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AN OVERVIEW ON ANTIOXIDANT ACTIVITY

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ABSTRACT

An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that can produce free radicals, leading to chain reactions that may damage cells. Antioxidants such as thiols or ascorbic acid (vitamin C) terminate these chain reactions. Most oxidations occur with the liberation of large amounts of energy. They are mainly derived from oxygen (reactive oxygen species/ROS) and nitrogen (reactive nitrogen species/RNS), and are generated in our body by various endogenous systems, exposure to different physicochemical conditions or patho-physiological states. Free radicals can adversely alter lipids, proteins and DNA (Deoxyribose nucleic acids) and have been implicated in aging and a number of human diseases including cancer. Antioxidants applications in this regard have become an interesting and introspective arena of research. Antioxidants can fight against these free radicals and protect the body from the various adversaries. The beneficial influence of many foodstuffs and beverages including fruits, vegetables, tea, coffee, and cocoa on human health has been recently recognized to originate from their antioxidant activity. Plant foods are known to protect against degenerative diseases and ageing due to their antioxidant activity (AOA) attributed to their high polyphenolic content (PC). This review article explains on antioxidants and methods used for determination of activity.

Keywords: Free radical, Antioxidant activity, Evaluation methods.

INTRODUCTION

An antioxidant is a molecule stable enough to donate an electron to a rampaging free radical and neutralize it, thus reducing its capacity to damage. These antioxidants delay or inhibit cellular damage mainly through their free radical scavenging property [1]. These low-molecular-weight antioxidants can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. Some of such antioxidants, including glutathione, ubiquinol, and uric acid, are produced during normal metabolism in the body [2]. Other lighter antioxidants are found in the diet. Although there are several enzymes system within the body that scavenge free radicals, the principle micronutrient (vitamins) antioxidants are vitamin E (α -tocopherol), vitamin C (ascorbic acid), and B-carotene. The body cannot manufacture these micronutrients, so they must be supplied in the diet [3].

History

The term antioxidant originally was used to refer specifically to a chemical that prevented the consumption of oxygen. In the late 19th and early 20th century, extensive study was devoted to the uses of antioxidants in important industrial processes, such as the prevention of

metal corrosion, the vulcanization of rubber, and the polymerization of fuels in the fouling of internal combustion engines [4]. Early research on the role of antioxidants in biology focused on their use in preventing the oxidation of unsaturated fats, which is the cause of rancidity [5]. Antioxidant activity could be measured simply by placing the fat in a closed container with oxygen and measuring the rate of oxygen consumption. However, it was the identification of vitamins A, C, and E as antioxidants that revolutionized the field and led to the realization of the importance of antioxidants in the biochemistry of living organisms. The possible mechanisms of action of antioxidants were first explored when it was recognized that a substance with antioxidative activity is likely to be one that is itself readily oxidized [6]. Research into how vitamin E prevents the process of lipid peroxidation led to the identification of antioxidants as reducing agents that prevent oxidative reactions, often by scavenging ROS before they can damage cells [7].

Antioxidant defense system

Antioxidants act as radical scavenger, hydrogen donor, electron donor, peroxide decomposer, singlet oxygen quencher, enzyme inhibitor, synergist, and metal-

chelating agents. Both enzymatic and nonenzymatic antioxidants exist in the intracellular and extracellular environment to detoxify ROS [8].

Mechanism of action of antioxidants

Two principle mechanisms of action have been proposed for antioxidants [9]. The first is a chain-breaking mechanism by which the primary antioxidant donates an electron to the free radical present in the systems. The second mechanism involves removal of ROS/reactive nitrogen species initiators (secondary antioxidants) by quenching chain-initiating catalyst. Antioxidants may exert their effect on biological systems by different mechanisms including electron donation, metal ion chelation, co-antioxidants, or by gene expression regulation [10].

Levels of antioxidant action

The antioxidants acting in the defense systems act at different levels such as preventive, radical scavenging, repair and *de novo*, and the fourth line of defense, i.e., the adaptation.

The first line of defense is the preventive antioxidants, which suppress the formation of free radicals. Although the precise mechanism and site of radical formation *in vivo* are not well elucidated yet, the metal-induced decompositions of hydroperoxides and hydrogen peroxide must be one of the important sources. To suppress such reactions, some antioxidants reduce hydroperoxides and hydrogen peroxide beforehand to alcohols and water, respectively, without generation of free radicals and some proteins sequester metal ions.

Glutathione peroxidase, glutathione-S-transferase, phospholipid hydroperoxide glutathione peroxidase (PHGPX), and peroxidase are known to decompose lipid hydroperoxides to corresponding alcohols. PHGPX is unique in that it can reduce hydroperoxides of phospholipids integrated into biomembranes. Glutathione peroxidase and catalase reduce hydrogen peroxide to water.

The second line of defense is the antioxidants that scavenge the active radicals to suppress chain initiation and/or break the chain propagation reactions. Various endogenous radical-scavenging antioxidants are known: some are hydrophilic and others are lipophilic. Vitamin C, uric acid, bilirubin, albumin, and thiols are hydrophilic, radical-scavenging antioxidants, while vitamin E and ubiquinol are lipophilic radical-scavenging antioxidants. Vitamin E is accepted as the most potent radical-scavenging lipophilic antioxidant.

The third line of defense is the repair and *de novo* antioxidants. The proteolytic enzymes, proteinases, proteases, and peptidases, present in the cytosol and in the mitochondria of mammalian cells, recognize, degrade, and remove oxidatively modified proteins and prevent the accumulation of oxidized proteins. The DNA repair systems also play an important role in the total defense system against oxidative damage. Various kinds of enzymes such as glycosylases and nucleases, which repair the damaged DNA, are known. There is another important function called adaptation where the signal for the production and reactions of free radicals induces formation

and transport of the appropriate antioxidant to the right site [11].

ENZYMATIC

Types of antioxidants

Cells are protected against oxidative stress by an interacting network of antioxidant enzymes. Here, the superoxide released by processes such as oxidative phosphorylation is first converted to hydrogen peroxide and then further reduced to give water. This detoxification pathway is the result of multiple enzymes, with superoxide dismutases catalyzing the first step and then catalases and various peroxidases removing hydrogen peroxide [12].

Superoxide dismutase

Superoxide dismutases (SODs) are a class of closely related enzymes that catalyze the breakdown of the superoxide anion into oxygen and hydrogen peroxide. SOD enzymes are present in almost all aerobic cells and in extracellular fluids. There are three major families of superoxide dismutase, depending on the metal cofactor: Cu/Zn (which binds both copper and zinc), Fe and Mn types (which bind either iron or manganese), and finally the Ni type which binds nickel [13].

Catalase

Catalase is a common enzyme found in nearly all living organisms, which are exposed to oxygen, where it functions to catalyze the decomposition of hydrogen peroxide to water and oxygen. Hydrogen peroxide is a harmful by-product of many normal metabolic processes: to prevent damage, it must be quickly converted into other, less dangerous substances. To this end, catalase is frequently used by cells to rapidly catalyze the decomposition of hydrogen peroxide into less reactive gaseous oxygen and water molecules [14].

Glutathione systems

The glutathione system includes glutathione, glutathione reductase, glutathione peroxidases, and glutathione S-transferases. This system is found in animals, plants, and microorganisms. Glutathione peroxidase is an enzyme containing four selenium-cofactors that catalyze the breakdown of hydrogen peroxide and organic hydroperoxides. There are at least four different glutathione peroxidase isozymes in animals. These enzymes are at particularly high levels in the liver and also serve in detoxification metabolism [15].

NON ENZYMATIC

Ascorbic acid

Ascorbic acid or "vitamin C" is a monosaccharide antioxidant found in both animals and plants. As it cannot be synthesized in humans and must be obtained from the diet, it is a vitamin. Most other animals are able to produce this compound in their bodies and do not require it in their diets. In cells, it is maintained in its reduced form by reaction with glutathione, which can be catalyzed by protein disulfide isomerase and glutaredoxins. Ascorbic acid is a reducing agent and can reduce and thereby

neutralize ROS such as hydrogen peroxide. In addition to its direct antioxidant effects, ascorbic acid is also a substrate for the antioxidant enzyme ascorbate peroxidase, a function that is particularly important in stress resistance in plants [16].

Glutathione

Glutathione is a cysteine-containing peptide found in most forms of aerobic life. It is not required in the diet and is instead synthesized in cells from its constituent amino acids. Glutathione has antioxidant properties since the thiol group in its cysteine moiety is a reducing agent and can be reversibly oxidized and reduced [17]. Due to its high concentration and central role in maintaining the cell's redox state, glutathione is one of the most important cellular antioxidants.

Melatonin

Melatonin, also known chemically as N-acetyl-5-methoxytryptamine, is a naturally occurring hormone found in animals and in some other living organisms, including algae. Melatonin is a powerful antioxidant that can easily cross cell membranes and the blood-brain barrier [18]. Therefore, it has been referred to as a terminal (or suicidal) antioxidant [19].

Tocopherols and tocotrienols (Vitamin E)

Vitamin E is the collective name for a set of eight related tocopherols and tocotrienols, which are fat-soluble vitamins with antioxidant properties. Of these, α -tocopherol has been most studied as it has the highest bioavailability, with the body preferentially absorbing and metabolizing this form [20]. It has been claimed that the α -tocopherol form is the most important lipid-soluble antioxidant, and that it protects membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction [21].

Uric acid

Uric acid accounts for roughly half the antioxidant ability of plasma. In fact, uric acid may have substituted for ascorbate in human evolution [22]. However, like ascorbate, uric acid can also mediate the production of active oxygen species.

PLANTS AS A SOURCE OF ANTIOXIDANTS

Synthetic and natural food antioxidants are used routinely in foods and medicine especially those containing oils and fats to protect the food against oxidation. There are a number of synthetic phenolic antioxidants, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) being prominent examples. These compounds have been widely used as antioxidants in food industry, cosmetics, and therapeutic industry. However, some physical properties of BHT and BHA such as their high volatility and instability at elevated temperature, strict legislation on the use of synthetic food additives, carcinogenic nature of some synthetic antioxidants, and consumer preferences have shifted the attention of manufacturers from synthetic to natural antioxidants. In

view of increasing risk factors of human to various deadly diseases, there has been a global trend toward the use of natural substances present in medicinal plants and dietary plants as therapeutic antioxidants. It has been reported that there is an inverse relationship between the dietary intake of antioxidant-rich food and medicinal plants and incidence of human diseases. The use of natural antioxidants in food, cosmetic, and therapeutic industry would be promising alternative for synthetic antioxidants in respect of low cost, highly compatible with dietary intake and no harmful effects inside the human body. Many antioxidant compounds, naturally occurring in plant sources have been identified as free radical or active oxygen scavengers [23]. Attempts have been made to study the antioxidant potential of a wide variety of vegetables like potato, spinach, tomatoes, and legumes. There are several reports showing antioxidant potential of fruits. Strong antioxidant activities have been found in berries, cherries, citrus, prunes, and olives. Green and black teas have been extensively studied in the recent past for antioxidant properties since they contain up to 30% of the dry weight as phenolic compounds.

Apart from the dietary sources, Indian medicinal plants also provide antioxidants and these include (with common/ayurvedic names in brackets) *Acacia catechu* (kair), *Aegle marmelos* (Bengal quince, Bel), *Allium cepa* (Onion), *A. sativum* (Garlic, Lahasuna), *Aleo vera* (Indian aloe, Ghritkumari) are also useful for their antioxidant activity.

ANTIOXIDANT POTENTIAL OF INDIAN FUNCTIONAL FOODS

In the last decade, preventive medicine has undergone a great advance, especially in developed countries. Research has demonstrated that nutrition plays a crucial role in the prevention of chronic diseases, as most of them can be related to diet. Functional food enters the concept of considering food not only necessary for living but also as a source of mental and physical well-being, contributing to the prevention and reduction of risk factors for several diseases or enhancing certain physiological functions. A food can be regarded as functional if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way which is relevant to either the state of well being and health or reduction of the risk of a disease. The beneficial effects could be either maintenance or promotion of a state of well being or health and/or a reduction of risk of a pathologic process or a disease. Whole foods represent the simplest example of functional food. Broccoli, carrots, and tomatoes are considered functional foods because of their high contents of physiologically active components (sulforaphen, B-carotene, and lycopene, respectively). Green vegetables and spices like mustard and turmeric, used extensively in Indian cuisine, also can fall under this category. "Nutraceutical" is a term coined in 1979 by Stephen DeFelice. It is defined "as a food or parts of food that provide medical or health benefits, including the prevention and treatment of disease." Nutraceuticals may

range from isolated nutrients, dietary supplements, and diets to genetically engineered “designer” food, herbal products, and processed products such as cereals, soups, and beverages. A nutraceutical is any nontoxic food extract supplement that has scientifically proven health benefits for both the treatment and prevention of disease. The increasing interest in nutraceuticals reflects the fact that consumers hear about epidemiological studies indicating that a specific diet or component of the diet is associated with a lower risk for a certain disease. The major active nutraceutical ingredients in plants are flavonoids. As is typical for phenolic compounds, they can act as potent antioxidants and metal chelators. They also have long been recognized to possess anti-inflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic activities [24].

Indian dietary and medicinal plants as functional foods

Ingredients that make food functional are dietary fibers, vitamins, minerals, antioxidants, oligosaccharides, essential fatty acids (omega-3), lactic acid bacteria cultures, and lignins. Many of these are present in medicinal plants. Indian systems of medicine believe that complex diseases can be treated with complex combination of botanicals unlike in west, with single drugs. Whole foods are hence used in India as functional foods rather than supplements. Some medicinal plants and dietary constituents having functional attributes are spices such as onion, garlic, mustard, red chilies, turmeric, clove, cinnamon, saffron, curry leaf, fenugreek, and ginger. Some herbs as *Bixa orellana* and vegetables like amla, wheat grass, soyabean, and *Gracinia cambogia* have antitumor effects. Other medicinal plants with functional properties include *A.marmelos*, *A. cepa*, *Aloe vera*, *A. paniculata*, *Azadirachta india*, and *Brassica juncea* [25].

FREE RADICAL

A free radical can be defined as any molecular species capable of independent existence that contains an unpaired electron in an atomic orbital. The presence of an unpaired electron results in certain common properties that are shared by most radicals. Many radicals are unstable and highly reactive. They can either donate an electron to or accept an electron from other molecules, therefore behaving as oxidants or reductants. The most important oxygen-containing free radicals in many disease states are hydroxyl radical, superoxide anion radical, hydrogen peroxide, oxygen singlet, hypochlorite, nitric oxide radical, and peroxy nitrite radical. These are highly reactive species, capable in the nucleus, and in the membranes of cells of damaging biologically relevant molecules such as DNA, proteins, carbohydrates, and lipids. Free radicals attack important macromolecules leading to cell damage and homeostatic disruption. Targets of free radicals include all kinds of molecules in the body. Among them, lipids, nucleic acids, and proteins are the major targets [26].

Production of free radicals in the human body

Free radicals and other ROS are derived either from normal essential metabolic processes in the human

body or from external sources such as exposure to X-rays, ozone, cigarette smoking, air pollutants, and industrial chemicals. Free radical formation occurs continuously in the cells as a consequence of both enzymatic and nonenzymatic reactions. Enzymatic reactions, which serve as source of free radicals, include those involved in the respiratory chain, in phagocytosis, in prostaglandin synthesis, and in the Cytochrome P450 system. Free radicals can also be formed in non-enzymatic reactions of oxygen with organic compounds as well as those initiated by ionizing reactions. Free radical reactions are expected to produce progressive adverse changes that accumulate with age throughout the body. Such “normal” changes with age are relatively common to all. However, superimposed on this common pattern are patterns influenced by genetics and environmental differences that modulate free radical damage. These are manifested as diseases at certain ages determined by genetic and environmental factors. Cancer and atherosclerosis, two major causes of death, are salient “free radical” diseases. Cancer initiation and promotion is associated with chromosomal defects and oncogene activation [27]. It is possible that endogenous free radical reactions, like those initiated by ionizing radiation, may result in tumor formation. The highly significant correlation between consumption of fats and oils and death rates from leukemia and malignant neoplasia of the breast, ovaries, and rectum among persons over 55 years may be a reflection of greater lipid peroxidation. Studies on atherosclerosis reveal the probability that the disease may be due to free radical reactions involving diet-derived lipids in the arterial wall and serum to yield peroxides and other substances. These compounds induce endothelial cell injury and produce changes in the arterial walls.

Free radical and aging

The human body is in constant battle to keep from aging. Research suggests that free radical damage to cells leads to the pathological changes associated with aging. An increasing number of diseases or disorders, as well as aging process itself, demonstrate link either directly or indirectly to these reactive and potentially destructive molecules. The major mechanism of aging attributes to DNA or the accumulation of cellular and functional damage [28]. Reduction of free radicals or decreasing their rate of production may delay aging. Some of the nutritional antioxidants will retard the aging process and prevent disease. Based on these studies, it appears that increased oxidative stress commonly occurs during the aging process, and antioxidant status may significantly influence the effects of oxidative damage associated with advancing age. Research suggests that free radicals have a significant influence on aging, that free radical damage can be controlled with adequate antioxidant defense, and that optimal intake of antioxidant nutrient may contribute to enhanced quality of life. Recent research indicates that antioxidant may even positively influence life span.

CONCEPT OF OXIDATIVE STRESS

The term is used to describe the condition of oxidative damage resulting when the critical balance

between free radical generation and antioxidant defenses is unfavorable. Oxidative stress, arising as a result of an imbalance between free radical production and antioxidant defenses, is associated with damage to a wide range of molecular species including lipids, proteins, and nucleic acids. Short-term oxidative stress may occur in tissues injured by trauma, infection, heat injury, hypertoxia, toxins, and excessive exercise. These injured tissues produce increased radical generating enzymes (e.g., xanthine oxidase, lipogenase, cyclooxygenase) activation of phagocytes, release of free iron, copper ions, or a disruption of the electron transport chains of oxidative phosphorylation, producing excess ROS. The initiation, promotion, and progression of cancer, as well as the side-effects of radiation and chemotherapy, have been linked to the imbalance between ROS and the antioxidant defense system. ROS have been implicated in the induction and complications of diabetes mellitus, age-related eye disease, and neurodegenerative diseases such as Parkinson's disease [29].

Oxidative stress and human diseases

A role of oxidative stress has been postulated in many conditions, including atherosclerosis, inflammatory condition, certain cancers, and the process of aging. Oxidative stress is now thought to make a significant contribution to all inflammatory diseases (arthritis, vasculitis, glomerulonephritis, lupus erythematosus, adult respiratory diseases syndrome), ischemic diseases (heart diseases, stroke, intestinal ischemia), hemochromatosis, acquired immunodeficiency syndrome, emphysema, organ transplantation, gastric ulcers, hypertension and preeclampsia, neurological disorder (Alzheimer's disease, Parkinson's disease, muscular dystrophy), alcoholism, smoking-related diseases, and many others. An excess of oxidative stress can lead to the oxidation of lipids and proteins, which is associated with changes in their structure and functions.

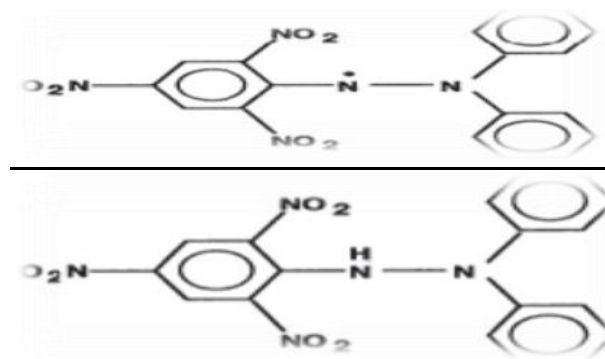
IN VITRO AND IN VIVO METHODS FOR EVALUATION OF ANTIOXIDANT ACTIVITY

In Vitro Methods

In vitro test procedures are carried out for evaluating antioxidant activities with the samples of interest. Generally in vitro antioxidant tests using free radical traps are relatively straightforward to perform. Among free radical scavenging methods, DPPH method is furthermore rapid, simple (i.e. not involved with many steps and reagents) and inexpensive.

DPPH Scavenging Activity

The molecule 1, 1-diphenyl-2-picrylhydrazyl (α,α-diphenyl-β-picrylhydrazyl; DPPH) is characterized as a stable free radical by virtue of the delocalisation of the spare electron over the molecule as a whole, so that the molecule does not dimerize, as would be the case with most other free radicals. The delocalization of electron also gives rise to the deep violet color, characterized by an absorption band in ethanol solution centered at about 517 nm.



$$\% \text{ inhibition of DPPH radical} = \frac{(\text{Abr} - \text{Aar})}{\text{Aar}} * 100$$

Hydrogen Peroxide Scavenging (H₂O₂) Assay

The ability of plant extracts to scavenge hydrogen peroxide can be estimated according to the method of Ruch *et al.* A solution of hydrogen peroxide (40 mM) is prepared in phosphate buffer (50 mM pH 7.4). The concentration of hydrogen peroxide is determined by absorption at 230 nm using a spectrophotometer. Extract (20–60 μg/mL) in distilled water is added to hydrogen peroxide and absorbance at 230 nm is determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging is calculated as follows:

$$\% \text{ scavenged [H}_2\text{O}_2] = \frac{[\text{A}_i - \text{A}_t]}{\text{A}_i} * 100$$

Superoxide Radical Scavenging Activity (SOD)

Although superoxide anion is a weak oxidant, it ultimately produces powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative stress. The superoxide anion scavenging activity can be measured as described by Gryglewski. The superoxide anion radicals are generated in 3.0 mL of Tris-HCl buffer (16 mM, pH 8.0), containing 0.5 mL of nitroblue tetrazolium (NBT) (0.3 mM), 0.5 mL NADH (0.936 mM) solution, 1.0 mL extract and 0.5 mL Tris-HCl buffer (16 mM, pH 8.0). The reaction is initiated by adding 0.5 mL phenazine methosulfate (PMS) solution (0.12 mM) to the mixture, incubated at 25 °C for 5 min and then the absorbance is measured at 560 nm against a blank sample. Other methods include hydroxyl radical scavenging activity, trolox equivalent antioxidant activity (TEAC), ferric reducing antioxidant power activity etc.

In Vivo Methods

For all in vivo methods the samples that are to be tested are usually administered to the testing animals (mice, rats, etc.) at a definite dosage regimen as described by the respective method. After a specified period of time, the animals are usually sacrificed and blood or tissues are used for the assay. It includes Glutathione reductase (GR) assay, c-Glutamyl transpeptidase activity (GGT) assay, LDL assay, Reduced glutathione (GSH) estimation, Superoxide dismutase (SOD) method etc [30].

CONCLUSION

Free radicals damage contributes to the etiology of many chronic health problems such as cardiovascular

and inflammatory disease, cataract, and cancer. Antioxidants prevent free radical induced tissue damage by preventing the formation of radicals, scavenging them, or by promoting their decomposition. Synthetic antioxidants are recently reported to be dangerous to human health. Thus the search for effective, nontoxic natural compounds with antioxidative activity has been intensified in recent years. In addition to endogenous antioxidant defense systems, consumption of dietary and plant-derived antioxidants appears to be a suitable alternative. Dietary and other components of plants form a major source of antioxidants. The traditional Indian diet, spices, and medicinal plants are rich sources of natural antioxidants; higher intake of foods with functional attributes including

high level of antioxidants in antioxidants in functional foods is one strategy that is gaining importance. Newer approaches utilizing collaborative research and modern technology in combination with established traditional health principles will yield dividends in near future in improving health, especially among people who do not have access to the use of costlier western systems of medicine.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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