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Antioxidants – A Pharmacological Overview

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Abstract

It is ironic that oxygen, an element indispensable for life, under certain situations has deleterious effects on the human body. Most of the potentially harmful effects of oxygen are due to the formation and activity of many chemical compounds, known as ROS, which have a tendency to donate oxygen to other substances. Free radicals and antioxidants have become commonly used terms in modern discussions of disease mechanisms [1]. In this modern world, due to the rapid advancement of civilization, industrialization, and overpopulation, scientific knowledge on antioxidants is important since most of the diseases are mediated through Reactive Oxygen Species (ROS).

Keywords: Antioxidants; Antioxidant Therapy; Free Radicals; Oxidative Stress

Introduction

Antioxidants have gained so much popularity mainly because they are known to be involved with so many biological processes such as tissue protection, immunity, health, maintaining homeostasis, aging, growth and development. Antioxidant has been defined as any substance that delays, prevents or removes oxidative damage to a target molecule [2,3] defined antioxidants as "any substance that directly scavenges ROS or indirectly acts to up-regulate antioxidant defenses or inhibit ROS production". In other words, we can define antioxidants as any molecule that inhibits the oxidation of another molecule. A chemical reaction involving the loss of electrons and increase in the oxidative state is termed as "oxidation." Oxidation results in the formation of free radicals that are unstable atoms and molecules deficit in electrons. 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]. 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 biochemistry of living organisms [6,7] that led to the identification of antioxidants as reducing agents that prevent oxidation reactions, often by scavenging reactive oxygen species before they can damage cells [8].

Classification of Antioxidants

Antioxidants may also be classified as enzymatic or non-enzymatic antioxidants [9].

Enzymatic (Endogenous)

The antioxidant enzymatic system directly/indirectly contributes to defense against the ROS. Catalase, superoxide dismutase (SOD), glutathione peroxidase, glutathione reductase, etc., are enzymatic antioxidants.

Non-Enzymatic

These antioxidants are quite a few, namely vitamins (A, C, E, and K), enzyme cofactors (Q10), minerals (Zn, Se, etc.), organosulfur compounds (allium and allium sulfur), nitrogen com- pounds (uric acid), peptides (glutathione), and polyphenols

(flavonoids and phenolic acid)

Mechanism of Action

Antioxidants neutralize free radicals by donating one of their electrons, which ends the electron stealing reaction. Antioxidants have been reported to work through single or combined mechanisms, namely, free radical scavenging, reducing activity, complexing of pro-oxidant, scavenging lipid peroxyl radicals, and quenching of singlet oxygen [9].

Two principle mechanisms in which antioxidants scavenge free radical are:

1. Chain-breaking mechanism by which the primary antioxidant donates an electron to the free radical present in the systems, or it simply decays into a harmless product. These antioxidants target free radicals and disrupt the chain reaction in the oxidation propagation phase. These make up most antioxidants in the industry [1].

2. Preventive antioxidant: These antioxidants block the formation of free radicals. This group includes metal chelators, which add to the efficacy of secondary or chain-terminating antioxidants. It prevents oxidation by reducing the rate of chain initiation. They can also prevent oxidation by stabilizing transition metal radicals such as copper and iron [10].

1.	Carotenoids (a form of vitamin A)	Apricots, peaches, broccoli, pumpkin, cantaloupes, carrots, spinach and sweet potatoes
2.	Beta-Carotene	Fruits, grains, oils and vegetables (carrot, green plants, spinach)
3.	Lycopene	Tomatoes
4.	Alpha Tocopherol (Vitamin E)	Nuts & seeds, whole grains, green leafy vegetables, vegetable oil and liver oil, eggs, poultry meat.
5.	Ascorbic acid (Vitamin C)	Citrus fruits like oranges and lime etc, green peppers, broccoli, green leafy vegetables, strawberries and tomatoes
6.	Selenium	Fish & shellfish, red meat, liver, yeast, grains, eggs, chicken and garlic
7.	Flavonoids	Green tea, grapes, apple, cocoa, berries, onion, broccoli.
8.	Resveratrol	Grapes, red wine, purple grape juice, peanuts, and some berries.

Most Commonly Known Antioxidants & Their Food Sources. [10]

Beneficial Effects of Antioxidants

Protect Against Heart Disease

The American Heart Association recommends a diet high in fruits, vegetables and other foods that contain antioxidants to help fight cardiovascular disease. They do not recommend antioxidant supplements, however, because there is no scientific evidence to support the idea that they have any beneficial effect on heart disease [11].

Protect Against Cancer

Lycopene is concentrated in tomato soups, sauces, tomato paste and other tomato products, and is also available in smaller amounts in fresh tomatoes, watermelon and pink grapefruit. Cancers of the mouth, pharynx, esophagus, stomach, colon and rectum can be prevented by lycopene and lutein may help decrease your risk of macular degeneration.

Boost Immunity

Vitamin C's ability to reduce the severity of the common cold is indicative of its effect on the immune system, according to experts at the Cleveland Clinic. Most fruits and vegetables provide some Vitamin C. Citrus fruits, kiwi, tomatoes and sweet peppers are particularly good sources.

Fight Aging

While it has not been shown that antioxidants actually increase anyone's lifespan, they do protect against some of the degenerative effects on the body of age-related diseases that can lead to early death. Studies on laboratory animals at the U.S. Agricultural Research Service suggest that a diet high in antioxidants, especially those found in blueberries, strawberries and spinach may also help fight the loss of brain function associated with aging

Eating a diet that includes a variety of fresh, deeply colored fruits and vegetables, such as broccoli, spinach, tomatoes, sweet peppers, carrots, mangoes, kiwi, berries and cantaloupe and other plant foods, such as grains, legumes (beans, lentils, and split peas) and nuts, is the safest and most effective way to boost your antioxidant supply and reap the health benefits these substances may convey [12].

Why Are They Used in Foods?

- **To Control Lipid Oxidation**

ROS attack unsaturated fatty acids which contain multiple double bonds and methylene groups.

Antioxidants scavenge radical and terminate chain reaction.

- **To Minimize Protein Modification**

ROS cause protein modification by nitration or chloration of amino acids.

Antioxidants scavenge O^{2-} And inhibit the formation of radicals causing nitration and chloration.

Free Radicals

They have unpaired electrons and are extremely reactive and are capable of initiating chain reactions that destabilize other molecules and generate free radicals. These free radicals are also termed as reactive oxygen species or ROS and create a homeostatic imbalance that generates oxidative stress and causes cell death and tissue injury. Free radicals are known to be formed as a result of environmental pollution, stress, cigarette smoke, UV Light, ionizing radiations, and xenobiotics. Toxic effect of the free radicals causes oxidative stress and results in the pathogenesis of diseases [13].

Ros are Generated by a Number of Pathways

Most of the oxidants produced by cells occur as:

1. A consequence of normal aerobic metabolism: approximately 90% of the oxygen utilized by the cell is consumed by the mitochondrial electron transport system.
2. Oxidative burst from phagocytes (white blood cells) as part of the mechanism by which bacteria and viruses are killed, and by which foreign proteins (antigens) are denatured.
3. Xenobiotic metabolism, i.e., detoxification of toxic substances. Consequently, things like vigorous exercise, which accelerates cellular metabolism; chronic inflammation, infections, and other illnesses; exposure to allergens and the presence of "leaky gut" syndrome; and exposure to drugs or toxins such as cigarette smoke, pollution, pesticides, and insecticides may all contribute to an increase in the body's oxidant load [14].

Molecular Damage Induced by Free Radicals

All the biological molecules present in our body are at risk of being attacked by free radicals. Such damaged molecules can impair cell functions and even lead to cell death eventually resulting in diseased states [15].

Lipids and Lipid Peroxidation

Membrane lipids present in subcellular organelles are highly susceptible to free radical damage. Lipids when reacted with free radicals can undergo the highly damaging chain reaction of lipid peroxidation (LP) leading to both direct and indirect effects. During LP a large number of toxic byproducts are also formed that can have effects at a site away from the area of generation, behaving as 'second messengers'. The damage caused by LP is highly detrimental to the functioning of the cell [16].

Carbohydrates

Free radicals such as $^{\bullet}OH$ react with carbohydrates by randomly abstracting a hydrogen atom from one of the carbon atoms, producing a carbon-centered radical. This leads to chain breaks in important molecules like hyaluronic acid. In the synovial fluid surrounding joints, an accumulation and activation of neutrophils during inflammation produces significant amounts of oxyradicals, that is also being implicated in rheumatoid arthritis.

DNA

Oxidative damage to DNA is a result of interaction of DNA with ROS or RNS. Free radicals such as $^{\bullet}OH$ and $H^{\bullet}$ react with DNA by addition to bases or abstractions of hydrogen atoms from the sugar moiety. The C4-C5 double bond of pyrimidine is particularly sensitive to attack by $^{\bullet}OH$, generating a spectrum of oxidative pyrimidine damage products, including thymine glycol, uracil glycol, urea residue, 5-hydroxydeoxyuridine, 5-hydroxydeoxycytidine, hydantoinand others. Similarly, interaction of $^{\bullet}OH$ with purines will generate 8-hydroxydeoxyguanosine (8-OHdG), 8-hydroxydeoxyadenosine, formamidopyrimidines and other less characterized purine oxidative products [17].

Proteins

Oxidation of proteins by ROS/RNS can generate a range of stable as well as reactive products such as protein hydro peroxides that can generate additional radicals particularly upon interaction with transition metal ions. Although most oxidised proteins that are functionally inactive are rapidly removed, some can gradually accumulate with time and thereby contribute to the damage associated with ageing as well as various diseases. Lipofuscin, an aggregate of per oxidized lipids and proteins accumulates in lysosomes of aged cells and brain cells of patients with Alzheimer's disease [18].

Antioxidant Protection

To protect the cells and organ systems of the body against reactive oxygen species, humans have evolved a highly sophisticated and complex antioxidant protection system. It involves a variety of components, both endogenous and exogenous in origin, that function interactively and synergistically to neutralize free radicals. Epidemiological researches strongly suggest that foods containing antioxidants and scavengers have a potential protective effect against disorders caused by ROS.

These components include|:

1. Nutrient-derived antioxidants like ascorbic acid (vitamin C), tocopherols and tocotrienols (vitamin E), carotenoids, and other low molecular weight compounds such as glutathione and lipoic acid.

2. Antioxidant enzymes, e.g., superoxide dismutase, glutathione peroxidase, and glutathione reductase, which catalyze free radical quenching reactions.
3. Metal binding proteins, such as ferritin, lactoferrin, albumin, and ceruloplasmin that sequester free iron and copper ions that are capable of catalyzing oxidative reactions.

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 [19]. 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 [20].

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 [21].

Oxidative Stress Test

In this advanced materialistic life, monitoring the levels of free radicals and oxidative stress is important in case of clinical practice. FORD (Free Oxygen Radicals Defense) is an easy, cheap and reliable diagnostic device to monitor oxidative stress [22,23]. It discriminates the high risk of oxidative damage on sick or healthy individuals, monitoring with precise laboratory parameters in the clinical situation at the baseline and in the follow-up of a medical prescription.

Ford (Free Oxygen Radicals Defense)

It is a colorimetric test based on the influence of antioxidants present in plasma to reduce the activity of free radicals. The principle of the assay is that at an acidic pH (5.2) and in the presence of a suitable oxidant solution ($FeCl_3$), 4-aminon, n- diethylaniline, the FORD chromogen, can form a stable and colored radical cation.

Antioxidant molecules (AOH) present in the sample which are able to transfer a hydrogen atom to the FORD chromogen radical cation, reduce it, quenching the color and producing a discoloration of the solution which is proportional to their concentration in the sample. This instrument will be helpful in understanding the problem of the individual bioavailability of each antioxidant molecule which can be monitored during the administration, with a pre-post measure of the oxidative balance. In order to achieve the evidence of the oxidative background related to the outcome of specific symptoms and diseases, epidemiological studies can be encouraged, and the role of nutrition and targeted antioxidant therapy can be better defined [19].

Pro-oxidants

Pro-oxidants are defined as chemicals that induce oxidative stress, usually through the formation of reactive species or by inhibiting antioxidant systems. Free radicals are considered pro-oxidants, but sometimes, antioxidants can also have pro-oxidant behavior. Vitamin C is a potent antioxidant, but it can also become a pro-oxidant when it combines with iron and copper reducing Fe^{3+} to Fe^{2+} (or Cu^{3+} to Cu^{2+}), which in turn reduces hydrogen peroxide to hydroxyl radicals [24].

α-Tocopherol is a powerful antioxidant, but in high concentrations, it can become a prooxidant. When vitamin E reacts with a free radical, it becomes a radical itself, and if there is not enough ascorbic acid for its regeneration, it will remain in this highly reactive state and support the autoxidation of linoleic acid [25].

Although not much evidence is found, it is proposed that carotenoids can also display prooxidant effects especially through autoxidation in the presence of high concentrations of oxygen-forming hydroxyl radicals [26]. Flavonoids may also serve as pro-oxidant [27].

Pro-oxidant Effect of Antioxidant Under Certain Conditions

Antioxidants also have the potential to act as prooxidants under certain conditions. For example, ascorbate, in the presence of high concentration of ferric iron, is a potent potentiator of lipid peroxidation. Recent studies suggest that ascorbate sometimes increase DNA damage in humans.

Recent mechanistic studies on the early stage of LDL oxidation show that the role of vitamin E is not simply that of a classical antioxidant. Unless additional compounds are present, vitamin E can have antioxidant, neutral or prooxidant activity. Beta-carotene also can behave as a prooxidant in the lungs of smokers.

The paradoxical role (pro-oxidant effect) of antioxidants is also directly related to the recently described 'redox signaling' of the antioxidants. The functional role of many antioxidants depends on redox cycling. For example, the best-described intracellular

antioxidant vitamin E supplementation in the face of infarcted myocardium exerted prooxidant effects resulting in the rupture of the plaques. When a cell is attacked by environmental stress, the cell's defense is lowered because of massive generation of ROS. The cell immediately responds to this stress by upregulating its antioxidant defense. During the induction process ROS function as signaling molecules.

It should be easily understood that in these pathophysiological conditions even though the antioxidants are lowered and supplementation of the antioxidants are warranted, the antioxidants should be harmful because they will prevent the function of the ROS to perform signal transduction to induce intracellular antioxidants [15].

Antioxidant Therapy

It is a way of treating patients with a variety of natural vitamins and nutritional elements in order to try to limit some kinds of degenerative conditions. Antioxidant therapy may include dietary changes as well as specific dietary supplements.

Antioxidants in Cosmetics

Antioxidants are very useful active ingredients for the manufacturing of cosmetics. Antioxidants are useful in two ways: On the one hand they prevent degradation of natural ingredients (proteins, sugars, lipids) in the cosmetic product. On the other hand, antioxidants protect the skin cells from being damaged and slow down the aging process.

Skin Care

Antioxidants protect the skin against sun damage and skin cancer. And they may actually reverse some of the discoloration and wrinkles associated with aging. These antioxidants work by speeding up the skin's natural repair systems and by directly inhibiting further damage

Hair Care

Antioxidants are extremely beneficial in the prevention of hair loss as well as stimulating new, healthy hair growth. "Some of the most powerful ones are green tea, blueberries and grape seed extract." More and more hair products are incorporating these ingredients into their formulas

Antioxidant Therapy: In Animals

Dog and cat foods, which often contain significant levels of fat, are especially susceptible to oxidation. The most common artificial antioxidants used in the pet food industry are ethoxyquin, butylated hydroxytoluene (BHT), and butylated hydroxyanisole (BHA). Commonly used natural antioxidants include tocopherols (vitaminE), ascorbic acid (vitamin C), citric acid, and rosemary. Ethoxyquin, which has been approved for use in animal feeds for over 30 years. It is currently allowed in dog foods at levels of up to 150 parts per million (ppm), or 0.015%. [28].

Assays of Determination

TAC assays may be broadly classified as electron transfer (ET)- and hydrogen atom transfer (HAT)-based assays

The DPPH (4-2,2-Diphenyl-1-Picrylhydrazyl) Method

A rapid, simple and inexpensive method to measure antioxidant capacity of food involves the use of the free radical 2,2-Diphenyl-1-picrylhydrazyl (DPPH). DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity of foods. It has also been used to quantify antioxidants in complex biological systems in recent years. The DPPH method can be used for solid or liquid samples and is not specific to any particular antioxidant component, but applies to the overall antioxidant capacity of the sample. A measure of total antioxidant capacity helps understand the functional properties of foods [29].

The FRAP (Ferric Reducing Antioxidant Power) Method

This method is simple, speedy, inexpensive, and robust does not require specialized equipment. FRAP assay also takes advantage of electron-transfer reactions. Here, a ferric salt, Fe (III) (TPTZ)2Cl3 (TPTZ) 2,4,6-tripyridyl*s*-triazine), is used as an oxidant. This method relies on the reduction by the antioxidants, of the complex ferric ion-TPTZ (2,4,6-tri(2-pyridyl)- 1,3,5-triazine). The binding of Fe^{2+} to the ligand creates a very intense navy blue color. The absorbance can be measured to test the amount of iron reduced and can be correlated with the amount of antioxidants. Trolox or ascorbic acid were used as references [29].

The ORAC (Oxygen Radical Absorption Capacity) Assay

This procedure is used to determine antioxidant capacities of fruits and vegetables. This method measures the antioxidant scavenging activity against the peroxyl radical, induced by 2,2'-azobis-(2-amidino-propane) dihydrochloride (AAPH), at 37°C. Fluorescein was used as the fluorescent probe. The loss of fluorescence was an indicator of the extent of the decomposition, from its reaction with the peroxyl radical. The advantage of the AUC approach is that it implies equally well for both antioxidants that exhibit distinct lag phase and those that have no lag phases. ORAC assay has been broadly applied in academy and in the food and dietary supplement industries as a method of choice to quantify AOC [29].

ORAC Values

One way of checking the antioxidant ability of vegetables and fruits is measuring its ORAC value or oxygen radical absorbance capacity. Some fruits/vegetables with their ORAC values/100 g in (brackets) are raisins (2830), black berries (2036), strawberries (1540), oranges (750), grapes (739), cherries (670), spinach (1260), beets (840), onion (450) and eggplant (390). Intake of fruits and vegetables with ORAC values between 3000 and 5000 per day is recommended to have significant impact of the beneficial effect of antioxidants [30].

The HORAC (Hydroxyl Radical Averting Capacity) Assay

This technique relies on the measurement of the metal-chelating activity of antioxidants, under the conditions of Fenton-like reactions. The method uses a Co(II) complex and hence evaluates the protecting ability against the formation of hydroxyl radical. Fluorescein is incubated with the sample to be analysed, then the Fenton mixture (generating hydroxyl radicals) was added. The initial fluorescence was measured, after which the readings were taken every minute after shaking. Gallic acid solutions were used for building the standard curve [31,32].

The TRAP (Total Peroxyl Radical Trapping Antioxidant Parameter) Assay

The luminol-enhanced chemiluminescence (CL) was exploited to monitor the reactions involving the peroxyl radical. The CL signal is driven by the production of luminol derived radicals, resulted from the thermal decomposition of AAPH. The TRAP value was determined from the duration of the time period during which the sample quenched the chemiluminiscence signal, due to the presence of antioxidants [32,33].

The Lipid Peroxidation Inhibition Assay

The lipid peroxidation inhibition assay method uses a Fenton-like system (Co(II) + H_2O_2), to induce lipid (e.g. fatty acid) peroxidation. α-linolenic acid was chosed as a model substrate. It was mixed with the analysed sample, as well as with the Fenton-like mixture, to induce lipid peroxidation. After the end of the incubation, the concentration of thiobarbituric acid-reactive substances (TBARS) was measured, as the index of lipid peroxidation. Lipid peroxidation was expressed in nmoles of TBARS per 1 ml of mixture α-linolenic acid/analysed sample [32,34].

The PFRAP (Potassium Ferricyanide Reducing Power) Method

An absorbance increase can be correlated to the reducing ability of antioxidants/antioxidant extracts. The compounds with antioxidant capacity react with potassium ferricyanide, to form potassium ferrocyanide. The latter reacts with ferric trichloride, yielding ferric ferrocyanide, a blue coloured complex, with a maximum absorbance at 700nm [35,36].

The CUPRAC (Cupric Reducing Antioxidant Power) Assay

The standard antioxidants or extracts are mixed with $CuSO_4$ and neocuproine. After 30 min, the absorbance was measured at 450 nm. In the assay, Cu(II) is reduced to Cu(I) through the action of electron donating antioxidants. Results are expressed in milligrams of Trolox per liter of extract [33].

Current Scenario

Combinations of antioxidants are proving to be better than using them separately, combination treatments are also becoming more popular individually. These have shown to include a large variety of antioxidant compounds such as carotenoids, polyphenols, vitamins and polysaccharides

- Antioxidant therapy in cows - Improves immune response which decreases mastitis in dairy cows and infectious disease incidences arising in stressed cattle following shipping

Newer and Novel Approaches to Reduce Free Radical Damage and Future Prospects

- Attention needs to be drawn on focusing more on disease-specific, target-directed, highly bioavailable antioxidants.
- Combination therapy of antioxidants.
- Targeted delivery of antioxidants.
- Antioxidant gene therapy.
- Development of genetically engineered plants.

There are several novel approaches in the study of free radicals/antioxidants for the improvement of human health. The total evidences from experimental, clinical, and epidemiological studies support the notion that consumption of foods obtaining high levels of dietary antioxidants, in addition to exerting several health benefits, may prevent or reduce the risk of oxidative stress.

Targeted delivery of antioxidants to mitochondria is a new exciting field of research that seeks to concentrate antioxidants on the inner membrane of mitochondria in order to protect against mitochondrial oxidative stress [37].

Antioxidant gene therapy has also recently been proposed as a treatment strategy that can overcome the problem of poor availability of the antioxidant at its target [38].

Development of genetically engineered plants, to yield vegetables with higher level of certain compounds is another approach to increase antioxidant availability. Tomatoes with up to 3 times lycopene concentration as well as with longer shelf life were developed [30].

Conclusion

Several decades have passed since the idea of antioxidant therapy was introduced for the first time. The field of antioxidants turned out to be much more challenging than what was presumed in the beginning. Much effort has been directed to the study of the efficacy of different antioxidants in human diseases, but unfortunately the products of this long process have not been satisfactory.

However, the lack of clear cut success in clinical trials does not disprove the crucial role of oxidative stress in diseases. We have learned many things along this way. Once we apply our experience to select the right disease and the right population, design optimized, and highly bioavailable antioxidants directed at specific and appropriate targets and choose optimal treatment times and durations, useful therapeutics could emerge for various diseases.

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