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A Review of In-Vivo and In-Situ Antioxidant Activity Models

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ABSTRACT

Because of our hectic lives and lack of physical activity, antioxidants are being heralded as a miracle cure for a wide range of lifestyle disorders, including aging, cancer, diabetes, cardiovascular disease, and other degenerative conditions. Pollution and exposure to dangerous chemicals add to these already devastating impacts. The buildup of dangerous free radicals may be caused by all of the aforementioned. This article delves further into several antioxidant activity models, both in vitro and in vivo.

INTRODUCTION:

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 1. An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals, which start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions by being oxidized themselves. As a result, antioxidants are often reducing agents such as thiols, ascorbic acid or polyphenols2. Although oxidation reactions are crucial for life, they can also be damaging; hence, plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C, and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxidases. Low levels of antioxidants, or inhibition of the antioxidant enzymes, causes oxidative stress and may damage or kill cells. As oxidative stress might be an important part of many human diseases, the use of antioxidants in pharmacology is intensively studied, particularly as treatments for stroke and neurodegenerative diseases. However, it is unknown whether oxidative stress is the cause or the consequence of disease. Antioxidants are also widely used as ingredients in dietary supplements in the hope of maintaining health and preventing diseases such as cancer and coronary heart disease. Although initial studies suggested that antioxidant supplements might promote health, later large clinical trials did not detect any benefit and suggested instead that excess supplementation may be harmful3. Antioxidants are classified into two broad divisions, depending on whether they are soluble in water (hydrophilic) or in lipids (hydrophobic). In general, water-soluble antioxidants react with oxidants in the cell cytosol and the blood plasma, while lipid-soluble antioxidants protect cell membranes from lipid peroxidation1. These compounds may be synthesized in the body or obtained from the diet4. The different antioxidants are present at a wide range of concentrations in body fluids and tissues, with some such as glutathione or ubiquinone mostly present within cells, while others such as uric acid are more evenly distributed. Some antioxidants are only found in a few organisms and these compounds can be important in pathogens and can be virulence factors5. In general, antioxidant systems either prevent these reactive species from being formed, or remove them before they can damage vital components of the cell6. However, since reactive oxygen species do have useful functions in cells, such as redox signaling, the function of antioxidant systems is not to remove oxidants entirely, but instead to keep them at an optimum level7.

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Free radicals may be defined as chemical species associated with an odd or unpaired electron. They are neutral, short lived, unstable and highly reactive to pair up the odd electron and finally achieve stable configuration. They are capable of attacking the healthy cells of the body, causing them to lose their structure and function. Cell damage caused by free radicals appears to be a major contributor to aging and degenerative diseases of aging such as cancer, cardiovascular disease, cataracts, immune system decline, liver diseases, diabetes mellitus, inflammation, renal failure, brain dysfunction and stress among others. 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, that functions interactively and synergistically to neutralize free radicals. Thus, antioxidants are capable of stabilizing or deactivating, free radicals before they attack cells 8. Antioxidants are absolutely critical for maintaining optimal cellular and systemic health and well-being. Free radicals are types of Reactive oxygen species (ROS), which include all highly reactive, oxygen-containing molecules. The reactive oxygen species produced in cells include hydrogen peroxide (H2O2), hypochlorous acid (HOCl), and free radicals such as the hydroxyl radical (•OH) and the superoxide anion (O2−)9. All these are capable of reacting with membrane lipids, nucleic acids, proteins and enzymes and other small molecules, resulting in cellular damage. In living organisms various ROSs can be formed in different ways, including normal aerobic respiration, stimulated polymorphonuclear leukocytes and macrophages and peroxisomes. These appear to be the main endogenous sources of most of the oxidants produced by cells. Exogenous sources of free radicals include tobacco smoke, ionizing radiation, certain pollutants, organic solvents and pesticides.

Naturally there is a dynamic balance between the amount of free radicals produced in the body and antioxidants to scavenge or quench them to protect the body against deleterious effects. The amount of antioxidant principles present under normal physiological conditions may be insufficient to neutralize free radicals generated. Therefore, it is obvious to enrich our diet with antioxidants to protect against harmful diseases. Hence there has been an increased interest in the food industry and in preventive medicine in the development of "Natural antioxidants" from plant materials. That is why plants with antioxidant properties are becoming more and more popular all over the world. Considering the importance of this area, we have listed some important in-vivo and in-vitro models for evaluating antioxidant activity.

In-vivo models for evaluating antioxidant activity:

Treatment: Rats are weighed at the beginning and at the end of experiment. Test drugs at various concentration depeding on the design of the experiment are administered for 3 days. Control rats are treated with the same volume of distilled water. Animals are stunned and decapitated 24 hr after the last dose. Killing is carried out at the same time of the day, to avoid the circadian variation in the level of tissue GSH. The livers and kidneys are rapidly removed, weighed, and part there of used for ascorbic acid determination. The rest of the tissues were frozen for not more than 72 hr to await analysis of reduce glutathione (GSH) and lipid peroxide (LP) concentrations. Care is to be taken to use the same parts of the tissue for the three measurements in each animal10.

Biochemical Determination: Assay of malondialdehyde (MDA): Lipid peroxidation was estimated in terms of Thiobarbituric acid reactive species (TBARS), using Malondialdehyde (MDA) as standard. The homogenized liver tissue (400 µl) was mixed with 10% TCA and incubated for 15 min at 4°C and then centrifuged at 2, 200 g for 15 min at 4°C. To 1 mL of protein-free supernatant, 1 mL of fresh TBA reagent was added, mixed thoroughly and incubated at 60°C for 1 h in water bath. Then optical density was measured at 532 nm for the assay of MDA. Lipid peroxide is expressed in terms of nM of MDA mg-1 of liver tissue11.

Assay of total tissue sulfhydryl group (reduced glutathione level): The soluble sulphydryl content of liver was determined according to the method of Grunert and Phillips 12. The metaphosphoric acid extract of liver or fractions, was saturated with NaCl and allowed to stand for 15-30 min and centrifuged at 3000 rpm for 10 min at 4°C. Take 1 mL of the aliquot of the supernatant and add to 3 mL saturated NaCl solution, allow it to stand for 10 min at 25°C. The nonspecific absorption in the sample was eliminated by reading the sample against a blank containing 2% metaphosphoric acid and Sodium nitroprusside. The colored complex developed is measured immediately at 520 nm on a colorimeter using blank tube.

Catalase assay: Catalase (CAT) activity was measured by monitoring decomposition of H2O2 according to the method of Johansson and Borg 13. The reaction was initiated by adding 50 µL of homogenized liver sample to the reaction mixture containing 250 mM PBS with 12 M methanol and 44 mM H2O2 and incubated at room temperature for 20 min. The reaction was terminated with addition of Purpald (22.8 mM) and again incubated at room temperature for 20 min. After adding potassium

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periodate (65.2 mM), the absorbance of the sample was measured at 550 nm. Catalse concentration was estimated by a standard graph plotted using known concentrations of formaldehyde and results expressed IU mg**-1** protein.

In-vitro models for evaluating antioxidant activity:

Conjugated diene assay: This method allows dynamic quantification of conjugated dienes as a result of initial PUFA (Poly unsaturated fatty acids) oxidation by measuring UV absorbance at 234 nm. The principle of this assay is that during linoleic acid oxidation, the double bonds are converted into conjugated double bonds, which are characterized by a strong UV absorption at 234 nm. The activity is expressed in terms of Inhibitory concentration (IC50)14-16.

DPPH Method (1, 1 diphenyl 2, picryl hydrazyl): This is the most widely reported method for screening of antioxidant activity of many plant drugs. DPPH assay method is based on the reduction of methanolic solution of colored free radical DPPH by free radical scavenger. The procedure involves measurement of decrease in absorbance of DPPH at its absorption maxima of 516 nm, which is proportional to concentration of free radical scavenger added to DPPH reagent solution. The activity is expressed as effective concentration EC50 17-19.

Hydroxyl radical scavenging activity: Hydroxyl radical scavenging capacity of an extract is directly related to its antioxidant activity. This method involves in-vitro generation of hydroxyl radicals using Fe3+ /ascorbate/EDTA/H2O2 system using Fenton reaction. Scavenging of this hydroxyl radical in presence of antioxidant is measured. In one of the methods the hydroxyl radicals formed by the oxidation is made to react with DMSO (dimethyl sulphoxide) to yield formaldehyde. Formaldehyde formed produces intense yellow color with Nash reagent (2M ammonium acetate with 0.05M acetic acid and 0.02M acetyl acetone in distilled water). The intensity of yellow color formed is measured at 412nm spectrophotometrically against reagent blank. The activity is expressed as % hydroxyl radical scavenging20.

Nitric oxide radical inhibition activity: Nitric oxide, because of its unpaired electron, is classified as a free radical and displays important reactivity's with certain types of proteins and other free radicals. In vitro inhibition of nitric oxide radical is also a measure of anti oxidant activity. This method is based on the inhibition of nitric oxide radical generated from sodium nitroprusside in buffer saline and measured by Griess reagent. In presence of scavengers, the absorbance of the chromophore is evaluated at 546 nm. The activity is expressed as % reduction of nitric oxide20.

Reducing Power Method: This method is based on the principle of increase in the absorbance of the reaction mixtures. Increase in the absorbance indicates increase in the antioxidant activity. In this method antioxidant compound forms a colored complex with potassium ferricyanide, trichloro acetic acid and ferric chloride, which is measured at 700nm. Increase in absorbance of the reaction mixture indicates the reducing power of the samples22.

Phospho molybdenum Method: It is a spectroscopic method for the quantitative determination of antioxidant capacity, through the formation of phospho molybdenum complex. The assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and subsequent formation of a green phosphate Mo (V) complex at acidic pH23.

Peroxynitrite radical scavenging activity: Peroxynitrite is now recognized by researchers as the culprit in many toxic reactions that were previously ascribed to its chemical precursors, superoxide and nitric oxide. Hence, an in vitro method for scavenging of peroxy radical has been developed to measure antioxidant activity. The scavenging activity is measured by monitoring the oxidation of dihydrorhodamine on a microplate fluorescence spectro-photometer at 485 nm24.

ABTS (2, 2-azinobis (3-ethyl benzothiazoline- 6-sulfonic acid) diamoniumsalt) Method: This is a measure of antioxidant activity as opposed to antioxidant concentration which might include a proportion of biologically inactive antioxidants. It also permits the measurement of antioxidant activity of mixtures of substances and hence helps to distinguish between additive and synergistic effects. The antioxidant activity of wines was measured by using this method. The assay is based on interaction between antioxidant and ABTS+ radical cation which has a characteristic color showing maxima at 645, 734 and 815nm25-28.

DMPD (N, N-dimethyl-p-phenylene diamine dihydrochloride) Method: This assay is based on the reduction of buffered solution of colored DMPD in acetate buffer and ferric chloride. The procedure involves measurement of decrease in absorbance of DMPD in presence of scavengers at its absorption maxima of 505nm. The antioxidant activity of wines was measured by using this method. The activity was expressed as percentage reduction of DMPD25-28.

Oxygen Radical Absorbance Capacity (ORAC): ORAC is an exciting and revolutionary new test tube analysis that can be utilized to test "Antioxidant Power" of foods and other chemical substances. It calculates the ability of a product or chemical to protect against potentially damaging free radicals. This analytical procedure measures the ability of a food, vitamin, nutritional supplement, or other chemicals to protect against the attack by free radicals, or to act as an Antioxidant. The test is performed using Trolox (a water-soluble analog of Vitamin E) as a standard to determine the Trolox Equivalent (TE). The ORAC value is then calculated from the Trolox Equivalent and expressed as ORAC units or value. The higher the ORAC value, the greater the "Antioxidant Power". This assay is based on generation of free radical using AAPH (2, 2-azobis 2-amido propane dihydrochloride) and measurement of decrease in fluorescence in presence of free radical scavengers. Hong et.al, (1996) have reported automated ORAC assay. In this assay b-phycoerythrin (b-PE) was used as target free radical damage, AAPH as a peroxy radical generator and Trolox as a standard control. After addition of AAPH to the test solution, the fluorescence is recorded and the antioxidant activity is expressed as trolox equivalent29-31.

b-Carotene Linoleate model: This is one of the rapid method to screen antioxidants, which is mainly based on the principle that Linoleic acid, which is an unsaturated fatty acid, gets oxidized by "Reactive Oxygen Species" (ROS) produced by oxygenated water. The products formed will initiate the b carotene oxidation, which will lead to discoloration. Antioxidants decrease the extent of discoloration, which is measured at 434nm and the activity is measured32.

Xanthine oxidase Method: This is one of the recent methods for evaluation of anti oxidant activity .The percentage inhibition in the xanthine oxidase activity in presence of anti oxidants is measured. Xanthine oxidase enzyme produces uric acid together with super oxide radicals from xanthine and the amount of uric acid is measured at 292nm33-34.

FRAP Method: FRAP (Ferric Reducing Ability of Plasma) is one of the most rapid test and very useful for routine analysis. The antioxidative activity is estimated by measuring the increase in absorbance caused by the formation of ferrous ions from FRAP reagent containing TPTZ (2, 4, 6 – tri (2 – pyridyl) – s – triazine) and FeCl36H2O. The absorbance is measured spectrophotometrically at 595nm35.

TRAP Method: This method is defined as total radical trapping antioxidant parameter. The fluorescence of R-Phycoerythrin is quenched by ABAP (2, 2'-azo–bis (2 -amidino- propane) hydrochloride) as a radical generator. This quenching reaction is measured in presence of antioxidants. The antioxidative potential is evaluated by measuring the delay in decoloration36.

Cytochrome C test: Superoxide anions were assayed spectrophotometrically by a cytochrome reduction method described by McCord and Fridovich (1969). Xanthine oxidase converts xanthine to uric acid and yields superoxide anions and these radicals directly reduce ferri- cytochrome C to ferro- cytochrome C, having an absorbance change at 550 nm. When test compounds showed superoxide scavenger activity, there was a decrease in the reduction of ferri-cytochrome C 37.

Erythrocyte ghost system: This method involves isolation of erythrocytes ghost cells and the induction of lipid peroxidation using erythrocyte ghosts and the induction of tetra-butyl hydroxyperoxide (t-BHP). TBARS (thio barbituric acid reactive substance) produced during the reaction is measured at 535 nm38.

Microsomal lipid peroxidation or Thiobarbituric acid (TBA) assay: TBA test is one of the most frequently used tests for measuring the peroxidation of lipids. Method involves isolation of microsomes from rat liver and induction of lipid peroxides with ferric ions leading to the production of small amount of Malonaldehyde (MDA). TBA reacts with MDA to form a pink chromagen, which can be detected spectrophotometricaly at 532 nm 39-40.

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