brahminoside (Ramaswamy *et al.*, 1970). *Centella asiatica* is best avoided in pregnancy. Hyperglycemic and hypercholesterolemic effects have been reported for asiaticoside in humans (Newall *et al.*, 1996), and caution should be exercised with the concomitant use of hypolipidemic and hypoglycemic therapies.

An alcoholic extract was found to be nontoxic up to a dose of 350 mg/kg intraperitoneally in rats. No mortality was found up to a dose of 5 g/kg in mice. In the doses commonly used no adverse reactions are reported (Aithal & Sirsi, 1961).

Asparagus racemosus Wild.

Asparagus racemosus is commonly known as "Shatavari", shatmuli, Kilavari, satavar, Pilli. Shatavari means "Who possess a hundred husbands. Shatavari is the main Ayurvedic rejuvenative tonic for female, as is Ashwagandha for the male. Shatavari is used for sexual

debility and infertility in both sexes. It is also used for menopausal symptoms and to increase lactation (Thakur *et al.,* 1989) Romans used *A. racemosus* for food and medicinal purposes. It was first cultivated in England at the time of Christ and brought to America by the early colonists. *A. racemosus* has also been used in traditional Indian ayurvedic medicine. The drug consists of dried tuberous roots of *A. racemosus.* The plant (Fig 4), grows in Himalayan and Sub-Himalayan ranges from 1300-1400 m height in India.

The root are cylindrical, fleshy tuberous, tapering towards the base and swollen in the middle. They are white to buff colored measuring 5-15 cm. in length and 1-2 cm. in diameter. When soaked in water the root becomes soft and swells considerably. The drug has a bitter taste (Handa & Kapoor, 2003).

Chemical Constituents- Steroidal saponins collectively known as shatavarins I-IV are present to the extent of 0.1-0.2%. The basic aglycone is sarsapogenin to which three glucose and one rhamnose molecules are attached constituting shatavarin – I whereas in shatavarin IV (Fig 5), two glucose and one rhamnose molecule are attached (Shah & Qadry, 1996; Handa & Kapoor, 2003).

Shatavarin–I is the main active glycoside of sarsapogenin. Shatavarin- IV is structurally related to shatavarin-I (Shah & Qadry, 1996; Handa & Kapoor, 2003).

Medicinal Uses

Shatavarin-I has been reported to have antioxytocic activity. The drug is also reported for galactagogue activity (Atal & Kapur, 1982; Shah & Qadry, 1996; Handa & Kapoor, 2003). Its petroleum ether extract showed diuretic activity, Shatavari has great reputation in Ayurveda in uterine diseases, as an antacid and tonic (Shah & Qadry, 1996; Atal & Kapur, 1982). Juice from the fresh root is given orally in dysentry. Root is pounded with root of *Smilax prolifera* Roxb. and is prescribed as drink to cure urinary disorder as well as discharge of blood in the urine. The root paste is applied externally on the feet to heal the wound (Atal & Kapur, 1982c).

Contraindications and Toxicity

Shatavari is rich in phytoestrogens a group of naturally occurring compounds that have a chemical structure very similar to estrogen female hormone thus can even bind to estrogen receptors and displace estrogen molecule from these receptors. Estrogen is a hormone that is necessary for the normal sexual development and growth of the breasts, uterus and ovaries, plays significant role in controlling

Fig 4. *Asparagus racemosus*

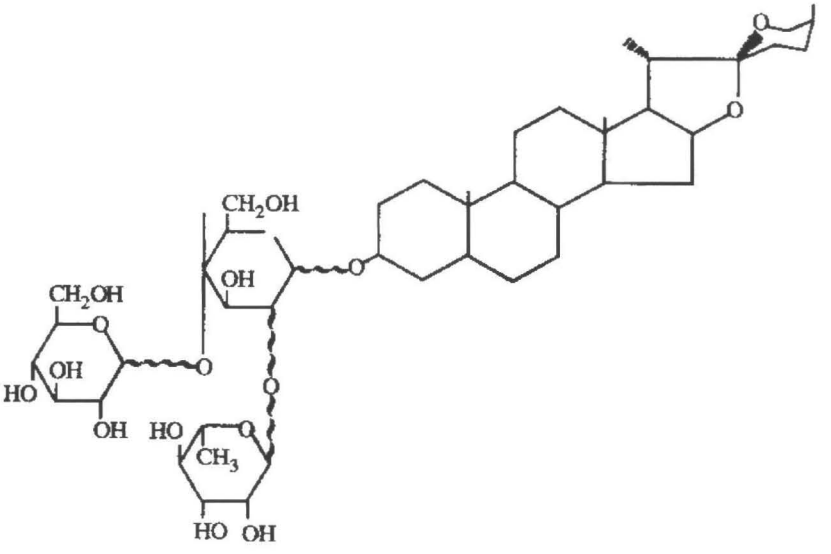


Fig 5. Shatavarin IV (Shah & Qadry, 2004)

woman's menstrual cycles and is essential for reproduction. Estrogen also helps maintain the cardiovascular system and prevent osteoporosis (disease in which the bones become extremely porous, subject to fracture, tend to heal slowly and are subject to infection). Estrogen's access to reproductive tissue *i.e.* breast and endometrial is controlled by estrogen receptors. Only estrogen or substances with a close structural resemblance to estrogen are permitted to bind. This explains how the similar phytoestrogens can bind to the estrogen receptors and displace the estrogen. Drug has no toxicity

Acorus calamus

A. calamus is known as Vacha. It is a aromatic marsh herb found in wild as well as cultivated throughout India. Medicinally useful part of the plant is rhizome. Essential oil consists of a range of sesquiterpene hydrocarbons, alcohols and ketons (*e.g.* acorone, acoragermacrone, calamendiol) besides eugenol, methyl isoeugenol and phenyl propane derivatives α and β-asaron. In Ayurveda, vacha has been acclaimed for treatment of epilepsy and as a tranquilizer. Juice of the herb is also recommended in Sushruta Samahita for enhancing intellectual vigour and longevity. Ethanolic extract of the rhizome has been shown to possess neuroprotective action.

Bacopa monnieri

It is commonly found in wet marshy and damp places through out India. The drug contains alkaloid-brahmine, and herpestine, saponins, bacosides A and B. The plant is used as nervine tonic, diuretic and commonly used to treat asthma, epilepsy, insanity and hoarseness. It is a major constituent of Medhya rasayana formulations, which facilitates learning and improves memory (Chatterjee *et al.,* 1963; Garai *et al.,* 1996). Studies using 50% ethanolic extract of the whole plant without roots demonstrated its effects on short and long term memory retention. Effects of *B. monnieri* extract on Alzheimer's disease using rat model have been demonstrated. Oral administration of 5-10 mg extract per kg body weight markedly reduced the memory deficits along with reduction in acetylcholine concentrations, choline acetylase activity, and muscuranic receptor binding in hippocampus and frontal cortex.

Celastrus peniculatus

Celastrus peniculatus along with *W. somnifera*, shilajit and *Convolvulus pleuricaulis* showed CNS activity (Salil & Gupta, 1998) Seed Oil is known to affect CNS and is effective in psychiatric patients. Stimulatory effects of *C. peniculatus* have been also reported

(Nadkarni, 1976). Its role in memory enhancement and improvement in IQ of mentally retarded children has been proved.

Convolvulus pleuricaulis

Convolvulus pleuricaulis is used in traditional systems of medicine in anxiety, neurosis, insanity, epilepsy, and also as a brain tonic. It is also one of the most important Medhya Rasayana drugs in Ayurveda. *C. pleuricaulis* is used traditionally to treat nervous debility, insomnia, fatigue, low energy level and as a brain tonic, alterative and febrifuge. The whole herb is used medicinally in the form of decoction with cumin and milk in nervous debility, and loss of memory. The plant is known as psychostimulant, tranquilizer, and useful in reducing mental tension. Methanolic extract of *C. microphyllus* Sieb. Ex Spreng (*C. pleuricaulis* Choisy) shown to enhance release of nerve growth factor (NGF). NGF prevents experimentally induced or age related degeneration of basal forebrain cholinergic cell bodies in adult rats and can also restore lesion induced loss of cognitive functions.

Crocus sativus

C. satvus is called as Kunkumam or Keshara in Ayurveda. It is small perennial cultivated in certain parts of Jammu and Kashmir, Himachal Pradesh and Uttarakhand in India. Medicinally useful part is the stigma which is dried and marketed as Saffron. Important constituent of Saffron are its pigments and essential oil. The major component is Crocin-1 along with Crocin-2, 3 and 4. Four crocusatins (F, G, H, I) have been also isolated (Li & Wu, 2002). Alcoholic extract of saffron ameliorated the impairment effects of ethanol on learning and memory processes. Crocin inhibits neuronal death induced by both internal and external apoptotic stimuli (Soeda *et al.,* 2001) thus act as neuroprotective. Crocin prevents activation of c-jun kinase phosphorylation, which is involved in the signalling cascade downstream ceramide for neuronal death (Ochai *et al.,* 2004).

Curculigo orchioides

Also called Taalmusli. Grow all over India. Pharmacological investigations revealed that 70% ethanol extract of the rhizomea are sedative and anticonvulsant.

Curcuma longa

Curcuma known as Haldi is perennial rhizome plant, growns all over India. *C.* powder or extract of Curcuma is curcumin which show strong antioxidant activity. Methanol extract of turmeric led to the

isolation of calebin-A and the curcumins which effectively protected neuronal cells against α-amyloid deposition (Park & Kim, 2002). In another study an oral administration of curcumin to alcohol-fed rats caused a significant reversal of brain lipid peroxidation indicating its neuroprotective role (Rajakrishnan *et al.,* 1999). *In vivo* experiments showed that oral intake of curcumin significantly reduce the duration and clinical severity of demylenation in experimental allergic encephalitis.

Cyprus rotundus

In Ayurveda known as Mustaka. It is a perennial weed, grow almost throughout India. Medicinally useful part is tuber and constituents are essential oil sesquiterpenoids, monoterpenes, aliphatic alcohols and acetate. Receptor binding assay demonstrated that isocurcuminol a constituernt of *C. rotundus* modulate GABAergic neurotransmission via enhancement of endogenous receptor ligand binding thus affecting epilepsy (Ha *et al.,* 2002).

Ficus religiosa

Ficus religiosa possesses anticonvulsant activity. The extract obtained from the leaves of the plant was evaluated for its activity against pentylenetetrazole (60 mg/kg *i.p.*) induced convulsions in albino rats. The study revealed 80 to 100% protection against PTZ induced convulsions when given 30-60 min prior to induced convulsions.

Ginkgo biloba

Extract from green leaves EGB761 was identified as therapeutically useful as neuroprotective agent, sustainable for the therapy of patients with cerebrovascular disorders or cerebral insufficiencies. Ginkgo is widely used in Europe for treating dementia. It improves blood flow in the brain and contains flavonoids that act as antioxidants. It is presumed that Ginkgo may improve thinking, learning and memory, and results are very encouraging in people with AD. More than forty components of Ginkgo have been identified and isolated. Two of the most important groups of active chemicals are flavonoids (*quercetin*, kaempferol, isorhemnetin) and terpenes (lactones or terpenoids which include bilobalide and several ginkgolides-A, B, C, J and M). Individual constituents have been studied in *in vitro*, animals and human experimental systems (Chavez & Chavez, 1998; Van Beek *et al.,* 1998). The mechanism of Ginkgo's therapeutic effects are not fully understood but attributed to the synergistic effects of its constituents (Behl *et al.,* 1999; Maitra *et al.,* 1995).

Mucuna pruriens

M. pruriens in Ayurveda called Atmagupta or Kapikacchu. It is herbaceous creeper grow in several parts of India. Seeds, root and pod bristles are medicinally useful parts. Important chemical constituent of the plant is the non protein amino acid, levodopa which is present is seeds. Besides, B-sitosterol, lecithin, glutathione, gallic acid are other important constituents (Vaidya *et al.,* 1978). Beans of this plant are used as nutritive food in some parts of India. It is also used as therapeutic agent in various reproductive and nervous diseases (Nadkarni *et al.,* 1908; Damodaran & Ramaswamy, 1937; Dutt, 1980). An Ayurvedic formulation containing Mucuna beans is used in Parkinson's.

Nardostachys jatamanasi

The plant is used by Santhal tribals in madness, epilepsy, unconsciousness, convulsions etc. The decoction of root is also reported to be useful in mental disorders, insomnia etc. *N. jatamanasi* is reported to yield 2% volatile oil containing an ester, an alcohol and two alkaloids (Jain *et al.,* 1970). The rhizome of jatamanasi yield jatamanashic acid (Chaudhary *et al.,* 1951). Various extracts of jatamanasi root showed sedative actions in rat. Ethanolic extract reduced the rat brain serotonin, and showed no effect on CNS. Oil from rhizome showed depressant action on the CNS. A preparation comprising *N. jatamanasi* and *C. asiatica, A. calamus, R. serpentina, S. lappa* and *V. wallichii* showed significant improvement in Schizophrenic patients (Chopra *et al.,* 1954). Methanol extract showed potent inhibition of acetylcholinesterase reaction rate.

Plumbago zeylanica

Also called Chitraka in Ayurveda. It is a perennial shrub grows wildly in hotter parts of India. Roots and root bark are medicinally useful. The chief constituent is Plumbagin. Ethanol extract of the root has shown spontaneous motility in rats with concomittent increase in dopamine and its metabolite homovanillic acid level in striatum, indicating a dopaminergic pathway for stimulatory action on the CNS. Plant has been also useful in treatment of Schizophrenia.

Semecarpus anacardium

Commonly known as Bhallataka. It is a tree commonly grown in hotter areas of India and in outer Himalayas. Fully developed nut is recognized medicinally. A phenolic glycoside anacardoside has been isolated. Besides the phenol, several bisflavoinioids have been obtained from defatted nut. A cytological and ultrastructural study on Swiss

rats from this laboratory has shown antioxidant and neuroprotective effects of the ethanolic extract (Shukla *et al.,* 2002; Bopaiah & Pradhan, 2001; Bhatnagar *et al.,* 2005).

Swertia chirayita

Called as Kiraatatikta in Ayurveda. Chiraytta is Indian trade name. It grows in temperate Himalayas from Kashmir to Bhutan. Whole herb is used medicinally. But root is considered as more potent. More than 20 polyhydroxylated xanthones were characterized such as swertinin, swerchrin and mangiferrin. Mengiferrin has been shown to posses free radical scavenginmg activity. Mengiferrin has been also shown to be superpoxide scavenger and an inhibitor of the expression of inducible nitric oxide synthatase and TNF–genes, thus revealing its potential for the treatment of neurodegenerative disorders (Leiro *et al.,* 2003).

Galanthus wornorii

Galanthamine is a pure unaltered extract of *Galanthus*. A recent study of Wilcock and coworkers (Wilcock *et al.,* 2000) has shown that galanthamine appears to slow the progression of neurodegeneration condition. It also reversibly and competitively inhibits acetylcholinesterase and enhances the response of nicotinic receptors to acetylcholine. Study in 653 Alzheimer patients have shown that galanthamine slows down the decline of the functional abilities, as well as cognition.

Lavandula stoechus

Lavandula has been used for a long time in traditional medicine as anticonvulsant. Gilani *et al.* (2000) validated its anticonvulsant effects. The study revealed that aqueous methanolic extract (600 mg/kg) significantly reduces the severity of the disease and increased the latency of onset of convulsions induced by pentylenetetrazole (Gilani *et al.,* 2000).

Paederia foetida Linn.

Ethanolic extract has been showed significiant antioxidant activity (Swain *et al.*, 2008).

RATIONALE FOR USING MULTIPLE ANTIOXIDANTS

Persons over 50-65 years of age, who have suffered brain trauma or have been exposed to high levels of pesticides or herbicides or have a family history of PD are called high risk population. Because of potentially increased oxidative stress in the brains of members of

these population groups, oral supplementations with appropriate antioxidants appears to be one of the rational choices for the prevention of PD among them. Conventional experimental designs for prevention are not suitable for maximal efficacy of antioxidant therapy due to the varied actions of antioxidants, environments and varied nature of free radicals. Almost all antioxidants, when oxidized, can act as free radicals; therefore, the use of a single antioxidant in any clinical trial can not be considered a rationale for improving the disease outcome. L-dopa therapy is one of the common therapies for advanced PD. However, the severe side-effects of this therapy appear in about five years (Sax *et al.*, 1971; Pentland *et al.*, 1982). The reasons for this are not known; however, L-dopa can generate free radicals during its own oxidation as well as during oxidative metabolism of its product dopamine. Thus, it appears rational to propose that an excessive quantity of free radicals are generated and this may be one of the factors which contribute to the side-effects of levo-dopa therapy. Selegiline used in combination with levodopa may reduce free radical levels by reducing the oxidative metabolism of dopamine; however, it would not affect the level of free radicals generated by the oxidation of L-dopa. Therefore, it is possible that supplementation with appropriate multiple antioxidants may help improving the disease.

CONCLUSIONS

In the present review an attempt has been made to congregate the antioxidant and CNS properties along with phytochemical, and toxicological information on selected medicinal plants used in Indian traditional medicinal system. During the past decade, the traditional system have gained importance in the field of medicine and in many developed as well as developing countries, A large proportion of population now relies heavily on traditional formulations and preparations. Since the usage of herbal medicines has increased, the issues related to their safety, quality and efficacy in industrialized and developing countries have also cropped up. Researchers are scientifically screening various claims regarding properties and uses of the material plants/products/preparations. No doubt in present time, healthcare professionals, manufacturers and consumers seek updated information on various aspect of medicinal plants and their properties.

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Free Radical Scavenging and DNA Damage Preventing Properties of *Amaranthus tricolor* L.

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ABSTRACT

The leaves of Amaranthus tricolor L. are consumed as food. Aqueous extracts of freeze dried leaves, made at room temperature (RTAE) and by boiling in water for 5 min (BAE) scavenged DPPH radical, superoxide radical and hydroxyl radical in a dose dependent manner. Different fractions of aqueous methanolic crude extract (CE) of fresh leaves e.g. ethyl acetate soluble fraction (EAF), methanol soluble fraction (MSF) and water soluble fraction (WSF) were also assayed for DPPH radical scavenging activity. WSF was found to have highest activity. CE and WSF were further assayed for their superoxide radical scavenging activity, hydroxyl radical scavenging activity, total antioxidant capacity (equivalent to ascorbic acid) and DNA damage preventing property. The extracts scavenged superoxide radical and hydroxyl radical in a dose dependent manner. Amaranthine, which is reported to have antioxidant activity, in CE (1.21%) was present in lower amount than that in WSF (10.4%). WSF scavenged DPPH radical, hydroxyl radical and prevented DNA damage at lower concentration than CE probably because of the higher amaranthine content in this fraction.

Key words : *Amaranthus tricolor*, amaranthine, antioxidant, DNA damage

INTRODUCTION

Reactive oxygen species (ROS), which include free radical species like superoxide radical ($O_2^{-\bullet}$) and hydroxyl radical ($^{\bullet}OH$) and non free

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radical apecies like hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) can be formed in living organisms in different ways. These oxygen radicals induce some oxidative damage to biomolecules like carbohydrates, proteins, lipids, DNA (Gulcin *et al.*, 2003; Lai & Piette, 1977; Kellog & Fridovich, 1975; Wiseman & Halliwell, 1996). Oxidative stress or excessive production of ROS accelerates aging, cancer, cardiovascular diseases, neurodegenerative diseases, inflammation etc. (Ames, 1983; Stadtman, 1992; Sun, 1990). The harmful action of the ROS can however be blocked by antioxidant substances which scavenge the free radicals and detoxify the organism. Immune system is vulnerable to oxidative stress. The antioxidants preserve adequate function of immune cells against homeostatic disturbances (De la Fuente & Victor, 2000). Phytochemicals in common fruits and vegetables can have complementary and overlapping mechanisms of action, including scavenging oxidative agents, stimulation of the immune system, regulation of gene expression in cell proliferation and apoptosis, hormone metabolism, and antibacterial and antiviral effects (Waladkhani & Clemens, 1998).

The leaves of *Amaranthus tricolor* L. (syn. *A. gangeticus*) of family Amaranthaceae are consumed as food in India and other Southeast Asian countries. The leaves are red-violet due to the presence of water soluble betacyanins amaranthine and isoamaranthine (Piatelli *et al.*, 1964; Piatelli *et al.*, 1969; Mabry & Dreiding, 1968). Historically, the pigments of *Amaranthus* plants have been used to colour foods, beverages and bread products in numerous new world locations, the southwestern United States, Mexico, Bolivia, Ecuador and Argentina. Natural pigments from *A. tricolor* can be legally used as food ingredients in China (Cai *et al.*, 1998). Amaranthine and isoamaranthine isolated from *A. tricolor* inhibited DPPH radical (Cai *et al.*, 2003). Antioxidant activity of raw and blanched material was studied by β-carotene bleaching assay and DPPH radical scavenging assay (Amin *et al.*, 2006). Three galactosyl diacylglycerols isolated from the leaves and stems of *A. tricolor* were reported to have potent cyclooxygenase and human tumor cell growth inhibitory activities (Jayaprakasa *et al.*, 2004). *In vitro* and *in vivo* studies *A. gangeticus* showed anticancer potential (Sani *et al.*, 2004). The leaf extract proved beneficial for clinical use as a radioprotector (Verma *et al.*, 2002). In this paper superoxide radical scavenging activity, hydroxyl radical scavenging activity and the property to scavenge DNA damage and lipid peroxidation by diferent extracts of *A. tricolor*, have been reported

MATERIALS AND METHODS

Plant Material

Leaves of *A. tricolor* were collected from the local market. Both fresh and dried (in lyophilizer) materials were used for the study.

Reagents

Chemicals such as ethylenediamine tetra acetic acid (EDTA), butanol, ammonium molybdate, sodium dodecyl sulphate were purchased from E. Merck (India) Limited. 1,1 diphenyl-2-picrylhydrazyl and catechin were procured from Sigma, USA. Thiobarbituric acid (TBA) was purchased from Spectrochem PVT. Ltd., India. Nitroblue tetrazolium, agarose, bromophenol blue were obtained from Sisco Research Laboratories Pvt. Ltd., India. Plasmid DNA (pBR322) was procured from Genei, India. All other reagents were of analytical grade.

Extraction

Aqueous extracts of dried leaves were made either at room temperature or by boiling in water for 5 min, centrifuged and the supernatants were used for analyzing antioxidant activity *in vitro*. The concentrations of the extracts were expressed in terms of dried material used to make extract/volume (mg/mL).

Fresh plant materials were also homogenized and extracted with 80% methanol (Cai *et al.*, 2001). The homogenate was kept at 4 – 8°C for 50 min. The homogenate was filtered and the filtrate was evaporated to dryness under reduced pressure at room temperature to obtain the crude extract (CE). The aqueous solution of the crude extract was then further fractionated (Fig 1). Aqueous solution of CE was first extracted with ethyl acetate (x3). The combined ethyl acetate fraction (EAF) was evaporated to dryness. The aqueous fraction was evaporated to dryness under reduced pressure in a rotary evaporator. As this fraction shows a yellow spot on TLC apart from the red betacyanin spot, the flask was rinsed with methanol to obtain a yellow coloured methanol soluble fraction (MSF) that was evaporated to dryness. The unidentified MSF shows a single spot on TLC that is not phenolic in nature as it does not respond to 5% $FeCl_3$ reagent. The remaining extract was water soluble fraction (WSF) that is red in colour due to presence of amaranthine.

DPPH Radical Scavenging Activity

The antioxidant activity of the extracts on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical

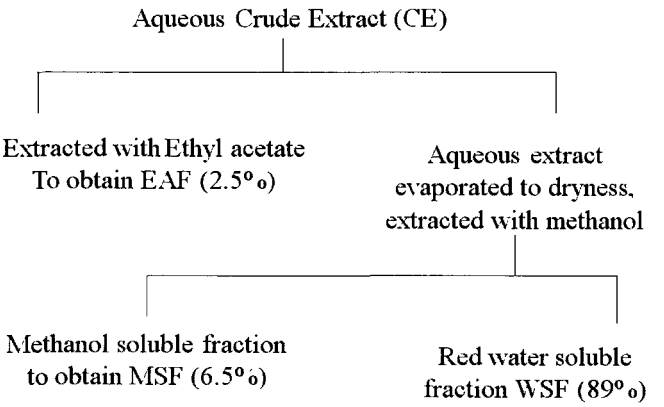

Fig 1. Extraction procedure for *A. tricolor* crude extract

was determined following the method described by Braca *et al.* (2001). Aqueous / methanolic extracts were added to 0.004% MeOH solution of DPPH. Absorbance at 517 nm was determined after 30 min and the percent inhibition activity was calculated as

$$\frac{Ao - Ae}{Ao} \times 100$$

(Ao = Absorbance without extract; Ae = absorbance with extract).

Assay of Superoxide Radical ($O_2^{-\bullet}$) Scavenging Activity

The method used by Martinez *et al.* (2001) for determination of superoxide dismutase was followed after modification (Dasgupta & De, 2004) in the riboflavin-light-nitrobluetetrazolium (NBT) system (Beauchamp & Fridovich, 1971). Each 3 mL reaction mixture contained 50 mm phosphate buffer (pH 7.8), 13 mm methionine, 2 mm riboflavin, 100 mm EDTA, NBT (75 mm) and 1 mL sample solution. The production of blue formazan was followed by monitoring the increase in absorbance at 560 nm after 10 min illumination from a fluorescent lamp.

Assay of Hydroxyl Radical ($^{\bullet}OH$) Scavenging Activity

The assay was based on benzoic acid hydroxylation method (Chung *et al.*, 1997). Hydroxyl radicals were generated by direct addition of iron (II) salts to a reaction mixture containing phosphate buffer. In a screw capped tube 0.2 mL sodium benzoate (10 mm) and 0.2 mL of $FeSO_4$, $7H_2O$ (10 mm) and EDTA (10 mm) were added. Then the sample solution and a phosphate buffer (pH 7.4, 0.1 M) were added to give a total volume of 1.8 mL. Finally, 0.2 mL of an H_2O_2 solution

(10 mm) was added. The reaction mixture was then incubated at 37°C for 2 h. After that the fluorescence was measured at 407 nm emission (Em) and excitation (Ex) at 305 nm. Measurement of spectrofluorometric changes has been used to detect damage by hydroxyl radical.

$$\text{•OH-scavenging activity (\%)} = \frac{1 - (\text{F.I.s} - \text{F.I.o})}{\text{F.I.c} - \text{F.I.o}} \times 100$$

where F.I.o: fluorescence intensity at Ex 305 and Em 407 nm with no treatment, F.I.c: fluorescence intensity at Ex 305 and Em 407 nm of treated control, F.I.: fluorescence intensity at Ex 305 and Em 407 nm of treated sample.

Lipid Peroxidation Assay

A modified (Dasgupta & De, 2004) thiobarbituric acid reactive species (TBARS) assay (Ohkowa *et al.*, 1979) was used to measure the lipid peroxide formed using egg yolk homogenates as lipid rich media (Ruberto *et al.*, 2000). Lipid peroxidation was induced by $FeSO_4$. Malondialdehyde (MDA), produced due to oxidation of polyunsaturated fatty acids, reacts with two molecules of thiobarbituric acid (TBA) yielding a pinkish red chromogen with an absorbance maximum at 532 nm that was measured. Egg homogenate (0.5 mL of 10% v/v) and 0.5 mL of extract were added to a test tube. 0.05 mL of $FeSO_4$ (0.07 M) was added to induce lipid peroxidation and incubated for 30 min. Then 1.5 mL of 20% acetic acid (pH 3.5) and 1.5 mL of 0.8% (w/v) thiobarbituric acid in 1.1% sodium dodecyl sulphate were added and the resulting mixture was vortexed and then heated at 95°C for 60 min. After cooling, 5.0 mL of butan-1-ol were added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532 nm. The leaves of *A. tricolor* contain high concentration of betacyanin that also absorbs at 532 nm. To eliminate this non-MDA interference, another set of samples was treated in the same way incubating without $FeSO_4$ to subtract the absorbance for betacyanin. Inhibition of lipid peroxidation (%) by the extract was calculated according to [(1 – E/C) x 100 where C is the absorbance value of the fully oxidised control and E is in presence of extract [Abs532(+$FeSO_4$) – Abs532(-$FeSO_4$)].

Prevention of DNA Damage

Plasmid DNA (pBR322) damage was induced by hydroxyl radical after addition of 50 mm $FeSO_4$, 100 mm EDTA and 0.5 mm H_2O_2. Mixtures of plasmid DNA (2 ml), plant extract (4 ml), $FeSO_4$ and EDTA (2 ml) and H_2O_2 (2 ml) were incubated at 37°C for 30 min. After incubation

2 ml of loading buffer (0.25% bromophenol blue and 40% sucrose in water) was added to the sample and loaded on to a 1% agarose gel prepared in Tris –acetic acid-EDTA buffer (4.84 g Trizma, 1.14 mL glacial acetic acid and 2 mL 0.5 M EDTA, pH 8.0) to which 2 ml of 1% ethidium bromide was added. Horizontal gel electrophoresis was performed at 150 V for 3 h. DNA strands were visualized under UV light. Images were analyzed using Quantity One 4.4.0 (BIORAD) software. The percentage protective effect of extracts was calculated following the equation (absorbance of supercoiled DNA in sample/ absorbance of supercoiled DNA in control) X 100.

Determination of Total Antioxidant Capacity

The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH (Prieto *et al.*, 1999).

Quantitative Determination of Pigment Content

Pigment content for the crude aqueous extracts and dried extracts were determined spectrophotometrically and expressed as amaranthine (Cai *et al.*, 1998) using e (molar absorptivity) for amaranthine = 5.66×10^4 cm $^{-1}$ mol^{-1} and MW = 726.6. Absorbance was measured at 536 nm.

RESULTS AND DISCUSSION

Aqueous extracts of dried leaves made at room temperature (RTAE) and made by boiling (BAE) inhibited DPPH radical (Fig 2) in a dose dependent manner (r = 0.9907 for RTAE and 0.9927 for BAE). The activity of the two extracts for scavenging DPPH radical differs

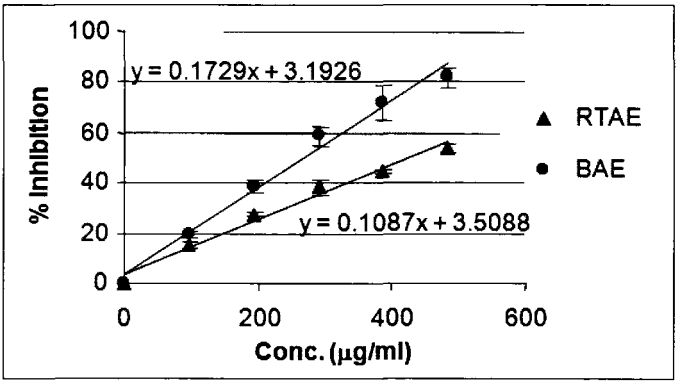

Fig 2. DPPH radical scavenging activity of A. *tricolor* leaf extract

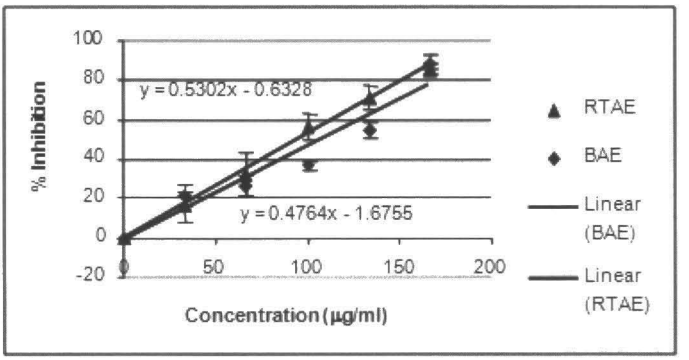

Fig 3. Superoxide radical scavenging activity of *A. tricolor* leaf

significantly ($p>0.01$), IC_{50} values being lower (270.72 mg/mL) in BAE than that in RTAE (427.7 mg/mL). IC_{50} value is inversely related to the activity. The extracts also scavenged superoxide radicals (Fig 3) generated by photochemical reduction of flavins and percentage inhibition was proportional to the concentrations ($r = 0.964559$ for RTAE, $r = 0.997572$ for BAE). The activity was found to be higher (not statistically significant) in RTAE (IC_{50} value = 95.5 mg/mL) than BAE (IC_{50} value = 108.47 mg/mL). The aqueous extracts also scavenged hydroxyl radicals generated by addition of iron (II) salts to a reaction mixture containing phosphate buffer (Guttridge, 1984). Benzoate is hydroxylated to hydroxybenzoates. Benzoate is weakly fluorescent, but after monohydroxylation forms highly fluorescent products (Gutteridge, 1987). Measurement of spectrofluorometric changes has been used to detect damage by hydroxyl radical. RTAE showed higher activity (IC_{50} value = 729.35 mg/mL) than BAE (IC_{50} value = 850.41) (not statistically significant). There was a linear correlation (Fig 4) between concentration of extract and •OH scavenging activity ($r = 0.9987$ for RTAE; $r = 0.9833$ for BAE). Total antioxidant activity of plant material

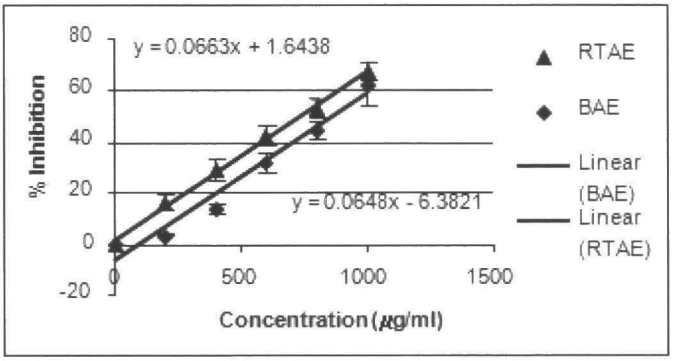

Fig 4. Hydroxyl radical scavenging activity of *A. tricolor* leaf

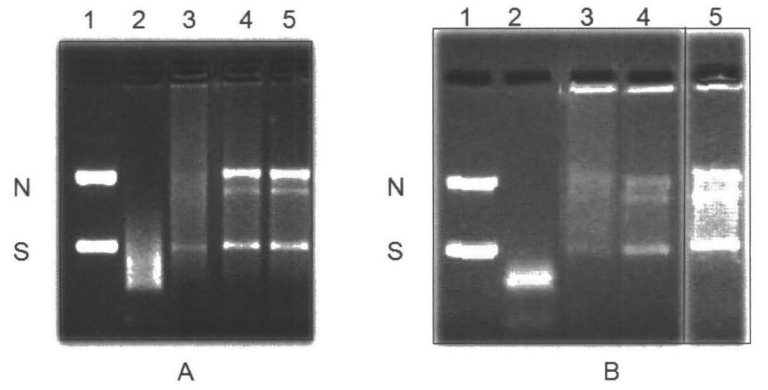

Fig 5. Effect of *A. tricolor* extract in preventing DNA damage induced by Fenton reaction
S, supercoiled DNA; N, nicked DNA
(A) Water soluble fraction (WSF): 1 = DNA + TAE buffer, 2 = DNA after Fenton reaction, 3 = damage preventing effect of WSF (0.42 µg/µl), 4 = damage preventing effect of WSF (0.83 µg/µl), 5 = damage preventing effect of WSF (1.66 µg/µl). (B) Crude extract (CE): 1 = DNA + TAE buffer, 2 = DNA after Fenton reaction, 3 = damage preventing effect of CE (3.3 µg/µl), 4 = damage preventing effect of CE (5 µg/µl), 5 = damage preventing effect of CE (6.7 µg/µl).

to make RTAE was equivalent to 53.97 µg ascorbic acid and that of BAE was equivalent to 44.19 µg ascorbic acid.

Different fractions of aqueous methanolic extract of fresh leaves were also assayed for DPPH radical scavenging activity (Table 1). Activities of different fractions were proportional to concentrations. All the fractions showed antioxidant activity. This suggests that apart from amaranthine and isoamaranthine which inhibited DPPH radical (Cai *et al.*, 2003), there are other constituents in the leaves of *A. tricolor* which also have antioxidant activity. However, the activity as measured by the IC_{50} value was higher in the crude extract (CE) and the water soluble fraction (WSF) than in the ethyl acetate soluble

Table 1. Free radical scavenging activity of different fractions of aqueous methanolic extract of fresh leaves

Assay	IC_{50} value (µg/mL)			
	CE	WSF	ESF	MSF
DPPH radical	100.28	89.78	312.14	296.95
Superoxide radical	82.48	83.35	–	–
Hydroxyl radical	342.76	265.08	–	–

– not done

Table 2. Prevention of DNA damage by *A. tricolor* extract

Concentration of extract (µg/µl)	WSF			CE		
	0.42	0.83	1.66	3.3	15	20
% inhibition of DNA damage	49.59±2.52	66.93±4.46	83.9±0.4	73.2±1.70	82.2±7.14	96.08±7.33

fraction (ESF) and the methanol soluble fraction (MSF). The crude extract and the water soluble fraction also scavenged superoxide radical and hydroxyl radical (Table 1) in a dose dependent manner. Total antioxidant activity of 1 mg CE was equivalent to 0.016 µg ascorbic acid and that of 1 mg WSF was equivalent to 253.6 µg ascorbic acid.

Since DNA damage is one of the most serious damage occurring due to oxidative stress in living organisms. Therefore, it is important to study the role of edible vegetables to prevent DNA damage *in vitro*. In the present study aqueous methanolic extracts of the leaves of *A. tricolor* were evaluated for their capacity to prevent DNA supercoiled strand scission. The reactive oxygen species involved in the damage of DNA by Fenton reagents were mainly hydroxyl radicals (Shih & Hu, 1996). The presence of hydroxyl radical generated by Fenton reaction resulted in 100% scission of supercoiled DNA. This could be clearly seen in lane 2 of Fig 5A, 5B, where the reaction mixture did not contain any plant extract. Presence of plant extract prevented DNA damage (Table 2). The DNA damage preventive property of plant extract was probably due to their hydroxyl radical scavenging property. Amaranthine content in CE (1.21%) is lower than that in WSF (10.4%). WSF prevented DNA damage at lower concentration probably because of the higher amaranthine content in this fraction.

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11

Free Radical Scavenging Activity of Methanolic Bark Extract of *Limonia acidissima*

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ABSTRACT

The antioxidant activity of methanolic bark extract of Limonia acidissima, (Rutaceae) from Burma, was determined by measuring the scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. The results showed dose-dependent free radical scavenging activity at the extract concentration of 0.25, 0.75 and 1.50 mg/mL. The free radical scavenging potency of the extract at 1.5 mg/mL was comparable to that of pure quercetin (0.1 mg/mL) and higher than that of ascorbic acid (0.1 mg/mL). The Fourier transform infra red spectroscopy (FTIR) fingerprints of the stem bark powder showed broad hydroxyl band, aromatic domain bands and carboxylic C-O band of triterpenoid and phenolic acids.

Key words : Antioxidant activity, free radical scavenging, FTIR, *Limonia acidissima*

INTRODUCTION

Limonia acidissima (Rutaceae) commonly known as wood apple, is distributed in dry warm regions of Burma, India, Malaya and Sri Lanka (Macleod & Moeller, 1989). The plant has several chemically active constituents. The stem bark of *L. acidissima* has been found to contain coumarins, alkaloids, sterols, triterpenoids and flavone glycosides (Macleod & Moeller, 1989; Chatterjee *et al.*, 1980; Khan *et al.*, 1975). Traditionally, it is believed that the regular application of

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the bark powder of the tree on the skin helps to keep skin smooth, fair and well-textured complexion. It is also known to protect the skin against cancer by blocking UV rays. Oil and constituents derived from the leave, bark, roots and fruit pulp are used as topical application on venomous wounds and against snakebite. It has been reported that the leaves and fruit of *L. acidissima* contain fungicides, bactericides and insecticides at high concentration (Bandara *et al.*, 1988; Adikaram *et al.*, 1989).

There is increasing interest in the role of antioxidant activity of natural extracts in prevention of diseases such as cancer, senile dementia, inflammation, and atherosclerosis (Sohal & Allen, 1985). Natural plant extracts, have been reported to have multiple biological effects including potent antioxidant activity that protect plants from oxidative damage and perform the same function for humans with the ability to inhibit oxidation of human Low Density Lipoprotein (LDL) (Robards *et al.*, 1999; Rice-Evans & Miller, 1996). Studies on antioxidant activities of *L. acidissima* crude extracts are limited. The present report describes free radical scavenging activity of the stem bark of *L. acidissima*.

MATERIALS AND METHODS

Chemicals

Folin-Ciocalteu reagent, Na_2CO_3, quercetin, Gallic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (St. Louis, MO). All other solvents and chemicals were analytical grade.

Plant Materials

The stem bark was obtained from Burma. Specimen was labeled, numbered and deposited at Organic Chemistry Laboratory, School of Pharmacy, University College Sedaya International (UCSI).

Sample Preparation

Dried stem bark powder (10 g) was extracted with methanol (500 mL) for 4 days using soxhlet apparatus. The extracts were filtered through filter paper (Whatman No. 1) and cooled to room temperature. The extracts were concentrated using rotary evaporator and kept in a refrigerator at –20 °C until further use.

Determination of Total Phenolic Content of Methanol Extracts

The concentration of total phenolics in extracts was determined by using Folin-Ciocalteu reagent and external calibration with gallic

acid. Briefly, 0.5 mL of extract solution in a test tube and 0.5 mL of Folin-Ciocalteu reagent was added and the contents mixed thoroughly. After 4 min, 1 mL of 10% Na_2CO_3 and 8 mL of distilled water were added, then the mixture was allowed to stand for 2 h at room temperature. The absorbance was measured at 760 nm. The concentration of the total phenolics was determined as mg of gallic acid equivalent by using an equation obtained from gallic acid calibration curve, determined by linear regression.

Free Radical Scavenging Activity of Extracts Using the DPPH Assay

The method for estimating the free radical scavenging activity of the methanol extracts of *L. acidissima* was adapted from that of Hatano *et al.* (1988) with some modifications. 2 mL methanolic solution of DPPH (0.1 mM) was mixed with 2.8 mL of *L. acidissima* extract at the 0.25, 0.75, 1.50 mg/mL respectively, and made up with methanol to a final volume of 3 mL. After 60 min standing, the absorbance of the mixture was measured at 517 nm against methanol as blank using Perkin-Elmer Lambda 45 spectrophotometer. Quercetin (0.01 mg/mL) and ascorbic acid (0.01 mg/mL) were used as standards. The radical scavenging activity (%) of the tested samples was evaluated by comparison with a control (2.8 mL DPPH solution and 1 mL of methanol). Each sample was measured in triplicate and averaged. The radical scavenging activity (RSA) was calculated using the formula:

$$RSA = \frac{A_C - A_S}{A_C} \times 100$$

Where A_C is the absorbance of the control and A_S is the absorbance of the tested sample after 60 min.

FTIR Analysis of Stem Bark Powder

The stem bark powder was used for Fourier Transform Infra Red (FTIR) spectroscopy analysis recorded in KBr disc. A known amount (1 mg) of the dry stem bark powder was mixed with KBr (50 mg) and ground to fine powder. The mixture was transferred to a die of a hydraulic press and compressed to produce a disc which was used for FTIR spectroscopy in the mid-IR range of 4000 – 600 cm^{-1}. The FTIR spectrum was recorded on Nicolet spectrometer.

Statistical Analyses

Experimental results were mean ± S.D. of three parallel measurements and analyzed by SPSS (version, 10.0 for Windows 98,

SPSS Inc.). Differences between means were determined using Tukey multiple comparisons. P values < 0.05 were regarded significant.

RESULTS AND DISCUSSION

The FTIR spectrum of *L. acidissima* stem bark powder is shown in Fig 1. The FTIR spectrum showed a great intensity of hydroxyl band (3400–3500 cm^{-1}) and aromatic domain bands (1634–1500 cm^{-1}) due to phenolics and flavone glycosides present in the bark. Carboxylic esteric band (1750–1730 cm^{-1}) was not observed but the presence of weak band at 1402 cm^{-1} was observed probably due to carboxylic C-O band due triterpenoid and phenolic acids present in stem bark. The total phenolic content of the methanol extracts of *L. acidissima* methanolic stem bark extract was 13.6 mg gallic acid equivalent/g dry weight of extract.

The results of free radical scavenging activity of *L. acidissima* methanolic stem bark extracts by the DPPH method are shown in Fig 2. A purple-colored DPPH is a stable free radical, which is reduced to α,α-diphenyl-β-picryl hydrazine (yellow colored) by reacting with an antioxidant. Antioxidants interrupt the free radical chain oxidation by donating hydrogen from hydroxyl groups to form a stable end product, which does not initiate or propagate further oxidation of lipids (Sherwin, 1978). The methanol extracts demonstrated a significant dose-dependent inhibitory activity against the DPPH radical at the extract concentration of 0.25, 0.75, 1.5 mg/mL. There was a similar radical scavenging activity for the reference compounds, quercetin and gallic (0.1 mg/mL). The results indicated that the methanol extracts are free radical inhibitors and primary antioxidants that react with free radicals.

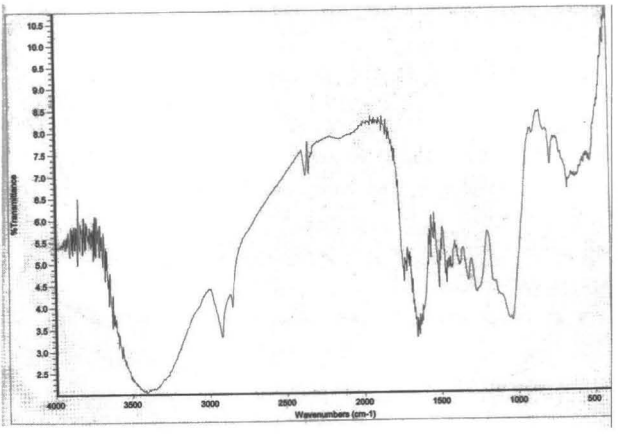

Fig 1. FTIR spectra of stem bark powder of *L. acidissima*

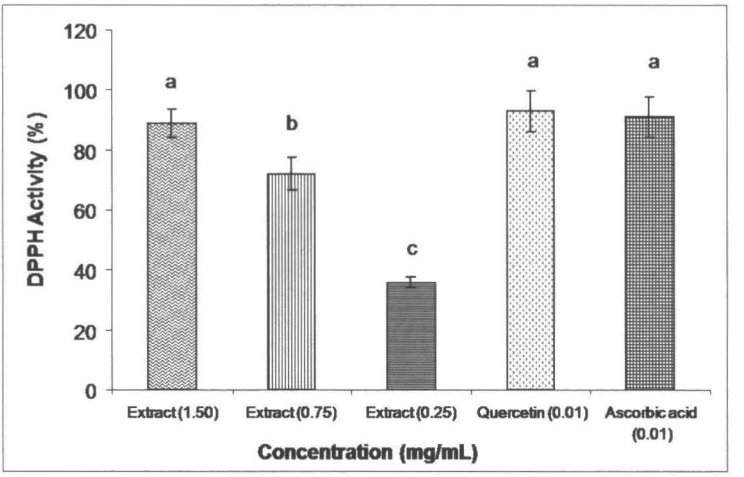

Fig 2. Free radical scavenging activity and total phenolic contents of methanolic extracts of *L. acidimissia* stem bark[a].
[a]Means with different letters indicate significantly different values ($p<0.05$)

L. acidissima contains several chemically active constituents, including flavone glycosides, coumarins, benzoquinone, sterol, hydroxyl and aromatic acids (Patra *et al.,* 1988). The antioxidant activity could be attributed to these chemical constituents of the extract which are effective free radical scavengers and their antioxidant activities are well documented (Evans & Miller, 1996; Curvelier *et al.,* 1996). Traditionally, *L. acidissima* stem bark powder is used as natural skin conditioner and facial cosmetic. Regular application to the skin helps to prevent excessive dryness and also acts as a sunscreen to prevent sunburn. *L. acidissima* paste derived from crushed branches and stem bark is a popular local cosmetic used as carrier for insect repellent and a facial cosmetic in Burma (McGready *et al.,* 2001). The medicinal and cosmetic properties may be ascribed to the free radical activities of the extract observed in this experiment. That is, the extract is capable of preventing cell damage caused by free radical reactions which are generally accepted to be involved in the ageing process.

To conclude, the methanolic stem bark extracts *L. acidissima*, were potent with respect to free radical scavenging activity determined by the DPPH radical method system. The antioxidative potency of the extract (1.5 mg/mL) was comparable to that of pure quercetin (0.1 mg/mL) and synthetic antioxidant ascorbic acid (0.1 mg/mL), thus presenting an alternative source for natural additives. Scavenging effects on superoxide anion and *in vivo* studies to assess the antioxidant effect in biological systems are going on in our laboratory.

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Sorghum arundinaceum, A Wild Cereal Grain with Potential: Comparison of its Antioxidant Potential with that of Domestic Cereals in the Same Family

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ABSTRACT

Sorghum arundinaceum, also known in English as common wild sorghum, is a wild grass which produces a grain that has been consumed in many parts of Zimbabwe. The phenolic comound content and antioxidant capacity of the wild cereal grain was determined and compared with that of Sorghum bicolor (Red type) and Sorghum bicolor (white type), which are traditionally cultivated grains. Sorghum arundinaceum was found to contain approximately 6.18 mgGA/100 mg sample ($p>0.05$) a significantly high concentration of total phenolic compounds compared to 3.98 and 2.10 mgGA/100mg sample found in Sorghum bicolor (Red type) and Sorghum bicolor (white type) respectively. The antioxidant activity was tested using the DPPH radical assay and the model system which involves testing the ability of the antioxidant extracts to inhibit phospholipids peroxidation. After 4 min, the percentage of DPPH$^{\bullet}$ left in solution of Sorghum arundinaceum extract was approximately 41.61%. The respective percentage DPPH radical concentrations in solution for Sorghum bicolor (Red type), Sorghum bicolor (white type) and ascorbic acid were 48%, 70.66% and 87.88%.

Key words : Wild cereals, Antioxidant, *Sorghum arundinaceum*, Free radicals

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INTRODUCTION

In Zimbabwe, there is evidence that with the expansion of organized agriculture and the land clearing and deforestation resulting from increasing fuel-wood demands, several species of wild plants are rapidly disappearing from the rural diet (Gomez, 1989). Cereals, together with oil seeds and legumes, supply a majority of the dietary protein, calories, vitamins, and minerals to the bulk of populations in developing nations (Chaven & Kadam, 1989). Cereal grains are grown in greater quantities worldwide than any other type of crop and provide more food energy to the human race than any other crop. In some of the poorest families in Zimbabwe, cereal food is almost entirely their source of nutrition because other sources of nutrition are very expensive. Persistent droughts and unreliable rainfall patterns in Southern Africa however, have made it difficult to plant the traditional cereal grains like maize, wheat and rice. There are some wild cereal grains however, which can be consumed in place of the traditional cereals because some are drought resistant and most of them take a very short time to mature. *Sorghum arundnaceum*, known in English as common wild sorghum is a robust tufted grass with thick culms of between 500-3000 mm tall. Its leaves are wide with a conspicuous white midrid and the inflorescence is an open panicle with loose branches.

Sorghum arundnaceum grows in damp soils and has no rhizomes. It flowers from January to June. The wild cereal has been reported to be consumed in Zimbabwe in times of drought (Shava, 2003).

Recently, natural plants have received much attention as sources of biologically active substances including antioxidants, antimutagens and anticarcinogens (Toshihiko Osawa *et al.*, 1992). Antioxidants play a crucial role in preventing diseases because of their ability to capture, deactivate or repair the damage caused by a group of molecules or atoms called free radicals that are implicated in many diseases (Alonso *et al.*, 2004). In food processing, lipidic oxidation not only causes a loss in nutritional and gustative quality of foods but also generates oxidized products such as free radicals which lead to various undesirable chemical reactions. To avoid or delay this autoxidation process, antioxidants have been used for over 50 years (Marie-Elisabeth Cuvelier, 1994). Synthetic antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene have been used as antioxidants for foods since the beginning of this century. The use of these synthetic antioxidants, however, has begun to be restricted because of their toxicity (Ito *et al.*, 1983). There is a pressing need

to find safe, economic antioxidants to replace these synthetic chemicals.

It is with this background that this paper seeks to find cheaper sources of 'natural' antioxidants in wild cereal grains, *Sorghum arundnaceum* in this case. This paper also seeks to compare antioxidant capabilities of the traditionally cultivated *Sorghum bicolor* (Red type) and *Sorghum bicolor* (White type) with that found in the common wild sorghum (*Sorghum arundnaceum*)

MATERIALS AND METHODS

Chemicals

Gallic acid, 1- diphenyl – 2 picrylhydrazyl (DPPH$^{\bullet}$) radical, trichloroacetic acid (TCA) and ascorbic acid were obtained from Sigma – Aldrich Chemie (Steinheim, Germany). Sodium carbonate, methanol (analytical HPLC grades were obtained locally.

Folin-Ciocalteu reagent (1 N), 20% sodium carbonate, standard gallic acid (0.5 mg/mL), 50% methanol in distilled water (1:1 v/v). TBA, TCA, $FeSO_4$ the reagents used were of chemical grade

Extraction of Phenolic Compounds

Total phenolic compounds were extracted from the cereal grains as described by Makkar (1999). The sample (2 g) was extracted twice with cold 50% aqueous methanol (10 mL). The two extracts were combined, made up to 20 mL with 50% aqueous methanol, centrifuged at 3000 rpm for 10 min and transferred into small sample bottles for analysis.

Follin C. Assay for Total Phenolics

To a sample (50 µl), distilled water (950 µl) was added to make up to 1 mL then 1N Folin C (500 µl) was added followed by sodium carbonate (2.5 mL). At the end of 40 min absorbencies at 725 nm were read using a spectronic 20® genesys™ spectrophotometer against a blank which contained methanol instead of sample. The method used was according to Makkar (1999). Gallic acid (0.5 mg/mL) was used as the standard and concentration of sample was expressed as mgGA/100 mg.

DPPH Radical Scavenging Activity

The method by Kuda *et al.* (2005) was used to determine the DPPH radical scavenging activity. Freshly prepared DPPH in methanolic solution (1.5 mL, 1 mm) was incubated with sample (0.5 mg/mL) for

20 min at room temperature after which, absorbance was read at 30 second intervals for 180 seconds at 517 nm on a spectronic 20® genesys™ spectrophotometer. Ascorbic acid (0.5 mg/mL) was used as a positive control. The percentage activity was calculated as follows: $(A_{t=x}/ A_{t=0})$ *100. Where $A_{t=x}$ is the absorbance at time x and $A_{t=0}$ is the absorbance at time 0 (initial absorbance). A blank with sample (80 µl) and buffer (2920 µl) was used.

Ability to Inhibit Phospholipid Peroxidation

Female Sprague Dawley rats (*Rattus norvegicus*) were obtained from the Animal House, University of Zimbabwe and dissected in the Physiology Department to obtain the brain. The rat brains were stored at –85°C until used. Homogenization of rat brain (2 g) was done in a chloroform:methanol mixture (2:1, v/v) followed by centrifugation at 3000x g for 5 min. The supernatant obtained was used as the source of phospholipids. The test run contained the phospholipids solution (50 µl), the sample extract (0.5 mL), 50% methanol (0.2 mL) and $FeSO_4$ (0.5 mL). The blank contained the phospholipid solution (50 µl) mixed with distilled water (0.5 mL) instead of the phenolic compound containing sample and methanol (0.2 mL, 50%). Ascorbic acid (0.5%) was used as the control. Incubation of the reaction mixture at 37°C for 1 h was followed by the addition of thiobarbituric acid (TBA) (0.5 mL) and trichloroacetic acid (TCA) (4 mL) and the solution was then heated in a boiling water bath for 15 min. After cooling the sample on ice, absorbance was read at 532 nm on a Shimadzu UV-1601 uv-visible spectrophotometer (Shimadzu Corporation, Australia).

Statistical Analysis

Results are expressed as the means ± standard deviation (vertical error bars) of three replicates. One way analysis of variance (ANOVA) and the Student's *t* test were used to determine the statistical difference. Statistical significance was $p<0.05$, unless otherwise stated.

RESULTS AND DISCUSSION

The contents of total phenolic compounds in methanolic extracts of *Sorghum arundinaceum*, *Sorghum bicolor* (Red type) and *Sorghum bicolor* (White type) are presented in Table 1.

The results obtained showed that the contents of total phenolic compounds in the methanolic extract of *Sorghum arundinaceum* were significantly higher ($p>0.05$) than in extracts of *Sorghum bicolor* (Red type) and *Sorghum bicolor* (White type). It is well known that plant polyphenols are widely distributed in the plant kingdom and

Table 1. Contents of total phenolic compounds in methanolic extracts of *Sorghum arundinaceum*, *Sorghum bicolor* (Red type) and *Sorghum bicolor* (White type)

Extract	Total phenolic compounds (mgGA/100 mg sample)
Sorghum arundinaceum	6.18 ± 0.36
Sorghum bicolor (Red type)	3.98 ± 0.24
Sorghum bicolor (White type)	2.10 ± 0.30

that they are sometimes present in surprisingly high concentrations (Harbone, 1993). This can be supported by the results obtained in Table 1 where *Sorghum arundinaceum,* which is the wild cereal exhibited the highest concentration of approximately 6.18 mgGA/100 mg of sample while *Sorghum bicolor* (Red type) and *Sorghum bicolor* (White type) followed with concentrations of 3.98 and 2.10 mgGA/ 100 mg of sample respectively. The results show us that the wild cereal (*Sorghum arundinaceum*) can also be used as a good source of phenolic compounds which can help in quenching free radicals in the body.

The results above were obtained using the Folin-Ciocalteu method. This method is not entirely specific to phenolic compounds (Wu *et al.*, 2005), since the method was designed to give a general measure of the phenolic composition not a specific one. It has also been reported that, not all phenolics exhibit the same extent of activity in the assay. It is therefore important to investigate further using other assays like the DPPH assay to measure radical scavenging activity and also ability to inhibit phospholipid peroxidation model system assay to measure ability of the extracts to reduce lipid peroxidation.

Radical scavenging capacity of antioxidants in Sorghum arundinaceum, Sorghum bicolor (Red type), *Sorghum bicolor* (White type) and ascorbic acid

The radical scavenging effects of *Sorghum arundinaceum*, *Sorghum bicolor* (Red type), *Sorghum bicolor* (white type) and ascorbic acid are represented in Fig 1. Scavenging of stable radicals such as the chromogen radical 1,1-diphenyl-2-picrylhydrazyl radical (DPPH$^{\bullet}$) quantified by spectrophotometry in methanol is extensively used for comparison of homologous series of antioxidants. The reaction stoichiometry of DPPH$^{\bullet}$ differs with the type of antioxidant. A 2:1 (radical/antioxidant) molar stoichiometry has been reported for

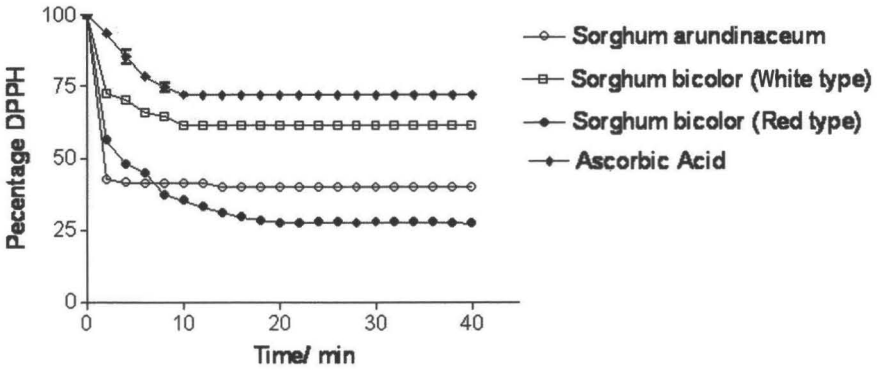

Fig 1. Effects of the antioxidants in *Sorghum arundinaceum*, *Sorghum bicolor* (Red type), *Sorghum bicolor* (White type) and ascorbic acid on DPPH. Values represent the mean ± SD (n=3)

ascorbic acid, trolox, α-tocopherol and several phenolic compounds (Arnao, 2000; Brandwilliams *et al.*, 1995).

The rate of depletion of DPPH radical in the extract of *Sorghum arundinaceum* was the fastest over time. After 4 min, the percentage of DPPH$^{\bullet}$ left in solution was approximately 41.61%. The respective percentage DPPH radical concentrations in solution for *Sorghum bicolor* (Red type), *Sorghum bicolor* (White type) and ascorbic acid were 48%, 70.66% and 87.88%. The percentages at this time represent the rate of depletion of the DPPH radical in the reaction mixture. *Sorghum arundinaceum* extract depleted the radical fastest with *Sorghum bicolor* (Red type), *Sorghum bicolor* (White type) and ascorbic acid following in the order *Sorghum bicolor* (Red type) > *Sorghum bicolor* (White type) > ascorbic acid. This result shows the potential that the wild cereal grains have as sources of free radical quenching phytochemicals.

Antioxidant activies of Sorghum arundinaceum, Sorghum bicolor (Red type), *Sorghum bicolor* (White type) and ascorbic acid

The ability of *Sorghum arundinaceum*, *Sorghum bicolor* (Red type), *Sorghum bicolor* (White type) and ascorbic acid to inhibit phospholipids peroxidation at different concentrations is shown in Fig 2. In biological systems, lipid peroxidation (oxidative degradation of polyunsaturated fatty acids in the membrane) generates a number of degradation products such as malonaldehyde (MDA). MDA was found to be important cause of cell membrane destruction and cell damage. Extensive studies on malonaldehyde have been carried out and MDA has been measured as an index of lipid peroxidation and as

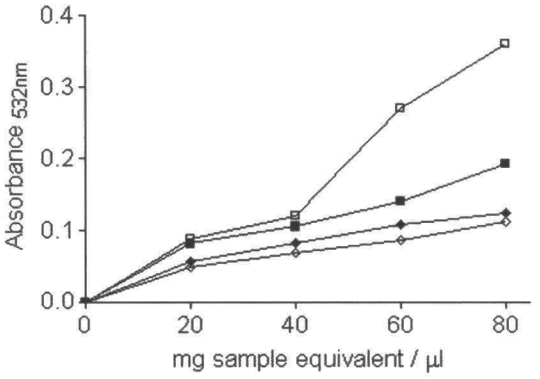

Fig 2. Antioxidant activities of *Sorghum arundinaceum* (□), *Sorghum bicolor* (Red type) (■), *Sorghum bicolor* (White type)(♦) and ascorbic acid (◊)

a marker of oxidative stress. The abilities of the extracts of the cereal grains to inhibit the peroxidation of lipids from the rat are shown in Fig 2. Iron sulfate ($FeSO_4$) was used to induce lipid peroxidation by forming hydroxyl radicals. The efficiency of the cereal extracts in preventing lipid peroxidation is inversely proportional to the amount of MDA formed. The more the MDA formed the less efficiency of the cereal extract as an inhibitor of phospholipid peroxidation.

Inhibition of lipid peroxidation was dependent on the concentration of the samples; high sample concentration resulted in limited MDA molecules being formed. The inhibition of phospholipid peroxidation shows the ability of antioxidant components in the cereal extracts to act as chain breakers. Chain breaking properties are as result of hydrogen and electron donation which was observed in the reducing power effects and the DPPH radical quenching abilities. The chain breaking properties are correlated to the total phenolic content as increase in phenolic content increased the extent of inhibition of peroxidation. Using phospholipids obtained from a biological specimen (in this case the rat liver) is important in depicting what happens in biological systems as the oxidation and radical chain initiation occurs in the body.

The ability of all the cereal extracts and ascorbic acid to inhibit lipid peroxidation was found to be dose dependant. All the samples showed high capacities to inhibit lipid peroxidation at high concentrations. *Sorghum arundinaceum* (Common wild sorghum) extract at all concentrations, exhibited very high antioxidant activity ($p>0.05$). At a concentration of 20 mg sample equivalent/µl, *Sorghum arundinaceum*, the wild cereal had an absorbance of 0.084 ± 0.02 mg sample equivalents/µl while *Sorghum bicolor* (Red type), *Sorghum*

bicolor (White type) and ascorbic acid had absorbencies of 0.057 ± 0.034, 0.090 ± 0.010 and 0.043 ± 0.03 mg sample equivalents/µl respectively for the same concentration of sample.

CONCLUSIONS

Sorghum arundinaceum, a wild cereal grain can be deemed as a good source of antioxidants as shown by a significantly high concentration ($P<0.05$) of 6.18 ± 0.36 mgGA/100mg sample compared to *Sorghum bicolor* (Red type) with 3.98 ± 0.24 and *Sorghum bicolor* (White type) with 2.10 ± 0.30 mgGA/100mg sample. *Sorghum arundinaceum* had the highest DPPH radical quenching capacity and also had the highest ability to inhibit phospholipids peroxidation. The ability to inhibit phospholipids was dose dependant for all samples. As concentration of extract increased, so did the ability to inhibit peroxidation. There was a positive correlation between the total phenolics and the ability to quench the DPPH radical. A positive correlation was also observed between total phenolics in all samples and the ability to inhibit lipid peroxidation.

ACKNOWLEDGEMENTS

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Antioxidant and Micronutrient Potential of Some Marketed Mango Varieties and Mango Products

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ABSTRACT

Micronutrient composition and total antioxidant capacity of 19 popular morphological types of mango along with 8 mango products were studied. Ripe mango exhibited 43-61% of the antioxidant activity as compared to bel fruit (Aegle marmelos) and 61-77% as compared to gooseberry (Emblica officinalis), the 2 fruits, with promising antioxidant potential, previously analyzed by us. Ripe mango had a potential to meet 42.7% of the recommended daily allowance (RDA) for β-carotene while unripe mango provided 3.2% of RDA. For lipid peroxidation as inhibition of thiobarbituric acid reactive substances (ITBARS), unripe varieties exhibited significantly higher values than ripe varieties ($t=3.71$, $p<0.05$). Superoxide scavenging activity (SOSA) as well as ferrous ion chelating ability (FICA) was found to be comparable between ripe and unripe varieties. The unripe varieties elicited higher values of total polyphenols than the ripe ones. The losses in the products, as compared with the ripe mango varieties for ITBARS ($t=2.27$, $p<0.05$) and FICA ($T=4.28$, $p<0.01$) were found to be significant.

Key words : Antioxidants, mangoes, micronutrients, qualities, varieties

INTRODUCTION

Mango (*Mangifera indica*) is one of the most important fruit crops grown in the tropics. It is known as the king of the tropical fruits

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on account for its delicious taste and excellent flavor. Over the decades, mango cultivation has gained significant importance due to its vast export potentialities. However of about 1000 varieties of mangoes cultivated in India, only 10-12 varieties carry commercial importance and are found suitable for table and processing purposes (Sairam *et al.*, 2003; Ahmed *et al.*, 2007). Among all varieties, *Alphonso* is the leading one in demand and fetches an extremely high price as table fruit.

Nutraceutical potential implies ability of the food material to have beneficial effects on health or healing effects in disease due to richness of some active principles like antioxidants, micronutrients, prebiotics, antimutagenic, antiatherogenic agents. Only limited studies have reported the nutraceutical potential of mango, using stem bark and leaf (Sanchez *et al.*, 2000; Arts *et al.*, 2000; Nunez-Selles, 2002; Garcia, 2003; Sanchez, 2003). However mango fruits have not received due attention for their varietal differences in nutraceutical potential (Iagher *et al.*, 2002; Sagar, 1998; Putturaju *et al.*, 1997; Anila *et al.*, 2002). Further, Pott *et al.* (2003) have quantified β-carotene stereoisomers in fresh and processed mango varieties. The studies on micronutrient variability in Indian mango types are scanty except few studies that report the total carotenoids and vitamin C content to check for the suitability for canned mango juice (Gowda & Ramanjaneya, 1995; Agte *et al.*, 2003; Williamson *et al.*, 1987; Yamaguchi *et al.*, 2003; Saucier & Waterhouse, 1999). Present study reports results of micronutrient composition and antioxidant capacity of 11 ripe varieties, 7 unripe varieties and 8 popular mango products. The objectives of the study were: 1. To assess the anti-oxidant capacity of selected mango types in terms of inhibition of thiobarbituric acid reactive substances (ITBARS), super oxide scavenging activity (SOSA) and ferrous ion chelating activity (FICA). 2. To evaluate the levels of ascorbic acid, beta carotene, riboflavin, thiamine, zinc, copper, iron, manganese and selenium as well as total polyphenols in these types.

METHODS AND MATERIALS

The ripe (11) as well as unripe (7) varieties of mango (Appendix 1) available in market were selected for the present study. We purposely chose marketed varieties since these are the only available varieties to common man and not the experimentally grown varieties. For the estimation of micronutrients and antioxidant capacity, intact, uniformly mature fruits free from canker and other visible symptoms of infection were chosen. The fruits were washed with distilled water;

Appendix 1. Names of different varieties of mango screened for the present study

Ripe varieties and their codes	Unripe varieties	Products
M01: *Aphoos* (Alphonso) Ratnagiri	*Rajapuri 1*	*Pulp (concentrate)*
M02: *Aphoos* (Alphonso) Devgad	*Rajapuri 2*	*Mawa*
M03: *Payari*	*Totapuri 1*	*Burfi*
M04: *Desi*	*Totapuri 2*	*Poli*
M05: *Raival*	*Langda*	*Chhunda*
M06: *Badam*	*Malgubba 1*	*Pickles*
M07: *Sakri*	*Malgubba 2*	*Sherbat*
M08: *Goti*		*Amchur*
M09: *Lalbaug*		
M10: *Neelam*		
M11: *Totapuri*		

Chhunda: grated pickle-, *aachar*: regular pickle, *amchur*: dry raw mango powder
sherbat- drink comprising of pulp, sugar, saffron and cardamom,
mawa: dehydrated and homogenized pulp, *poli and papad-sheets* made out of dehydrated and homogenized pulp,
burfi -a sweet prepared by mixing and condensed mango pulp, milk and sugar.

edible portions were collected and homogenized in a mixer to form a pulp, which was used for further experiments. Further, popular ripe mango products having frequent demand in the market such as burfi, canned pulp, mawa, *poli and papad* (round sheets made out of dehydrated and homogenized pulp) that represent ripe mango products were chosen for above said estimations. The products prepared using unripe mangoes mainly included grated pickle (*chhunda*), regular pickle (*aachar*), amchur powder and sherbat (drink comprising of pulp, sugar, saffron and cardamom).

Estimation of Antioxidant Activities and Micronutrients

The methods for measurements of antioxidant activities, micronutrients and polyphenols were as stated earlier (Agte *et al.*, 2003).

Measurement of ITBARS

This was done as per the method of Williamson *et al.* (1987) with either increasing amounts of α-tocopherol as standard or test material as methanolic extracts (1%). Briefly, the peroxide ions were generated by addition of thiobarbituric acid (0.67% in 0.25 M HCL) in a 10%

sunflower oil-water system containing Tween-20. The reaction tubes were placed in a boiling water-bath for 1 h. The tubes were cooled and the developed magenta-pink colour was read at 528 nm on a spectrophotometer. ITBARS were expressed as mm equivalents of α-tocopherol.

Measurement of SOSA

This was measured as per the method of Yamaguchi *et al.* (2000). Phenazine methosulphate (PMS, 60 µM in phosphate buffer -pH 7.4) and Nicotinamide Adenine Dinucleotide (NADH- reduced, 468 µM) acted as source for generation of superoxide radicals. The reaction was started with addition of PMS followed by incubation for 10 min at 37°C. The reduction in the developed blue colour on addition of nitroblue tetrazolium (NBT- 156 µM) was read at 560 nm on a spectrophotometer. The activity of tannic acid was measured as standard polyphenol. The activities of samples were expressed as tannic acid equivalents.

Measurement of FICA

This was measured as per Yamaguchi *et al.* (2000). The activity of the samples was expressed as EDTA equivalents. Ferrous sulphate (1 mm) and equal amount of test solution in 1% SDS were mixed. Tris buffer and 2 2' bipyridyl (0.1%) in 0.2M HCl were added along with 0.4% hydroxylamine hydrochloride. Absorbance was read at 522 nm in a spectrophotometer.

Micronutrient Profile and Polyphenol Content

Levels of β-carotene, ascorbic acid, riboflavin, thiamine, zinc, copper, iron manganese and selenium were estimated as per Agte *et al.* (2003) and briefly stated below.

Estimation of β-carotene

Alcoholic KOH extracts of food samples were incubated in water-bath at 60°C for 20 min, removed and allowed to cool at room temperature. The unsaponifiable matter was then extracted 3 times with petroleum ether. The petroleum ether extract was washed with water to remove the alkali, passed through anhydrous sodium sulfate and evaporated to dryness under vacuum at 40°C. The dry residue was immediately dissolved in cyclohexane. The absorbance of cyclohexane solution, measured at 460 nm on a spectrophotometer gave the value of β-carotene content of the sample. The values were expressed as micrograms/100 g of food material.

Estimation of Ascorbic Acid

The weighed food samples were extracted with 6% HPO_3 to which of acetate buffer was added. A sample blank and reagent blank (control) as well as standards were also run simultaneously. Finally 2, 6 DCPIP dye solution was added in the control and sample tubes just prior to reading the absorbance at 540 nm. A standard curve was constructed and based on the average slope value the concentration of vitamin C was calculated. The values were expressed as mg/100 g of food sample.

Estimation of Riboflavin

To the sample extract in water, caprylic alcohol was added followed by 4% $KMNO_4$ solution. The mixture was stirred and within 2 min, 1: 1 H_2O_2: H_2O mixture was added to discharge the permanganate colour and pH adjusted to 7 with NaOH. The volume was suitably made up and fluorescence measured on a spectrofluorometer with excitation at 440 nm and emission at 640 nm. A series of standards were also simultaneously run. The quantity of the vitamin was determined and expressed as mg/100 g of food sample.

Estimation of Thiamin

The food sample was extracted with acetate buffer. To the vitamin extract basic lead acetate solution was added and centrifuged. The aliquot was then treated with 30% H_2SO_4 followed by addition of 40% NaOH. K_3FeCN_6 solution followed by isobutyl alcohol was then added and the contents allowed standing for 2 min after shaking. The aqueous layer was rejected and the fluorescence of alcoholic layer was measured on a spectrofluorometer with excitation at 360 nm and emission at 485 nm. Thiamin content was calculated and expressed as mg/100 g of food sample.

Estimation of Trace Elements

Dried food sample was accurately weighed in a silica crucible and incinerated in a muffle furnace at 600°C for 3 h till the sample was completely ashed. The ash was then dissolved in concentrated hydrochlororic acid (HCl). The analysis of zinc, copper, iron, manganese and selenium were carried out on Atomic Absorption Spectrophotometer after diluting the samples suitably.

Rice flour samples from National Institute of Environmental Studies (NIES), Japan were used as reference standards to ensure quality of trace metal estimations.

Estimation of Polyphenols

Polyphenols were estimated as per the method described by Saucier *et al.* (17). Briefly, Folin-Ciocalteau's reagent (100 µl) was added to sample extracts (10% in distilled water) (10 µl) and a series of standard tannic acid (20-80 µl). After 30 seconds, 20% $NaHCO_3$ (300 µl) was added and the tubes were left in dark at room temperature for 2 h. The tubes were then read at 700 nm on a spectrophotometer. Values were expressed as mg/100 g of tannic acid.

Statistical Analysis

All the analyses were done in 3 replicates and expressed as mean ± S.D. Comparison of ripe and unripe varieties was done using Student's t test. Association of the antioxidant potential with total polyphenols and micronutrients were computed as Pearson's r value. The values were considered significant for $p<0.05$.

RESULTS AND DISCUSSION

Antioxidant Capacity of the Screened Mango Types

ITBARS in presence of test materials represents the antioxidant activity against peroxides (ROO.) while SOSA measures antioxidant activity against superoxide (O_2^-). In presence of other chelating agents, the complexing ability of 2,2' bipyridyl gets diminished allowing reduction as estimate of iron chelating activity (FICA). As these different systems represent different aspects of antioxidative action, it was thought worthwhile and meaningful to use multiple indices for determination of antioxidant capacity.

Antioxidant and Micronutrient Contents of Fresh Fruits

There was a large variability among all the mango types for the 3-antioxidant indices (% CV=8.7-68.7) as well as for content of polyphenols (% CV=44.1 for ripe and 18.3 for unripe). For inhibition of lipid peroxidation, the unripe varieties exhibited higher values than the ripe varieties (Table 1 & Fig 1). Fig 2 and Fig 3 shows the SOSA and FICA for ripe mango varieties. SOSA and FICA values were comparable within the ripe and unripe varieties ($p>0.1$). Unripe varieties elicited higher values of total polyphenols than the ripe varieties ($p<0.05$) (Table 3).

Amidst all the ripe varieties, the ITBARS was found to be highest in *M1* followed by *M2* whereas M6 elicited lowest values (Fig 1). For SOSA and FICA, yet again, *M1* followed by *M2* was found to be the most promising (Fig 2, 3). M8 for SOSA whereas M7 for FICA elicited low values, demonstrating M1 to be the most promising to improve

Table 1. Antioxidant activities and polyphenol content in ripe and unripe mango varieties (Values have been given as mean ± S.D.)

Type of fruit	ITBARS mm vit E eq./100 g	SOSA mm tannic acid eq./100 g	FICA mm EDTA eq./100 g	Polyphenols mg/100 g
Ripe (11)	0.37 ± 0.17	35.42 ± 12.01	37.73 ± 7.46	47.9 ± 18.38
Unripe (7)	0.54 ± 0.047	37.26 ± 3.67	35.11 ± 7.08	83.5 ± 14.88
Processed mango products (8)	0.16 ± 0.11	33.2 ± 8.76	18.65 ± 8.14	58 ± 27.42
Gooseberry (2)	0.60 ± 0.06	48.3 ± 1.21	61.67 ± 1.23	1324 ± 3.38
Bael fruit (2)	0.86 ± 0.10	77.19 ± 1.06	62.24 ± 0.98	104.4 ± 2.13

ITBARS – Inhibition of thiobarbituric acid reactive substances; SOSA- Superoxide ion scavenging activity; FICA- Ferrous ion chelating ability

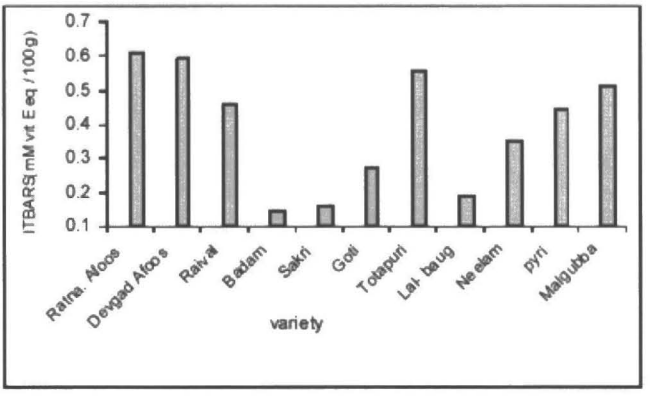

Fig 1. ITBARS of the ripe varieties of mango

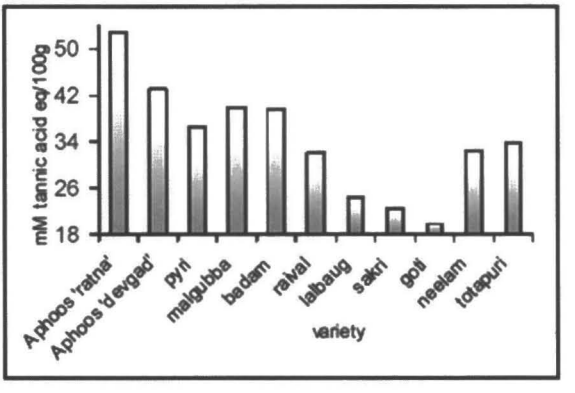

Fig 2. SOSA for ripe mango varieties

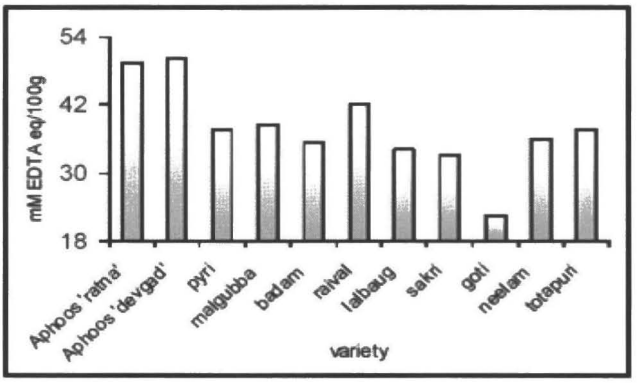

Fig 3. FICA for ripe mango varieties

Table 2. Micronutrient profiles of mango types

Parameter	RDA*	Ripe varieties	Contribution of % RDA from 100 g	Unripe varieties	Contribution of % RDA from 100 g
Vitamin C mg/100 g	40 mg	6.13 ± 1.55	15.3	10.95 ± 1.73	27.4
β-carotene µg/100 g	2400 µg	1025.5 ± 103.8	42.73	76.00 ± 6.24	3.16
Riboflavin mg/100 g	1.4 mg	0.09 ± 0.01	6.43	0.11 ± 0.01	7.85
Thiamine mg/100 g	1.2 mg	0.13 ± 0.03	10.83	0.1 ± 0.01	0.83
Zinc mg/100 g	15.5 mg	0.15 ± 0.03	0.97	0.23 ± 0.02	1.5
Copper mg/100 g	2.2 mg	0.06 ± 0.01	2.73	0.01 ± 0.03	1.13
Iron mg/100 g	28.0 mg	0.58 ± 0.10	2.1	0.56 ± 0.28	2.00
Manganese mg/100 g	5.5 mg	0.34 ± 0.14	6.2	0.3 ± 0.08	0.36

*Cross reference: RDA for Indians, Nutritive values of Indian foods, Indian Council of Medical Research, 2000, pg 94

Table 3. Polyphenol content of mango varieties and products

Code	Mango varieties	Polyphenols (mg/100 g)
	Ripe	
M01	Alphonso 'Ratnagiri'	71
M02	Alphonso 'Devgad'	47
M03	Pyri	65
M04	Langda	33
M05	Malgubba	40
M06	Desi	75
M07	Raival	50
M08	Badam	18
M09	Sakri	56
M10	Goti	24
M11	Lalbaug	48
	Mean	**47.90**
	Std. Deviation	**18.38**
	Unripe	
	Rajapuri (2)	98
	Totapuri (2)	67
	Langda	75
	Malgubba (2)	94
	Mean	**83.50**
	Std. Deviation	**14.89**
	Products	
	pulp	64
	burfi	55
	mava	53
	Aam papad (sheets)	28
	chuunda	43
	pickles	58
	sherbat	43
	Amchur	120
	Mean	**58**
	Std. Deviation	**27.42**

the overall antioxidant profile of the body. Secondly, this variety also was found to possess the highest content of β-carotene (1247 μg/ 100 g) of all the screened varieties, thereby demonstrating its superiority among the other varieties once again.

The antioxidant capacities as well as polyphenol content of ripe and unripe mango varieties were also compared with those of bael fruit (*Aegle marmelos*) and gooseberry (amla- *Emblica officinalis*), the most promising fruits of all the fruits previously screened by us (Tarwadi & Agte, 2005; Tarwadi & Agte, 2007). This was because bael fruit and gooseberry exhibited highest antioxidant values among all the commonly available fruits in Indian markets. When compared to these fruits, mango (ripe variety) was found to exhibit 43-61% of the antioxidant activity as compared to bael fruit and 61-77% as compared to gooseberry.

The contribution of for various antioxidant micronutrients from 100 g ripe mango as % RDA ranged from 0.97–42.73 (Table 2). Mango (ripe and unripe variety) was found to be poorer source of polyphenols (Tables 1, 3) as well as for vitamin C (Table 2) when compared with the above two promising fruits. Thus ripe mango has a potential to meet 42.73% of RDA for β-carotene and 40% RDA for selenium, thereby demonstrating it to be a promising source for these two micronutrients. Our results agree well with those reported by Sonia *et al.* (2007). Consumption of unripe mango however seems to be more proficient for supply of vitamin C (27.4% RDA) and selenium (80%) (Table 2). The contents of polyphenols (1324 mg/100 g) as well as ascorbic acid (445 mg/100 g) were found to be highest in gooseberry among all the commonly consumed fruits (Tarwadi & Agte, 2007).

The correlation matrix for associations of various antioxidant activities within each other and with contents of polyphenols and zinc has been stated in Table 4. Both SOSA and FICA as also zinc content showed significant association ($p<0.01$) with ITBARS. Surprisingly we could see only a marginal statistical association only between FICA and polyphenol content of the screened mango varieties ($p<0.05$). No other vitamin/trace element except zinc revealed any association with either of the antioxidant indice or polyphenols.

Antioxidant and Micronutrient Contents of Mango Products

The popular mango products available in the market such as *burfi*, *mawa*, *sheets* (made out of dehydrated and homogenized pulp), *chhunda* (grated sweet mango pickle), *aachar* (regular mango pickle),

Table 4. Correlation matrix for association within antioxidant indices, polyphenols and zinc for all mango varieties

	ITBARS	SOSA	FICA	Polyphenols	Zinc
ITBARS	1				
SOSA	0.50**	1			
FICA	0.57**	0.33	1		
Polyphenols	0.05	-0.18	0.36**	1	
Zinc	0.61**	0.23	0.19	0.23	1

* = $p<0.05$, ** = $p<0.01$

dried mango powder and *sherbet* were also analyzed for the antioxidant and micronutrient contents. Products in general exhibited lower antioxidant activities, indicating losses due to processing. The contents of polyphenols of the products were comparable with the ripe mango varieties and were considerably lower than the unripe mango varieties (Table 1).

Mango fruits have not received due attention so far, especially for their biodiversity in the nutraceutical potential. From this perspective, the outcome of our study has indicated to be moderate in terms of antioxidant profile and micronutrient content. But this fruit is eaten in plenty during the season, due to its pleasant taste, color and flavor as compared to Bael or Gooseberry. Thus it can be a good source of antioxidants. Variability in the data shows the scope for choosing the best performing varieties such as *Alphonso* having good health-promoting potential in terms of nutritional and antioxidant parameters important to naturally battle the oxidative stress and improvise the antioxidant status of the body.

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14

Vimang: Experiences from the Antioxidant Therapy in Cuban Primary Health Care

GILBERTO L. PARDO ANDREU[1,*], ALBERTO J. NÚÑEZ SELLÉS, MARIELA GUEVARA GARCÍA AND ALINA ALVAREZ LEÓN

ABSTRACT

Antioxidant therapy with Vimang in Primary Health Care is an attractive alternative for the complementary or direct treatment of diseases related to oxidative stress, inflammation or pain, with a high efficacy. The results of clinical studies on elderly subjects, breast dysplasia (mild or moderated), HIV and skin diseases are shown from ethnomedical evidences previously reported. HIV patients (seropositive with CD4 counts between 300 and 500) administered with Vimang for six months in a double-blind randomised and placebo controlled trial (68 patients) reached the same value of plasma oxidative stress biomarkers as the seronegative control group for total antioxidant status (TAS), hydroperoxides (HPO), superoxide dismuthase (SOD), malonyl dialdehyde (MDA) and DNA fragmentation. On elderly subjects (n = 31, Vimang tablets, 300 mg) the self-perception of their health status was improved in 8 from 9 evaluated parameters in terms of life quality being body pain the most significant (Health Questionaire SF-36). In the treatment of breast dysplasia (n = 100, Vimang tablets, 300 mg) it was found an efficacy higher than 85%, with similar or better results than Vitamin E. In the treatment of skin disorders (n = 590, Vimang cream, 1,2 %) an improvement by 86,8 and 96,7% was observed in treated patients with inflammation symptoms and pain, respectively, and more than

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90 % of patients were cured totally or partially. The most relevant results were observed in the recovery of skin pigmentation in pregnancy melasme and pitiriasis versicolor, (52 patients), infectious processes (53 patients), mycosis (169 patients) and atopic dermatitis (35 patients). Neither adverse reactions nor toxicity responses were observed during treatments.

Key words : *Vimang*, antioxidant therapy, Primary Health Care, elderly, breast dysplasia, skin disorder, clinical studies, Cuba

INTRODUCTION

The conservation of Redox homeostasis in the human body is essential to maintain a good health status. If the equilibrium between the oxidant systems (generators of reactive oxygen and nitrogen species, RONS) and antioxidants is affected towards the formers due to an excessive production/accumulation of RONS, the weakening of the antioxidants system or both; then a condition of oxidative stress will be established in the biological system. The excesses of RONS does promote an oxidative damage to the main biomolecules (lipids, proteins and DNA) initiating several biochemical events that may lead to the onset or aggravation of diseases and also they may alter the physical and psychical performance of an apparently healthy people. Fig 1 shows a molecular comprehensive scheme of the oxidative stress impact on human health. Over 100 diseases have associated in their aetiology or progression the occurrence of oxidative stress (Thomasset *et al.*, 2007; Halliwell, 2006; Cutter & Rodíguez, 2002; Schulz *et al.*, 2000; Miller *et al.*, 1997; Sharma & Agarwal, 1996; Ebadi *et al.*, 1996; O'Brien *et al.*, 1996; Portal *et al.*, 1995; Heliovaara *et al.*, 1994).

Despite all the evidences from the scientific literature about the relationship between the oxidative stress and disease progression, particularly those chronic; the administration of antioxidant products to patients (antioxidant therapy) is considered not relevant in the therapeutics methodologies. One reason to explain such tendency is that the regulatory health agencies do not consider antioxidants as drugs, instead they are classified as "nutritional supplements or natural products for health" since oxidative stress in not considered as a therapeutic category.

The most extended myth about oxidative stress is maybe its relation with a large number of diseases. Such association raises doubts in medical authorities concerning the efficacy of antioxidant therapy in the prevention or reduction of diseases progression. The oxidative

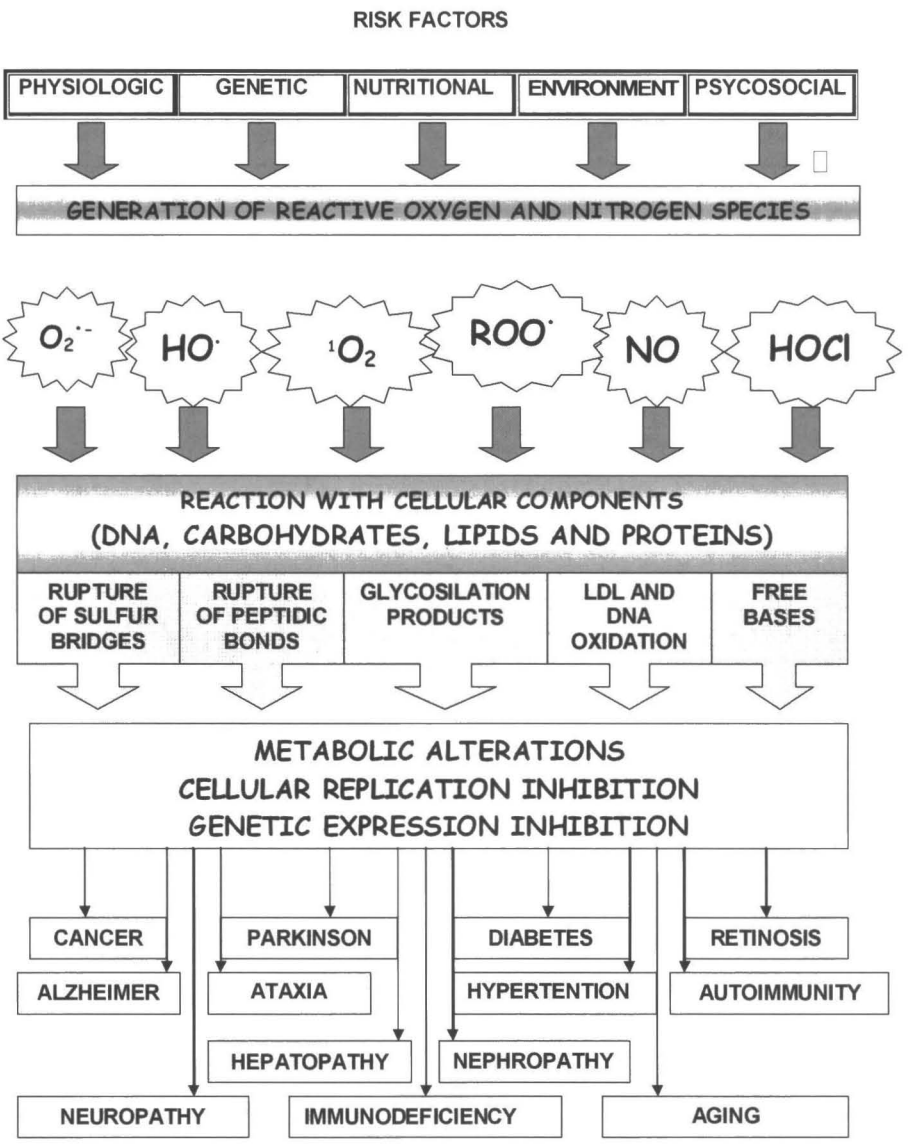

Fig 1. Chemical and biological aspects of oxidative stress. The risk factors stimulate the generation of RONS ($O_2^{\cdot -}$, HO·, $^1O_2$, ROO·, NO, HOCl) that attack cellular components producing metabolic alterations, inhibition of cellular replication and inhibition of gene expression associated to the pathophysiology of several neurodegenerative and immunological diseases and the aging process

stress is not considered as a disease because it is not possible its association with a specific syndrome as the diabetes, hepatopathies or hyperlipidemies do. This is another element that reinforces the misgivings about antioxidant therapies.

The point is that a growing number of clinical trials show the importance of antioxidant therapy against diseases like pre-clampsy in pregnancy (Rahman & Tomasi, 2003), arthritis (Matyska-Piekarska, 2006), prosthatytis (Pasqualotto *et al.*, 2000), diabetes (Manzella *et al.*, 2001), queratitis (Vertugno *et al.*, 2001) among others. Nevertheless, in contrasts with the lack of clinical trials with natural antioxidants that come out from traditional medicine or ethnomedical practice probably because of the scarceness of financial support for suitable pre-clinical and clinical investigations. On the other hand, there is an extended believe that pure compounds are the only possible pharmaceuticals (with few exceptions), and natural products are commonly rejected as drugs by regulatory health agencies because, most of the times, they are presented as crude extract mixtures with doubts about the reproducibility and standard manufacture.

This work shows some experimental results and the Cuban experience in the application of a new natural antioxidant known by its commercial name, Vimang in primary health care to demonstrate the benefits of antioxidant therapy associated to the improvement of patient's quality of life and the success in the treatment of some diseases.

Ethnomedical Evidences

Mango stem bark has been traditionally used in many countries for the treatment of menorrhagia, diarrhoea, syphilis, diabetes, scabies, cutaneous infections, anaemia, etc. using an aqueous extract obtained by decoction as reported in the *Napralert Database* (Napralert Database, 2007). The use of that mango stem bark extract (MSBE) in Cuba has been documented on more than 7 thousand patients in the last ten years by the Center of Pharmaceutical Chemistry (personal communication), with emphasis on patients with malignant tumours (Tamayo *et al.*, 2001). Initial *in vitro* tests demonstrated that MSBE had not cytotoxic effects on tumour cells. However, more than 95 % of cancer patients treated with MSBE (2286 patients) evidenced an improvement in terms of their quality of life (appetite, body weight, self-independence for the daily life, etc.); inflammation and/or pain were significantly reduced and several biochemical markers were improved in time (*i.e.* haemoglobin and transaminase, being the most significant) (Núñez-Sellés *et al.*, 2002). It was relevant that more than 60% of patients with diabetes mellitus (408 patients)

reduced the insulin dose by 20 IU after 6 months of MSBE oral administration; *ca.* 80% of patients with benign prostate hyperplasia (826 patients) improved the urine retention after 3 months of MSBE administration (oral and rectal); and 95% of patients with different types of dermatitis (1297 patients) were improved after one-week treatment with topical MSBE. Also significant was that 87% of patients with *Lupus erithematosus* (675 patients) improved their quality of life after the first month of MSBE treatment (oral and topical administration).

The working hypothesis was that MSBE had an antioxidant effect, probably connected to analgesic, anti-inflammatory, and immunomodulatory effects, which could explain the results observed from the ethnomedical studies. More than 10 years of scientific research have allow the corroboration of this hypothesis, supported by more than 50 papers published in peer-reviewed journals mainly in pre-clinical area (for review see Núñez-Sellés *et al.*, 2007).

Production and chemical composition of MSBE

Mango trees are gender broadly distributed in Cuba (273 varieties), but only 16 varieties could afford the fresh raw material (stem bark) with a reproducible chemical composition and no toxic effects. Trees or fruit production were not damaged from the collection of the stem bark, and it could be repeated every 2 to 4 years, depending on the variety, during a field study conducted from 1994 to 2004. The bark is carefully cut along the mango tree stem, without affecting the inner part of it, from the top (25 cm below the lowest branch) to the bottom (25 cm above the highest root). Cut width is not larger than 20 cm. Thus, the environmental impact of mango stem bark collection is minimal and the availability of raw material may assure a large production of the MSBE on an industrial scale (unpublished results). Furthermore, mango farmers were benefited from stem bark collection, giving a new value-added product to their plantations, with a three-fold increase in their incomes as compared to fruit production alone.

Industrial MSBE is obtained by decoction of the stem bark using water as solvent (no organic solvents are used thorough the industrial process) with subsequent concentration, drying and homogenization to yield between 10 and 15% of a homogeneous brown powder (30 – 60 μm particle size), which melts with decomposition from 215 to 218°C. This active principle is used further to produce several formulations like coated tablet, capsule, syrup, cream, ointment, suppository, vaginal oval, and ampoule for injection, which have been protected by a patent (Núñez-Sellés *et al.*, 2002) and registered as

phytodrug, food supplement or cosmetic by the Cuban health regulatory agencies.

MSBE chemical composition has been reported elsewhere (Núñez-Sellés *et al.*, 2002) having polyphenols as the main fraction (*ca.* 45 %). Mangiferin (MF) is the major component of MSBE, with a typical isomeric composition (MF + isoMF + homoMF), different from other MFs extracted from other regions or natural sources (data no published). Biologically active terpenoids like beta-elemene, beta-selinene, alpha-guaiene, hinesol, and beta-eudesmol have been also identified. A recent report about elements composition described the presence of calcium and selenium at concentrations within the Daily Recommended Allowance (DRA) given by nutritional regulatory bodies, with a significant contribution of Cu and Zn as important biological elements (Núñez-Sellés *et al.*, 2007b). Other components as free sugars, polyalcohols, sterols, and unsaturated fatty acids have been also reported and quantified. Quality control specifications for the industrial production of MSBE have been established after an exhaustive study of its chemical composition in different sites, soils, varieties, and tree age (data no published).

Clinical Evidences

Relevant controlled clinical trials with MSBE formulations (*Vimang*) have been conducted on HIV/AIDS, geriatrics and skin disorders in Primary Health Care with significant results in terms of the improvement of the patient quality of life. HIV patients (seropositive with CD4 counts between 300 and 500) were administered with 8 *Vimang* tablets/day (2.4 g MSBE daily before meals) for six months in a double-blind randomised and placebo controlled trial (68 patients). Seven of nine oxidative stress (OS) biomarkers were improved in 58 % of the *Vimang* group, against 3 % of the placebo group, both diet-controlled. The *Vimang* group reached the same value of plasma OS biomarkers as the seronegative control group for total antioxidant status (TAS), hydroperoxides (HPO), superoxide dismuthase (SOD), malonyl dialdehyde (MDA) and DNA fragmentation (Fig 2). Statistical trends were observed for the increase of CD4 and the decrease of CD95 counts. Moreover, antigen p24 disappeared in patients treated with *Vimang* for 6 months, whereas it had the same concentration or even it was increased in the placebo group. The trend for the reduction of transaminase, uric acid, and erythrosedimentation in the *Vimang* group was also observed. No toxic-, neither side-effects, were found for the *Vimang* group in plasma, kidney, and liver (Peres Santo *et al.*, 2003). A second trial on 120 HIV seropositive patients of 12-months duration is ongoing at present.

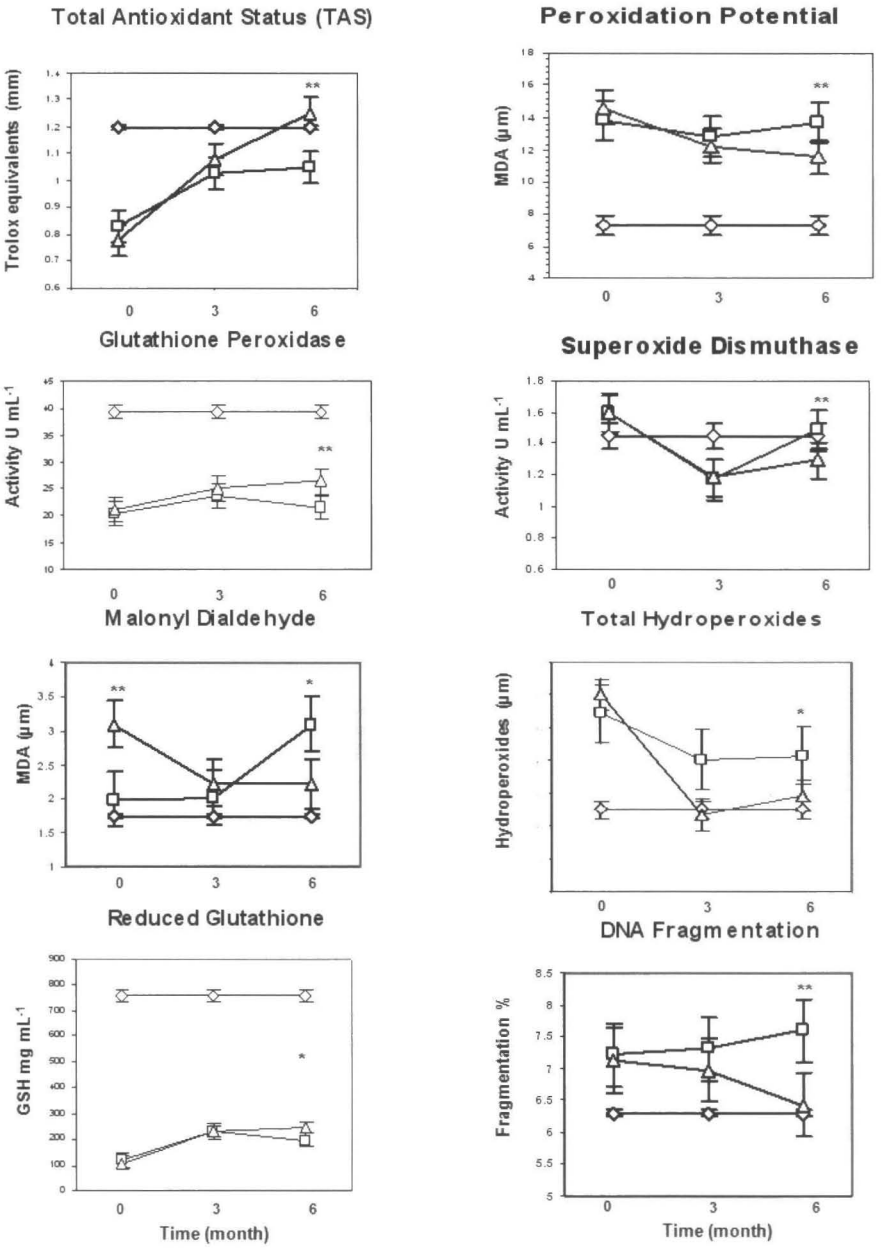

Fig 2. Influence of supplementation with *Vimang* (8 tablets/day) on oxidative stress markers of HIV/AIDS patients (n=82) in a double-blind randomized clinical trial[19,22]. Analyses were done at times 0, 3 and 6 months (◊ = Seronegative control group, Δ = *Vimang* group, □ = Placebo group). ** $p<0.01$; * $p<0.05$ *Vimang* treated vs. Placebo group

A group of 31 adults older than 65 years were administered daily with 3 *Vimang* tablets before meals for 2 months in a pilot controlled trial in Primary Health Care in order to measure the improvement on their serum redox status and their health-associated quality of life. The supplementation increased the extracellular superoxide dismutase (SOD) activity and serum total antioxidant status. It also decreased serum thiobarbituric reactive substances and GSSG levels (see Table 1) (Pardo Andreu *et al.*, 2006). Such intervention improved the self-perception of health in the elderly (Fig 3). Eight of nine evaluated parameters from SF-36 Health Questionnaire, got better at the end of the trial, but the most significant was "body pain", which started to improved significantly after 15 days of the initial treatment.

A field study in Primary Health Care (590 patients) was conducted with the *Vimang* cream formulation (1.2 % MSBE) on skin disorders, mainly related to skin damage, inflammation and pain in several pathologies. Approximately 86.8 % and 96.7 % of all patients improved in terms of inflammation and pain, respectively, in times ranging from 7 to 90 days (2 daily applications) depending on the skin disorder or pain location (Fig 4). Relevant results were observed in skin

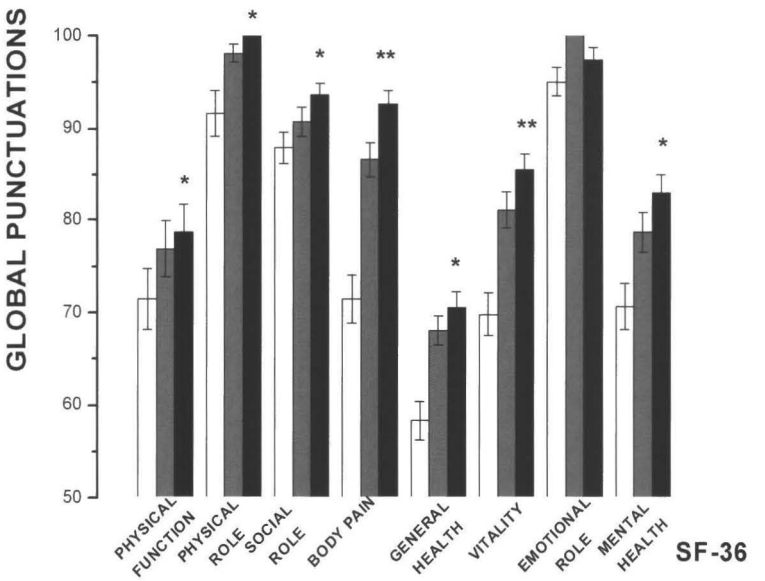

Fig 3. Influence of supplementation with *Vimang* (3 tablets/day) on the different dimensions from the SF-36 Health Questionnaire of elderly people (n=31) with 65 or more years-old in a controlled clinical trial in Primary Health Care[19]. Analyses were done at times (□) 0, (▩), 30 and (■) 60 days. Values are means ± SEM. *p<0.05; **p<0.01 respect to Time zero punctuations

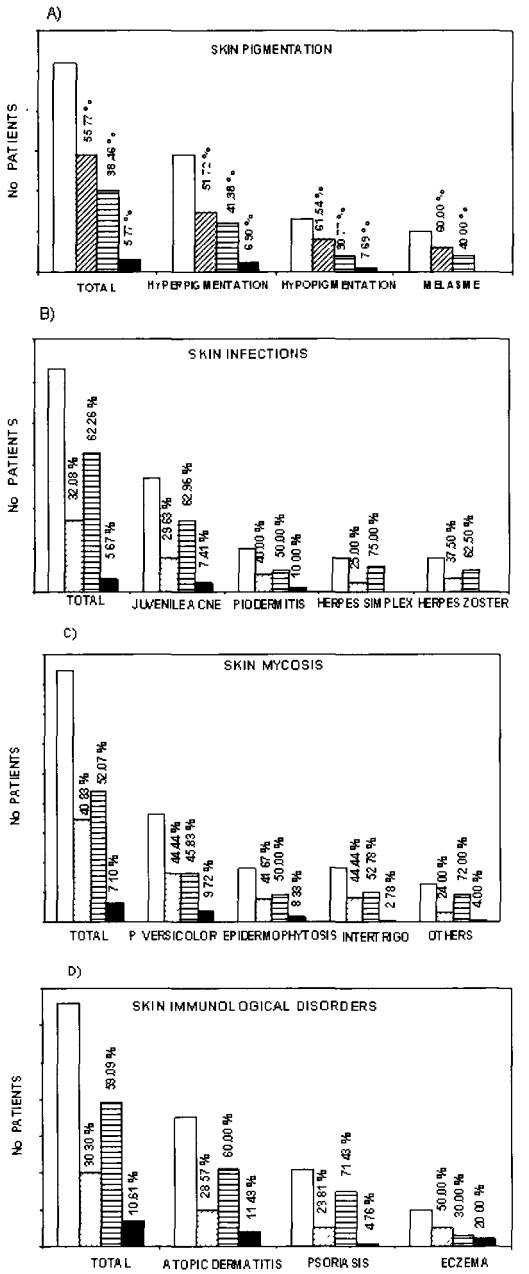

Fig 4. Results of a field study in Primary Health Care (San Agustín, Havana) with the *Vimang* cream (1.2%) on 340 patients[19]. A) Skin pigmentation (n=52); B) Skin infections (n=53); C) Skin mycosis (n=169 patients); D) Skin immunological disorders (n=66 patients). The legends are: (□) Total number of patients, (▨) Patients cured, (▤) Partially cured, (■) Not cured

Table 1. Effects of age and *Vimang* supplementation on serum antioxidant status[23]

Variable	Young	Elderly-non-supplemented	Elderly-supplemented [days]		
			15	30	60
Total glutathione	451.06 ± 19.47	446.64 ± 17.24	474.48 ± 15.32	452.52 ± 19.12	462.30 ± 23.04
GSH [ng mL^{-1}]	440.77 ± 19.47	430.66 ± 10.4	461.92 ± 11.85	440.10 ± 13.65	449.18 ± 15.78
GSSG [ng mL^{-1}]	10.29 ± 2.17	15.98 ± 2.05[a*]	12.56 ± 1.82[b*]	12.42 ± 2.11[b*]	13.12 ± 1.09[b*]
$\frac{2GSSG}{GSH+2GSSG} \times 100$	4.46 ± 1.08	6.91 ± 1.01[a*]	5.16 ± 0.92[b*]	5.34 ± 0.78[b*]	5.52 ± 1.02[b*]
SOD activity [U $mL^{-1}min^{-1}$]	5.64 ± 1.08	3.84 ± 0.413[a*]	10.1 ± 0.498[b**]	7.08 ± 0.325[b**]	7.01 ± 0.355[b**]
Total antioxidant status [mmol of Trolox l^{-1}]	1.31 ± 0.058	1.02 ± 0.057[a**]	1.35 ± 0.039[b**]	1.39 ± 0.057[b**]	1.59 ± 0.052[b**]
TBA reactants [µmol of MDA l^{-1}]	3.55 ± 0.094	4.67 ± 0.345[a**]	3.48 ± 0.089[b**]	3.21 ± 0.157[b**]	2.73 ± 0.123[b**]
Peroxidation Potential [µmol of MDA l^{-1}]	14.91 ± 1.01	14.70 ± 0.593	15.07 ± 0.449	14.67 ± 0.742	14.70 ± 0.553

Values are means ± SEM; [a]represents significant differences between young and elderly-non-supplemented groups; [b]represents significant differences between elderly-non-supplemented and elderly-supplemented groups. *$p<0.05$, **$p<0.01$

pigmentation (52 patients), skin infections (53 patients) and skin mycosis (169 patients), with more than 90% of patients cured or partially cured (unpublished results).

A clinical study was also conducted with women diagnosed with slight to moderate breast dysplasia. They were divided into two groups: one treated with 500 U Vitamin E daily and other treated with three *Vimang* tablets 300 mg each. The main objective of the study was the evaluation of Vimang antioxidant therapy against Vitamin E during three months in breast dysplasia (Ricardo *et al.*, 2005). The patients were physically examined (including an ultrasound test) and they also answered a questionnaire at 0, 30, 60 and 90 days after the treatments. The results showed that *Vimang* treatment reduced the breast area with nodular tendency more extensively than Vitamin E, probably associated to a higher antioxidant efficacy of the natural extract. In this regard a recent pre-clinical investigation showed that *Vimang* was more effective than several classical antioxidants like Vitamins C and E and β-carotene in the prevention of lipid peroxidation and tissues oxidative damage (Martinez *et al.*, 2000).

Other double-blind randomized clinical trials have just started on bronchial asthma, atopic dermatitis, and diabetes mellitus; clinical protocols for the treatment of post-acute brain infarct and post-myocardial infarct have been recently approved all of them under the supervision of the Cuban health regulatory body (CECMED). MSBE formulations, *Vimang* (tablet and cream), were added to the Basic Drug List of the Cuban Health System as anti-inflammatory and analgesic on December, 2004, from the request of the National Divisions of Primary Health Care and Epidemiology, Ministry of Public Health. There is an increasing demand of *Vimang* by both physicians and population. Thus, MSBE, used as an active principle in different pharmaceutical formulations, has proved to be effective and reproducible as antioxidant, anti-inflammatory and immunomodulator without being an isolated "monoceutical" for medical uses, on the basis of scientific evidence from the basic to the clinical research.

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15

Novel Nutraceutical Properties of *Stevia rebaudiana* (Bertoni) Bertoni

SHARMILA CHATTOPADHYAY[1]

ABSTRACT

The antioxidant potential and oxidative DNA damage preventive activity of Stevia rebaudiana (Bertoni) Bertoni has been investigated which may be useful as a novel potential exogenous source of natural antioxidant as functional food called nutraceutical. The main object of the present study is the prevention of oxidative DNA damage brought about by ROS and scavenging of those harmful free radicals. Another subject of the study is the scientific evaluation of the strikingly potential antioxidant nature of this popular herb. The dried crude extract showed varied range of antioxidant activity in terms of 50% inhibitory concentrations (IC_{50}), as measured by DPPH method, for initial screening. In addition to the DPPH method, the bioactive fraction was much active in scavenging $ABTS^+$, the free radical. The IC_{50} value of the bioactive fraction was 3.04 µg/mL as compared to the crude extract, which had an IC_{50} value of 28.6 mg/mL. Both the crude alcoholic extract as well as the bioactive fraction had impressive hydroxyl radical-mediated DNA damage preventive activity, in vitro. The crude alcoholic extract prevented DNA damage at a concentration of 1 mg/mL while that of bioactive fraction has at 0.1 mg/mL. Hence, this frontline herb may be explored further as natural dietary antioxidant or 'nutraceutical' along with its increasing demand as natural sweetener.

Key words : Antioxidant, nutraceuticals, oxidative DNA damage, ROS, *Stevia rebaudiana* (Bertoni) Bertoni

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"Let Food Be Thy Medicine and Medicine Be Thy Food"
— Hippocrates

INTRODUCTION

Nutraceuticals, are the functional foods with potentially disease-preventing and health-promoting properties. They also include naturally occurring dietary substances in pharmaceutical dosage forms. Thus, they include "dietary supplements" as defined by the Dietary Supplement Health and Education Act of 1994 (DSHEA).

Functional foods or nutraceuticals are assuming a middle ground between food and drugs due to a growing body of evidence that supports their role in maintaining health and contributing to the treatment of diseases. Since Hippocrates advised to "Let food be thy medicine and medicine be thy food," we have defined medicines and foods based on what is known about each substance in terms of efficacy, safety and the significance of its perceived contribution to health. Over time, we tend to redefine these substances as our experience and expectations change. The ancient Greeks, for example, looked upon garlic as a performance-enhancing drug and officially sanctioned it for this use during the first Olympic games. During the age of sailing ships, lemons were dispensed to sailors to prevent and treat scurvy. John Woodall, the father of naval hygiene and a "Master in Chirurgerie," published "The Surgeon's Mate" in 1636 in which he wrote, "The juice of lemmons is a precious medicine... It is to be taken each morning two or three teaspoonfuls, and fast after it 2 h".

Modern functional foods or nutraceuticals became available in the 1920's, when iodine was added to salt to prevent goiter. This was followed by vitamin D milk. Today, many Americans start their day with calcium-fortified orange juice (to strengthen their bones). Then, they spread a margarine that lowers cholesterol on folate-enriched toast (to protect their hearts and prevent birth defects). Hence, nutraceuticals may be considered as 'over-the-counter nutritional supplements'. In another sence, nutraceuticals are naturally occurring components in food or a food supplement. They have a positive effect and are therefore beneficial to health. All of these naturally existing chemicals find their source either directly or indirectly from the age-old 'Herbalism'. The term Herbalism refers to the practice of the making and using of folk and traditional medicines by using plants and plant extracts. The more recent name given to it is phytotherapy.

The generation and release of excess reactive oxygen species (ROS) are strongly implicated in the pathogenesis of inflammatory periodontitis. The destructive activities of ROS are neutralized by

anti-oxidants, which constitute the body's natural defence against excess ROS released. Anti-oxidant mechanisms vary, but radical scavenging species such as uric acid and reduced glutathione are believed to be the most effective in protecting vital cell components from structural damage during hyper-inflammation (Stadtman, 1992; Ames *et al.*, 1993; Frei, 1994; Wiseman & Halliwell, 1996; Shahidi, 1997).

It is a well known fact that anti-oxidants work in concert, and the study of individual species in relation to inflammatory diseases can provide a distorted and misleading picture of their role in the pathobiology of anti-oxidant mediated disease conditions. This opens up the potential for a new generation of novel, dual-action host-modulation therapies, or 'nutraceuticals', as adjuncts in the management of anti-oxidant mediated disease conditions (Craig, 1999; Kumar & Chattopadhyay, 2007).

Stevia rebaudiana (Bertoni) Bertoni is a perennial herb of the Asteraceae (Compositae) family and is valued for natural source of sweetener production. It is native to Paraguay, where it grows wild in sandy soils. It is often referred to as "the sweet herb of Paraguay". Stevioside, the main sweet component in the leaves of *S. rebaudiana* (Bertoni) Bertoni tastes about 300 times sweeter than sucrose (0.4% solution). The remarkably high yield of several high-potency low-calorie sweeteners in its leaf tissues makes the plant economically important. The leaf extract of this plant has also been used traditionally in the treatment of diabetes (Hanson & Oliveira, 1993). It has particular advantages for those suffering from obesity, diabetes mellitus, heart disease, and dental caries (Kinghorn & Soejarto, 1985).

Stevia is gaining significance in different parts of the world and is expected to develop into a major source of high-potency sweetener, for the growing natural food market. A descriptive study focusing on the sweet and non-sweet constituents of the genus *Stevia*, modifications of the naturally occurring sweeteners to improve the taste and its botany, can be found in the recent excellent book by Prof. A.D. Kinghorn. In the USA powdered *Stevia* leaves and refined extracts from the leaves have been used as a dietary supplement since 1995. *Stevia* has also been approved as a dietary supplement in Australia, New Zealand and Canada. In Japan and South American countries, *Stevia* may also be used as a food additive. *Stevia* is currently banned for use in food in the European Union. It is also banned in Singapore and Hong Kong. The advantages of stevioside as a dietary supplement for human subjects are manifold, it is stable, it is non-caloric, and it maintains good dental health by reducing the intake of sugar and opens the possibility for use by diabetic and phenylketonuria patients and obese persons.

Taken together we have studied in details the bioactivity profile. We are intrigued to find out the it's antioxidant potential and oxidative DNA damage preventive activity of the leaf of *S. rebaudiana* (Bertoni) Bertoni with a view to develop this amazing herb as "Nutraceutical'.

MATERIALS AND METHODS

Fresh leaves of *Stevia rebaudiana* (Bertoni) Bertoni were washed in cold tap water to remove any dirt or extraneous matter followed by shade-drying at 30-35°C for period of 72 h. The grinding of dried leaves of *Stevia rebaudiana* was done with an electrical grinder followed by extraction of the above-mentioned grinded leaves with 85% methanol (3 × 1 day) and filtering the 85% alcoholic extract through Whatman filter paper to get a particle-free extract. The pooled methanol portion of the extract was evaporated under reduced pressure in a rotary vacuum evaporator at 40°C and the aqueous portion was lyophilized to produce a dry crude extract. The rest aqueous portion was fractionated with hexane, chloroform, and ethyl acetate, serially. Each fraction was concentrated resulting in hexane, chloroform, and ethyl acetate fractions using a rotary vacuum evaporator at 40°C. The ethyl acetate fraction was used for further analysis. The aqueous residue was lyophilized and also used for DPPH test along with the hexane, chloroform and ethyl acetate fractions.

The ethyl acetate fraction (2.5 g) was separated into further fractions by SiO_2 column chromatography using a mixed solvent of ethyl acetate: ethyl methyl ketone: methanol: water (5:3:3:1). Six fractions were sequentially obtained from this column, which are the mixtures of flavonoids. These six fractions were further separated by HPLC using the same solvent system to obtain bioactive fraction. Compounds of the bioactive fraction were characterized by LC-MS/MS and ^{1}HNMR analysis.

The free radical scavenging activity of crude alcoholic extract and four fractions was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH). Stock solution of the crude extract and the four fractions at 10 mg/mL and a freshly prepared DPPH solution (100 mm) were used as described previously (Kumar & Chattopadhyay, 2007). The control solution did not contain any test sample. Quercetin was used as a standard. The percent radical scavenging activity (% RSC) was calculated

$$\% \text{ RSC} = \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \times 100\%$$

The ABTS stock solution was prepared by reacting ABTS (7 mM) and potassium persulphate (2.45 mM) and allowed to stand for at

least 16 h to generate $ABTS^+$ free radicals (Re *et al.*, 1999). The working solution was prepared by diluting the stock solution with methanol such that its absorbance reaches 0.7 ± 0.02 at 734 nm ($A_{Control}$). The reaction was performed in 1 mL volume containing different concentrations of the extracts in 10 µl volumes and 990 µl ABTS working solution. Their absorbance (A_{Sample}) was noted at 734 nm exactly 6 min after the reaction mixture was prepared. Quercetin was used as a standard.

pBluescript II SK (-) supercoiled DNA maintained in *E. coli* XL-1 strain was used for Fenton reaction-induced damage assay (Ghanta *et al.*, 2007). 100 ng of plasmid pBluescript II SK (–) was treated with $FeSO_4$, H_2O_2, and phosphate buffer (pH 7.4) to final concentrations of 0.5 mM, 25 mM, and 50 mM, respectively along with test samples. After the incubation, the extent of DNA damage and the preventive effect of the test samples were analyzed on 1% agarose gel.

RESULTS AND DISCUSSION

The relative antioxidant activity, in a concentration-dependant manner, is shown in Fig 1. Preliminary screening was performed using DPPH assay to find out the extract having the best antioxidant activity. The IC_{50} values of the crude extract as well as the four fractions were determined. The IC_{50} values ranged from 9.26–327.17 µg/mL, with the ethyl acetate fraction having the best radical scavenging activity. The average contents of total polyphenols and total flavonoids in the ethyl acetate fraction were 0.86 mg gallic acid

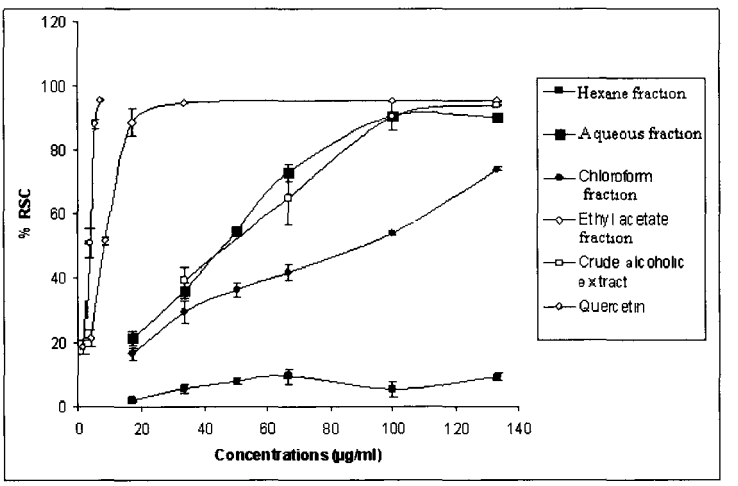

Fig 1. The concentration-dependant (5-130 µg/mL) DPPH° scavenging activity of crude alcoholic extract and different fractions (Mean ± SD, n=3)

equivalents/mg of dry weight and 0.83 mg of quercetin equivalents/ mg of dry weight respectively. Since the ethyl acetate fraction had the highest radical scavenging activity and since it has been found to be rich in flavonoids (Rajbhandari & Roberts, 1983) further bioactivity studies were carried out with this fraction.

2,2´-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) or ABTS radical scavenging activity of the crude alcoholic extract as well as the ethyl acetate fraction was performed. The % RSC was calculated as described above. The free radical scavenging potential of the crude extract and the ethyl acetate fraction was also determined by $ABTS^+$ scavenging activity. Their comparative profile is shown in Fig 2.

The protective effect of the crude extract and the ethyl acetate fraction was checked on Fenton reaction-induced damage of pBluescript II SK (-) supercoiled DNA maintained in *E. coli* XL-1 strain. Control pBS DNA showed two bands, one of open circular that was hardly visible and the other of supercoiled. $FeSO_4$ + H_2O_2 treatment in the absence of any extract led to the formation of open circular DNA by the strand scission of supercoiled DNA whereas the presence of crude extract or the ethyl acetate fraction prevented this strand scission to a considerable extent in comparison to quercetin (Fig 3a). Densitometric analysis confirmed the experimental data (Fig 3b). Stevioside, the principal sweetening agent in *Stevia*, was also used to check its activity against the prevention of DNA strand scission.

The term 'Nutraceuticals' very obviously shows its birth from the combination of two words - nutrition and *pharmaceuticals*.

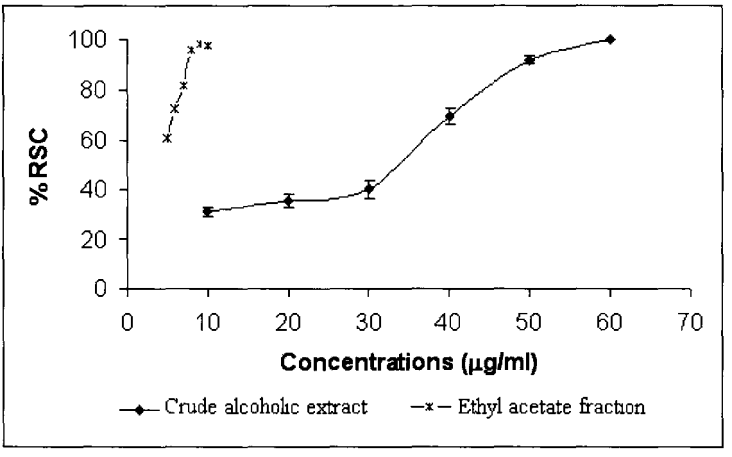

Fig 2. $ABTS^+$ scavenging activity in a concentration-dependant manner of crude alcoholic extract (10-60 µg/mL) and ethyl acetate fraction (5-10 µg/mL) of *Stevia rebaudiana* (Mean ± SD, n=3)

Nutraceuticals are mainly ingredients that serve the function of improving health or merely preventing diseases when mixed with food or food supplements in correct proportions. These nutraceuticals are naturally occurring, *i.e.* they find their sources from plants. However, to make their beneficial properties extremely effective, they need to be processed and manufactured appropriately. The international market for nutraceuticals is rapidly expanding and new research is constantly being conducted. New products are appearing on the market, some with novel therapeutic applications.

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. As oxidative stress might be an important part of many human diseases, the use of antioxidants has

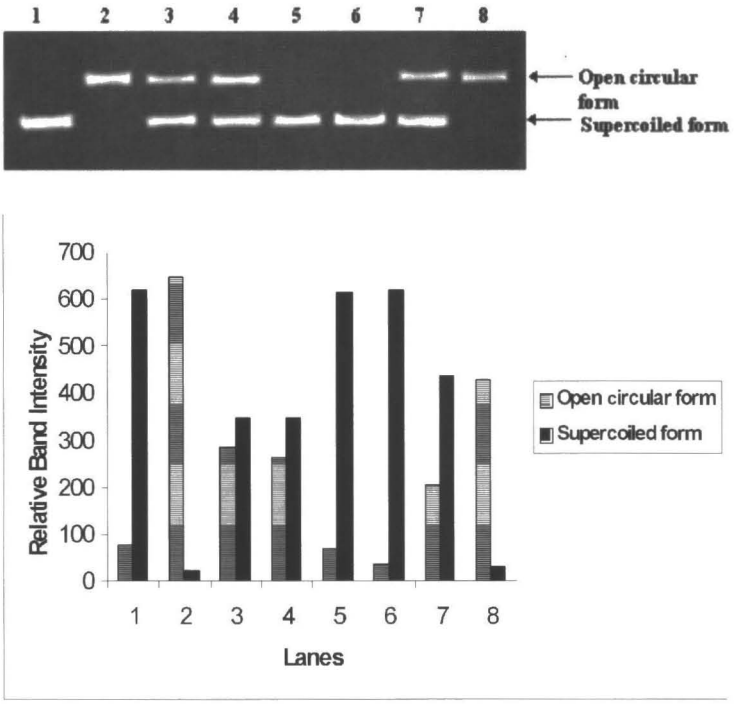

Fig 3a. Electrophoretic pattern of pBluescript II SK (–) DNA breaks by •OH generated from Fenton reaction and prevented by crude alcoholic extract and ethyl acetate fraction of *Stevia rebaudiana*. Lane 1: Untreated Control DNA (250 ng), Lane 2: $FeSO_4$ (0.5 mm) + H_2O_2 (25 mm) + DNA (250 ng), Lane 3: Only H_2O_2 (25 mM) + DNA (250 ng) Lane 4: Only $FeSO_4$ (0.5 mM) + DNA (250 ng), Lanes 5-8: $FeSO_4$ (0.5 mM) + H_2O_2 (25 mM) + DNA (250 ng) in presence of ethyl acetate fraction (1 µg), crude alcoholic extract (10 µg), Quercetin (1 mM) and Stevioside (100 µg) respectively (n=3). **b.** Densitometric analysis of open circular and supercoiled DNA damage induced by •OH generated from the Fenton reaction in presence or absence of CAE and EAE (Mean ± SD, n=3)

been intensively studied, particularly as treatments for stroke and neurodegenerative diseases. However, it is unknown whether oxidative stress is the cause or the consequence of disease. Antioxidants are also widely used as ingredients in dietary supplements in the hope of maintaining health and preventing diseases such as cancer and coronary heart disease. Although some studies have suggested antioxidant supplements have health benefits, other large clinical trials did not detect any benefit for the formulations tested, and excess supplementation may be harmful. In addition to these uses in medicine, antioxidants have many industrial uses, such as preservatives in food and cosmetics and preventing the degradation of rubber and gasoline.

At least two major human problems aging and cancer, involve ROS mediated DNA damage (Cerutti, 1994; Wiseman & Halliwell, 1996). The novelty of this investigation is the preventive activity of its bioactive fraction against DNA strand-scission by oxidative stress. Considering this fact, this frontline herb may be investigated further as potential source of natural anticancer lead. Antioxidant potential of this bioactive fraction of *Stevia rebaudiana* (Bertoni) Bertoni against ROS scavenging activity also showed potentially significant activities. Taken together, *Stevia* may be explored further as potential source of natural dietary antioxidant or 'Nutraceuticals'.

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Oligosaccharides and Total Polyphenols Contents in Italian Common Bean (*Phaseolus vulgaris* L.) Landraces: A Quality Evaluation

FIBIANI M.[1,*] AND LO SCALZO R.[1]

ABSTRACT

Common bean (Phaseolus vulgaris L.) represents one of the most important components of human diet, due to its significant content in macronutrient, mainly complex carbohydrates and proteins. However, it contains some phytochemicals that are involved in biological actions for human health. In common bean, a positive action is characterized by the polyphenols content, for their antioxidative potential, and the negative one by raffinose family oligosaccharides, represented by raffinose and stachyose, that are involved in problems of digestion, also if their positive action on intestinal microflora has been established. In fact, genetic amelioration programs are aimed to maximize positive aspects and to minimize negative ones. On the other hand, the study of naturally developed genotypes, such as old landraces, coming from a restricted territory, could spontaneously give in a natural way the expected responses in terms of health contribution. The present work showed a study on the composition in raffino-oligosaccharides, polyphenols and antioxidant potential by DPPH quenching from common bean genotypes coming from South Italy, and their comparison with a commercially available variety. The old landraces showed a content in raffino-oligosaccharides significantly lower than the commercial variety, and the response for the content in polyphenols and antiradical activity was differentiated according to different typologies, generally showing a low amount in old landraces respect to commercial variety.

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Key words : Common bean (*Phaseolus vulgaris* L.), DPPH· antiradical capacity, landraces, polyphenols, raffinose, stachyose

INTRODUCTION

The need of a careful knowledge about the healthy properties of food products originated from plants is continously increasing in the last few years. The study of old cultivated varieties in a restricted territory, better known as landraces, does not only represent the safeguard of the agroecosystem, but could be also the starting base for programs of genetic amelioration. The main characters up to now studied for ameliorating plant products were strictly related to agronomical traits, but in the last few years the interest for healthy properties has been also increased. Often, the healthy traits investigated in plants and obtained by complex conventional breeding and genetic engineering approaches, can be easily naturally found in old genotypes selected in an appropriate and restricted territory.

An important plant product for human nutrition is represented by legumes, among which common bean (*Phaseolus vulgaris* L.) is one of the most representative, and in some parts of the world they are the main source of proteins, also if they are deficient in sulphur-containing amminoacids compared with meat. Besides, beans are also rich in antinutritional factors, especially proteins and carbohydrates (Wang *et al.*, 2003). Among the soluble carbohydrates, a special role is represented by the raffinose family of oligosaccharides (RFOs) that are considered responsible for flatulence (Price *et al.*, 1988): this property is related to their α-1-6 galactosidic configuration, an unusual linkage for human glycosidic enzymes, but suitable for bacterial degradation. On the other hand, recent data reconsidered RFOs in order to their prebiotics properties (Aranda *et al.*, 2000). Furthermore, beans are generally good sources of several phytochemicals with health promoting effects (Geil & Anderson, 1994), such as polyphenols, responsible for the antioxidant capacity against free radical (Beninger & Hosfield, 2003), although they inhibit Fe absorption and, with phytate, reduce the bioavailability of the high minerals content (Sandberg, 2002).

Previous studies have been made on chemometric traits of dry beans, especially considering their content in RFOs (Sánchez-Mata *et al.*, 1998) and polyphenol profile (Heimler *et al.*, 2005; Lin *et al.*, 2008). RFOs were also used as index for a geographical characterization of different bean genotypes (Muzquiz *et al.*, 1999). Surprisingly, no literature was specifically found on the consideration of both traits, that could be very important in the characterization

of old landraces grown in Italy, that have been studied for their genomic differences (Piergiovanni *et al.*, 2000a, 2000b; Lioi *et al.*, 2005).

The aim of the present work was to evaluate the RFOs and polyphenol profile of some bean landraces coming from Southern Italy, compared to a commercially available sample, such as "Borlotto" that represents one of the most traditional Italian grain typology (Ranalli *et al.*, 2005). The characterization comprised the HPLC analysis of sucrose and, for RFOs, raffinose and stachyose. As for healthy potential, bean samples were also assayed for their total polyphenol content and for the index of antiradical capacity, by the quenching of 1,1-diphenyl-2-picrylhydrazyl (DPPH·) radical.

MATERIALS AND METHODS

A dry organic "Borlotto" bean, cultivated in southern Italy, was marketly purchased and analyzed, in order to refer to a commonly used control data obtained from landraces.

Landrace beans were collected in commercial fields located in Sicilia (Polizzi and Sinagra), in Calabria (Mormanno) and in Basilicata (Rotonda, Sarconi and Paterno).

These fields are located in mountain regions (260 – 917 m), in the *Parco delle Madonie* (Polizzi), close to the *Parco dei Nebrodi* (Sinagra), in the *Parco del Pollino* (Mormanno and Rotonda), and in *Alta Val d'Agri* (Sarconi and Paterno).

From Sicilia, the samples analyzed were as follows: landraces "Badda bianco" (4 samples: Ph05, Ph06, Ph07, Ph08), "Badda rosso" (1 sample: Ph09) and "Badda nero" (4 samples: Ph10, Ph11, Ph12, Ph13) from 4 different farms located in Polizzi; and landrace "Monaco Mussu niuro" (1 sample: Ph14) from Sinagra.

From Calabria and Basilicata, the samples analyzed were as follows: landrace "Poverello bianco" (6 samples) from 6 different farms located in Mormanno (Ph15, Ph16, Ph17) and in Rotonda (Ph18, Ph19, Ph20); landrace "Riso (or Tondino) bianco" (1 sample: Ph21) from Sarconi; landrace "Tabacchino" (2 samples: Ph22, Ph23) from Sarconi and Paterno respectively; and landrace "Castelluccisa" (3 samples: Ph24, Ph25, Ph26) from 3 different farms located in Rotonda. For more exact sample identification, the samples list was exposed in Table 1.

These landraces were characterized by different seed colors and weights (http://old.alsia.it/agrifoglio/monografia/quaderno2/schede.pdf; http://www.ba.cnr.it/~germap14/ilcb/fs_intro.html). "Badda" beans showed a bicolor seed coat pattern, light brown and white, violet and

Table 1. Analyzed common bean samples with corresponding origin and cultivation source

Sample	Typology	Region	Field	Farm
Control	Borlotto	South Italy	not specified	-
Ph05	Badda bianco	Sicilia	Polizzi Generosa	a
Ph06	Badda bianco	Sicilia	Polizzi Generosa	b
Ph07	Badda bianco	Sicilia	Polizzi Generosa	c
Ph08	Badda bianco	Sicilia	Polizzi Generosa	d
Ph09	Badda rosso	Sicilia	Polizzi Generosa	a
Ph10	Badda nero	Sicilia	Polizzi Generosa	a
Ph11	Badda nero	Sicilia	Polizzi Generosa	b
Ph12	Badda nero	Sicilia	Polizzi Generosa	c
Ph13	Badda nero	Sicilia	Polizzi Generosa	d
Ph14	Monaco Mussu niuro	Sicilia	Sinagra	e
Ph15	Poverello bianco	Calabria	Mormanno	f
Ph16	Poverello bianco	Calabria	Mormanno	g
Ph17	Poverello bianco	Calabria	Mormanno	h
Ph18	Poverello bianco	Basilicata	Rotonda	i
Ph19	Poverello bianco	Basilicata	Rotonda	j
Ph20	Poverello bianco	Basilicata	Rotonda	k
Ph21	Riso (or Tondino) bianco	Basilicata	Sarconi	l
Ph22	Tabacchino	Basilicata	Sarconi	l
Ph23	Tabacchino	Basilicata	Paterno	m
Ph24	Castelluccisa	Basilicata	Rotonda	n
Ph25	Castelluccisa	Basilicata	Rotonda	j
Ph26	Castelluccisa	Basilicata	Rotonda	k

white, black and white, and about 50, 46, 51 g per 100 seeds for "bianco", "rosso" and "nero" respectively; "Monaco Mussu niuro" showed a white coat with a dark brown pattern around hilum, and 69 g per 100 seeds; the white "Poverello" and "Riso" showed 50 and 68-73 g per 100 seeds for Mormanno and Rotonda landraces respectively; "Tabacchino" showed a tobacco coloured seed coat and 46-49 g per 100 seeds; "Castelluccisa" showed a bicolor seed coat, cream and white, and 56-59 g per 100 seeds.

Polizzi "Badda" bean is a "Slow Food" presidium; Sarconi and Paterno landraces obtained the Protected Geographical Indication (PGI) European Community quality mark (EU, 1992) as "Fagioli di

Sarconi", while Rotonda "Poverello" bean is included in a requested PGI named as "Fagioli bianchi di Rotonda".

Beans were collected in the summer 2006 and for the samples Ph05 and Ph09 in the summer 2007 as well. Dry beans were reduced in fine particles grounding 50 to 100 seeds per sample in a flour mill.

For sugars determination, samples of 100 mg of bean flour were extracted with 1.4 mL of ethanol 75% aqueous solution vortexing for 2 min, sonicating for 30 min at room temperature and vortexing for 30 sec again; after each extraction the samples were centrifuged for 10 min at 6000 rpm.

Aliquots of supernatants, diluted to 1:1 with mobile phase and sonicated 1 min before injection, were analyzed in a Jasco HPLC system equipped with an 880 pump, a 1550 sampler and a 930 RI detector. The chromatographic column was a Supelco Li Chrosorb Amino (5 μm, 150 mm x 4, 6 mm). The mobile phase was acetonitrile 75% aqueous solution, flow rate 0.9 mL/min at 25° C. Chromatograms were recorded with a Shimadzu C-R6A Chromatopac DANI. Sugars identification was made by comparison of retention times of commercial standards (sucrose, raffinose and stachyose, 5.7, 8.7, and 13.8 min retention times, respectively) and quantification was made by comparison with calibration curve of authentic standard solutions; sugars amounts were expressed as mg/g flour.

For total polyphenols content (TPC) and antioxidant activity determinations, samples of 100 mg of bean flour were extracted with 1.0 mL of HCl 0.01 N in ethanol 50% aqueous solution, vortexing for 2 min, sonicating for 15 min at room temperature and vortexing for 30 sec again; after each extraction the samples were centrifuged for 10 min at 6000 rpm.

The TPC was determined using the Folin-Ciocalteu method, described by Singleton *et al.* (1999), with modifications. A 0.2 mL aliquot of supernatant was subsequently added to 2.0 mL of deionized water, and with 0.5 mL of Folin-Ciocalteu reagent. The solution was mixed, then 1.0 mL of Na_2CO_3 20% aqueous solution was added. The mixed solution was allowed to stand for 90 min in the dark at room temperature then the absorbance was measured at 730 nm in a 1 cm-path length cuvette (UNICAM UV/Vis spectrometer). The blank was also measured by assaying the pure extraction solution. TPC was expressed as gallic acid equivalents (mg GAE/100 g flour) by comparison with a calibration curve of pure gallic acid standard solutions.

The antioxidant capacity was evaluated by the DPPH· quenching assay, following the rationale by Brand-Williams *et al.* (1995). In a 1

cm-path length spectrophotometer cuvette, 1.8 mL of absolute methanol was added with 0.3 mL of supernatant and with 0.11 mL of ethanolic DPPH· solution (40 mg in 50 mL), and the quenching reaction was kinetically recorded measuring the absorbance at 517 nm up to 3 min (Jasco UVIDEC-320 spectrophotometer). The blank was made measuring the absorbance of the pure extraction solution in absence of bean extract. The DPPH· percentage decrease was calculated by the ratio of sample absorbance versus blank one, taking the data after a fixed time of 3 min reaction at 25°C. The scavenging activity was expressed as gallic acid equivalents (mg GAE/100 g sample) by comparison with a calibration curve obtained plotting the concentrations of gallic acid standard solutions (range 0.063–0.126 mg GA/mL) against relative DPPH· percentage decreases (R^2=0.956).

The discussed data referred to the average from each bean typology, clustering the data both from different samplings and from different harvest year.

RESULTS AND DISCUSSION

Sugars Analysis

Oligosaccharides profile (Fig 1) showed in the "Borlotto" control variety a medium value for sucrose (25.5 mg/g) and raffinose (4.3 mg/g), and the highest value for stachyose (38.6 mg/g). The present values, if compared to those found in literature (Sánchez-Mata *et al.,* 1998; Muzquiz *et al.,* 1999), were close for sucrose and raffinose, while stachyose was found to be higher in our control than in the general amount found in the samples from references.

"Badda" typology generally had an amount of sucrose close to "Borlotto" in all types: "bianco", "rosso" and "nero". The amount of RFOs was different from "Borlotto" especially for the content in stachyose, averaging at 16.2 mg/g that is decisively lower than control value. Among "Badda" types, the "nero" shows a slightly higher amount of raffinose respect to other types and a further decrease in stachyose, arriving at 12.2 mg/g, the absolutely lowest detected value. As regards "Monaco Mussu niuro", the only analyzed sample had a sucrose amount lower than the control, a raffinose one equal and a stachyose one at 16.2 mg/g, lower than control.

The "Poverello bianco" types comprised two samples coming from two different locations of production, sometimes considered as the same ecotype (www.ba.cnr.it/~germap14/ilcb/others/fs_others.html). These two types did not differ between them for raffinose and stachyose, both resulting in a lower amount than control, but they resulted differ for sucrose: Rotonda type was higher than corresponding one from Mormanno (29.0 and 20.0 mg/g, respectively).

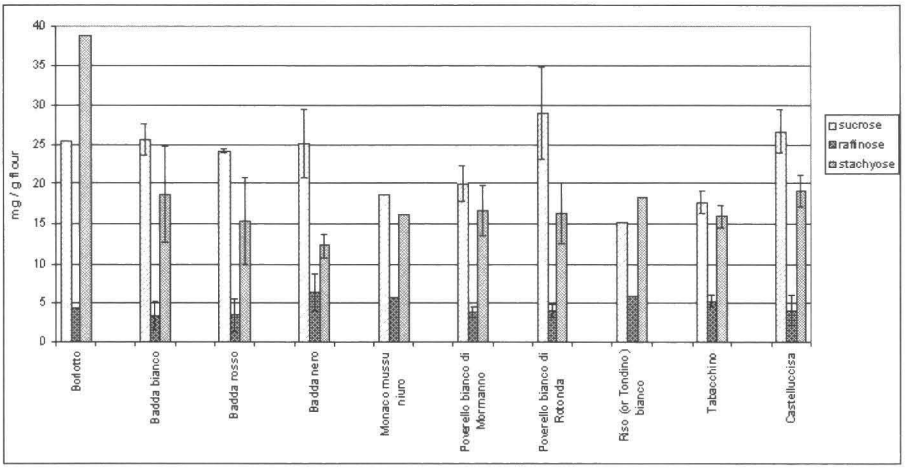

Fig 1. Sucrose, raffinose and stachyose contents (mg/g flour) in analyzed common bean samples (means of each typology ± std. dev.)

The "Riso (or Tondino) bianco" typology stood out for the lowest content in sucrose (15.1 mg/g), while raffinose and stachyose were in the same range of "Poverello" samples, confirming the lower amount of stachyose than control.

"Tabacchino" showed a sucrose content lower than "Castelluccisa"; the latter content was close to control, while raffinose and stachyose were in the same range for both landraces typologies, and their amount resembled what previously found for the other landrace samples.

Nutraceutical Potential

Bean samples were also evaluated for nutraceutical potential, analyzing their content in total polyphenols and the index of antiradical capacity, by the DPPH· assay. A significant correlation (r_{xy} = 0.83) was found between these two quality parameters, with a total average of 137.6 mg GAE/100 g for TPC, and a value of 222.9 mg GAE/100 g for antiradical activity. Similar values were found for TPC by other authors on Italian bean landraces (Heimler *et al.*,2005).

The single values were plotted in a two-dimensional graph (Fig 2) in order to better check the differences among the assayed typologies. The points located in the bottom, left side, represented a low index both in TPC and in antiradical activity, while a shift towards the top and right side of the graph is for an increase of these traits. The purple striped "Borlotto" control sample is located in the zone of high amount of both TPC and DPPH· activity (203.3 and 315.3 mg GAE/100 g, respectively).

"Badda" types were clustered in the central part of the graph, meaning intermediate values, lower than control, especially for TPC. Among these types, "Badda rosso" showed a slight increase in nutraceutical potential respect to the other types, namely "bianco" and "nero". The "Monaco Mussu niuro" bean showed a decreased value respect to "Badda" ones.

The "Poverello" typology was clearly placed in the zone at low TPC and DPPH· quenching index respect to control: not so great differences were found between the two samples from Rotonda and Mormanno; the average for TPC and DPPH· quenching resulted 76.1 and 108.6 mg GAE/100 g, respectively.

The "Riso" typology was also placed in the left-bottom part of the graph, showing the lowest index in anti-DPPH· activity (47.7 mg GAE/100 g).

At the completely opposite side, "Tabacchino" samples showed the highest values in both parameters (243.4 for TPC and 341.7 mg GAE/100 g for DPPH· quenching), higher than control. "Castelluccisa" was placed in the middle of the graph, in the zone of "Badda" samples.

TPC and anti-DPPH· activity seemed to be strictly related to seed coat color, as previously reviewed by Salunkhe *et al.* (1982), showing the highest values for fully coloured typologies, medium values for the bicolor typologies and the lowest ones for the white typologies.

CONCLUSIONS

The content of RFOs, the flatulence factor of bean, was decreased in all beans landrace varieties, respect to a "Borlotto" control sample

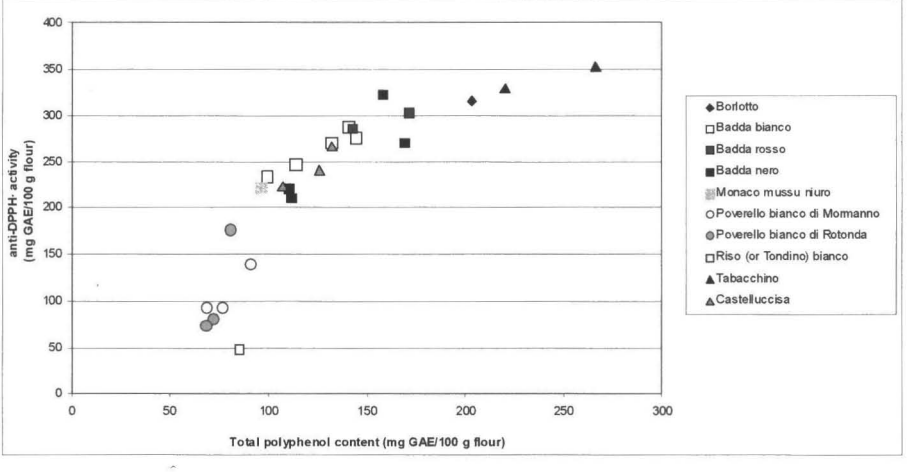

Fig 2. Total polyphenol content *versus* anti-DPPH· activity (mg GAE/100 g flour) in analyzed common bean samples

purchased in a market. The decrease was especially due to the stachyose amount, present at about 5-10 fold more than raffinose.

As regards to nutraceutical profile, the situation was more differentiated: "Borlotto" control showed a higher index, with "Badda" and especially "Poverello" typologies clearly lower. "Tabacchino" beans had a very high nutraceutical potential, and togheter with their low content in RFOs, could be considered for future breeding programmes based on the quality for the consumer.

Future studies are needed to better understand the changes of these measured parameters for different locations and harvest time, as well as after cooking, that is essential for the eating of these legumes.

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17

Biological Activities and Main Compounds of Fruits

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ABSTRACT

Many studies have shown that the consumption of fruits and vegetables is associated with a reduced risk of many diseases, including cancer, atherosclerosis, and neurovegetative diseases, which are related to elevated levels of oxidative stress. Antioxidant compounds can decrease oxidative stress, minimizing the incidence of these diseases. Fruits supply several antioxidant compounds, as for example vitamin C, carotenes, and/or polyphenols. On the other hand, some compounds present in fruits have themselves been identified as being mutagenic. This chapter reviews the major compounds and their corresponding biological activities of 23 fruits commonly consumed in the world.

Key words : Fruits, Main compounds, Biological activities

In the last years, there has been a growing interest in nutraceuticals and functional foods. Plants, including food plants (fruits and vegetables), synthesize a vast array of secondary chemical compounds that, although not involved in primary metabolism, are important for a variety of ecologic functions that enhance the plant's ability to survive. Interestingly, these compounds may be responsible for the multitude of beneficial effects that have been reported for fruits with an array of health-related bioactivities (Joseph *et al.*, 2005). Many

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studies (Joseph *et al.*, 1999; Joseph *et al.*, 1998; Prior *et al.*, 1998; Cao *et al.*, 1996; Wang *et al.*, 1996) have suggested that the most important benefits of such compounds may be derived from their antioxidant, antimutagenic, anticarcinogenic, and anti-inflammatory properties.

Fruits present a large spectrum of constituents. Besides carbohydrates, lipids, and proteins (for review see Spada *et al.*, 2008), carotenoids, vitamins and polyphenols are the most widely and best-studied compounds of fruits (Table 1).

Many fruits present high levels of carotenoids, for example acerola, mango, papaya and Surinam cherry (Table 1). About fifty to sixty different carotenoids are typically present in the human diet, and the most abundant forms found in plasma are β-carotene (precursor of vitamin A), lycopene, lutein, β-cryptoxanthin and zeaxanthin (Halsted, 2003). The biological effects of carotenoids are related to their antioxidant properties (Faulks & Southon, 2001), which can prevent the appearance of serious diseases such as cancer, pulmonary disorders, cataract (Tapiero, 2004) and atherogenesis (Faulks & Southon, 2001; Voutilainen *et al.*, 2006).

Vitamins, mainly C and E, can also be found in fruits (Table 1). Vitamin C or ascorbic acid is ubiquitous in fruits. This compound is an important antioxidant (Fenech & Ferguson, 2001), antimutagenic (Kojima *et al.*, 1992; Guha & Khuda-Bukhsh, 2002) and a regulator of DNA-repair enzymes (Cooke *et al.*, 1998; Lunec *et al.*, 2002). It is also involved in wound healing, tyrosine metabolism, conversion of folic acid to folinic acid, carbohydrate metabolism, synthesis of lipids and protein, iron metabolism, and resistance to infections (Suntornsuk *et al.*, 2002, Saffi *et al.*, 2006).

Vitamin E can be found in cashew apple, mango, red grapes, and peaches (Table 1). This vitamin is able to donate its hydrogen to free radicals, thereby forming a stable species (Rimbach *et al.*, 2002). Vitamin E radical can be regenerated by ascorbate, resulting in the formation of an ascorbyl radical (Rimbach *et al.*, 2002). There is epidemiologic and clinical evidence that high intake of vitamin E may be associated with a decreased risk of coronary heart disease (Diaz *et al.*, 1997; Kohlmeier *et al.*, 1997). Chronic oral administration of vitamin E prevented the loss of mitochondrial function and reduced ROS-induced damage in aging mice (Navarro *et al.*, 2005). These beneficial effects were paralleled by an increased lifespan, better neurological performance and higher exploratory activity (Panetta *et al.*, 2004).

Phenols (hydroxybenzenes) and especially polyphenols (containing two or more phenol groups) are ubiquitous in plant foods and, apart

Table 1. Compounds with potential biological activity in different fruits

Fruit	Compounds with potential biological activities	Reference(s)
Acai (*Euterpe oleracea* L.)	Vitamin C[1], carotenoids[1], polyphenols (cyanidin[2,4,5], procyanidin[2,3], peonidin[2], pelargonidin[2], catechin[2], epicatechin[2,3], resveratrol[2], protocatechuic acid[3])	[1]Spada *et al.*, 2008; [2]Rocha *et al.*, 2007; [3]Rodrigues *et al.*, 2006; [4]Lichtenthaler *et al.*, 2005; [5]Del Pozo-Insfran *et al.*, 2004
Acerola (*Malpighia glabra* L.)	Vitamin C[1], carotenoids[1], polyphenols[1,2]	[1]Spada *et al.*, 2008; [2]Mezadri *et al.*, 2006
Apple (*Malus domestica* B.)	Vitamin C[1,5], polyphenols (procyanidins[2,3], anthocyanins[3], catechin[3,4], epicatechin[4], quercetin[5])	[1]Spada *et al.*, 2008; [2]Kahle *et al.*, 2005; [3]Vrhovsek *et al.*, 2004; [4]Sanoner *et al.*, 1999; [5]Ballot *et al.*, 1987
Black mulberry (*Morus nigra* M.)	Vitamin C[1], carotenoids[1], polyphenols (coumaric acid[2], salicylic acid[2], caffeic acids[2])	[1]Spada *et al.*, 2008; [2]Zadernowski *et al.*, 2005
Cashew apple (*Anacardium occidentale* L.)	Vitamin C[1], vitamin E[2], carotenoids[1], polyphenols[3] (quercetin, procyanidin, anacardic acid)	[1]Spada *et al.*, 2008; [2]Ryan *et al.*, 2006; [3]Melo Cavalcante *et al.*, 2003
Coconut (*Cocos nucifera* L.)	Vitamin C[1,3], polyphenols (catechin[4], phenolic acids[2])	[1]Spada *et al.*, 2008; [2]Dey *et al.*, 2005; [3]Mantena *et al.*, 2003; [4]Kirszberg *et al.*, 2003
Cupuacu (*Theobroma grandiflorum* W.)	Vitamin C[1], polyphenols (catechin[2], epicatechin[2], quercetin[2], kaempferol[2])	[1]Spada *et al.*, 2008; Yang *et al.*, 2003
Kiwi fruit (*Actinidia chinensis* P.)	Vitamin C[1,3], Carotenoids[1], polyphenols[1,2]	[1]Spada *et al.*, 2008; [2]Chang & Case, 2005; [3]Kvesitatdze *et al.*, 2001

Table 1. *Contd.*

Fruit	Compounds with potential biological activities	Reference(s)
Lemon (*Citrus limon* B.)	Vitamin C[1,2], polyphenols (eriocitrin[2,3], hesperidin[2,3], diosmetin[2,3], diosmin[2,3], diosmetin[2,3,4], quercetin[4], apigenin[4], hesperetin[4], homoeriodictyol[4])	[1]Spada *et al.*, 2008; [2]González-Molina *et al.*, 2008; [3]Miyake *et al.*, 2007; [4]Gil-Izquierdo *et al.*, 2004
Mango (*Mangifera indica* L.)	Vitamin C[1,2,10], vitamin E[3], carotenoids[1,3,4,5,8], polyphenols (quercetin[4,8], rhamnetin[5,8], gallotannins[6], flavonols[8], kaempferol[8], xanthone[8], isomangiferin[8], galloyl derivatives[8], mangiferin[8,9], catechin[9], epicatechin[9], tannic acid[7], caffeic acid[7], gallic acid[9], benzoic acid[9])	[1]Spada *et al.*, 2008; [2]Ribeiro *et al.*, 2007; [3]Ornelas-Paz Jde *et al.*, 2007; [4]Berardini *et al.*, 2005; [5]Chen *et al.*, 2004; [6]Berardini *et al.*, 2004; [7]Singh *et al.*, 2004; [8]Schieber *et al.*, 2003; [9]Nunez Selles *et al.*, 2002; [10]Ballot *et al.*, 1987
Melon (*Cucumis melo* L.)	Vitamin C[1,3,4], carotenoids[1,2], polyphenols[1]	[1]Spada *et al.*, 2008; [2]Portnoy *et al.*, 2008; [3]Lester, 2008; [4]Gil *et al.*, 2006
Orange (*Citrus aurantium* L.)	Vitamin C[1], carotenoids[1], polyphenols[2] (neoeriocitrin, narirutin, naringin, hesperidin, neohesperidin, naringenin, hesperetin)	[1]Spada *et al.*, 2008; [2]Pellati *et al.*, 2004
Papaya (*Carica papaya* L.)	Vitamin C[1,4], carotenoids[1-3], polyphenols[1]	[1]Spada *et al.*, 2008; [2]Mutsuga *et al.*, 2001; [3]Cano *et al.*, 1996; [4]Osato *et al.*, 1993
Passion fruit (*Passiflora alata* L.)	Vitamin C[1], carotenoids[1,2]	[1]Spada *et al.*, 2008; [2]Mourvaki *et al.*, 2005

Table 1. *Contd.*

Fruit	Compounds with potential biological activities	Reference(s)
Peach (*Prunus persica* L.)	Vitamin C[1,3,4,5], vitamin E[3], carotenoids[1], polyphenols (catechin[2], epicatechin[2], quercetin[3], eriodictyol[2], naringenin[2], kaempferol[2], isoehamnetin[2], protocatechuic acid[2], vanillic acid[2], coumaric acid[2])	[1]Spada *et al.*, 2008; [2]Wijeratne *et al.*, 2006; [3]Carbonaro *et al.*, 2002; [4]Gil *et al.*, 2002; [5]Ballot *et al.*, 1987
Pineapple (*Ananas* ssp)	Vitamin C[1], carotenoids[1,3], polyphenols[1,2]	[1]Spada *et al.*, 2008; [2]Wen *et al.*, 1999; [3]Ballot *et al.*, 1987
Raspberry (*Rubus idaeus* L.)	Vitamin C[1], carotenoids[1], polyphenols (anthocyanins[2-4], procyanidins[3], ellagitannins[3,4], kaempferol[4], quercetin[4], ellagic acid[4])	[1]Spada *et al.*, 2008; [2]Fang Chen *et al.*, 2007; [3]Beekwilder *et al.*, 2005; [4]Mullen *et al.*, 2002
Red grape (*Vitis vinifera* L.)	Vitamin C[1], vitamin E[2,3], tocoteienol[2,3], carotenoids[1], polyphenols (malvidin[2], quercetin[4,5,6], catechin[4,5,6], epicatechin[4,5,6], resveratrol[2,4,5,6], delphinidin[2,5], cyanidin[2,5], petunidin[4], peonidin[2,5], malvidin[5], procyanidin[5], epicatechin gallate[5], *trans*-polydatin[5], isorhamnetin[5], kaempferol[5], gallic acid, protocatechuic acid[5], caftaric acid[5], *p*-hydroxybenzoic acid[5], caffeic acid[5], *p*-coumaric acid[5], ferulic acid[5], ellagic acid[6])	[1]Spada *et al.*, 2008; [2]Dani *et al.*, 2008; [3]Horvatha *et al.*, 2006; [4]Chafer *et al.*, 2005; [5]Kammerer *et al.*, 2004; [6]Yilmaz & Toledo, 2004
Red guava (*Psidium guajava* L.)	Vitamin C[1,5], carotenoids[1,4], polyphenols (guajadial[2], quercetin[3], myricetin[3], kaempferol[3], apigenin[3])	[1]Spada *et al.*, 2008; [2]Carasek *et al.*, 2006; [3]Miean *et al.*, 2001; [4]Mercadante *et al.*, 1999; [5]Ballot *et al.*, 1987
Soursop (*Annona muricata* L.)	Polyphenols[1,2]	[1]Spada *et al.*, 2008; [2]Augusto *et al.*, 2000

Table 1. *Contd.*

Fruit	Compounds with potential biological activities	Reference(s)
Strawberry (*Fragaria vesca* L.)	Vitamin C[1,3], carotenoids[1,2], polyphenols[1,2]	[1]Spada *et al.*, 2008; [2]Kiselova *et al.*, 2006; [3]Ballot *et al.*, 1987
Surinam cherry (*Eugenia uniflora* O.)	Polyphenols[1,2]	[1]Spada *et al.*, 2008; [2]Almeida *et al.*, 1995
Tangerine (*Citrus reticulata* L.)	Vitamin C[1], carotenoids[1,2,4], polyphenols[1,3]	[1]Spada *et al.*, 2008; [2]Ricón *et al.*, 2005; [3]Gil-Izquierdo *et al.*, 2004; [4]Nogata *et al.*, 2003

from known vitamins and minerals, may be one of the most widely marketed groups of dietary supplements. This class of plant metabolites contains more than 8000 known compounds, ranging from simple phenols such as phenol itself through to materials of complex and variable composition such as tannins (Bravo, 1998). Phenolic compounds in fruits (Table 1) include flavonoids (mainly quercetin, hesperidin, anthocyanins, catechins, and kaempferol), phenolic acids (salicylic acid), hydroxycinnamic acids (coumaric and caffeic), and stilbenes (resveratrol).

Much of the literature on polyphenolic compounds concerned about the deleterious effects associated with the ability of certain phenols to bind and precipitate macromolecules including protein and carbohydrates, thereby reducing the digestibility of foods (Singleton & Rossi, 1983). More recently, interest has been rekindled in the recognition that many polyphenols, although non-nutrients, show antibacterial effects (Avorn, 1994), ability to reduce blood pressure (Lampe, 1999), antioxidant, anti-inflammatory, antimutagenic and/or anticarcinogenic effects, at least in *in vitro* systems (Saiko *et al.*, 2008; Rodrigues *et al.*, 2006; Sairam *et al.*, 2003; Miyazawa *et al.*, 1999; Bravo, 1998). A prospective study of 800 elderly men showed that the ingestion of flavonoids, mainly in tea, onions, and apples, was associated with significant reduction in mortality from coronary heart disease (Hertog *et al.*, 1993). In addition, polyphenols can also inhibit platelet aggregation and vascular relaxation through the production of nitric oxide (Dubick *et al.*, 2001).

Almost all the fruits present in this review show antioxidant activity (Table 2), which can be associated with the presence of carotenoids, vitamins, and mainly, polyphenols. The mechanisms of the antioxidant action of polyphenols are complex and they are still been studied. In a general way, they can avoid reactive species formation either by inhibition of enzymes or by chelation of trace elements involved in free radical production, scavenging reactive species, and up-regulating or protecting antioxidant defense (Halliwell & Gutteridge, 1999). Some compounds can also act in a similar way to the enzymatic defenses, since they are able to neutralize reactive species such as superoxide anion and hydrogen peroxide (Silalahi, 2002).

Many fruits (Table 2) can also present antimutagenic activity. There are a number of different mechanisms, which have been implicated in the antimutagenic effects of polyphenols. Some of these are non-specific as for example, polyphenols can exert an antioxidant action (Hartman & Shankel, 1990; Hoensch & Kirch, 2005; Anisimov *et al.*, 2006; Valcheva-Kuzmanova & Belcheva, 2006, Srinivasan, 2007) or inhibit the uptake of mutagens such as benzo[a]pyrene (Hatch *et al.*,

2000). Different polyphenols may act to upregulate the activity of glutathione S-transferase and/or may directly interfere with DNA adduct formation (Ferguson, 2001).

Although many polyphenols can present antimutagenic effects, some of them can act as a weak mutagenic agent (Ferguson, 2001). The exact reason why a polyphenol can be a mutagenic or an antimutagenic compound is not known, but structure-activity relationships among the flavonoids suggested that bacterial mutagenicity required a double bond between positions 2 and 3 and a hydroxyl group at position 3 (Nagao *et al.*, 1981). It is also known that a number of polyphenols, including quercetin, can bind to DNA (Alvi *et al.*, 1986) and this direct interaction may be an important mechanism of bacterial mutagenicity. Interestingly, some fruits (cashew apple, coconut and kiwi fruit) can present both mutagenic and antimutagenic activities (Table 2). It is known that high concentrations of ascorbic acid (Franke *et al.*, 2005) and some kinds of polyphenols can induce mutagenic effects (De Flora, 1998; De Flora *et al.*, 2001) depending on factors such as pH and the presence of Cu(II) and Fe(III) in the media (Wang *et al.*, 1996; Ferguson, 2001; De Flora, 1998).

Intensive research conducted over the last few years has shown that polyphenols, carotenoids, and vitamins derived from fruits interfere with tumor progression by acting directly on tumor cells as well as by modifying the tumor's microenvironment (stroma) and creating physiological conditions that are hostile to tumor growth (Béliveau & Gingras, 2007). Anticarcinogenic activity can also be related to the antioxidant effect (Béliveau & Gingras, 2007). Some fruits, like coconut, kiwi fruit, lemon, mango, and red grape can present anticarcinogenic activities *in vivo* assays (Table 2).

Various plant polyphenols have profound effects on the function of immune and inflammatory cells (Middleton Jr. *et al.*, 1992). Polyphenols present in green tea (mainly epigallocatechin gallate) can inhibit the inducible nitric oxide (NO) synthase and block NO-associated DNA damage (Bartsch *et al.*, 1996). Acai, black mulberry, mango, and raspberry have shown important anti-inflammatory effects (Table 2).

Plants have developed sophisticated active defense systems against pathogens, among them the production of antibiotic compounds. Centuries of folk wisdom have identified certain fruits or vegetables as having antibacterial potential (Lampe, 1999). Cashew apple, red grape, red guava, lemon, mango and papaya present antibacterial and/or antifungal actions (Table 2) acting against *Bacillus subtilis, Enterobacter cloacae, Escherichia coli, Salmonella typhi,*

Table 2. Biological activities of fruits

Fruits	Biological activities	Reference(s)
Acai	Antioxidant activity[1-5] Vasodilatory activity[2] Anti-inflammatory effect[3] Mutagenic activity[1]	[1]Spada *et al.*, 2008; [2]Rocha *et al.*, 2007; [3]Rodrigues *et al.*, 2006; [4]Schauss *et al.*, 2006; [5]Lichtenthaler *et al.*, 2005
Acerola	Antioxidant activity[1,2]	[1]Spada *et al.*, 2008, [2]Hanamura *et al.*, 2005
Apple	Antioxidant activity[1,2]	[1]Spada *et al.*, 2008, [2]Leu *et al.*, 2006
Black mulberry	Antioxidant activity[1,2] Anti-inflammatory effect[2]	[1]Spada *et al.*, 2008, [2]Kim & Park, 2006
Cashew apple	Antioxidant activity[1-3] Mutagenic/comutagenic activities[1,4,5] Antibacterial activity[2] Antimutagenic activity[4,5]	[1]Spada *et al.*, 2008, [2]Green *et al.*, 2007; [3]Konan *et al.*, 2006; [4]Melo Cavalcante *et al.*, 2003; [5]Trevisan *et al.*, 2006
Coconut	Antioxidant activity[1,5,6] Mutagenic activity[2-4] Antimutagenic activity[3,5] Anticarcinogenic activity[5]	[1]Spada *et al.*, 2008, [2]Sandhya & Rajamohan, 2006; [3]Petta *et al.*, 2004; [4]Narasimhamurthy *et al.*, 1999; [5]Nalini *et al.*, 1997; [6]Bell & Kamens, 1990
Cupuacu	Antioxidant activity[1,2] Antimutagenicity[1]	[1]Spada *et al.*, 2008; [2]Yang *et al.*, 2003
Kiwi fruit	Comutagenic activity; low antimutagenic and mutagenic effects[1-3] Anticarcinogenic activity[4]	[1]Spada *et al.*, 2008; [2]Deters *et al.*, 2005; [3]Tang & Edenharder, 1997; [4]Edenharder *et al.*, 1994.
Lemon	Antifungal activity[1] Antimutagenic activity[2-4] Anticarcinogenic activity[5]	[1]Ben-Yehoshua *et al.*, 2008; [2]Spada *et al.*, 2008; [3]Higashimoto *et al.*, 1998; [4]Bala & Grover, 1989; [5]National Toxicology Program, 1990.
Mango	Antioxidant activity[1,3-5] Anti-inflammatory activity[2] Anticarcinogenic activity[4] Antimutagenic activity[1,6] Antidiarrhoeal activity[7] Antibacterial activity[7]	[1]Spada *et al.*, 2008; [2]Knödler *et al.*, 2007; [3]Mahattanatawee *et al.*, 2006; [4]Rodriguez *et al.*, 2006; [5]Percival *et al.*, 2006; [6]Pardo-Andreu *et al.*, 2006; [7]Sairam *et al.*, 2003.
Melon	Antioxidant activity[1-4]	[1]Spada *et al.*, 2008; [2]Lester, 2008; [3]Vouldoukis *et al.* 2004; [4]Lester *et al.*, 2004.
Orange	Antioxidant activity[1,3,5,6]	[1]Spada *et al.*, 2008; [2]Nelson *et*

Table 2. *Contd.*

Fruits	Biological activities	Reference(s)
	Adrenergic activity[2] Antigenotoxic activity[4] Antimutagenic activity[1,7]	*al.*, 2007; [3]Jayaprakasha *et al.*, 2007; [4]Franke *et al.*, 2006; [5]Deyhim *et al.*, 2006; [6]Hosseinimehr & Karami, 2005; [7]Miyazawa *et al.*, 1999
Papaya	Antioxidant activity[1,2,4,5,8,14] Antibacterial activity[3,11,14] Inhibitory effect on sperm motility[6] Antifertility activity[7,9,10,12,15] Androgenic activity[13]	[1]Spada *et al.*, 2008; [2]Lohiya *et al.*, 2008; [3]Nayak *et al.*, 2007; [4]Gambera *et al.*, 2007; [5]Mehdipour *et al.*, 2006; [6]Rahmat *et al.*, 2004; [7]Lohiya *et al.*, 1999; [8]Imao *et al.*, 1998; [9]Lohiya *et al.*, 1994; [10]Chinoy *et al.*, 1994, [11]Osato *et al.*, 1993; [12]Lohiya & Goyal, 1992; [13]Chinoy & Ranga Geetha, 1984; [14]Emeruwa, 1982; [15]Gopalakrishnan & Rajasekharasetty, 1978
Passion fruit	Antioxidant activity[1,2] Anticonvulsant effect[2]	[1]Spada *et al.*, 2008; [2]Nassiri-Asl *et al.*, 2007.
Peach	Antioxidant and antimutagenic activities[1]	[1]Spada *et al.*, 2008
Pineapple	Antioxidant activity[1-3] Antifertility activity[4]	[1]Spada *et al.*, 2008; [2]Herraiz & Galisteo, 2003; [3]Sun *et al.*, 2002; [4]Garg *et al.*, 1970
Raspberry	Antioxidant activity[1,2-6] Antimutagenic activity[1] Vasodilatory activity[3]	[1]Spada *et al.*, 2008; [2]Viljanen *et al.*, 2004; [3]Wada & Ou, 2002; [4]Mullen *et al.*, 2002; [5]Wang & Jiao, 2000; [6]Kalt *et al.*, 1999
Red grape	Antioxidant activity[1,2,4,6,8,9,13,14] Antimutagenic activity[1] Antibacterial activity[3] Anticarcinogenic activity[5,9] Antiarrhythmic and cytoprotective effects[7] Protective effects against ischemia-reperfusion[10,15] Radioprotective effects[14] Antiexudative and capillaritonic effects[16] Vasodilatory activity[11,12]	[1]Spada *et al.*, 2008; [2]Kedage *et al.*, 2007; [3]Thimothe *et al.*, 2007; [4]El-Ashmawy *et al.*, 2007; [5]Lala *et al.*, 2006; [6]Devi *et al.*, 2006; [7]Al-Makdessi *et al.*, 2006; [8]Janisch *et al.*, 2006; [9]Stagos *et al.*, 2005; [10]Nakagawa *et al.*, 2005; [11]Madeira *et al.*, 2005; [12]Soares *et al.*, 2004, [13]Shafiee *et al.*, 2003; [14]Castillo *et al.*, 2000; [15]Maffei Facinó *et al.*, 1996; [16]Zafirov *et al.*, 1990
Red guava	Antioxidant activity[1,5] Antibacterial activity[2,4]	[1]Spada *et al.*, 2008; [2]Pelegrini *et al.*, 2008; [3]Rai *et al.*, 2007;

Table 2. *Contd.*

Fruits	Biological activities	Reference(s)
	Antimutagenic effect[1,6] Hypoglycemic effects[3,7]	[4]Abdelrahim *et al.*, 2002; [5]Jime'nez-Escrig *et al.*, 2001; [6]Grover & Bala, 1993; [7]Cheng & Yang, 1983
Strawberry	Antioxidant activity[1-4] Mutagenic activity[1]	[1]Spada *et al.*, 2008; [2]Kiselova *et al.*, 2006; [3]Rababah *et al.*, 2005; [4]Kahkonen *et al.*, 2001.
Soursop	Antioxidant and antimutagenic activities[1]	[1]Spada *et al.*, 2008.
Tangerine	Antioxidant and antimutagenic activities[1]	[1]Spada *et al.*, 2008.

Staphylococcus aureus, Proteus vulgaris, Pseudomonas aeruginosa, and *Klebsiella pneumonia* (Sairam *et al.*, 2003; Osato *et al.*, 1993). The microbial activity of fruits is related to the presence of different types of polyphenols, mainly procyanidins (Taguri *et al.*, 2004).

Briefly, this review compiles data about biological activity and the main secondary compounds of fruits, reinforcing the idea that a diet rich in fruits could be used to prevent many kinds of pathologies, providing a genuine beneficial effect on human populations.

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18

Grape Juice: Its Compounds and Health Benefits

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ABSTRACT

Experimental data have increasingly suggested that cellular oxidative damage has a relevant pathophysiological role in several types of human diseases, such as atherosclerosis and cancer (Ames et al., 1993). In order to minimize oxidative stress our cells have developed a complex biochemical redox mechanism, consisting of both enzymatic and non-enzymatic components (Park et al., 2003). Moreover, the diet, especially the consumption of fruits and vegetables, also has an important role in the maintenance of physiological redox equilibrium. These foods supply several antioxidants, including several polyphenolic compounds to the body. Grapes are rich in phenolic compounds, such as flavonoids (catechin, epicatechin, quercetin, anthocyanins, procyanidins), and resveratrol (3,5,4'-trihydroxy-stilbene), which are mainly found in red grape products (Wang et al., 2002; Soleas et al., 1997; Fuleki & Ricardo da-Silva, 2003). In this chapter we review the main constituents of purple and white grape juices and their health benefits. All findings suggest that grape juices induce an important antioxidant, antiplaquetary, antitumoral and antimutagenic activities, and this may be an important issue for further investigations in the area of biochemical functional foods.

Key words : Grapes juices, Phenolic compounds, Biological activities

GRAPE JUICE AND ITS CONSTITUENTS

Grapes are the most widely grown fruit in the world, second to oranges, and represent an essential component in the Mediterranean

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diet and culture (Olalla *et al.*, 2004). In North America, purple commercial grape juice is primarily made from *Vitis labrusca* cv. Concord grapes. Niagara grapes, another *labrusca*-type cultivar, are responsible for the typical flavor of commercial white grape juice. Both of these cultivars are extensively grown for juice production in the Niagara region of the province of Ontario, Canada (Sun *et al.*, 2001). Wine, grape and grape products contribute to $162 Billion to US economy, according to study by MKF Research LLC of Napa Valley unveiled on Capitol Hill by the Congressional Wine Caucus on January 17 (2007). Research documenting many positive health benefits associated with the consumption of grapes and grape products are increasing the market for these products.

Actually there are several types of grape juices in worldwide markets. At first, grape juice can be manufactured with any variety of grapes, since they reach an appropriate maturation. Grape juices produced in traditional wine countries are elaborated with *Vitis vinifera* grapes, from white or purple cultivars. On the other hand, the Brazilian grape juices are manufactured with *Vitis labrusca* grapes, known as American or hybrid, mainly Bordo and Concord (purple types), Niagara (white ones) and Rose (Goethe) (Rizzon *et al.*, 1998; Dani *et al.*, 2007).

The chemical composition of grape juice slightly differs from the fruit, except for the higher amounts of raw fiber and oil, found in seed. The technology of preparation, mainly related to temperature and extraction time, regulates the solubility and diffusion intensity of the compounds, from the skin into the must. This is an outstanding influence on the chemical composition and on the type of the final product (Rizzon *et al.*, 1998). In general, white, purple and rose grape juices with different nutritional characteristics and phenolic content can be obtained, although there is little study about it.

Besides the different varieties of grapes, the actual market counts on the conventional and the organic juice classes. This last juice belongs to the organic farming, which is currently practiced worldwide, and does not use chemical substances, such as pesticides and synthetic fertilizers. Some studies have reported differences in phenolic and nutritional contents of fruits (strawberry, peach and plum) conducted to the traditional and organic methods (Asami *et al.*, 2003; Lombardi-Boccia *et al.*, 2004). However, there isn't an agreement on which method is better and neither on how the agricultural practice could influence on the product's final compound.

A recently study with eight different types of grape juices, white (Niagara) or purple (Bordo), manufactured with organically- or conventionally-produced grapes were used to assess polyphenol content

of different kinds of juices (Dani *et al.*, 2007). Within agricultural method, organic juices presented higher polyphenol content when compared to juices manufactured with conventionally-grown grapes (Dani *et al.*, 2007). This fact could be explained because phenolic compounds are secondary metabolites produced and accumulated in plant tissues, during stress situation. As pesticides are not used in organic farming, plants are more susceptible to the action of phytopathogens, and this causes the plant to produce higher amounts of phenolic compounds as a means to defend itself (Soleas *et al.*, 1997).

Different methodologies are applied in grape juice manufacturing. When purple juices are produced, the pulp is heated along with the skin and seed, resulting in a higher incorporation of phenolic compounds into the juice (Fuleki & Ricardo-da-Silva, 2003). Purple grape juices produced with skin heating showed a higher phenolic compound content when compared to white juices (Table 1), and also high carbohydrate and caloric levels (Dani *et al.*, 2007).

Phenolic constituents are very important to enology because they are directly or indirectly related to wine and juice's quality, especially to their color and astringency, and have also nutritional and pharmacological interest (Riberéau-Gayon *et al.*, 2003). The polyphenol structure has at least one aromatic ring, in which (at least), one hydrogen is replaced with a hydroxyl group. They can be classified as flavonoid and non-flavonoid compounds (Riberéau-Gayon *et al.*, 2003; Ferguson, 2001).

Flavonoids include the anthocynins, quercetin, catechin, epicatechin and procyanidins (Ferguson, 2001). Grape juice presents mainly (+)-catechin, (-)-epicatechin and four procyanidins (B1, B2, B3 e B4) (Table 1). The concentration of these compounds can change according to the pressing method (hot or cold maceration), to the cultivar and, to a lesser degree, pasteurization and vintage (Fuleki & Ricardo-da-Silva, 2003). Anthocyanins are responsible for many of the fruit and floral colors observed in nature. In Concord grape juice the major anthocyanins are delphinidin, cyanidin, petunidin, malvidin, and peonidin, in this order of quantity (Wang *et al.*, 2003).

Among the compounds named non-flavonoid, stilbenes, benzoic and *cinnamic acid* derivatives deserve special attention. Resveratrol, a stilbene, is the major component of the polyphenols from grapes and their products (Sun *et al.*, 2001). It is a phytoalexin present in grapevines (Flanzy, 2003), which was originally identified as the active ingredient of an Oriental herb (Kojo-kan), used for treatment of a wide variety of diseases including *dermatitis, gonorrhea*, fever, hyperlipidemia, arteriosclerosis, and inflammation (Sun *et al.*, 2001).

Table 1. Phenolic contents of different varieties of grape juices

Species of cultivar	Variety	Main characteristic	Catechin (ppm)	Epicatechin (ppm)	Procyanidin B1 (ppm)	Procyanidin B2 (ppm)	Procyanidin B3 (ppm)	Procyanidin B4 (ppm)	Resveratrol content (ppm)	Total phenolic	Authors
Vitis labrusca	Bordo	Conventional purple grape juice	2.06	22.13	1.33	1.83	7.95	4.66	0.075	119.59*	Dani *et al.*, 2007
Vitis labrusca	Niagara	Conventional white grape juice	7.39	5.95	7.53	1.32	13.06	2.45	ND	48.05*	Dani *et al.*, 2007
Vitis labrusca	Bordo	Organic purple grape juice	33.89	2.72	7.53	2.32	10.03	0.64	0.213	262.50*	Dani *et al.*, 2007
Vitis labrusca	Niagara	Organic white grape juice	0.90	1.81	3.45	1.58	18.5	3.59	ND	60.20*	Dani *et al.*, 2007
Vitis labrusca	Concord	Conventional purple grape juice	5.53	6.89	18.03	11.61	1.53	1.01	ND	145.81*	Fuleki & Ricardo -da-Silva, 2003
Vitis vinifera	Vincent	Conventional white grape juice	18.41	33.11	32.14	17.99	6.55	11.12	ND	ND	Fuleki & Ricardo-da-Silva, 2003

Table 1. *Contd.*

Species of cultivar	Variety	Main characteristic	Catechin (ppm)	Epicatechin (ppm)	Procyanidin B1 (ppm)	Procyanidin B2 (ppm)	Procyanidin B3 (ppm)	Procyanidin B4 (ppm)	Resveratrol content (ppm)	Total phenolic	Authors
Vitis labrusca	Niagara	Conventional white grape juice 1	0.98	1.07	ND	0.35	0.04	0.16	ND	32.81**	Fuleki & Ricardo-da-Silva, 2003
Vitis labrusca	Concord	Conventional purple grape juice	ND	ND	ND	ND	ND	ND	ND	2.06***	Seeram *et al.*, 2008
Vitis vinifera	ND	Conventional white grape juice	ND	ND	ND	ND	ND	ND	ND	327**	Frankel *et al.*, 1998
Vitis labrusca	Concord	Conventional purple grape juice	ND	ND	ND	ND	ND	ND	ND	1742**	Frankel *et al.*, 1998
Vitis labrusca	Concord	Conventional Purple grape juice concentrate	ND	ND	ND	ND	ND	ND	ND	977***	Singletary *et al.*, 2003

ND = not determined; *in mg catechin/mL; **mg of galic acid/L; ***mg of galic acid/100 mL

Although non-flavonoids contents, especially resveratrol, are well-known in wines (Fuleki & Ricardo-da-Silva, 2003) there are very few studies about the content of these compounds in grape juices (Table 1), opening an interesting possibility of new studies about this issue.

Phenolic content of different varieties of grape juices, most of them originated from *Vitis labrusca* grapes, are shown in Table 1. It is possible to notice that polyphenol content can be modify according to the variety and the elaboration process of the juices.

GRAPE JUICE AND ITS HEALTH BENEFITS

It has been already reported that grape juice can prevent: (i) platelet aggregation, (ii) LDL oxidation and oxidative damage to DNA, (iii) coronary disease and atherosclerosis (Table 2). The most studied biological effect of the grapes juices is their antioxidant activity, which can be observed in *in vitro, ex vivo* and *in vivo* assays. In *in vitro* and *ex vivo* assays, purple grape juices, mainly the organic ones, showed a better antioxidant activity, which is positively correlated to resveratrol, catechin, and total phenolic contents (Dani *et al.*, 2007; Ferguson, 2001). On the other hand, in *in vivo* assays (using the *Saccharomyces cerevisiae* yeast model), white juices present a better protection activity against damages generated by hydrogen peroxide. Among the purples grape juices, the organic ones showed a better antioxidant activity, which is positively correlated to resveratrol content (unpublished data from our group).

The disparities related to results obtained through *in vitro* and *in vivo* assays could be attributed, at least, in part, to phenols metabolism. *In vivo* antioxidant effects depend on polyphenols bioavailabity and metabolism (Vinson *et al.*, 2004), which can be influenced by their structure, absorption and interaction with other compounds (Manach *et al.*, 2004).

Oxidative stress is considered as a major risk factor that contributes to age-related increase in lipid peroxidation and declined antioxidants in the central nervous system during aging (Balu *et al.*, 2005). Several reports have shown that long term polyphenols supplementation improves cognitive performance in old Wistar rats, mainly because the capacity to these polyphenol in preventing the oxidative stress damage (Joseph *et al.*, 1999; Bastianetto & Quirion, 2002).

The results from a study of Barbara Shukitt-Hale *et al.* (2006), which evaluates the effects of Concord grape juice on cognitive and motor deficits in aging, suggest that it may take a higher concentration of grape juice to enhance motor performance, whereas lower concentration may be sufficient to alter cognitive performance. A study with striatum and *substantia nigra* isolated from adult Wistar

Table 2. Beneficial effects related for different grape juices

Species of cultivar	Variety	Main characteristic	Biological activity	Reference(s)
Vitis labrusca	Bordo	Convetional purple grape juice	Antioxidant activity	Dani *et al.*, 2007; Dani *et al.*, 2008a; Dani *et al.*, 2008b
Vitis labrusca	Niagara	Conventional white grape juice	Antioxidant activity	Dani *et al.*, 2007
Vitis labrusca	Bordo	Organic purple grape juice	Antioxidant activity	Dani *et al.*, 2007; Dani *et al.*, 2008a; Dani *et al.*, 2008b
Vitis labrusca	Niagara	Organic white grape juice	Antioxidant activity	Dani *et al.*, 2007
Vitis vinifera	ND	Conventional purple grape juice	Antioxidant activity	Sanchez-Moreno *et al.*, 1999
Vitis vinifera	*ND*	Conventional purple grape juice	Antioxidant activity	Day *et al.*, 1997
Vitis labrusca	Concord	Conventional purple grape juice	Antioxidant activity	Osman *et al.*, 1998; Freedman *et al.*, 2001; O'Byrne *et al.*, 2002; Dávalos *et al.*, 2005; Wang *et al.*, 1996
Vitis labrusca	Bordo and Niagara	Conventional purple and white, respectively grape juice	Antioxidant activity	Dani *et al.*, 2007; Fuhrman *et al.*, 1995; Carbonaro *et al.*, 2002
Vitis labrusca	Concord	Conventional purple grape juice	Inhibition of platelet aggregation	Keevil *et al.*, 2000; Osman *et al.*, 1998; Demrow *et al.*, 1995

Table 2. *Contd.*

Species of cultivar	Variety	Main characteristic	Biological activity	Reference(s)
Vitis labrusca	Concord	Conventional purple grape juice	Antioxidant activity	Abu-Amsha *et al.*, 1996; Durak *et al.*, 1999; Frankel *et al.*, 1998; Stein *et al.*, 1999
Vitis vinifera	ND	Conventional purple grape juice	Antioxidant activity	Day *et al.*, 1997
Vitis labrusca	Concord	Conventional purple grape juice	Inhibiton of atherosclerosis	Vinson *et al.*, 2001
Vitis labrusca	Concord	Conventional purple grape juice	Antithrombotic and vasodilatory activities	Folts *et al.*, 2002
Vitis labrusca	Concord	Conventional purple grape juice	Antithrombotic activity	Demrow *et al.*, 1995
Vitis labrusca	Concord	Conventional purple grape juice	Antiinflammatory activity	Albers *et al.*, 2004
Vitis vinifera	*ND*	Conventional purple grape juice	Vasodilatory activity	Takahara *et al.*, 2005
Vitis labrusca	Concord	Conventional purple grape juice	Vasodilatory activity	Chou *et al.*, 2001; Stein *et al.*, 1999
Vitis labrusca	Concord	Conventional purple grape juice	Benefits on cognitive and motor deficits in aging	Barbara Shukitt-Hale *et al.*, 2006; Park *et al.*, 2003
Vitis labrusca	Concord	Conventional purple grape juice	Antitumoral activity	Park *et al.*, 2003
Vitis labrusca	Concord	Conventional purple grape juice	Antitumoral activity	Singletary *et al.*, 2003

rats was the pioneer to show that purple grape juices can reduce oxidative stress in brain structures (Dani *et al.*, 2008b). This result is corroborated by Balu *et al.* (2005), whom found normal levels of lipid peroxidation and antioxidant defenses in grape seed extract-supplemented aged rats.

Additionally, some studies showed that the intake of approximately 125-480 mL/day of conventional purple grape juice elaborated from *Vitis vinifera* grapes is able to increase antioxidant levels in men (Day *et al.*, 1997; Osman *et al.*, 1998; Freedman *et al.*, 2001; O'Byrne *et al.*, 2002). This demonstrates that the diet is one defense strategy to prevent, intercept, or repair age-induced oxidative stress. In fact, all kind of fruit intake is associated with a lowered risk of degenerative disease, whereas the lack of adequate consumption of fruits and vegetables is linked to cancer incidence (Ames *et al.*, 1993).

It is also attributed to the grape juice the decrease of cardiovascular diseases (Vinson *et al.*, 2001; Singletary *et al.*, 2003; Sanchez-Moreno *et al.*, 1999). Platelet aggregation can be reduced by the intake (5-7.5 mL/kg/ day) of grape juice for one week, which is not observed for orange or grapefruit juices intake (Keevil *et al.*, 1999). Purple grape juice presents a concentration of total polyphenol three times higher than citric juices, which indicates the potential effect of polyphneols on platelet aggregation. This effect could consequently reduce thrombosis coronary and myocardium infarct risks (Keevin *et al.*, 2000). Intake of purple grape juice improves, also, endothelium function in patients with atherosclerotic vascular disease (Chou *et al.*, 2001).

Inhibition of chemically induced rat mammary tumorigenesis was observed for Concord grape juice constituents, suggesting a potential breast cancer prevention (Singletary *et al.*, 2003). Park *et al.* (2003) showed that grape juice consumption result in a pronounced reduction in the levels of DNA damages, when compared to the pre-supplementation level. Additionally, both purple and white grape juices showed antimutagenic activity in *Saccharomyces cerevisiae* yeast, which is positive correlated with the phenolic content of the juices. In fact, polyphenol antimutagenic activity (flavonoids or non-flavonoids) has been already reported in literature (Ferguson, 2001).

Briefly, this review shows that grape juices, both purple and white, are rich in several bioactive compounds, which are able to decrease oxidative stress damages and also assisting in checking many important diseases such as cancer, coronary heart disease and neurological diseases. Besides, grape juice is a non-alcoholic beverage, which can be included in children and elder peoples diets, when the alcoholic beverages is not prescribed.

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Nutraceutical Potential of Commonly Consumed Fruits and Vegetables

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ABSTRACT

The prevalence of non communicable diseases has shown profound increase due to the oxidative stress and rapid changes in diet and lifestyle. Due to this, the focus of food science has been shifted to maximization of both life expectancy and quality by identifying food ingredients that improve the capacity to resist disease and enhance health. Recent studies have emerged with the health attributes for fruits and vegetables with their antioxidant action and have been implicated to offer protective role in majority of oxidative stress related disorders. There are also reports on use of fruits and vegetables for improving the micronutrient status, methods for assessment of nutraceutical potentials and the linkages of the intakes of fruits and vegetables with health and non communicable diseases The active principles may be various carotenoids, flavonoids, vitamins, trace metals, polyphenols, enzyme inhibitors, organic acids etc. Literature on the active principles having antioxidant, anticarcinogen, hepato-protective, antidiabetic, cardio-protective activity for commonly consumed fruits and vegetables has been reviewed.

Key words : Fruits, vegetables, nutraceutical potential, oxidative stress

INTRODUCTION

The prevalence of non communicable diseases has shown profound increase due to the oxidative stress and rapid changes in diet and

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lifestyle. Due to this, the focus of food science has been shifted to maximization of both life expectancy and quality by identifying food ingredients that improve the capacity to resist disease and enhance health. In comparison to herbs, the medicinal values of fruits and vegetables might be moderate but the fact remains that these are consumed routinely, can be safely brought into practice for those having lower consumption, can be identified by a common man *i.e.*, do not need expertise for their identification and are easily available in the market. The active principles may have antioxidant, anticarcinogen, hepatoprotective, antidiabetic, cardioprotective, prebiotic, antibacterial activity. Recent studies have emerged with the health attributes for fruits and vegetables with their antioxidant action and have been implicated to offer protective role in majority of health disorders which are now considered to be primarily related to oxidative stress.

The poor intake of fruits and vegetables with nutraceutical potential may ultimately result in inefficient protection of human body from events that lead to health disorders. The documented data on various aspects of nutraceutical potential for the fruits and vegetables of Indian origin is limited. A review of majority of reports covering 1992-2008 for commonly consumed fruits and vegetables has been presented.

ANTIOXIDANT POTENTIAL OF FRUITS AND VEGETABLES

The antioxidant properties of fruits and vegetables could be attributed to carotenoids, flavonoids, vitamins, trace metals and polyphenols. Plant derived antioxidants function as oxygen quenchers, free radical scavengers, peroxide decomposers and enzyme inhibitors. The intake of antioxidant compounds present in food are now considered as important as vitamins for health promotion and protection against the damage due to oxidative stress related disorders such as cataract, besides improving the overall antioxidant status. The levels of antioxidants in fruits and vegetables vary depending upon the type and agro-climatic conditions. The growing interest in the substitution of synthetic food antioxidants by natural antioxidants and in the health implications of antioxidants as nutraceuticals has fostered research on vegetable sources and the screening of raw materials for identifying antioxidants. Plant and plant products have been used as a source of medicine for a long time. Among the more important constituents of edible plant products, low molecular weight antioxidants are the most important species. It is known that consumption of fruits and vegetables is essential for normal health of human beings.

Green leafy vegetables (GLV) offer a rich and inexpensive source of many micronutrients and phytochemicals with antioxidant

properties. The potential of GLV in cooked and uncooked forms was assessed. There was a large variability in all the 3 antioxidant indices *viz.* for inhibition of lipid peroxidation (ITBARS), superoxide scavenging activity (SOSA) and ferrous ion chelation activity (FICA). Leaves of coriander, colocasia (green variety) and drumstick showed high values. Differences between cooked and uncooked values were highly significant for all the 3 indices (Tarwadi & Agte, 2003).

Commonly consumed 12 fruit-vegetables and 15 root-vegetables of the Indian subcontinent in cooked and uncooked states were assessed for antioxidant and micronutrient potential. There were significant cooking losses for all the 3 antioxidant indices. Levels of ascorbic acid in cooked fruit vegetables and root vegetables were high (61.9 & 31.3% of RDA). Root vegetables showed higher levels of zinc, selenium and polyphenols. Popular fruits and vegetables such as guava, spinach, bitter gourd, yam, ginger, beet root as also the less common ones like bael (*Aegle marmelos*), kokum (*Garcinia indica*) and mango ginger (*Curcuma ameda*) showed potential to combat stress *in vitro* (Tarwadi & Agte, 2005, 2007).

In another study, 14 grape hybrids, 7 marketed varieties *i.e.*, *Thompson seedless, Sonaka, Kishmish chorni, Malaga, Catauba, Concord, Large White*, 2 raisin types and 5 juice samples were analysed for antioxidant and micronutrient quality parameters. Purple hybrids and marketed types showed promising ITBARS and SOSA. FICA was highest in market whites and lowest in purple hybrid types. Juice samples showed highest values for SOSA. Raisins showed highest content of polyphenols. The levels of micronutrients from 100 g of grapes would amount to 11% RDA for ascorbic acid, 10% RDA for riboflavin, 6% RDA for thiamine and 3-4% RDA for manganese and selenium. Grapes however seemed to be poor sources for β-carotene, iron and zinc (Agte *et al.*, 2003).

Polyphenol-rich dietary foodstuffs, consumed as an integral part of vegetables, fruits, and beverages have attracted attention due to their antioxidant and anticancer properties. Ellagic acid (EA), a polyphenolic compound widely distributed in fruits and nuts, has been reported to scavenge free radicals and inhibit lipid peroxidation. Chronic consumption of alcohol potentially results in serious illness including hepatitis, fatty liver, hypertriglyceridemia, and cirrhosis. EA exerts beneficial effects at the dosage of 60 mg/kg body wt. against alcohol-induced damage, and it can be used as a potential drug for the treatment of alcohol-abuse ailments in the near future (Devipriya *et al.*, 2008).

The aerial parts of *Coriandrum sativum, Spinacia oleracea, Trigonella corniculata* and *Trigonella foenum-graecum* when studied

for their nutritional composition, antioxidant and free radical scavenging activities, showed lower inhibitory concentration values (4.1-7.9 mg/mL, efficiency concentration values (178-321 mg/mg DPPH) and higher values of anti-radical power (0.31-0.51) as compared with their seeds. The leaves of *C. sativum* were found with good amounts of caffeic acid, ferulic acid, gallic acid and chlorogenic acid (Bajpai *et al.,* 2005).

The total antioxidant activity of selected natural food materials by an *in vitro* method involving the measurement of oxidation of linoleic acid by fluorimetry was evaluated. Pomegranate peel gave the maximum antioxidant activity due to the presence of its high polyphenolic content. At a concentration of 60 ppm, pomegranate peel powder reduced lipid peroxidation by 65% in an *in vitro* assay (Kelawala & Ananthanarayan, 2004). Ferulic acid (FA) is a phytochemical commonly found in fruits and vegetables such as tomatoes, sweet corn and rice bran. It arises from metabolism of phenylalanine and tyrosine by Shikimate pathway in plants. It exhibits a wide range of therapeutic effects against various diseases like cancer, diabetes, cardiovascular and neurodegenerative disorders. A wide spectrum of beneficial activity for human health has been advocated for this phenolic compound, at least in part, because of its strong antioxidant activity. FA, a phenolic compound is a strong membrane antioxidant and known to positively affect human health. FA is an effective scavenger of free radicals and it has been approved in certain countries as food additive to prevent lipid peroxidation. It effectively scavenges superoxide anion radical and inhibits the lipid peroxidation. It possesses antioxidant property by virtue of its phenolic hydroxyl group in its structure. The hydroxy and phenoxy groups of FA donate electrons to quench the free radicals. The phenolic radical in turn forms a quinone methide intermediate, which is excreted via the bile (Srinivasan *et al.,* 2007).

CHEMOPREVENTION AGAINST ENVIRONMENTAL TOXICANTS

Chemoprevention has emerged as a very effective preventive measure against carcinogenesis. Fruits and vegetables contain a variety of ingredients that exhibit a natural strategy for chemopreventive effects against an array of xenobiotics. Several bioactive compounds present in fruits and vegetables have revealed their cancer curative potential on benzo(a)pyrene (B(a)P) induced carcinogenesis.

Ionizing radiation is known to induce oxidative stress through generation of reactive oxygen species (ROS) resulting in imbalance of the pro-oxidant and antioxidant activities ultimately resulting in cell death. Ferulic acid (FA) helps in protecting the hepatocytes against γ-radiation induced cellular damage and can be developed as

a effective radioprotector during radiotherapy (Srinivasan *et al.,* 2006). The antigenotoxic potential of lupeol, a triterpene, and mango pulp extract (MPE) was evaluated in Swiss albino mice. Benzo[a]pyrene (B[a]P), a well-known mutagen, was given at a single dose of 100 mg/kg body weight intraperitoneally, a significant decrease in B[a]P-induced clastogenicity was recorded in supplemented groups. The incidence of aberrant cells and micronuclei was found to be reduced by both lupeol and MPE when compared to the B[a]P-treated group. The anti-cytotoxic effects of lupeol or MPE were also evident, as observed by significant increase in mitotic index (Prasad *et al.,* 2008).

The efficacy of quercetin on the level of lipid peroxides, activities of antioxidant enzymes and tumor marker enzymes in B(a)P induced experimental lung carcinogenesis in Swiss albino mice was assessed. In lung cancer bearing animals, there was an increase in lung weight, lipid peroxidation and marker enzymes such as aryl hydrocarbon hydroxylase, gamma glutamyl transpeptidase, 5'-nucleotidase, lactate dehydrogenase and adenosine deaminase with subsequent decrease in body weight and antioxidant enzymes-superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase, glutathione reductase, reduced glutathione, vitamin E and vitamin C. Quercetin supplementation (25 mg/kg body weight) attenuated all these alterations, which indicates the anticancer effect that was further confirmed by histopathological analysis. Overall, the above data show that the anticancer effect of quercetin is more pronounced when used as an chemopreventive agent rather than as a chemotherapeutic agent against B(a)P induced lung carcinogenesis (Kamaraj *et al.,* 2007). Administration of quercetin, a common polyphenolic component of many vascular and edible plants including vegetables, fruits and tea significantly reduced the tumor volume in rats induced for mammary carcinoma using dimethyl benz (a) anthracene (DMBA) (Devipriya *et al.,* 2006).

Apigenin, a bioflavonoid, is abundantly present in fruits and vegetables and possesses potential chemopreventive properties against a wide variety of chronic diseases. The anti-genotoxic effects of apigenin against a known genotoxicant, benzo(a)pyrene (B(a)P) (125 mg kg(-1) orally) toxicity in Swiss albino mice were studied. B(a)P administration led to induction of cytochrome P-450 (CYP), aryl hydrocarbon hydroxylase (AHH) and DNA strand breaks ($p<0.001$), which was suppressed by apigenin (2.5 and 5 mg kg(-1) orally) dose dependently ($p<0.001$) restored the level of reduced glutathione (GSH), quinone reductase (QR) and glutathione-S-transferase (GST) (Khan *et al.,* 2006).

ANTICANCER ACTIVITY

Cancer continues to be a major health problem despite advances in medical technology for its diagnosis and treatment. Hence prevention strategies are needed to decrease the burden of the disease. Of all the environmental factors, dietary components appear to play an important role in the initiation/progression of the disease. Nutrients and non-nutrients in the diet can influence the carcinogenic process at various stages, from initiation to overt manifestation. The National Institute of Nutrition has conducted studies on several aspects of diet-cancer inter-relationships. These include studies on metabolic susceptibility, case-control approach to determine the risk factors and intervention studies to determine the role of nutrients and non-nutrient components on preneoplastic events.

Extensive work has been carried out demonstrating the antimutagenic/anticarcinogenic potential of some commonly consumed spices and vegetables such as turmeric, mustard, green leafy and allium species of vegetables. Dietary intervention for cancer prevention is needed to control the disease besides avoiding risk factors such as smoking and alcoholism and exposure to genotoxicants. Public education and awareness about the beneficial effects of consuming a healthy diet including plenty of fresh vegetables and fruits with spices such as turmeric in adequate amounts to prevent cancer are required (Krishnaswamy & Polasa, 1995).

Diet has been implicated in prostate cancer risk and there is evidence of risk reduction with a healthy diet. To examine whether a low fat diet rich in fruits and vegetables can reduce the risk of developing prostate cancer, microscopically proved cases of prostate cancer (n=594) and the controls were investigated for consumption of oil/fat, fruits and vegetable and other probable confounding factors. Controlling for age and probable confounding factors, a statistically significant protective effect for prostate cancer was observed for those who consumed fruits and vegetables 2 to 3 kg (OR 0.5, 95% CI 0.3-0.8) and more than 3 kg (OR 0.4, 95% CI 0.3-0.6) per week compared to those who consumed less than 2 kg per week. The linear trend for the protective effect was highly significant with increase in the consumption of fruits and vegetables ($p = 0.001$) (Sunny, 2005).

Although tobacco is the primary etiologic factor for oral precancerous lesions in India, evidence from other sources indicates that diet may modify risk. One case-control study was designed to minimize a variety of biases in its attempt to investigate the relation between diet and oral precancerous lesions. After controlling for tobacco use, intake of fruits, vegetables, and β-carotene evidenced inverse trends in risk ($p<0.05$), with an average reduction of over

10% per quartile of exposure. Associations with certain micronutrients appeared to differ according to gender, with an apparent 20% reduction in risk per mg of zinc consumed per day among men and the suggestion of an increased risk among those women in the lowest quartile of iron intake (an increase of approximately 2.5-fold) and ascorbic acid intake (an increase of approximately 70% increase) compared with other women ($p<0.10$). Consumption of vegetables, fruits, and several micronutrients may inhibit precancerous lesions of the oral cavity (Gupta *et al.,* 1999).

The diets of 158 tobacco/betel quid-chewing women diagnosed with oral premalignant lesions and 155 quid-chewing but lesion-free controls, frequency matched for age, tobacco/betel habits, and socioeconomic status, were assessed using a food frequency survey. Index scores generated from the food frequency survey indicated that the mean levels of consumption for foods of animal origin ($p<0.001$), total vegetables and fruit ($p = 0.001$), vegetables alone ($p = 0.006$), fruits alone ($p = 0.006$), and green leafy vegetables ($p = 0.015$) were significantly lower in cases than in controls (Carley *et al.,* 1994).

Cancer of the gallbladder is rare but fatal, and has an unusual geographic and demographic distribution. Gallstones and obesity have been suggested as possible risk factors and diet is known to influence both these factors. A significant reduction in odds ratio was seen with the consumption of radish (OR 0.4; 95% CI 0.17-0.94), green chilli (OR 0.45; 95% CI 0.21-0.94) and sweet potato (OR 0.33; 95% CI 0.13-0.83) among vegetables, and mango (OR 0.4; 95% CI 0.16-0.99), orange (OR; 0.45; 95% CI 0.22-0.93), melon (OR 0.3; 95% CI 0.14-0.64) and papaya (OR 0.44; 95% 0.2-0.64) among fruits. A reduction in odds was also seen with the consumption of cruciferous vegetables, beans, onion and turnip, however the difference was not statistically significant. On the other hand, an increase in the odds was observed with consumption of capsicum (OR 2.2), beef (OR 2.58), tea (OR 1.98), red chilli (OR 1.29) and mutton (OR 1.2), however the difference was statistically not significant. In conclusion, the results show a protective effect of vegetables and fruits on gallbladder carcinogenesis, but red meat (beef and mutton) was found to be associated with increased risk of gallbladder cancer (Pandey & Shukla, 2002).

The effects of different intake levels of vegetables and fruit (VF) on some cancer-relevant biomarkers such as DNA damage and oxidative stress were investigated. In a randomized controlled trial, 64 nonsmoking male subjects were asked to consume a diet with 2 servings of VF/day for 4 wk. Then subjects were randomly assigned to 1 of 3 groups with either a low (2 servings/day), medium (5 servings/day), or high (8 servings/day) intake level of VF for another

4 wk. At the end of study, the plasma lutein, zeaxanthin, alpha-carotene, and β-carotene but not cryptoxanthin and lycopene concentrations were significantly higher in subjects consuming 8 servings/day than in those receiving 2 servings/day. Different levels of VF consumption and plasma carotenoid concentrations did not result in differences in the levels of endogenous DNA strand breaks, oxidative DNA damage, antigenotoxic capacity of lymphocytes, plasma markers for lipid peroxidation (malondialdehyde, 8-iso-prostaglandin-F2α) and antioxidant capacity [trolox-equivalent antioxidant capacity assay]. Thus, although consumption of 8 servings vs 2 servings/day of VF for 4 wk significantly increased the carotenoid level in plasma, there were no differences in DNA damage, lipid peroxidation, and antioxidant capacity markers among healthy, well-nourished, nonsmoking men (Briviba *et al.,* 2008).

Among the many carotenoids present in nature, lycopene has been of special interest and has received attention in recent times due to its suggestive association in reducing risk for cancer at many sites including breast, prostate and pancreas. Several studies have attempted to determine the bioactive levels of this carotenoid in human tissues and the influence of plant food and cancer on carotenoid levels. Experimental studies have also implicated the protective role of lycopene during carcinogenesis. These observations should justify further exploration and evaluation of the biological function of lycopene alone or in combination with other chemical compounds present in tomato fruit for their use in cancer prevention (Sengupta & Das, 1999). Vital ingredients used in Indian cooking include garlic. Following garlic treatment, significant inhibition of cell proliferation and induction of apoptosis, as well as suppression of cyclooxygenase-2 activity were observed, associated with significant reduction in the incidence of aberrant crypt foci. The study points to combined protective effects of garlic components on colon carcinogenesis (Sengupta *et al.,* 2004).

The polyphenolic antioxidants, consumed as an integral part of vegetables, fruits and beverages, are suggested as possessing anticarcinogenic properties. The anticarcinogenic potential of plant polyphenols ellagic acid (EA) and quercetin against N-nitrosodiethylamine-induced lung tumorigenesis was investigated in mice model. Ellagic acid was able to significantly reduce tumor incidence to 20% from the control value of 72.2%. Quercetin (QR) caused the tumour incidence to decrease from 76.4% to 44.4% when fed until the third dose of carcinogen. Both of the polyphenols suppressed the tumour incidence mainly by acting at the initiation phase of the carcinogenesis. Besides this, ellagic acid was found to be a better chemopreventor than quercetin. Ellagic acid was found

to be more effective in decreasing the lipid peroxidation and increasing the GSH. This may be one of the reasons for its observed better anticarcinogenic property as compared to quercetin (Khanduja *et al.,* 1999).

Colon cancer is the second most common cancer among men and women worldwide. The effect of red chilli (*Capsicum annum* L.), cumin (*Cuminum cyminum* L.), and black pepper (*Piper nigrum* L.) on colon cancer induced in rats by a colon-specific carcinogen, 1,2-dimethylhydrazine (DMH) was investigated. Chilli supplementation promotes colon carcinogenesis, whereas cumin or black pepper suppresses colon carcinogensis in the presence of the procarcinogen DMH (Nalini *et al.,* 2006). The anticarcinogenic properties of some commonly consumed spices and leafy vegetables were investigated.

Although the incidence rate of colorectal cancer is very low, and rectal cancer remains more common in India, a significant increase in its incidence has been reported for both men and women over the last 2 decades. The MTHFR genetic susceptibility and common environmental risk factors were evaluated in the development of colon and rectal cancer, and assessed the interactions between gene and environmental factors with colorectal cancer in 59 colon cancer cases, 243 rectal cancer cases and 291 controls. High intake of nonfried vegetables or fruits was inversely associated with both colon and rectal cancer risk. Especially, the combination of a high intake of nonfried vegetables and MTHFR 1298CC genotype was associated with the lowest rectal cancer risk (OR = 0.22, 95% CI 0.09-0.52) (Wang *et al.,* 2006).

Cancer of the larynx is fourteenth most common cancer in the world. Limited data are available from India on associations with risk factors. Three hundred and five laryngeal cancer patients and an equal number of healthy controls matched for their age within 2 years, sex and place of residence constituted the study population. In the univariate analysis a lower consumption of roots and tubers green leaf vegetable other vegetables and fruits, and higher consumption of milk, eggs, meat, tea, alcohol, smoking, consumption of betel leaf with tobacco as well as a preference for spicy and fried foods emerged as significant positive variables. After adjusting for education, years of use of alcohol, smoking, chewing of betel leaf with tobacco in the model, low green leafy vegetables and preference for spicy foods were found to be positively related to the risk of laryngeal cancer (Kapil *et al.,* 2005).

Gallbladder cancer (GBC) is the prominent malignancy of hepato-biliary tract, and epidemiological studies world wide have implicated dietary factors in the development of gallbladder cancer. The

nutritional preventive effect against GBC could be attributed to high content of vitamins, carotenes and fibers (Rai *et al.,* 2004).

Calcium glucarate (Cag), Ca salt of D-glucaric acid is a naturally occurring non-toxic compound present in fruits, vegetables and seeds of some plants, and suppress tumor growth in different models. Topical application of Cag suppressed mouse skin tumor development (Singh & Gupta, 2003).

The incidence rates of most digestive cancers in India are moderate or low. The highest rates are recorded in the urban population of Mumbai and the lowest in the rural population of Barshi in Maharashtra state. The rates will rise as the life expectancy of Indians increases along with urbanization and, within the next few decades, may reach those recorded in Indians living abroad. Indians should be encouraged to retain their traditional protective diets, eat more fruits and vegetables, do more physical activity, and abstain from tobacco. Gastroenterologists can also help in secondary prevention by screening high-risk individuals, *e.g.*, patients with chronic liver disease for liver cancer and relatives of patients with familial bowel cancer (Mohandas & Jagannath, 2000) .

Two-hundred and six breast cancer cases were histologically confirmed breast cancer diagnoses at the Cancer Institute in Chennai (Madras), India. One-hundred and fifty hospital controls were patients who had cancer at any site other than breast and gynecological organs, and 61 healthy controls were persons accompanying patients in the Cancer Institute. Serum levels of carotenoids such as β-carotene, lycopene, cryptoxanthin, and zeaxanthin & lutein were determined by HPLC. Serum levels of total carotenes and total carotenoids including β-carotene, which reflects food intake of colored vegetables and fruits and has a protective role for certain sites of cancer, were significantly lower among breast cancer cases and hospital controls compared to healthy controls, especially in post-menopausal women (Ito *et al.,* 1999).

HEPATOPROTECTIVE ACTIVITY

The different fractions of alcoholic extract and one phenolic compound AB-IV of seeds of *Cichorium intybus* Linn were screened for antihepatotoxic activity on carbon tetrachloride (CCl(4))-induced liver damage in albino rats. The methanol fraction and compound AB-IV were found to possess a potent antihepatotoxic activity comparable to the standard drug Silymarin (Silybon-70) (Ahmed *et al.,* 2003).

The effects of feeding the plant products on the induction of squamous cell carcinomas in the stomachs of Swiss mice by feeding

benzo[a]pyrene(B[a]P) and on the induction of hepatomas in Wistar rats by feeding 3'-methyl-4-dimethylaminoazobenzene (3'MeDAB) were investigated. Among the nine plant products tested, cumin seeds (*Cuminum cyminum* Linn) and basil leaves (*Ocimum sanctum* Linn) significantly decreased the incidence of both B[a]P-induced neoplasia and 3'MeDAB-induced hepatomas. Poppy seeds (*Papaver somniferum* Linn) significantly inhibited B[a]P-induced neoplasia alone, while the other plant products, asafoetida, kandathipili, turmeric, drumstick leaves, solanum leaves and alternanthera leaves were ineffective. These results suggest that cumin seeds, basil leaves and to a lesser extent poppy seeds, which are all widely used in Indian cooking, may prove to be valuable anticarcinogenic agents (Aruna & Sivaramakrishnan, 1992).

Linkages of the Consumption of Fruits and Vegetables with Health Disorders

As part of a joint Indian Council of Medical Research/WHO initiative, survey was done on 1260 men and 1304 women 15-64 years of age living in urban slum in Faridabad district, Haryana. The mean number of servings per day of fruits and vegetables was 2.7 for men compared with 2.2 for women. Overall, only 7.9% and 5.4% of men and women, respectively took > or = 5 servings per day of fruits and vegetables (Anand *et al.,* 2007).

To examine the relationship between fruit and vegetable intake (g/d) and CVD risk factors in 983 urban south Indians of Chennai, fruit and vegetable intake (g/d) were measured using a validated semi-quantitative FFQ. The data revealed that after adjusting for potential confounders such as age, sex, smoking, alcohol, BMI and total energy intake, the highest quartile of fruit and vegetable intake (g/d) showed a significant inverse association with systolic blood pressure (beta = - 2.6 (95% CI - 5.92, - 1.02) mmHg; p= 0.027). A higher intake of fruit and vegetables explained 48% of the protective effect against CVD risk factors. Increased intake of fruits and vegetables could play a protective role against CVD in Asian Indians who have high rates of premature coronary artery disease (Radhika *et al.,* 2008).

Habitual food and nutrient intakes of 140 Indian cataract patients and 100 age- and sex-matched controls (50-75 years), from high income group and low income groups, were assessed by food frequency questionnaire and data were examined for linkages with blood/lens parameters of oxidative stress through a case-control study. Plasma levels of oxidative stress, antioxidant enzymes and antioxidant micronutrients were also assessed. Intake of animal foods and fried

snacks was significantly higher while vegetables, green leafy vegetables, fruit, tea and micronutrient intakes were lower in patients than controls ($p<0.001$). Lens oxidative stress and opacity showed a significant negative association with fruit intake ($p<0.05$). Multiple regression analysis indicated association of intakes of iron, β-carotene, ascorbic acid, tannic acid and inositol pentaphosphate with plasma oxidative stress ($p<0.01$) and association of intakes of iron, ascorbic acid and inositol triphosphate with lens oxidative stress ($p<0.01$). Weighted least square regression for lens opacity revealed that intakes of ascorbic acid, folic acid and inositol pentaphosphate explain to 59.7% of the total variation ($p<0.01$). Dietary deficiency of antioxidant micronutrients was greater for patients than controls. Deficiency of β-carotene, ascorbic acid, folic acid, iron, phytate and polyphenols increased oxidative stress in blood and lens (Tarwadi & Agte, 2004; Tarwadi *et al.,* 2008).

Dietary patterns of 232 men and 223 women (20-65 years) from rural, industrial and urban regions of Western India were evaluated by food frequency questionnaire along with RBCMZn, hemoglobin, ceruloplasmin, plasma zinc and SOD. A significant positive correlation was observed between intakes of green leafy vegetables, other vegetables and milk products with RBCMZn status ($p<0.05$) but not with plasma zinc ($p>0.2$). Fruit and other vegetable intake were positively correlated with SOD ($p<0.05$) (Agte *et al.,* 2005).

To examine interrelationships between (1) dietary habits, (2) socioeconomic, and (3) environmental factors, and their impact on plasma retinol and plasma ascorbic acid, a total of apparently healthy and non-anemic 214 men and 108 women (20-50 years) were examined. Logistic regression showed education, environment, green leafy vegetables (GLV) and milk intake as predictors of plasma retinol deficiency, while non-sweet fruit intake influenced plasma ascorbic acid. Subnormal status of retinol and vitamin C emphasizes the need to increase consumption of fruit, GLV and milk products, and also better education and environment. Avoiding passive smoking demands attention in order to improve levels of these vitamins (Chiplonkar *et al.,* 2002).

Supplementation study was carried out in 66 children of 10-12 years of age for a period of about 4 months. Feeding of 100 g/day of cauliflower leaves powder supplements *i.e.* biscuits and *shakarpara* improved the Hb, serum retinol, height, weight and nutritional status in deficient subjects. On the basis of blood analysis, 33 children were taken as deficient having low level of both Hb (<10 g/dl) and serum retinol (<20 microg/dl). Similar number of children (33) were selected as control purposively who had Hb>10 g/dl and serum retinol>20 microg/dl. The increase in Hb, serum retinol, weight and height in

supplemented group was 14.61, 33.27, 4.48 and 7.06%, respectively (Jood *et al.,* 2001).

Between 1996 and 1999, a study was carried out in Southern India on risk factors for oral cancer on 591 incident cases of cancer of the oral cavity (282 women) and 582 hospital controls (290 women). Frequent consumption of fish, eggs, raw green vegetables, cruciferous vegetables, carrots, pulses, apples or pears, citrus fruit, and overall consumption of vegetables and fruit decreased oral cancer risk. The risk associated with low consumption of vegetables was higher among smokers than among non-smokers (Rajkumar *et al.,* 2003).

Methods for Assessing the Efficacy and Safety of Nutraceuticals

Micronutrient contents and antioxidant capacity get affected by food processing and cooking indicating the need for data on locally consumed cooked preparations.

Erythritol (INS 968) is an important four-carbon sugar alcohol in the food industry which also occurs naturally in certain fruits, vegetables, and fermented foods. Currently, HPLC and GC methods are in use for the quantification of erythritol in natural/processed foods. However, an immunoassay for erythritol has also been developed (Sreenath & Venkatesh, 2008).

Increasing consciousness about future sustainable agriculture and hazard free food production has lead organic farming to be a globally emerging alternative farm practice. The accumulation of air-borne heavy metals in edible parts of vegetables and in cultivated soil horizon in organic farming system in a low rain fall tropical region of India. Concentrations of heavy metals in cultivated soil horizon and in edible parts of open field grown vegetables increased over time. Vegetable concentrations of heavy metal appeared in the order Zn > Pb > Cu > Ni > Cd and were maximum in leaves (spinach and amaranths) followed by fruits (tomato and egg plant) and minimum in roots (carrot and radish). Multiple regression analysis indicated that the major contribution of most heavy metals to vegetable leaves was from atmosphere. For roots however, soil appeared to be equally important (Pandey & Pandey, 2008).

To determine antioxidant phenolics and flavonoids in commonly consumed Indian foods, 85 food-stuffs comprising of cereals, pulses, nuts, oilseeds, vegetables, fruits and beverages were chemically analyzed. Total phenolics were measured biochemically and flavonoids were measured as a sum of quercetin, kaempferol, luteolin and pelargonidin. High flavonoid content (>100 mg/100 gm) was present in tea, coffee, apple, guava, terminalia bark, fenugreek seeds, mustard seeds, cinnamon, red chili powder, cloves and turmeric. Medium levels

(50-100 mg) were found in Indian gooseberry, omum, cumin, cardamom, betel leaf and brandy. Small but significant amounts were also present in food-items of large consumption such as kidney beans, soyabeans, grapes, ginger, coriander powder, bajra and brinjal (Nair *et al.,* 1998).

During recent years importance of B complex vitamins, beta-carotene and vitamin C has been realised in terms of their antioxidative and anticarcinogenic properties. Fruits and vegetables are the rich sources of these vitamins. However, there are considerable cooking losses of vitamins, and information on vitamin contents of cooked foods is essential for assessing the adequacy of vitamin intakes. Secondly, there is a growing trend to consume ready to eat foods such as stuffed pancakes (*samosa, patties*), pastries, French fries; replacing traditional foods for lunch or dinner like *roti*, vegetable curry, bread, non-vegetarian items. Ready-to-eat foods are considered to give empty calories rather than a balanced diet. A study was undertaken to estimate ascorbic acid, folic acid, riboflavin, thiamine and β-carotene of 263 cooked food samples and 260 meals. Irrespective of whether it is ready-to-eat or a lunch/dinner food item, the contribution of vegetables in the preparations was found to make a marked impact on the vitamin profile. While results justify the concept of a food pyramid, emphasis needs to be given to types of fruits and vegetables rich in vitamins; preferably in their uncooked form, rather than considering their total consumption (Agte *et al.,* 2002).

In another study, a prospective human trial was undertaken to investigate the effect of GLV as a natural fortificant of multiple micronutrients. A short term (0-4 h) response (AUC) of single dose of 7.9 mg β-carotene and 130 mg ascorbic acid (through a spinach carrot meal) against a standard meal without GLV plus 10 mg β-carotene and 150 mg ascorbic acid tablets was studied in 2 groups with 4 volunteers each. In a second trial of 3 weeks supplementation, 5 groups of young adults (n=40) were given either 100 g GLV/day alone or with tablets of vitamin E (100 mg/day) or vitamin C (100 mg/day) or more oil (5 g/day) or non–GLV meal with tablet of β-carotene (10 mg/day). Three week supplementation of GLV with more oil significantly increased plasma β-carotene (51%) and haemoglobin (9%). GLV with vitamin E increased plasma β-carotene (40%), haemoglobin (8%) and plasma vitamin C (6%). Supplementing β-carotene without GLV significantly increased haemoglobin (11%), plasma zinc (14%) in addition to β-carotene (Agte *et al.,* 2005).

HORMONAL EFFECTS

The combined effects of *Trigonella foenum-graecum* and *Allium sativum* extracts were evaluated for their ameliorative potential in

the L-thyroxine-induced hyperthyroidic rat model to contribute to an understanding of interaction between the two extracts. The findings reveal *Trigonella foenum-graecum* and *Allium sativum* extracts may be used individually and not together in the regulation of hyperthyroidism (Tahiliani & Kar, 2003).

ANTIDIABETIC EFFECTS

Vegetables are among the numerous plant adjuncts tried for the treatment of diabetes mellitus. A few vegetables that are commonly consumed in India have been claimed to possess antidiabetic potency. In recent years, there has been a renewed interest to screen such plant food materials, for a possible beneficial use. Considerable amount of work has been carried out in this regard with bitter gourd (*Momordica charantia*) and ivy gourd (*Coccinia indica*) both in experimental animals and human diabetic subjects. Majority of these studies have documented the beneficial effect of the fruit of bitter gourd and leaf of ivy gourd when administered orally as a single dose. The hypoglycaemic influence is claimed to be mediated through an insulin secretagogue effect or through an influence on enzymes involved in glucose metabolism. The limited number of studies on other vegetables such as cabbage (*Brassica oleracia*), green leafy vegetables, beans and tubers has shown the beneficial hypoglycaemic influence in both experimental animals and humans. There is scope for more extensive research in this area, especially to examine the long term beneficial effect of dietary vegetables, to identify the active principle, and to understand the mechanism of action, which is at present unclear. Since diet forms the mainstay in the management of diabetes mellitus, there is scope for exploiting the antidiabetic potency of vegetables to the maximum extent. Such plant food adjuncts possessing hypoglycaemic activity appear to hold promise as potential antidiabetic agents (Platel & Srinivasan, 1997).

Although amylase inhibitors were considered as antinutritional factors earlier, in recent years these factors have proved to be having health potential in control of diabetes and obesity. Pigeon pea (*Cajanus cajan L*) seeds were analysed quantitatively for amylase inhibitor (AI) activity and qualitatively, by an in-gel-detection method on polyacrylamide gels. At least four AI isoforms were identified in pigeon pea seeds. The AIs inhibit human salivary and bovine pancreatic amylase but fail to inhibit bacterial, fungal and endogenous amylase (Giri & Kachole, 1998).

Brassica juncea (BJ; Hindi name: *Rai*) seeds and *Murraya koenigii* (MK; English names: Curry leaves) leaves, used as food ingredients and also by diabetics in India, were assessed in a fructose-mediated

non-genetic model of insulin resistance. Feeding of fructose diet containing 10% *Brassica juncea* seeds powder for 30 days significantly decreased fasting serum glucose, insulin and cholesterol levels but did not normalize them but not, a diet containing 15% *Murraya koenigii* leaves powder. Results of the study suggest that BJ can play a role in management of pre-diabetic state of insulin resistance and should be promoted for use in patients prone to diabetes (Yadav *et al.*, 2004).

Quercetin, a constituent present in fruits and vegetables, was studied in two different doses (50 and 80 mg/kg body weight) for 45 days to assess its effect on streptozotocin induced diabetes. It resulted in a decrease in the levels of blood glucose, plasma thiobarbituric acid reactive substances and hydroperoxides. Quercetin also resulted in the activities of superoxide dismutase, catalase coming to near normal, along with the levels of vitamin C and vitamin E. Quercetin at lower doses was found to be more effective. These result indicate that quercetin ameliorated the diabetes-induced changes in oxidative stress (Mahesh & Menon, 2004).

CARDIOVASCULAR EFFECTS

Cardiovascular diseases (CVD) are growing contributors to global disease burdens, with epidemics of CVD advancing across many regions of the world which are experiencing a rapid health transition. Diet and nutrition have been extensively investigated as risk factors for major cardiovascular diseases like coronary heart disease (CHD) and stroke and are also linked to other cardiovascular risk factors like diabetes, high blood pressure and obesity. Regular frequent intake of fruits and vegetables is protective against hypertension, CHD and stroke. Sufficient knowledge exists to recommend nutritional interventions, at both population and individual levels, to reduce cardiovascular risk. That knowledge should now be translated into policies which promote healthy diets and discourage unhealthy diets. This requires coordinated action at the level of governments, international organizations, civil society and responsible sections of the food industry (Srinath Reddy & Katan, 2004).

South Asians have high rates of acute myocardial infarction (AMI) at younger ages compared with individuals from other countries but the reasons for this are unclear. Standardized case-control study of 1732 cases with first AMI and 2204 controls matched by age and sex from 15 medical centers in 5 South Asian countries and 10,728 cases and 12,431 controls from other countries were recruited to the study. Protective factors were lower in South Asian controls than in controls from other countries (moderate- or high-intensity exercise, 6.1% vs 21.6%; daily intake of fruits and vegetables, 26.5% vs 45.2%; alcohol

consumption > or =once/wk, 10.7% vs 26.9%). However, some harmful factors were more common in native South Asians than in individuals from other countries (elevated apolipoprotein B (100)/apolipoprotein A-I ratio, 43.8% vs 31.8%; history of diabetes, 9.5% vs 7.2%) (Joshi *et al.,* 2007).

There is evidence that inclusion of high fiber foods such as oats, fruits and vegetables in the diet can decrease fat intake and modulate blood lipids. To test this hypothesis, 61 group A and 59 group B patients with essential hypertension were administered guava fruit preferably before meals in a foods-to-eat approach rather than foods to restrict, in a randomized and single-blind fashion for 12 weeks. There was a significant net decrease in serum total cholesterol (9.9%), triglycerides (7.7%) and blood pressures (9.0/8.0 mm Hg) with a significant net increase in high-density lipoprotein cholesterol (8.0%) after 12 weeks of guava fruit substitution in group A than in group B (Singh *et al.,* 1992).

The effects of administration of guava and papaya fruit (100 g/day), vegetables, and mustard oil (5 g/day) (group A); antioxidant vitamins C (50 mg/day) and E (30 mg/day), plus β-carotene (10 mg/day) (group B); a high-fat (5-10 g/day) (group C); or a low-fat (4-5 g/day) diet (group D) were compared over 24 diet weeks in a randomized fashion, while all groups of rabbits (five in each of four groups) received a hydrogenated fat diet (5-10 g/day) for a period of 36 weeks. After 12 weeks on the high-fat diet, each group of rabbits had an increase in blood lipoproteins. The fruit and vegetable-enriched prudent diet (group A) caused a significant decline in blood lipids at 24 and 36 weeks, whereas the lipid levels increased significantly in groups C and D. Group A also had a significant rise in vitamin E (2.1 Umol/l), C (10.5 Umol/l), A (0.66 Umol/l), and carotene (0.08 Umol/l) and a decrease in lipid peroxides (0.34 nmol/mL at 36 weeks, whereas the levels were unchanged in groups C and D. Group B rabbits had a significant and greater increase than group A in plasma vitamins E, C, A, and carotene; a rise in HDL cholesterol; and a greater decrease in lipid peroxides after 24 and 36 weeks of treatment (Singh *et al.,* 1995). Cardiovascular diseases are major causes of mortality and disease in the Indian subcontinent, causing more than 25% of deaths. It has been predicted that these diseases will increase rapidly in India and this country will be host to more than half the cases of heart disease in the world within the next 15 years. Coronary heart disease and stroke have increased in both urban and rural areas. Case-control studies indicate that tobacco use, obesity with high waist: hip ratio, high blood pressure, high LDL cholesterol, low HDL cholesterol, abnormal apolipoprotein A-1:B ratio, diabetes, low consumption of fruits and vegetables, sedentary lifestyles and psychosocial stress are

important determinants of cardiovascular diseases in India. These risk factors have increased substantially over the past 50 years and to control further escalation it is important to prevent them. National interventions such as increasing tobacco taxes, labelling unhealthy foods and trans fats, reduction of salt in processed foods and better urban design to promote physical activity may have a wide short-term impact (Gupta *et al.,* 2008).

Elevated plasma homocysteine level is a risk factor for atherosclerotic disease. Plasma homocysteine levels are influenced by genetic, physiological and lifestyle factors. Folate status is the major determinant of plasma homocysteine level and there is a strong inverse correlationship between plasma homocysteine level and serum or erythrocyte folate levels. To maintain low plasma homocysteine concentration, people should be advised to increase their consumption of pulses, eggs, green leafy vegetables and fruits which are rich in B vitamins (Krishnaswamy & Lakshmi, 2002).

A study was designed to test the efficacy of the administration of fruits and vegetables for 12 weeks as an adjunct to a prudent diet in decreasing blood lipids in 310 (intervention; group A) and 311 (control; group B) patients with risk factors of coronary artery disease (CAD) in a parallel, single-blind fashion. Fruits and vegetables decreased total cholesterol level by 6.5% and low-density lipoprotein cholesterol level by 7.3% in group A, whereas the levels were unchanged in group B (Singh *et al.,* 1992).

To test whether a fat reduced diet rich in soluble dietary fibre, antioxidant vitamins, and minerals reduces complications and mortality after acute myocardial infarction, 505 patients with suspected acute myocardial infarction were assigned to diet A (advised to eat more fruit, vegetables, nuts, and grain products, n=204) or diet B (n=202) within 24-48 h of infarction. Blood lipoprotein concentrations and body weight fell significantly in patients in group A compared with those in group B (cholesterol fell by 0.74 mmol/l in group A vs. 0.32 mmol/l in group B, 95% confidence interval of difference 0.14 to 0.70, and weight by 7.1 vs. 3.0 kg, 0.52 to 7.68). The incidence of cardiac events was significantly lower in group A than group B (50 vs. 82 patients, $p<0.001$). Group A also had lower total mortality (21 vs. 38 died, $p<0.01$) than group B (Singh *et al.,* 1992).

ANTIOXIDANT AND MICRONUTRIENT STATUS

To investigate if low micronutrient status is a predisposing factor for hypertension in traditionally lacto-vegetarian Indian population, blood micronutrient profile was assessed in 109 hypertensives (30-58 years) and 115 age sex matched normotensives. Food intakes were estimated

through food frequency questionnaire. Intake of omega-6 fatty acids were higher (p=0.08) and omega-3 fatty acids were lower in hypertensives than normotensives. Mean plasma ascorbic acid and folic acid were significantly higher (p<0.001) and ceruloplasmin and erythrocyte membrane zinc was marginally higher (p=0.07) in normal subjects. Low dietary intakes of ascorbic acid, folic acid and zinc emerged as possible risk factors for hypertension (Chiplonkar *et al.,* 2004). Status of lipid peroxidation was studied in rats induced high fat diet and some commonly used spices, *viz. Murraya koenigi* and *Brassica juncea*. The study revealed that these species alter the peroxidation (thiobarbituric acid reactive substances) level to a beneficial extent. Histological studies also focus on modulation of hepatic functions to near normal level (Khan *et al.,* 1997). Two-hundred and six breast cancer cases were histologically confirmed for breast cancer diagnoses at the Cancer Institute in Chennai (Madras), India. One-hundred and fifty hospital controls were patients who had cancer at any site other than breast and gynecological organs, and 61 healthy controls were persons accompanying patients in the Cancer Institute. Serum levels of carotenoids such as beta-carotene, lycopene, cryptoxanthin, and zeaxanthin and lutein were determined by HPLC. Serum levels of total carotenes and total carotenoids including β-carotene, which reflects food intake of colored vegetables and fruits and has a protective role for certain sites of cancer, were significantly lower among breast cancer cases and hospital controls compared to healthy controls, especially in post-menopausal women (Ito *et al.,* 1999). Apparent absorption of 8 micronutrients and degradation of phytic acid from Indian vegetarian meals were studied in human subjects who underwent ileostomy. Absorption of β-carotene, ascorbic acid, riboflavin and thiamine was 63% to 75.6%. There was a negative non significant trend in values of beta-carotene absorption with increased intake of beta-carotene (r=-0.51, p>0.1) and iron (r=-0.67, p=0.1) but a positive significant trend with riboflavin intake (r=0.84, p=0.018). Zinc, copper and iron showed a lower absorption (10%-20%). Patterns of phytic acid in the meals and output indicated partial degradation and absorption (34%) (Agte *et al.,* 2005).

SUMMARY

Fruits and vegetables are substantial contributors of carotenoids, flavonoids, vitamins, trace metals and polyphenols which attribute nutraceutical actions.

Although there are a number of reports about the claims of certain fruits and vegetables as effective strategy in health disorders such as cancer, CHD and diabetes, their potential as curative agents is

moderate and could be promising as preventive agents or as adjunct/ complimentary therapy.

The antioxidant potential of fruits and vegetables should be properly utilized and advocated by bringing more public awareness as natural agents to cope up the oxidative stress arising due to environmental pollutants and occupational health such as exposure to sunlight.

There is a need to explore under-utilized fruits and vegetables and to develop value added products using these food commodities.

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Bioactive Compounds and Functional Foods of Pseudocereals

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ABSTRACT

Food consumption trends show increased in using of non-conventional, non-cereal grains. This increase of non-cereal grains consumption exists not only because these grains commonly possess two to four times more protein than traditionally used cereal grains, but because their proteins are often of higher nutritional quality. In addition to that they contain bioactive compounds with valuable health impact. The most spread alternative grains are amaranth, buckwheat and quinoa which are referred as pseudocereals, have been shown to acquire great economic potential as well as high nutritional value. The grains of those pseudocereals can be processed into various healthy food products. In this article, the bioactive compounds of three grains and their health impact as functional foods has been reviewed.

Key words : Pseudocereals, amaranth, buckwheat, quinoa, bioactive compounds, functional food

INTRODUCTION

Plants make up 95% of the earth's food supply, with the common cereal grains such as wheat, rye, barley, rice, corn, sorghum and millet providing more than 75% of basic human protein requirements. Although it was clear that priority attention had to be given to the major cereals, other cereals and pseudocereals began to receive more attention. Consumption trends show increased use of non-

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conventional, non-cereal grains. This increased interest in non-cereal grains was achieved because these grains commonly possess two to four times more protein than traditionally used cereal grains, and their proteins are often show higher nutritional quality. Moreover, these grains found to contain good level of health promote components which play important roles in the human body.

Pseudocereals are broadleaf plants (non-grasses) that are used in much the same way as cereals (true cereals are grasses). Their seeds can be ground into flour and otherwise used as cereals. However, since they are dicotyledonous and not true cereals, the seeds are technically referred to as pseudocereals such as the edible seeds of dicots; amaranth (*Amaranthus sp.*) buckwheat (*Fagopyrum esculentum*) and quinoa (*Chenopodium quinoa*) (Kauffman, 1992).

AMARANTH

Amaranth is an under-exploited promising plant (Teutonico & Knorr, 1985) which has been cultivated as early as 4000 BC. known as "food of the god" (Pszczola, 1998). Grain amaranth types are believed to have originated from central and South America, whereas the main vegetable types are believed to have originated in South East Asia (Saunders & Becker, 1984).

The family Amaranthaceae (class dicotyledons, order Caryophyllales) is comprised of more than 60 genera and about 800 species of annual or perennial herbaceous plants. The members of genus *Amaranthus* are widely distributed throughout the world in tropical, sub-tropical and temperate regions as grain crops, potherbs, ornamentals and dye plants. Amaranth is hardy, wild, fast growing cereal-like plant with seeds yield of about 3 tons per hectare in 3-4 months (Singhal & Kulkarni, 1988). Although many species of amaranth are considered as opportunistic weeds and eventually used as food, very few species such as *A. hypochondriacus* L., *A. caudatus* L., and *A. cruentus* L., were mostly used for human consumption. They are believed to have light-coloured seeds and to possess high nutrition healthy foods.

Amaranth plant has many advantages to be widely used; its yield can reach as high as (3.3 tons/ha) under dry land conditions (Sooby *et al.,* 1998) comparing with wheat (2.54 tons/ha) and barley (2.33 tons/ha) (Dendy & Brockway, 2001). Amaranth seeds have high protein content (13 – 19%) with a high level of essential amino acids especially lysine (average 5.8 g/100 g protein) comparing to other grains like corn, rice, and wheat, which contains about 9.5, 7.5 and 12.0% protein contents and low amount of lysine (about 1.5, 3.5 and 2.0 g/100 g protein), respectively (Belitz & Grosch, 1999). It has 1.5 to 3 times

more oil content than other grains, and also has a cholesterol-lowering properties attributed to squalene, dietary fibres, tocotrienols and isoprenoid compounds (Lozoya-Gloria, 1994).

It has three times more fibres than wheat, and high level of calcium and iron as well as many other vitamins and minerals (Pszczola, 1998). It can successfully grow in adverse environmental conditions such as drought, high temperature, and saline soils, and also it is a crop with multiple uses such as food, forage, silage, green manure and animal feed (Lozoya-Gloria, 1994). The leaves appearance, texture, flavour, and overall eating quality of several *Amaranth* species are comparable with spinach. The leaves have high protein content (28-48% db.), ash content (33-40.7% db.), and fibres (11.1-23.2% db.) (Segura-Nieto *et al.*, 1994).

Bioactive Compounds of Amaranth

Dietary Fibers

From a nutritional and physiological viewpoint, soluble and insoluble polysaccharides other than starch and lignin are called dietary fibers (Belitz & Grosch, 1999). They are now recognized to have a beneficial effect on health. Their moderate digestion and absorption of other nutrients in the small intestine, provide substrate for fermentation in the colon, improve blood glucose control and lower low-density lipoprotein (LDL) cholesterol levels (Gray, 2003).

Amaranth seeds contain high level of non-fermentable fibers. The fibers are highly concentrated in the amaranth seed coat-embryo fraction (Betschart *et al.,* 1981). Gamel *et al.* (2006a) reported that total dietary fibers (TDF) represented about 14% in Amaranth seeds and *A. caudatus* had significantly higher dietary fibers content (14.03%) than that of *A. cruentus* (13.40%). The levels of total dietary fibers in pale and dark seeded varieties of *A. caudatus* reported to be 8% and 16%, respectively (Pedersen *et al.,* 1990). Soluble dietary fibers fraction (SDF) made up between 30 to 44% of the TDF in the pale seeded varieties, but it was only 18% in the black seeds. Bressani (1994) stated that the TDF content of *A. caudatus* and *A. hypochondriacus* was ranged from 7.6 to 16.4%, and the ratio of soluble to total dietary fibers was ranged from 18 to 48. Moreover, Tosi *et al.* (2001) obtained different dietary fibers levels from whole *Amaranthus cruentus* seeds with differential milling process. The TDF was 14.2% and about 40% of them were soluble dietary fibers.

Phytosterols

Phytosterols are encountered mainly in the unsaponifiable portion of plant oils and correlated with the level of serum total cholesterol

and low density lipoprotein (LDL). Amaranth sterols ranged from 0.27 to 0.32 mg/g dry weight, where spinasterol was the preponderant sterol in both weedy and vegetable amaranth, ranging from 48 to 53% of the total sterols followed by Delta 7 sigmasterol (Fernando & Bean, 1985). More than 50% of total sterols are compounds with D^7 structure.

Some varieties of amaranth are moderately good sources of tocotrienols. Amaranth seed tocols consist of 33% tocopherols, 61% β-tocotrienol, and 6% other tocotrienols while amaranth oil tocols consist of 43% tocopherol, 47% β-tocotrienol and 8% other tocotrienols (Ozer & Azzi, 2000). Tocopherols and tocotrienols (vitamin E isomers) are well-known natural antioxidants. Besides their known activity as antioxidants and free radical scavengers, they have also proved to be active against hypercholesterolemic arteriosclerosis (Ozer & Azzi, 2000). All amaranth species contain tocotrienols and squalene, which are known to affect cholesterol biosynthesis. The dietary supplementation of Amaranth seeds (*A. cruentus* and *A. hypochondriacus*) on cholesterogenesis to 6-week old female chickens caused 10-13% and 7-70% reduction in the cholesterol and low-density lipoprotein in blood serum, respectively (Qureshi *et al.,* 1996). High density lipoprotein-cholesterol and blood serum triglycerides were not affected by amaranth supplementation. The hypocholesterolemic effect of the amaranth diets may be attributed to the high tocotrienol content which has been reported to reduce the β-hydroxy-β-methylglutaryl-coenzyme A reductase (HMG Co-A reductase) activity and act as oxidized sterols reducing the cholesterol levels and also due to the high soluble fiber and high squalene contents of grain amaranth (Naber, 1983). It was reported that α-tocotrienol exerts a dose-dependent inhibition of the microsomal (HMG-CoA) reductase activity (Qureshi *et al.,* 1986) and that α-tocopherol caused an increase in its activity. Moreover, γ- and δ-tocotrienols are more potent cholesterol inhibitors than α-tocotrienol, while β-tocotrienol has a very low biological activity (Pearce *et al.,* 1992).

Squalene

Squalene, which represented 6% of amaranth oil, is isoprenoid 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene. It has higher amount than that present in other cereal grains oils (Saunders & Becker, 1984). Jahaniaval *et al.* (2000) reported that the percentage of the squalene in the triacylglycerols sample from *Amaranthus* accessions was ranged from 8.05% to 11.19%.

Han-Ping *et al.* (2002) found the concentration of squalene in four *Amaranthus* sp.; *A. cruentus, A. hypochondriacus, A. hybridus*, and

A. tricolor, ranging from 3.6% to 6.1% of total lipids. Gamel *et al.* (2007) reported 4.8 and 4.9% squalene in two amaranth species, *A. caudatus* and *A. cruentus*. They also stated that popping process reduce the squalene content by 26.5 and 14.5% respectively.

Sun *et al.* (1995) mentioned that the processing of milling, extrusion, and oil extraction of amaranth did not appear to cause any significant change in squalene content of the oil, while the bleaching and decolourizing processes increased the squalene from 6.96% to 8.01% as reported by Lyon and Becker (1987). The saponification process increased the squalene content from 4.2% in the crude oil to 43.3% in the unsaponifiables.

Squalene is not very susceptible to peroxidation and appears to function in the skin as a quencher of singlet oxygen, protecting human skin surface from lipid peroxidation due to exposure to UV and other sources of ionizing radiation. Supplementation of squalene to mice has resulted in marked increases in cellular and nonspecific immune functions in a dose-dependent manner (Kelly, 1999).

Commercially, this lucrative hydrocarbon is extracted from whale and shark liver oil and used as a skin penetrant and lubricant (Lehmann, 1996). Newmark (1997) proposed that the high squalene content of olive oil, as compared to other human foods, is a major factor in the cancer risk-reducing effect of olive oil. A mechanism is proposed for the tumor-inhibitory activity of squalene, based on its known strong inhibitory activity of HMG-CoA reductase activity. Amaranth seeds and oil could be an under utilize source for squalene as replacement of shark liver oil.

In long-term bioassay study, it have been shown that squalene can effectively inhibit chemically-induced colon, lung and skin tumourigenesis in rodents. The protective effect is observed when squalene is given before and/or during carcinogen treatment. The mechanisms involved for the chemopreventive activity of squalene may include inhibition of Ras farnesylation, modulation of carcinogen activation and anti-oxidative activities (Smith, 2000). Shin *et al.* (2004) examined the hypocholesterolaemic effect of amaranth grain and oil. Both amaranth grain and oil lowered serum and hepatic cholesterol and triglyceride levels. Faecal excretion of cholesterol and bile acid in the amaranth oil group increased, while amaranth grain affected only bile acid excretion. In the second part of experiment, rats were fed the cholesterol diet for four weeks and injected with saline (control), amaranth squalene or shark liver squalene (200 mg/kg) for seven days. The hypolipidaemic effects of amaranth squalene were evident in both serum and liver. In addition, amaranth squalene markedly increased faecal excretions of cholesterol and bile acid,

and slightly inhibited 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity. Amaranth grain and oil supplement, as an antioxidant therapy, may be beneficial for correcting hyperglycaemia and preventing diabetic complications. It is suggested that amaranth could be a valuable substitute for hypercholesterolemic patients allergic to cereals (Czerwiski *et al.,* 2003).

Phenolic Compounds

Polyphenols are abundant micronutrients in our diet, and evidence for their role in the prevention of degenerative diseases such as cancer and cardiovascular diseases is emerging. The health effects of polyphenols depend on the amount consumed and on their bioavailability.

Amaranth seed contain relatively good level of phenolic compounds. The total content of phenolic compounds was estimated by the Folin-Ciocalteu method to be ranged from 39.17 mg/100 g of *Amaranthus caudatus* to 56.22 mg/100 g of *A. paniculatus* seeds (Klimczak *et al.,* 2002). On the other hand Gamel *et al.* (2006b) reported higher level of phenolic compounds (5.2 mg/g) in *A. caudatus* and *A. cruentus* seeds. Gorinstein *et al.* (2008) reported average of 30 mg/100 g polyphenol content in three amaranth species. Ferulic acid was the dominat phenolic acid in amaranth seeds with average mount 310 mg/g dry matter. Water and acetone extract of amaranth seeds showed good antioxidant activity and free radicals scavenging capacity. Their antioxidant activities were comparatively assessed by total radical-trapping antioxidative potential (TRAP), ferric ion-reducing antioxidant power (FRAP), cupric-reducing antioxidant capacity (CUPRAC) and nitric oxide (NO•) assays (Gorinstein *et al.* (2008). The presence of phenolic compounds was highly correlated with the antioxidant effect of the amaranth seeds.

In another study, the antioxidant activity of ethanolic extracts obtained from two amaranth species was evaluated in a β-carotene-linoleic acid model system. The addition of amaranth extracts in the range of 0.01-0.1% inhibited degradation of a β-carotene in a model emulsion during incubation at 60°C; 0.05% addition of amaranth seeds extract was proposed as practically applicable (Klimczak *et al.,* 2002). It can be concluded that amaranth seeds and their phenolic compounds serve as good antioxidant constitute for free radicals scavenging in human body.

Functional Foods of Amaranth Seeds

Amaranth flour is commonly included as an ingredient in cereal-based foods to boost their nutritive value and health benefit. These

foods are often touted as, for example "Amaranth" breads, breakfast cereals, cookies, crackers, granola bars, or whatever when they only contain relatively small percentages (*e.g.* 5 to 15%) of Amaranth ingredient (Schnetzler & Breene, 1994). Amaranth can be eaten in many ways: in India popped Amaranth seeds are mixed with milk and/or honey or syrup, in order to make confections, or with milk. It can be used as breakfast cereal, cooked as gruel or baked as biscuits, cakes, and bread. As the seed does not contain gluten the flour must be mixed with wheat to produce bread. Moreover, whole grain amaranth food would be suitable for celiac patients. Tortillas and arepas, basic nutritional foods in several Latin American countries were prepared from whole flour of raw *A. cruentus* seeds using mixtures of 90:10, 80:10, and 50:50 with industrialized corn flour (Sanchez-Marroquin & Maya, 1985). Ratio of 50:50 and 60:40 blends of whole *A. cruentus* seed and its high protein fraction flours with oat flour respectively, were found to be highly suitable for development of low-cost infant formula (Sanchez-Marroquin *et al.,* 1986). Amaranth meals positively affect plasma lipid profile in rats fed cholesterol-containing diets. The degree of this positive influence is directly connected to the contents of the bioactive components and the antioxidant activities of the studied samples. It is suggested that amaranth could be a valuable substitute for hypercholesterolemic patients allergic to cereals (Czerwiski *et al.,* 2003). Bejosano and Corke (1998), studied the effect of replacement of isolated proteins obtained from *A. cruentus* and *A. hybridus,* on wheat dough properties and noodle quality. Addition of 2% Amaranth protein isolate increased the values of cooking loss, weight increase and volume increase of the noodle cooked in boiling water for 10 min.

Amaranth cereal products have high nutritive value and possess several health benefits such as improve blood glucose control, lower low-density lipoprotein (LDL) cholesterol levels and antioxidant.

BUCKWHEAT

Common or sweet buckwheat (*Fagopyrum esculentum* Moench) is a broad-leafed herbaceous annual. It belongs to the family Polygonaceae, which is generally referred to as the buckwheat, rhubarb or sorrel family. However, because its seed structurally and chemically resembles the cereal grains, buckwheat is usually handled and classed with the cereals. It is shown to have originated in the mountainous regions of Temperate Asia (northern India and China). In the new world buckwheat is produced in many parts of the world and has long been an important part of the human diet (Joshi & Rana, 1995).

There are many species of buckwheat in the world, and mainly nine species have agricultural value. Generally two types are used

around the world: common buckwheat (*F. esculentum*) and tartary buckwheat (*F. tataricum*). Common buckwheat is commonly grown and used, while tartary buckwheat is grown in mountainous regions (Li & Zhang, 2001; Bonafaccia *et al.,* 2003). Buckwheat bears triangular seeds with black hull covering the light green to white kernel. The colour gets lighter into the inner layers of the kernel. The hardness of the hull depends on the species of buckwheat (Li & Zhang, 2001).

Common buckwheat, the most widely consumed species, has the advantages of sweet taste, large seed size, and easy dehulling seed coat. Conversely, Tartary buckwheat has the disadvantages of bitter taste, small seed size, and tight seed coat that make dehulling difficult (Steadman *et al.,* 2001; Fabjan *et al.*, 2003; Chai *et al.,* 2004).

Buckwheat protein content varies from 13-15 % of the groat. The main protein fraction is globulin, which represents almost half of all proteins while prolamine represents the lowest content. The amino acid composition shows excellent amino acid proportions and in particular high amount of lysine which is often limiting in plant proteins. Starch is the major carbohydrate in buckwheat, and its amount may vary from 67 to 75%. Starch granules in the endosperm are polygonal or round in shape with diameter ranging from 2 to 12 μm with average 6-8 μm (Joshi & Rana, 1995). The total lipids of whole buckwheat grain range from 1.5-4%. The bran has the highest content (9.6-19.7%), while the endosperm contains 2-3%. Buckwheat oil contains 16-20% saturated fatty acids, 30-45% oleic acid and 31-41% of linoleic acid. The ash content of buckwheat varies from 2–2.2%, depending upon the variety. Moreover, Buckwheat flour contains various kinds of vitamins, such as B1, B2, and niacin, at relatively high levels (Pomeranz, 1983).

Bioactive Components of Buckwheat

Dietary Fibers

The major components of total dietary fiber (TDF), cellulose, non-starch polysaccharides, and lignins, are concentrated in the cell walls of starchy endosperm, aleurone and seed coat of buckwheat grain. The content of TDF in groats may range from 5 to 11% (Joshi & Rana, 1995; Zheng *et al.,* 1998; Steadman *et al.,* 2001). The bran fraction obtained by milling of buckwheat is enriched in dietary fiber (13-16%), but buckwheat flours contain considerably lower amounts of fiber (1.7-8.5%) with more portions to soluble fiber (Steadman *et al.,* 2001).

TDF can be classified into soluble- and insoluble dietary fiber. Soluble dietary fiber (SDF), and to a lesser extent insoluble dietary fiber (IDF), are fermented by microflora in the digestive system to produce short fatty acids, implicated in serum cholesterol and colon cancer reduction. Few data were available about the composition and properties of SDF in buckwheat. Water soluble non-starch polysaccharides were first isolated from buckwheat by Asano *et al.* (1970). They reported that xylose, mannose, galactose, and glucuronic acid are the main constitutes of that fraction. One of the most important characteristics of buckwheat water soluble non-starch polysaccharides is their very high molecular weight; as a consequence, they can form very viscous solutions when dissolved in water.

Flavonoids

Buckwheat contains many flavonoid compounds, known for their effectiveness in reducing the blood cholesterol, keeping capillaries and arteries strong and flexible, and assisting in prevention of high blood pressure (Santos *et al.,* 1999). Six flavonoids, rutin, orientin, vitexin, quercetrin, isovitexin and isoorientin, have been isolated and identified in buckwheat. Rutin and isovitexin are the only flavonoids components of buckwheat seeds, while the hulls contain all six compounds (Mazza & Oomah, 2005).

Rutin (quercetin-3-rutinosid) is a flavonol glycoside synthesized in higher plants as a protectant against ultraviolet radiation and disease (Gaberščik *et al.,* 2002; Rozema *et al.,* 2002). Rutin, the main buckwheat flavonoid was first discovered in the 19th century. Approximately 50 years ago, buckwheat was cultivated as a source of rutin for herbal drug production in the United States (Ohsawa & Tsutsumi, 1995). Among fruits, vegetables and grain crops, grapes and buckwheat are the most important rutin containing foods. No rutin was found in cereals and pseudocereals except buckwheat, which can be used as a good source of dietary rutin (Ohsawa & Tsutsumi, 1995; Watanabe, 1998; Kreft, *et al.,* 1999; Park *et al.,* 2000).

Buckwheat is still considered to be a major dietary source of rutin. However, there is a wide variation of rutin content in buckwheat seed depending on the species, variety, and the environmental conditions under which they are produced (Jiang *et al.,* 2007). The amount of flavonoids in general and rutine in particular of buckwheat varied between species. The grain of *F. tataricum* was reported to contain high level of rutine (1.6-1.8%) followed by *F. homotropicum* (0.07–0.14%) and *F. esculentum* (0.02-0.025%), while the average of flavonoids content was 2.0, 0.35, and 0.037%, respectively (Jiang *et al.*, 2007). The amount of rutin as dry weight

of dark buckwheat flour was (218 mg/kg), while the raw (uncooked) groats had 230 mg/kg of rutin (Kreft *et al.,* 2006).

The rutin present in our food and drinks has many interesting effects. The molecular structure of rutin shows that the phenolic part is linked with sugar and that makes the molecule more soluble. Rutin is a secondary plant metabolite that antagonizes the increase of capillary fragility associated with haemorrhagic disease, reduces high blood pressure (Abeywardena & Head, 2001), decreases the permeability of the blood vessels and has an anti-oedema effect, reduces the risk of arteriosclerosis and has antioxidant activity (Watanabe, 1998; Park *et al.,* 2000; Holasova *et al.,* 2001).

Lignans

Lignans are compounds with a dibenzylbutane skeleton, which have been found in many higher plants. These plant components act in mammalians as hormone-like phytoestrogens (Setchell, 1995). Buckwheat contains a considerable amount of these compounds and provided the third highest amount of excreted lignans among many cereals. (Thompson *et al.,* 1991). Secoisolariciresinol diglycoside (SDG) and matairesinol (MAT) are the main buckwheat lignans. The concentration of plant lignans acting as precursors of mammalian lignans is measured by subjecting a particular food ingredient to fermentation by intestinal microorganism and by measuring the amounts of enterodiol (ED) and enterolactone (EL) released (Setchell, 1995). Lignans have been shown to reduce mammary tumor size by more than 50% and tumor number by 37% in carcinogen treated rats (Setchell, 1995; Rickard & Thompson, 2000). Furthermore, it has been suggested that lignans have antimiotic, antiestrogenic, antiviral, antibacterial, antifungal, and antioxidant properties (Setchell, 1995; Thompson *et al.,* 1995; Rickard & Thompson, 2000).

Fagopyritols

Fagopyritols are specific carbohydrate compounds identified in buckwheat. Fagopyritols are mono-, di-, and trigalactosyl derivatives of D-*chiro*-inositol that accumulate especially in the embryo and the aleurone tissues of buckwheat. Among the plant sources, buckwheat is the richest in these carbohydrates. It has been reported that the bran milling fractions may contain 2.6 g of fagopyritols per 100 g of dry weight, whereas dark and light buckwheat flours contain 0.7g and 0.3 g/100 g, respectively. Published literature indicates that D-*chiro*-inositol could positively affect the blood glucose level and insulin activity (Ortmeyer *et al.,* 1993; Fonteles *et al.,* 2000).

Buckwheat extract has shown that to be equally efficient in lowering blood glucose level and activating insulin as synthetic D-*chiro*-inositol (Kawa *et al.,* 2003). There is also evidence that D-*chiro*-inositol can help in control development of polycystic ovary (Nestler *et al.,* 1999). However, the fate of fagopyritols in the human digestive system as well as the amount necessary to consume to achieve beneficial effects remain unknown and require further investigation.

Functional Foods of Buckwheat

A large variety of buckwheat foods have been produced traditionally in many countries in Asia, Europe and South Africa, in Canada, USA, Brazil and in certain other places around the world. Consequently, Dishes made from buckwheat seed are generally classified into two groups, flour dishes and groats dishes (Ikeda, 2002). Buckwheat grain is milled into flour or dehulled to produce groats. Two types of milling are used to produce flour. One is similar to wheat milling in which the grain is milled into flour.

The second type of milling is the dehulled buckwheat groat. Buckwheat grain is first hydrothermally treated and then dehulled. Different conditions and equipment are used for groat production, and the effects of these treatments on the final products have been reported by Pomeranz (1983). Buckwheat flour and groats are used for a wide variety of dishes. The flour is mixed with wheat flour for the production of buckwheat noodles called 'soba noodles' in Japan. The buckwheat flour content ranges from 50 to 80% depending on the type of noodle produced.

The groats are utilized in many dishes in through out the world. In Asia they are consumed as noodles, dumplings and as unleavened chapattis. In Europe, Kasha is used in dishes ranging from pilafs to mixtures with meat. In North America, the main use has been in pancakes; however, utilization of buckwheat has been increasing in the form of noodles and various ethnic dishes (Mazza & Oomah, 2005).

Buckwheat is also used in pastries and as a meat extender. In bioassay experiment, buckwheat was reported to have a prebiotic effect and provide healthy food. That was due to the increase in the rat-intestine of lactic acid bacteria, decease of total cholesterol, HDL cholesterol and, HDL phospholipids when rats were fed with buckwheat diet (Préstamo *et al.,* 2003).

Buckwheat has significant antioxidant activity and anti-inflammatory activity in mice and rats. In addition to the effectiveness

of improving glycemic control, reducing blood glucose level and inhibit LDL oxidation (Mazza & Oomah, 2005).

The presence of rutin and other phytochemicals such as polyphenolics and fagopyritols in buckwheat plants is one of the main reasons for the production of different kinds of buckwheat foods.

QUINOA

Quinoa (*Chenopodium quinoa*) is a pseudo-cereal with origins dating to the Incas. The pre-Colombian Andean people used the seed as a staple food component, and at times, replaced the animal protein in their diet with quinoa (Koziol, 1992). Today, quinoa is mainly cultivated in Argentina, Bolivia, Chile, Colombia, Ecuador, and Peru, locations that mostly coincide with the limits of the Inca Empire. Quinoa has also shown promise in tests of farm scale cultivation in high altitudes of Colorado, and near sea level in Washington and Oregon, as well as in England and in Scandinavia (National Research Council, 1989). The quinoa plant grows from 3 to 6 ft., in height and bears leaves that extend from the stalk. Quinoa seeds grow in large clusters at the end of the stalk, and seed color varies from pink, orange, red, purple, to black. Quinoa seeds, shape of which resembles sesame seeds, can be consumed whole or ground into flour.

Quinoa is commonly referred to as a pseudo-cereal since it is not a member of the grass family, but produces seeds that can be milled into flour and used much like a cereal crop. The crop has received consideration attention both within and out side south America, due to a number of attractive points. It is able to grow well under poor environmental conditions and can be combine-harvested. Quinoa can survive low rainfall, high altitudes, thin cold air, hot sun, sub-freezing temperatures, salinity, and poor sandy alkaline soils (Fleming & Galwey, 1995). The adaptability of different cultivars to salt stress merits special note (National Research Council, 1989). However, growth for most strains is optimized with short day lengths and cool temperatures.

The chemical composition of quinoa gives it high nutritional value. Quinoa has excellent reserves of protein and, unlike other grains, is not missing the amino acid lysine, so the protein is more complete (Ng *et al.,* 2007). The average protein content of the grain is 14.5% and in some varieties can reach up to 22% (Galway *et al.*, 1990). This protein is rich in histidine, lysine and isoleucine (Koziol, 1992). Moreover, the grains have high concentration of tryptophan, usually the second most deficient amino acid in cereals (Comai *et al.,* 2007). The quinoa grain is good source for oil, vitamins and minerals

compared to that of other cereals (Galway *et al.*, 1990). Several studies have revealed that the oil content in quinoa ranges from 1.8 to 9.5%, with an average of 5.0–7.2% (Mounts & Anderson, 1983). Quinoa oil is rich in essential fatty acids, like linoleate and linolenate (Koziol, 1992) and has a high concentration of natural antioxidants like α-tocopherol (5.3 mg/100 g) and γ-tocopherol (2.6 mg/100 g) (Ruales & Nair, 1992). No much data was available regarding the dietary fibers of quinoa. Dini *et al.* (2005) reported low dietary fibers content (4.0%) of quinoa which is extended with breeding to about 12% in case of sweet type (kancolla seeds). Starch is the main component representing about 60% of the grain (Atwell *et al.*, 1983). The Food and Agriculture Organization (FAO) observed that quinoa seeds have high quality proteins and higher levels of energy, calcium, phosphorus, iron, fibre and B-vitamins than barley, oats, rice, corn or wheat (Koziol, 1992). However, the seeds of most quinoa varieties contain saponins, located in the outer layers of the seed coat (Dini, *et al.*, 2002), most of which are bitter-tasting constituents. Because of this, they need to be washed or milled to remove the seed coat. The increased demand for quinoa has led researchers to produce several cultivars, selected and bred for their tolerance to heat and cold, resistance to disease, and for sweet taste. Perhaps the oldest and most widespread of the new varieties is kancolla (Dini *et al.*, 2005).

Research efforts in quinoa have been focused on its chemical composition, with considerable attention paid to saponins in quinoa. There has been some work accomplished in processing quinoa, focused mainly on the effects of dehulling and washing on changes in chemical composition; specifically removal of saponins. Usually, quinoa is processed by means of soaking, rubbing, rinsing, and boiling in the domestic setting. It is industrially processed by means of wet and dry milling (Becker & Hanners, 1990). Today's health conscious consumers are illustrating a preference towards value added products, and in general, more nutritious food items. The opportunity to supplement or completely replace common cereal grains (corn, rice and wheat) with a cereal of higher nutritional value (such as quinoa) is inherently beneficial to the public interests.

Bioactive Compounds of Quinoa

Betaines

A betaine in chemistry is any neutral chemical compound with a positively charged cationic functional group such as an ammonium ion or phosphonium ion (generally: onium ions) which bears no hydrogen atom and with a negatively charged functional group such as a carboxylate group which may not be adjacent to the cationic

site. Historically the term was reserved for trimethylglycine (glycine betaine).

Analysis of the polar extracts from a sweet variety of quinoa (Kancolla seeds) led to the isolation of five betaines: glycine betaine, trigonelline, trigonelline methylester, trigonelline glucosylester and 3-carboxy-1-(2-sulfoethyl)-pyridinium (Dini *et al.,* 2006). In mammals, glycine betaine (acts as an osmolyte in the inner medulla of the kidney, preserving osmotic equilibrium, thus maintaining the tertiary structure of macromolecules (Yancey & Burg, 1990; Yancey & Somero, 1979). In humans, glycine betaine can be readily absorbed through dietary intake or endogenously synthesised through the catabolism of choline in the liver (Flower *et al.,* 1972). Glycine betaine is also an important source of methyl groups, required for the formation of methionine and S-adenosylmethionine (Barak *et al.,* 1996; Chambers & Lever, 1996). Glycine betaine intake can lower plasma homocysteine levels in patients suffering from homocystinuria (Wilken *et al.,* 1983), and in chronic renal failure patients with hyperhomocysteinemia (McGregor *et al.,* 2002).

Phenolic Compounds

The general definition of phenolic compounds is any compound containing a benzene ring with one or more hydroxyl groups. Phenolics acids, flavonoids, condesed tannins, and alkylresorcinols are examples. All plant based foods have phenols, which affect their appearance, taste, odour and oxidative stability (Naczk & Sahidi, 2004). Nasimba *et al.* (2008) assessed the total phenolics content and the antioxidant properties of quinea and amaranth spp. Among the five plant materials, the ethanolic extract of the Japan sea-level type of *C. quinoa* demonstrated the highest phenolics content (148.0), followed by *A. hypochondriacus* (133.2) *A. cruentus* (130.4 and 99.8), and the Bolivia altiplano type of C. quinoa (94.3) mg/g tannic acid equivalents. The FRAP assay and the b-carotene method demonstrated the higher antioxidant activity for the Japanese *C. quinoa*. Dykes and Rooney (2007) reported the total phenolic level and the antioxidant activity (as determined by ABTS method) in red and black quinoa to be 4.5 and 4.0 mg gallic acid equivalent/g and 50 and 40 μm Trolox equivalent/ g, respectively. In another study, five flavonol glycosides and a vanillic acid glucosyl ester were found in the sample of sweet variety of quinoa (Kancolla seeds) and obtained by MeOH extract. The quercetin 3-O-β-D-apiofuranosyl-(1→2)-O-[α-L-rhamnopyranosyl-(1→6)]-β-D-galactopyranoside-3, 4-dimethyl ether was isolated for the first time in quinoa varieties (Dini *et al.,* 2004).

Functional Food of Quinoa Grains

Numerous studies have been conducted to investigate the feasibility of incorporating quinoa into foods. Advantages of using quinoa as an ingredient include raising the protein content and improving the taste of the product. Lorenz and Coulter (1991a) and Lorenz *et al.* (1995) evaluated the performance of blended quinoa and wheat flours in breads, cakes and cookies. Breads and cakes made with up to 10% quinoa flour were acceptable. When used up to 10% in cakes and 20% in cookies, quinoa contributed a favourable nutty taste to the products. Chauhan *et al.* (1992) investigated the baking performance and overall acceptability of quinoa/wheat breads using quinoa flour or quinoa meal. In general, breads with 10% of water-soaked quinoa meal were more acceptable than were other quinoa variations. Nutritional properties, sensory evaluation and physical characteristics were examined in extrusion studies blending quinoa and com grits (Coulter & Lorenz, 1991a, 1991b; Lorenz *et al.*, 1995). Quinoa flour was extruded with com grits to produce expanded snack products. Addition of quino increased product density and decreased product expansion and shears strength, and produced a darker, less yellow extruded product. The products were rated as moderately acceptable. Quinoa has been incorporated into wheat noodles (Lorenz *et al.*, 1993). No statistically significant difference was found between noodles made with 10% and 30% quinoa. Noodles with 50% quinoa content were ranked least acceptable.

Mahoney *et al.* (1975) reported that using cooked quinoa (boiled for 30 min) in animal diet improved the nitrogen efficiency for growth by 40%, the weight gain by 100% and the protein efficiency ratio by 29% relative to the values obtained with uncooked grain.

Present of saponins in quinoa limits some how the utilization of this crop in some extends. Breeding in quinoa focus mainly in development of a variety with high grain yield accompanied with high protein and low saponin content (Bhargava, *et al.*, 2006). Saponins can be removed either by the wet method, *i.e.* washing and rubbing in cold water, or by dry method, *i.e.* toasting and subsequent rubbing of the grains to remove the outer layers (Risi & Galwey, 1984). On commercial scale, saponins are removed by abrasive dehulling (Reichert *et al.*, 1986), but in this method, some saponin remains attached to the perisperm (Becker & Hanners, 1991). Saponin removal by dry method reduces the vitamin and mineral content to some extent, the loss being significant in case of potassium, iron and manganese (Ruales & Nair, 1992).

The development of a market of quinoa has involved investigation of novel ways in which it can be incorporated into food products.

Different types of bread, pasta and snacked food could be suitable products to be produced from this crop blended with other cereals. That definitely will improve the nutritional quality and health benefit of those food products.

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