University of Nigeria

Research Publications

Author	**NJOKU, Ugochi Olivia** **PG/M.Sc/03/34258**
Title	**Antioxidant Activity of Seed Extract and Fractions of Monodora Tenuifolia (Annonaceae)**
Faculty	**Medicine**
Department	**Pharmacology/Toxicology**
Date	**February, 2007**
Signature	

University of Nigeria

Virtual Library

Serial No.	
Author 1	
Author 2	
Author 3	
Title	
Keywords	
Description	
Category	
Publisher	
Publication Date	
Signature	

University of Nigeria

Curriculum Vitae

Name	
Specialisation	
Faculty	
Department	
Date	
Signature	

ANTIOXIDANT ACTIVITY OF SEED EXTRACT
AND FRACTIONS OF *Monodora tenuifolia*
(Annonaceae)

By

NJOKU, UGOCHI OLIVIA
PG/M.Sc/03/34258

A PROJECT SUBMITTED TO THE DEPARTMENT OF
PHARMACOLOGY/TOXICOLOGY, UNIVERSITY OF
NIGERIA, NSUKKA IN PARTIAL FULFILLMENT OF
THE REQUIREMENT FOR THE AWARD OF
MASTERS OF SCIENCE (M.Sc) DEGREE.

TITLE PAGE

ANTIOXIDANT ACTIVITY OF SEED EXTRACT
AND FRACTIONS OF *Monodora tenuifolia*
(Annonaceae)

A PROJECT SUBMITTED TO THE DEPARTMENT OF
PHARMACOLOGY/TOXICOLOGY, UNIVERSITY OF
NIGERIA, NSUKKA IN PARTIAL FULFILLMENT OF
THE REQUIREMENT FOR THE AWARD OF
MASTERS OF SCIENCE (M.Sc.) DEGREE

BY

NJOKU, UGOCHI OLIVIA
DEPARTMENT OF PHARMACOLOGY/TOXICOLOGY
UNIVERSITY OF NIGERIA, NSUKKA

SUPERVISOR: Prof. P. A. AKAH
           DEPARTMENT OF PHARMACOLOGY/TOXICOLOGY
           UNIVERSITY OF NIGERIA, NSUKKA

2007

CERTIFICATION

NJOKU, Ugochi Olivia, a postgraduate student in the Department of Pharmacology/Toxicology and with registration number PG/M.Sc/03/32458 has satisfactorily completed the requirement of research work for the degree of Masters of Science (M.Sc) in Pharmacology/Toxicology.

    This work embodied in this project is original and has not been submitted in part or full for any other diploma or degree of this or any other University.

Project Supervisor

Head of Department

DEDICATION

To my beloved husband, great and wonderful kids: Onyii, Obi. Jnr. &

Chioma, parents and loved ones.

ACKNOWLEDGEMENT

My most profound gratitude goes to my supervisor, Prof. P.A. Akah for his warmly guidance and supervision. His unreserved kindness and understanding not only inspired me but also encouraged me throughout the trying moments of this work.

I also wish to express my indebtedness to Dr. Charles Okoli of the Department of Pharmacology and Toxicology who by close supervision and assistance contributed in no small measure to the completion of this work.

My gratitude goes to Okoye and Ajaba for their unrelentful assistance throughout my laboratory work.

To my husband, Prof. O. U. Njoku, my kids: Onyinye, Obi Jnr., and Chioma, I really appreciate your moral and financial support.

My thanks also goes to my caring colleagues, friends who have contributed inestimably throughout the course of this work. In this respect, I am particularly grateful to Ada Onyirioha, Thelma Ihedioha, Ada Ibiam, Uju Udeogaranya, Prof. Mrs. E. Okeke, and a host of others.

I thank my parents: Elder and Elder (Mrs.) Ibe, my parents' in-law: Sir and Lady Rufus Njoku, my siblings: Paul, Uloma, Nwajiulo, Ngozi, Jude, Chizoba, and Tochukwu who have been an everlasting source of love, strength and encouragement.

Finally, above all, to my gracious heavenly father for the grace, strength and ability, for perfecting all that concerneth me to him be all the glory thanksgiving and honour.

TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

The antioxidant activity of the seed extract and fractions of *Monodora tenuifolia* (Fam. Annonaceae) was evaluated. The *Monodora tenuifolia* seed was extracted with pet ether 40–60°C to produce the crude extract. Fractionation of the extract by column chromatography using pet ether 60–80°C and diethyl-ether produced 2 fractions (F_1) and (F_2). Phytochemical analysis of *Monodora tenuifolia* seed extract showed the presence of some plant secondary metabolites, viz: alkaloids, flavonoids, proteins, carbohydrates, saponins, glycosides, cyanogenic glycosides, cardiac glycosides, tannins, steroidal aglycon while, O and C glycosides, anthracene glycosides and reducing sugar were absent. The 3 fractions showed the presence of vitamin A and vitamin E, The pet-ether extract and the fractions (F_1 and F_2) reduced CCl_4-induced lipid peroxidation in rat liver homogenate. They also exhibited significant antioxidant activity in nitric oxide induced lipid peroxidation. The crude extract and diethylether fraction (F_2) produced dose-dependent protective effect against lipid peroxidaiton and free radical generation in liver homogenate. The acute toxicity study with the crude extract showed no signs of obvious toxicity up to a dose level of 5000 mg/kg. These results suggest that *Monodora tenuifolia* seed extract possessed significant antioxidant properties and could be used for the treatment of diseases associated with free radical generation.

CHAPTER ONE

1.1 Antioxidant: An overview

Antioxidants are a group of substances, which when present at low concentrations, in relation to oxidizable substrates, significantly inhibit or delay oxidative processes, while often being oxidized themselves (Kanner et al., 1999).

The application of antioxidants are widespread, in industries they are used in preventing polymer from oxidative degradation, rubber and plastic from losing strength, gasoline from autooxidation, synthetic and natural pigments from discolouration and as additives to cosmetics, food (especially food with high fat content) beverages and baking products (Kanner *et al*, 1999).

In recent years there has been an increase in the application of antioxidant in medicine as information is constantly gathered linking the development of human diseases to oxidative stress (Halliwell *et al.*, 1999). The generally accepted hypothesis in any biological system is that, an important balance must be maintained between the formation of reactive oxygen and nitrogen species (ROS and RNS, respectively). The reactive species such as superoxide (O_2), hydrogen peroxide (H_2O_2), hydroxyl radical (OH), nitrogen oxide (NO), and hypochlorous acid (HOCl), are all products of normal metabolic pathways of human organs, but under certain condition, when in excess they can exert harmful effects.

To maintain an oxido/redox balance, organs protect themselves from the toxicity of excess ROS/RNS in different ways, including the use of endogenous and exogenous antioxidants.

OXIDATIVE STRESS   DEFENSE SYSTEM

BALANCE

Endogenous sources of Res/RNS	Exogenous Sources of ROS/RNS	Enzymatic	Non-Enzymatic
– Mitochondria	– Radiation	–Catalase (CAT)	
– Peroxisomes	– Ozone	– Paraoxonase	– Vitamin C
–Inflammatory Cells	– Xenobiotics	– Glutathione-peroxidase	– Flavonoids
		–Superoxide dimutase	– Glutathione
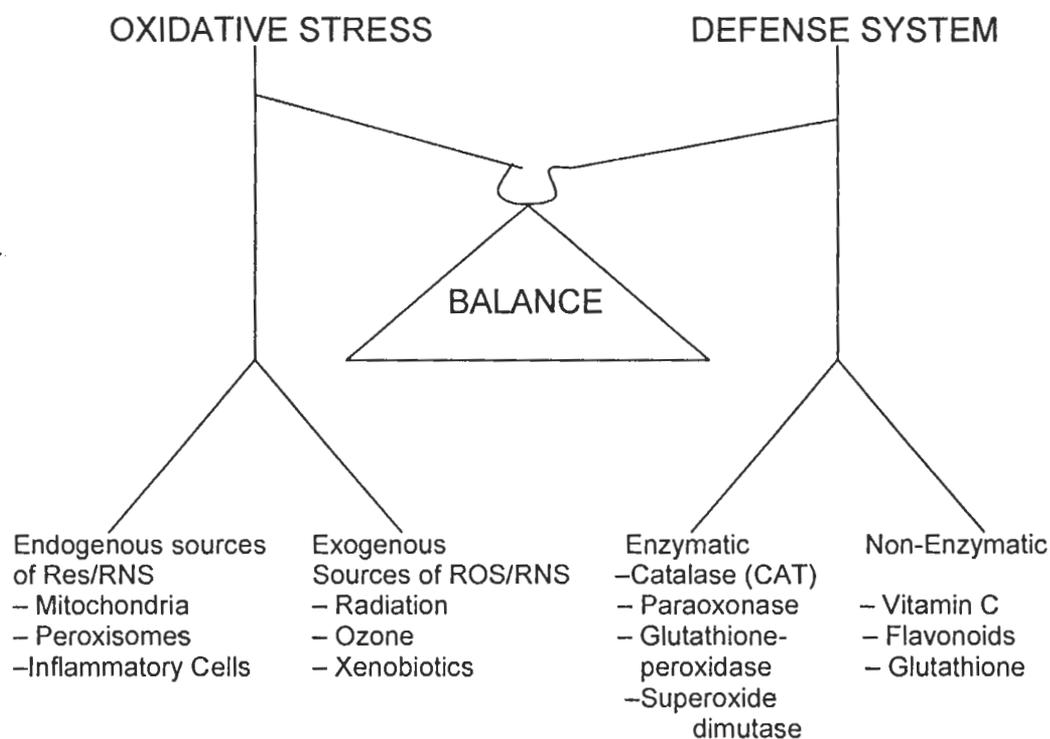
Fig. I: Shows a balance between oxidative stress and defense system

1.1.1 Natural antioxidant of low and high molecular weight

Naturally occurring antioxidants of high or low molecular weight, can differ in their mechanism and site of action (Sahart, 2001). They can be divided into the following categories: -

(a)   Enzymes

(b)   High molecular weight proteins

(c)   Low molecular weight antioxidants

a)   **Enzymes:** The best studied cellular antioxidants are the enzymes, superoxide dimutase (SOD), catalase and glutathione peroxidase (GPx). These attenuate the generation of reactive oxygen species by removing potential oxidants or by transforming ROS/RNS into relatively stable compounds. SOD, which was discovered in the late 60s, catalyzes the transformation of the superoxide radical into hydrogen peroxide, which can then be further transformed by the enzyme catalase into water and molecular oxygen (Sahart, 2001).   Glutathione peroxidase (GPx) reduces lipid peroxides (ROOH), formed by the oxidation of poly-unsaturated fatty acid (PUFA) to a stable, non-toxic molecule hydroxyl fatty acid (ROH) (Sahart, 2001). Less well studied (but probably just as important) enzymatic antioxidants are the peroxiredoxins and the recently discovered sulfiredoxin. Other enzymes that have antioxidant properties (though this is not their primary role) include Paraxonase, Glutathione – strainsferases, and aldehyde, dehydrogenases (Current Medicinal Chemistry, 2005).

b)   **High molecular weight proteins:** These preventive antioxidants hinder the formation of new ROS. These antioxidants are protein that binds ROS to protect essential proteins. The group includes albumin, metallothonine, transferring, ceruloplasmin, myoglobin, happtoglobin and ferritin (Current Medicinal Chemistry, 2005).

These are all present in plasma and bind to redox active metals and limit the production of metal – catalyzed free radicals (Current

Medicinal Chemistry, 2005). Metals such as iron, copper, chromium, vanadium and cobalt are capable of redox cycling in which a single electron may be accepted or donated by the metal (Current Medicinal Chemistry, 2005). Albumin and ceruloplasmin can bind copper, ions, and transferin binds free iron. Haptoglobin binds heme-containing protein and can thus clear them from the circulations (Current Medicinal Chemistry, 2005). Both free and heme associated protein have pro-oxidant properties due to their reaction with H_2O_2 to form ferryl species, which can easily initiate lipid peroxidation (Current Medicinal Chemistry, 2005).

c) **Low molecular weight antioxidants:** These are subdivided into lipid-soluble antioxidants (Tocopherol, carotenoids, quinines, bilirubin and some poly-phenols) and water-soluble antioxidant (ascorbic acid, uric acid and some polyphenols) (Niki, 1987).

α-Tocopherol (vitamin E) and β-carotene have considerable support as lipid-soluble antioxidants; tocopherol might act synergistically with ascorbate. Vitamin C in living organisms regenerates vitamin E by reducing the tocopherol radical that is produced when vitamin E scavenges a peroxyl radical (Niki, 1987). Uric acid is another antioxidant in primates as their blood has a higher concentration than that of other mammals; uric acid might serve to scavenge reactive free radicals (R·) and therefore account for the prolonged life span of humans (Ames et al., 1981). Some carotenoids, including β-carotene, quench highly

reactive singlet oxygen under certain conditions and can block free radical-mediated reactions (Bendich, 1989).

1.1.2 Mechanism of action of antioxidants

Two principle mechanisms of action have been proposed for antioxidant. The first is chain-breaking mechanism, by which primary antioxidants donate an electron to the free radical present in the system. The second mechanism involves removal of ROS/RNS initiators (secondary antioxidants) by eliminating chain-initiating catalyst (Murray, et al., 1990).

Electron donation

Primary antioxidants are compounds that are able to donate hydrogen atom rapidly to a lipid radical forming a new radical, more stable than the initial one (Murray et al., 1990). Biological organs contain many polyunsaturated fatty acids (PUFA), such as linoleic, linolenic and arachidonic acid, mainly in the form of ester with cholesterol. These PUFA can undergo lipid peroxidation that can be interrupted by the primary antioxidant by the donation of electrons.

The whole process can be depicted as follows.

$RH + O_2$ (Singlet oxygen)   - - - - -ROOH

$ROOH + Fe^{+2}$ - - - - - $RO. + HO^- + Fe^{+3}$

$ROOH + Fe^{+3}$ - - - - $ROO. + H^+ + Fe^{+2}$

$ROO. + \alpha - TO.$ - - - -   Non radical products.

RH =   Polyunsaturated fatty acid (PUFA)
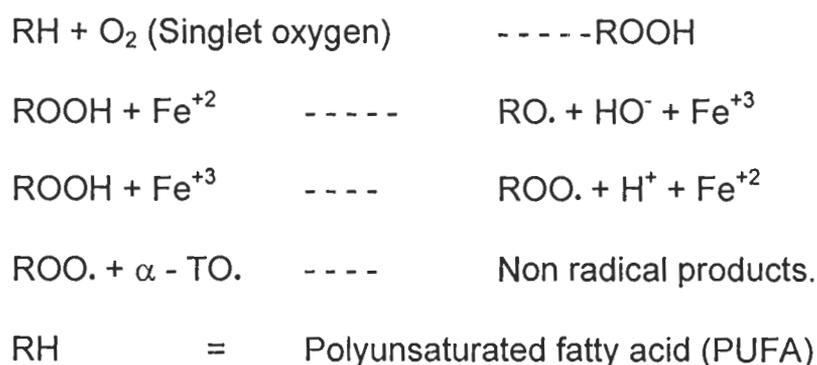
ROOH   =   PUFA hydroperoxide

RO.   =   Alkoxyl radical

ROO.   =   Peroxyl radical

α - TO.   =   Tocopheryl radical

Metal Chelation

Secondary antioxidant can retard the effect of ROS/RNS radical initiated action by means of initiator removal or elimination. This can be accomplished by deactivation of high-energy species, absorption of UV light, scavenging of oxygen and thus reducing its concentration (Omenn *et al*, 1996). Chelation of metal catalyzes free radical reaction or inhibits peroxidase, such as NADPH oxidase, xanthine oxidase, dopamine-β-hydroxylase or lipoxygenases (Omenn *et al.*, 1996).

The ability of antioxidant to chelate transition metal ions can be followed spectroscopically. High molecular weight proteins bind directly or indirectly to redox active metals and thus inhibit the production of metal-catalyzed free radicals. Some low molecular weight compounds, such as polyphenols, in addition to their ability to donate hydrogen atom and thus act as chain-breaking antioxidant, can also chelate transition metal ions and hence inhibit free radical formation (Omenn *et al.*, 1996).

1.2   Lipid peroxidation

Peroxidation (autooxidation) of lipids exposed to oxygen is responsible not only for deterioration of food (rancidity) but also for damage to tissues *in vivo*, where it may be a cause of cancer,

inflammatory diseases, atherosclerosis, aging, etc (Murray et al., 1990). The deleterious effects are initiated by free radicals (ROO., RO., OH.) produced during peroxide formation from fatty acids containing methylene interrupted double bonds, i.e., those found in the naturally occurring polyunsaturated fatty acid (PUFA) (Murray *et al.*, 1990).

Lipid peroxidation is a chain reaction providing a continuous supply of free radicals that initiate further peroxidation. Since the molecular precursor for the initiation process is generally the hydroperoxide product (ROOH), lipid peroxidation is a branching reaction with potentially devastating effects. To control and reduce lipid peroxidaiton both humans and nature involves the use of antioxidants.

1.3 Mechanisms of lipid peroxidation induction

Three different mechanisms are able to induce lipid peroxidation.

1.3.1 Autoxidation

This is a radical – chain process involving three sequences:

(a) **Initiation**: In a peroxide-free lipid system, the initiation of a peroxidation sequence refers to the attack of a ROS (with sufficient reactivity) able to abstract a hydrogen atom from a methylene group ($-CH_2-$); these hydrogen having very high mobility (Morel, 1997). This attack generates easily free radical from polyunsaturated fatty acids: OH is the most efficient ROS to do that attack, whereas O_2. is sufficiently reactive.

The carbon radical tends to be stabilized by a molecular rearrangement to form a conjugated diene. Under aerobic conditions conjugated dienes are able to combine with O_2 to give a peroxyl (or peroxyl) radical, $ROO^•$.

(b)   Propagation: A peroxyl radical is able to abstract hydrogen from another lipid molecule (adjacent fatty acid), especially in the presence of metals such as copper or iron, thus causing an autocalalytic chain reaction. The peroxyl radical combines with hydrogen to give a lipid hydroperoxide. This reaction characterizes the propagation stage (Morel, 1997).

(c)   Termination: Termination (formation of a hydroperoxide is most often achieved by reaction of a peroxyl radical with α-tocopherol, which is the main lipophilic "chain-breaking molecule" in the cell membranes. Furthermore, any kind of alkyl radical (lipid free radical) can react with a lipid peroxide to give non-initiating and non-propagating species such as the relatively stable dimers (Morel, 1997).

1.3.2 Photo-Oxidation

As singlet oxygen ($^•O_2•$) is highly electrophilic, it can react rapidly with unsaturated lipid but by a different mechanism than free radical autoxidation. In the presence of sensitizers (chlorophyll, porphyrins, myoglobin, riboflavin, methylene blue, etc.) a double bond interacts with singlet oxygen produced from O_2 by light. Oxygen is added at either

ends of carbon of a double bond, which takes the trans configuration. Thus, one possible reaction of singlet O_2 with a double bond between C_{12} and C_{13} of one fatty acid is to produce 12- and 13-hydroperoxide.

The inhibition of photosensitized oxidation is efficiently inhibited by an antioxidant present and rich in green leafy carotenoids, vegetables and many coloured fruits (Khachik *et al.,* 1986). The inhibitory mechanism is thought to be through an interference with the formation of singlet oxygen from the oxygen molecule. In contrast tocopherols inhibits its oxidation by quenching the previously formed singlet oxygen, and this forms stable additional products (Morel, 1997).

1.3.3 Enzymatic Peroxidation

Lipoxygenase enzymes (from plants and animals) catalyze reaction between O_2 and polyunsaturated fatty acid, such as arachidonic acid ($20 : 4_n - 6$), containing methylene interrupted double bonds. When $20: 4_n–6$ is the substrate, these hydroperoxides are known as H_PETES, which can be transformed into hydroxyl products (HETEs). These HETEs are also formed directly via cytochrome P450 induced reactions.

Cycloxygenase enzymes (in plants and animals) catalyzed the addition of molecular oxygen to various polyunsaturated fatty acid, they are thus converted into biologically active molecule called endoperoxide (PGG, PGH), intermediates in the transformation of fatty acid to prostaglandin (Morel, 1997).

1.4   General antioxidant actions

1.4.1 Free radicals

Every cell has chemical reactions involving the oxidation and reduction of molecules. These reaction or redox pathways can lead to the production of free radicals.

A free radical is any chemical species capable of independent existence possessing one or more unpaired electrons. Biological free radicals are thus highly unstable molecules that have electrons available to react with various organic substrates (Sahart, 2001).

Many free radicals are generated from naturally occurring processes such as oxygen metabolism and inflammatory processes. For example, when cells use oxygen to generate energy, free radicals are created as a consequence of ATP production by the mitochondria (Sahart, 2001). Exercise can increase the levels of free radicals as can environmental stimuli such as ionizing radiation (from industry, sun exposure, cosmic rays, and medical x-rays), environmental toxins, altered atmospheric conditions (e.g. hypoxia and hyperoxia), ozone and nitrous oxide (primarily from automobile exhaust). Lifestyle stressors such as cigarette smoking and excessive alcohol consumption are also known to affect levels of free radicals (Omenn *et al.,* 1996).

It has been noted that certain organ systems are predisposed to greater levels of oxidative or nitrosative stress. Those organ systems most susceptible to damage are the pulmonary system (exposed to high

levels of oxygen), the brain (exhibits intense metabolic activity yet has lower levels of endogenous antioxidants), the eye (constantly exposed to damaging UV light), circulatory system (victim to fluctuating oxygen and nitric oxide levels) and reproductive systems (at risk from the intense metabolic activity of sperm cells). Nearly every organ system can be found to have an Oxidative or Nitrosative "Achilles heel" (Omenn, *et al.*, 1996).

Reactive Oxygen Species (ROS)

ROS is a term collectively describing radicals and other non-radical reactive oxygen derivatives. These intermediates may participate in reactions giving rise to free radicals or that are damaging to organic substrates. ROS in living organisms include the following:

Radicals		Non-Radicals	
Hydroxyl	$OH \cdot$	Peroxynitrite	$ONOO^-$
Superoxide	$O_2 \cdot^-$	Hypochloric acid	$HOCl$
Nitric oxide	$NO \cdot$	Hydrogen peroxide	H_2O_2
Thyl	$RS \cdot$	Singlet oxygen	$^1\Delta_g (^1O_2)$
Peroxyl	$RO_2 \cdot$	Ozxone	O_3
Lipid peroxyl	$LOO \cdot$	Lipid peroxide	$LOOH$

Reactive Nitrogen Species (RNS)

RNS are nitrogen-based molecules that can act to facilitate nitrosylation reactions. Reactive nitrogen species (RNS) include:

Nitrous oxide	$NO \cdot$
Peroxynitrite	$OONO^-$
Peroxynitrous acid	$ONOOH$
Nitroxyl anion	NO^-
Nitryl chloride	NO_2Cl
Nitrosyl cation	NO^+
Nitrogen dioxide	$NO_2 \cdot$
Dinitrogen trioxide	N_2O_3
Nitrous acid	HNO_2

The most reactive and damaging free radicals are the $OH \cdot$ and $OONO^-$ (Sahart, 2001). Many other radical species can be formed by biological reactions, for example: phenolic and other aromatic species are often formed during xenobiotic metabolisms as part of natural detoxification mechanisms (Sahart, 2001). Most of the free radicals are produced by mitochondria and most of the free radical damage is to mitochondria membranes and mitochondrial DNA (Wei and Lee, 2002). Between one and five percent of the oxygen used by mitochondria to generate energy results in the formation of superoxide radicals (Wei and Lee, 2002).

Although mitochondria are the major source of free radicals, there are numerous other sources. A green peroxidase of phagocytic cells (as neutrophils and monocytes) are another source of free radicals. They (neutrophils and monocytes) assist in bacteriocidal activity by catalyzing

the oxidation of ionic halogen to free halogen (Buetner and Jurkiewicz, 1996).

Myeloperoxidase enzyme, which is a peroxidase found in the lysosomal granules of myeloid cells, particularly macrophages and neutrophils, responsible for generating potent bacteriocidal activity by the hydrolysis of hydrogen peroxide (produced in the metabolic burst) in the presence of halide ions (Buettner and Jurkiewicz, 1996). Free radicals are generated by eicosanoids from arachidonic acid during Ischemia-reperfusion injuries. During reperfusion the endothelial enzyme xanthine oxidase converts oxygen to superoxide, which can react with nitric oxide to produce peroxynitrite (Buettner and Jurkiewicz, 1996). Free radicals from tobacco smoke and air pollution can cause oxidative damage to lungs, blood vessels and other body tissue (Bendich and Olson, 1989).

Reactive free radicals $(R\cdot)^2$ appear to have a role in the general process of aging and in tissue damage that results from radiation, reactive oxygen metabolite and carcinogen metabolism (Rose and Bode, 1993).

Details of underlying chemistry of ascorbate (Levine and Morita, 1985) and free radical generation (Grisham and McCord, 1986) are available. Although many or most $R\cdot$ that are generated in the body are metabolized to non-reactive species (Fig. 2), cellular damage is initiated under some conditions.

Animals have evolved intricate and interrelated processes for protecting against the effects of R˙. The enzymatic reactions of superoxide dimutase (SOD), catalase, glutathione peroxidase are not 100% effective in eliminating the formation of all free radicals. For example, the very reactive hydroxyl free radical, HO˙, is not eliminated by these mechanisms (Rose and Bode, 1993).

At the body's non-enzymatic protective mechanisms is a scavenging reaction in which some endogenous compounds with the inherent trait of entering into redox reactions contributes an electron to fill the outer shell of R˙ and thereby neutralize it to a non-reactive species. In principle, many chemicals could serve this purpose because the high reactivity of R˙ results in it extracting an electron from almost any available molecule. A few of the compounds shown to have this property are: Mannitol (Caughey and Watkins (1985) heamoglobin (Giulivi and Davies, 1990), estrogens (Niki and Nakon, 1990), bile acids and derivatives (Stocker *et al.*, 1990) and serotonin (Jovanovic *et al.*, 1990).

For a substance to function biologically, it must do more than simply react with R˙. The present emphasis is on water-soluble compounds that might have been useful throughout the long evolutionary development from microbes to mammals. Particular emphasis is on primates, as they are subjected to threat from R˙ over long lifespan.

It must be considered that the source of R· changed over the last 10 years, with the threat from reactive oxygen species increasing (due to plant generation of O_2) and the threat from solar radiation diminishing (due to emergence of the earth's stratospheric ozone layer) (Rose and Bode, 1993). Carcinogen metabolites tend to be electron-deficient or electrophilic (Cavalieri and Rogan, 1984).

Properties of an ideal free radical scavenger

Protection is thought to be available in the form of endogenous compounds that react with and thereby "scavenge" the R˙. Because many R˙ are reactive forms of oxygen, an effective scavenger is often referred to as an antioxidant.

To be an effective antioxidant physiologically, a substance must have certain chemical and biological properties.

(a)   **It must be present in adequate amounts in the body**

In that most R˙ have a brief half-life in the body and diffuse only over short distances, the probability that they react with any given antioxidant is proportional to the antioxidant's concentration in the immediate environment where the R˙ is generated. Most potential scavengers are present in the mammalian body at a low concentration. Some have appeared only recently in evolution.

For instance, the introduction of hemoglobin coincided with the appearance of animals having a closed circulatory system; most earlier forms of aerobic life depended on cutaneous respiration and did not

have the possibility of respiratory pigments protecting them from free radical threats (Rose and Bode, 1993).

(b)   **It must be versatile**

The ideal scavenger should combine with a wide variety of free radicals, i.e., it must be readily oxidized. One limitation of superoxide dismutase (SOD) in eliminating free radicals is its lack of versatility; it has but one substrate (Rose and Bode, 1993).

(c)   **It must be suitable to be compartmentalized**

The antioxidant must be suitable for the body to translocate it between tissues and must accumulate within compartments where a need for protection exists at the time. A frequent cellular mechanism for directing substrates to specific sites of the body is membrane transport , e.g. through polarized cells of the gastrointestinal tract, renal tubule, liver, placenta etc. (Rose and Bode, 1993). Molecualr size of the antioxidant is important. Small molecules may be so permanent that even if they were recognized by a transport mechanism, they would readily diffuse out of any membrane-bound compartment. Very large compounds may not be transported across cellular membranes at rates great enough to be useful (Rose and Bode, 1993).

(d)   **It must have tolerable toxicity**

Ideally, the antioxidant would be non-toxic, both before and after it performs the scavenging reaction. If toxicity is a possibility, careful

management of the toxic form must be accomplished under normal conditions (Rose and Bode, 1993).

(e) **It must be available**

If the compound is to be accessible to all animal species, it should either be synthesized de novo or acquired in the diet. A particular antioxidant might be produced by some animal species or acquired in the diet by others. If some organisms became devoid of synthetic capability (e.g. primates, in the case of ascorbic acid) the compound must be suitable to be ingested as food. Therefore, it must exist in plant products and be stable for periods of days or weeks after harvest. It must also be suitable for the normal processes of ingestion, digestion, and intestinal absorption.

(f) **It might be suitable for regeneration**

The process of neutralizing a $R^\bullet$ results in the scavenger becoming oxidized to a form that has less capacity to react with additional $R\bullet$. Thus a scavenger would be particularly useful if it is recycled so that dietary acquisition does not become prohibitively expensive. The compound must have a biologically convenient reducing mechanism, which could be either a specific enzyme or a direct chemical reaction.

(g) **It must be conserved by the kidneys**

If the compound is filtered in the glomerular of the kidney, it must be suitable for reabsorption. Because renal clearance of small

compounds that are filtered but not reabsorbed is high in most animals (with the half-life of plasma disappearance < 1 hr), large urinary losses would occur in the absence of active reabsorption (Rose and Bode, 1993).

1.4.2 Antioxidant molecules

We can accept that many substances interact with free radicals or at least with the most reactive of them. This is not surprising, as some $R^\bullet$ (such as $HO^\bullet$) are so electrophylic that they strip an electron or hydrogen atom from almost any compound with which they come in contact. Some of these reactions immediately result in products that are stable, thus terminating the free radical activity. Many of those compounds, however, have few of the properties listed above. For instance, mannitol is present in plants but it is not synthesized in animals. It is not recognized by mammalian membrane receptors or transporters; thus it is not absorbed in the gastrointestinal tract or directed to specific sites of the body. Mannitol does not enter most animal cells, as evidenced by its use in research as an extracellular space marker (Rose and Bode, 1993).

α-Tocopherol (Vitamin E) and β-carotene have considerable support as lipid-soluble antioxidants; tocopherol might act synergistically with ascorbate. Vitamin C in living organisms regenerates vitamin E by reducing the tocopherol radical that is produced when vitamin E scavenges a peroxyl radical (Niki, 1987).
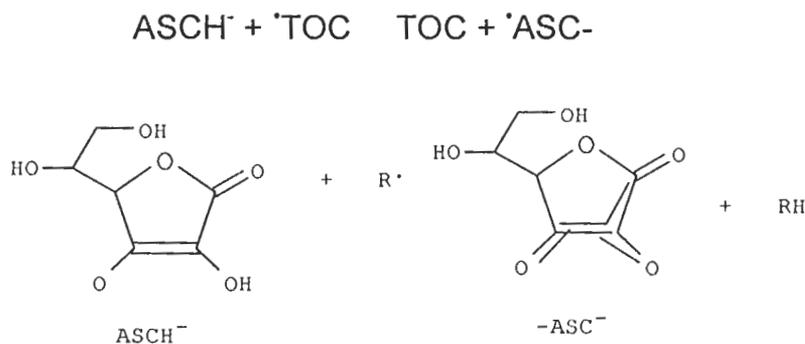
ASCH⁻ + ˙TOC   TOC + ˙ASC-

Fig. 2: Ascorbate free Radical Formation

This interaction is consistent with the results of a ^{13}C-NMR study, which showed that the phenolic head group of α-tocopherol in unilamellar vesicles is located very close to the lipid-water interface (Perly *et al.*, 1985).

Urate is another likely candidate for an antioxidant role in primates, as their blood has a higher concentration than that of other mammals. Urate might serve to scavenge R˙ and thereby account for the prolonged life span of humans (Ames *et al.*, 1981).

Free radicals can be listed by one-electron reduction potentials in millivolts (mV) at pH 7.0. The reduced form of each radical is capable of neutralizing (reducing) free radicals having a higher potential. As can be seen from the Table 1, the hydroxyl radical (˙OH) has the highest potential and is the most destructive (reactive) of biological free radicals.

Table I: Radical Reaction Potentials

Radical	mV
˙OH (hydroxyl)	+2300
˙LO (alkoxyl)	+1600
LOO˙	+1000
˙GS (glutathione)	+920
˙HU (Urate)	+590
˙TOC (Tocopherol)	+480
˙ASC (Ascorbate)	+282
Fe^{3+} - EDTA	+120

Vitamin C (Ascorbate ASCH⁻)

Vitamin C can donate a hydrogen atom to a free radical molecule (R˙) thereby neutralizing the free radical while becoming an ascrobate radical itself. It accumulates in many tissues, both in animal species that produce it and in those that absorb it as a vitamin. Considering the levels of ascorbate in humans compared with the plasma, it is highly concentrated in leukocytes, adrenal, pituitary and compartments of the eye (Evans *et al*, 1982).

Table I shows that the ascorbate radical/ascorbate thermodynamic couple is low compared with the reduction potential of the α-tocopherol free radical, the glutathione radical, the aliphatic alkoxyl and alkyl peroxyl radicals, and the hydroxyl free radical. Because of this, ascorbate will act as an antioxidant in each system, and also with

superoxide (Nishikimi, 1975), the urate free radical and other radicals not prevalent in the body such as nitroxides (Melhorn, 1991). The biological damaging reactive oxidative species come from a variety of sources, including ionizing radiation, oxygen metabolism and carcinogen metabolism. The ease with which ascorbate is oxidized has resulted in significant commercial utility; ascorbate, or its stereoisomers form, D-Isoascorbate, is effective in preventing (or reversing) oxidation in a wide variety of food products. The evidence is also strong that ascorbate has high reactivity with $R^{\cdot}$ in body fluids (Buettner and Jurkiewicz, 1993).

Ascorbate's efficacy as a scavenger depends on the reactivity of the ascorbyl free radical (AFR). If AFR were highly reactive with other substances at the biological pH, temperature, electrolyte composition, etc. the chain of free radical reactions would be propagated to completion as with other intermediate forms of $R^{\cdot}$ in the cell.

In addition to being well suited for an antioxidant role in biology, ascorbate has also been shown to have a pro-oxidant role *in vitro* (Borg and Schaich, 1989).

Chelation of Fe^{3+} with EDTA actually enhances the reactivity of iron toward superoxide, thus favouring the Haber-Weiss Reaction (Buettner and Jurkiewicz, 1996). Fe^{3+}–EDTA chelate can catalyze the Fenton Reaction to generate hydroxyl ion without reduction of Fe^{3+} to Fe^{2+} on the other hand, Fe^{3+} and Fe^{3+}–EDTA can be reduced to Fe^{2+} by ascorbate ($ASCH^{-}$) to generate the ascorbate radical ($^{\cdot}ASC^{-}$). The

reduced Iron can then generate a hydroxyl radical by the Fenton Reaction. Copper ion (Cu^{2+}) is 80 times more efficient at reacting with ascorbate than Fe^{3+} (Buettner and Jurkiewicz, 1996). Thus vitamin C can be a powerful antioxidant as long as metal ions are not present, but small amounts of vitamin C in the presence of metal ions can make vitamin C a dangerous pro-oxidant. Large amounts of vitamin C can restore the antioxidant function. (Vitamin C has been called an "oxymoron antioxidant") (Buettner and Jurkiewicz, 1996).

Ascorbate is present in many plants, microorganisms, and animals; it therefore appears to have been present throughout animal evolution. Evidence that early forms of life use ascorbate comes from the finding of ascorbate oxidase isoenzymes in tea leaves (Chen and Asada, 1989).

There are three (3) principal reasons for suggesting that ascorbate serves an important role as scavenger of free radicals in the human body:

(a) It is chemically suited to react with oxidizing free radicals;

(b) It is present in the body at sufficiently high concentrations to be effective;

(c) It fits into the physiology of cellular transport and metabolism. This combination of properties is well suited for this antioxidant molecule to contribute to the extended life span potential of humans (Cutler, 1984).

The most effective singlet oxygen quenchers are carotenoids, phytochemicals, which plants produce to protect themselves from singlet oxygen produced by ultraviolet light (Cutler, 1984).

Carotenoids

Of 600 carotenoids from natural sources that have been characterized, fewer than 10% serve as precursors of vitamin A. Many dietary carotenoids, both with and without provitamin A activity, are formed in the blood and tissues of humans (Bendich and Olson, 1989).

β-carotene, the most nutritionally active carotenoid, comprises 15–30% of total serum carotenoids (Bendich and Olson, 1989).

Green leafy vegetables and many coloured fruits are rich in carotenoids and polyenes, (Khachik *et al.*, 1986). In animal models, carotenoids have been implicated as chemo-protective or chemo-preventive agents in several kinds of cancer (Peto *et al.*, 1981), particularly skin cancer. Epstein (1977) first showed that injected β-carotene slowed the growth of skin tumors in hairless mice exposed to ultraviolet light (UV-A, UV-B). Similarly, feeding either β-carotene, canthaxanthin, or phytoene to hairless mice exposed to UV-B irradiation delayed the appearance of skin tumors and reduced their number (Mathews-Roth, 1982).

Carotenoids may protect cells from oxidative stress by quenching free radicals capable of causing cellular damage. Unsaturated lipids in cell membranes are prime targets for free radical reactions. A free

radical-mediated attack on lipid membranes can initiate a chain reaction that results first in lipid peroxidation and ultimately in functionally significant damage to membranes, enzymes and nucleic acids (Benedich and Olson, 1989).

Both *in vivo* and *in vitro*, β-carotene has been shown to protect isolated lipid membranes from peroxidation, LDL-containing lipids from oxidation, and liver lipids from oxidation induced by carbon tetrachloride-induced free radicals.

In chemical studies, the possible basis for the protective actions of carotenoids have been examined. Although β-carotene primarily has been studied, theoretically all carotenoids with a similar conjugated double bond system should act similarly (Krinsky and Deneke, 1982). In purely chemical studies, β-carotene interacts with peroxyl radicals irreversibly to form a carbon-centered carotenoid radical (Burton, 1989).

It is difficult to extrapolate directly from chemical and biological systems. For example, although antioxidant effects in a chemical system were noted at a carotenoid concentration of 50 μM, maximal inhibition of peroxidation was observed at 0.5 mM (Burton and Ingold, 1984). On the other hand, as singlet oxygen quenchers, low concentrations of carotenoids with nine or more conjugated double bonds can inhibit the peroxidation of linolenate (Burton, 1989).

At high oxygen tensions, α-tocopherol is the most effective antioxidant (Burton, 1989).

More than 20 epidemiological studies, both prospective and retrospective types, have shown that the risk of developing or dying from certain types of cancer (usually in both men and women) is inversely associated with the intake of carotenoid-containing fruits and vegetables and with higher levels of serum β-carotene concentration (Ziegler, 1989). In the case of lung cancer, which has been most closely associated with intake of fruits and vegetables, individuals with the lowest carotenoid intake or serum β-carotene concentrations were at a two- to sevenfold higher risk of developing neoplasms than those in the highest intake and highest serum level groups (Ziegler, 1989).

1.5   Oxidative stress

Oxidative stress is a medical term for damage to animal or plant cells (and thereby the organs and tissues composed of those cells) that occurs in normal metabolic processes through the production of "free radicals" caused by reactive oxygen species (Current Medicinal Chemistry, 2005). In the quest to find a "mate" and become stable, free radicals interact with the nearest molecule, targeting proteins, fats, or even DNA. These actions can be so violent that they create bursts of light within the body. If not neutralized rapidly, they may crate more free radicals or cause damage to vessel and cell walls, lipids, proteins and even the nucleus (DNA) of the cell, processes which can lead to cell death (apoptosis) by induction of mitochondrial membrane permeability

transition and release of apotogenic factors such as cytochrome C (Wei and Lee, 2002).

Oxidative stress could be defined as an imbalance between pro-oxidants prevailing (Current Medicinal Chemistry, 2005). Superoxide is produced deleteriously by I-electron transfers in the mitochondrial electron transfer chain. Other enzymes capable of producing superoxide are xanthine oxidase, NADPH oxidases and cytochrome P450(s). Hydrogen peroxide is produced by a wide variety of enzymes including monoxygenases and oxidases (Current Medicinal Chemistry, 2005).

Meals such as Iron, Copper, Chromium, Vanadium and Cobalt are capable of redox cycling in which a single electron may be accepted or donated by the metal. This action catalyzes reactions that produce reactive radicals and can produce reactive oxygen species such as hydroxyl radical in reactions like Fenton's reaction. The hydroxyl radical then can lead to modifications of amino acids (e.g. meta-tyrosine and ortho-tyrosine formation from phenyl-alanine) carbohydrates, initiate lipid peroxidation (Current Medicinal Chemistry, 2002).

Most enzymes that produce reactive oxygen species contain one of these metals. The presence of such metals in biological systems in an unsequestered form (not in an enzyme or other protein) can significantly increase the level of oxidative stress (Current Medicinal Chemistry, 2002). Under normal circumstances, there is a delicate balance between the production of oxidants and antioxidants. However,