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Antioxidant Activity: An Overview

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Abstract

With advent in knowledge prevalence regarding health issues, natural antioxidants are gaining popularity. These are compounds responsible for hindering autoxidation reactions in food system and reducing oxidative stress in human body. This review aims to crystallize the information on antioxidants with regards to its classification, role in food processing and determination techniques. The review also locates some commercially available antioxidants as applied in food processing.

Keywords: *Antioxidants, classification, antioxidant activity, determination assays, toxicity*

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DEFINITION OF ANTIOXIDANT

Antioxidant means "against oxidation". Any substance at low concentrations compared to that of an oxidizable substrate that significantly delays or prevents oxidation of that substrate is called as antioxidant. Antioxidants play vital role in preserving the quality of food and maintaining health of human being.

NEED OF ANTIOXIDANTS

Oxidation reaction depending upon site of occurrences presents specific repercussions. If the site of occurrence is food system, then food deteriorates. When oxidation occurs in biological cell system, it causes damage or death to the cell.

The oxidative deterioration of fats and oils, when present as a component in foods, is responsible for rancid odor and flavor with a consequent decrease in nutritional quality, sensory appeal and safety. This is caused by the formation of primary hydroperoxides and secondary potentially toxic compounds through auto-oxidation of unsaturated fatty acids consisting of a free radical chain mechanism (Figure 1). The direct oxidation of unsaturated lipids with the double bond in a singlet state (no unpaired electrons, paired electrons are in the same orbital and have opposite spin) by oxygen in its ground triplet state (two free electrons in separate orbitals with same spin direction) is spin forbidden.

To overcome this spin barrier, initiators or catalysts are required to start the lipid oxidation process by removing an electron from either the lipid or oxygen or by changing the electron spin of the oxygen (Figure 2). As only trace amounts of catalysts are needed, many situations that appear to be spontaneous or uncatalyzed are actually driven by contaminants or conditions that have gone undetected or unconsidered. Indeed, in most foods, biological systems, and laboratory experiments, it is fair to say that multiple catalysts and initiators are always operative.

Thus, exposure of lipids to initiators like light, metals, singlet oxygen and sensitizers (chlorophyll, hemoproteins, and riboflavin), or preformed hydroperoxide decomposition products causes generation of primary hydroperoxides. Lipoxygenase-catalyzed oxidation also produces hydroperoxides [1]. The addition of antioxidants is required to control the oxidative deterioration.

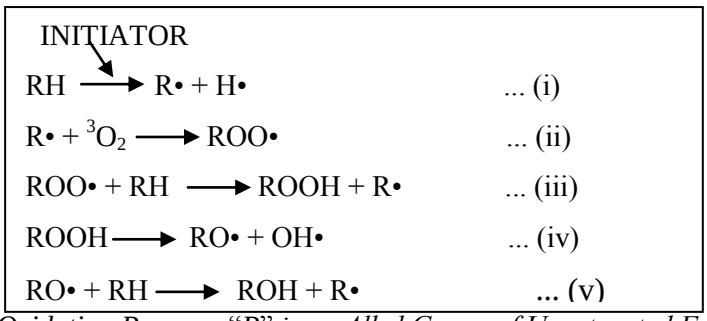

Fig. 1: *Reactions of Oxidation Process. "R" is an Alkyl Group of Unsaturated Fatty Acid, "H" is a α-methylenic Hydrogen Atom which is Easily Detachable, ''RO•' is Alkoxy Radical and 'ROO•' is Peroxy Radical.*

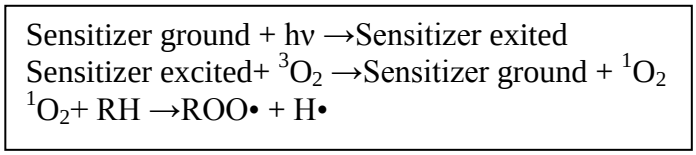

Fig. 2: *Formation of Hydroperoxides by Photoxidation of a Lipid with a Sensitizer (hv is Energy in the form of UV Light, Sensitizers That are Naturally Present in Photosensitive Pigments, their Degradation Products, or Polycyclic Aromatic Hydrocarbons Capable of Transferring Energy From Light to Chemical Molecules).*

In human body, about 5% of the inhaled oxygen is converted into reactive oxygen species which encompasses the hydroxyl radical, the superoxide anion radical, hydrogen peroxide, singlet oxygen, nitric oxide radical, hypochlorite radical, and various lipid peroxides. All are capable of reacting with membrane lipids, nucleic acids, proteins and enzymes, and other small molecules, resulting in cellular damage.

As a defense mechanism against reactive oxygen species, addition of antioxidants is required to food system. In human body, however, a variety of components, both endogenous (body's immune system) and exogenous in origin, function interactively and synergistically. As part of a healthy lifestyle and a well-balanced, wholesome diet, antioxidant supplementation is now being recognized as an important means of improving free radical protection.

CLASSIFICATION OF ANTIOXIDANT

Broadly, there are five major types of antioxidants [2] as described below:

- *Primary antioxidants* or chain breaking antioxidants are those compounds, mainly phenolic substances that terminate the free radical chains in lipid oxidation and function as hydrogen and electron donors. In addition, primary antioxidants chelate transition metals acting as catalyst in lipid oxidation.
- *Oxygen scavengers* are those substances which react with oxygen and can thus remove it in a closed system, e.g., ascorbic acid (vitamin C).
- *Secondary antioxidants* are those compounds which function by decomposing the lipid hydroperoxides into stable end products.
- *Enzymatic antioxidants* are those enzymes which function either by removing dissolved or head space oxygen, e.g., glucose oxidase, or by removing highly oxidative species, e.g., super oxide dismutase.
- *Chelating agents* are synergistic substances which greatly enhance the action of phenolic antioxidants. Most of these synergists exhibit little or no antioxidant activity, for example citric acid, amino acid, and phospholipids such as cephalin.

MECHANISM OF ACTION

Primary Antioxidant

Primary antioxidants work in three main ways to curb the oxidation reaction.

Chain Breakers or Free Radical Interceptors

Most of the primary antioxidants that act as chain breakers or free radical interceptors are mono or polyhydroxy phenols with various ring substitutions. As primary antioxidants (AH), they work on hydrogen atom transfer mechanism. In this, the antioxidant, reacts with highly reactive radical lipid and peroxy radicals (ROO•) and transfer one hydrogen atom to radical to form stable organic lipid derivatives and antioxidant radicals (A•) that are more stable and less readily available to participate in propagation reactions. Primary antioxidants have higher affinities for peroxy radicals than lipids and react predominantly with peroxy radicals (Figure 3) [3].

$$R\bullet + AH \longrightarrow RH + A\bullet \quad \text{... (vi)}$$

$$ROO\bullet + AH \longrightarrow ROOH + A\bullet \quad \text{... (vii)}$$

$$ROO\bullet + A\bullet \longrightarrow ROOA \quad \text{... (viii)}$$

$$RO\bullet + AH \longrightarrow ROH + AO\bullet \quad \text{... (ix)}$$

$$RO\bullet + A\bullet \longrightarrow ROH + R \quad \text{... (x)}$$

$$A\bullet + A\bullet \longrightarrow AA \quad \text{... (xi)}$$

Fig. 3: *Mechanism of Primary Antioxidant Activity (AH is an Antioxidant Molecule).*

Single Electron Transfer Mechanism

In single electron transfer mechanism (Figure 4), an electron is donated to free radical to form energetically stable anion, while the antioxidant forms a cation radical which is also a less reactive species.

$$R\bullet + AH \xrightarrow[\text{the highest occupied orbital}]{\text{electron abstraction form}} R^- + AH^+$$

Fig. 4: *Single Electron Transfer Mechanism of Antioxidant (AH).*

Metal Chelation

Third mechanism is metal chelation (Figure 5), as transition metals act as catalyst and also proxidants in oxidation reaction. The antioxidant forms a stable complex by ligating metal ions.

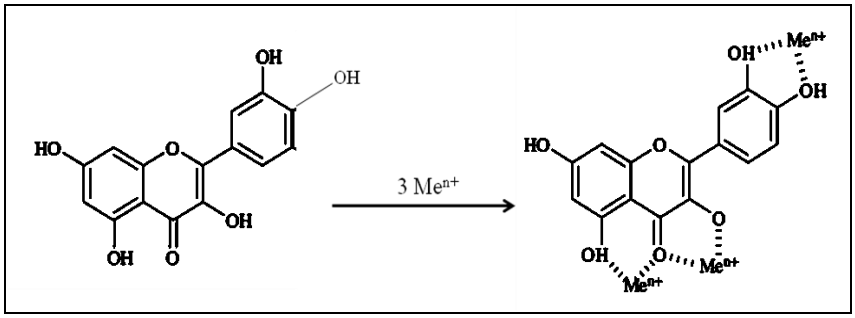

Fig. 5: *Transition Metal Chelation Mechanism of Antioxidant.*

Antioxidants Other Than Primary Antioxidants

Antioxidants other than primary antioxidants do not convert free radicals into stable molecules. They act as chelators for pro-oxidant or catalyst metal ions, provide H to primary antioxidants, decompose hydroperoxide to non-radical species, deactivate singlet oxygen, absorb ultraviolet radiation, or act as oxygen scavengers. They often enhance the antioxidant activity of primary antioxidants. Table 1 provides examples of some of these compounds.

Table 1: *Antioxidants with Mode of Action Excluding Free Radicals.*

Mode of Activity	Antioxidants
Metal chelation	Cirtic, Malic, Succinic and Tartaric acids Ethylenediaminetetraacetic acid, Phosphates
Oxygen scavenging and reducing agents	Ascorbic acid, Ascorbyl palmitate, Erythorbic acid, Sodium erythorbate, Sulfites
Singlet oxygen quenching	Carotenoids (β-Carotene, Lycopene and Lutein)

ANTIOXIDANT IN FOOD PROCESSING

Synthetic Antioxidants

Synthetic antioxidants are produced as pure substance, and therefore, can be applied as such or in mixtures with other pure substances in definite composition. Application is thus relatively easy, requiring no substantial modifications of the recipe and processing conditions. Among the synthetic types, the most frequently used antioxidants to preserve food are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tert-butyl hydroquinone (TBHQ).

The effectiveness of antioxidants varies depending on the food and conditions of processing and storage [4]. Antioxidants may lose their effectiveness during high-temperature treatment [5–7]. Decomposition products of TBHQ under frying temperatures in a model system were characterized by Kim and Pratt [8], where tert-butylbenzoquinone (TBBQ) was identified as the primary and major oxidation product. Such inter-conversion plays an important role in antioxidant effectiveness in food preservation. Antioxidants may even be lost through volatilization at high temperature, and the resulting volatiles may impair the stability of oils during thermal processing. Compounds with higher polarity have lower volatility. Consequently, due to its low polarity, BHT showed the highest volatility [9]. Depending on the type and concentration of individual components in mixture, interaction between antioxidants could lead to a negative or positive synergism during the high-temperature treatment. Food processing operations require antioxidants that survive high temperatures experienced during baking, cooling or frying and provide protection to finished products.

Nature Identical Antioxidant

Increasing concern over the safety of chemical additives in foods has given rise to an interest in replacing synthetic antioxidants with natural antioxidants. Most widely used natural antioxidants are not exactly purely natural, but nature identical. Their structure is the same as that of natural substances, but they are prepared by synthesis. They are supplied in a relatively pure state like other synthetic antioxidants and so can be added very easily in the amount desired. Antioxidants like tocopherol, ascorbic acid, citric acid and β-carotene belong in this group [10].

Botanical/Natural Antioxidant

Natural antioxidants are extracted, usually in a mixture of several compounds, from variable sources. The composition of the mixture containing active substance(s) and other compounds, which may be inactive or possessing negligible activities, depend on the plant variety, agro-technology, climatic conditions, degree of ripeness, and many other factors. Their composition should be determined in every batch, and if necessary, the procedure of their preparation or application, and the amount added to food products should be adapted according to analytical results.

Some botanicals which have long been known to exhibit antioxidant properties are shown in Figure 6.

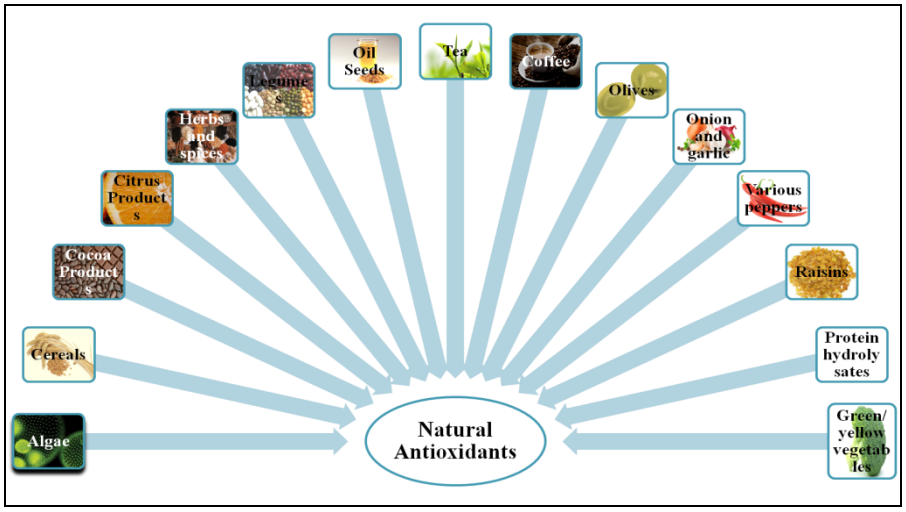

Fig. 6: *Some Sources of Natural Antioxidants among the Naturally Occurring Antioxidants, only a Few that are commercially Available.*

Food Extracts as Antioxidant

The most common natural antioxidants commercially exploited are tocopherols. They are present naturally in 0.02 to 0.2% by weight in edible oils and cereals [11]. It is recommended to keep α-tocopherol, the most potent of all tocopherols namely α, β, γ, and δ type, content at a level of 50 to 500 mg/kg of the substrate, depending on the nature of the foods.

Tocopherols present in crude vegetable oils get concentrated in the deodorizer distillate during the process of deodorization by the steam distillation of crude vegetable oils. The distillate is then separated into aqueous and organic layers. The organic layer contains most of the tocopherols transferred and is subsequently subjected to vacuum distillation to obtain the tocopherol concentrate. The extract can then be further concentrated by super critical (SC) CO_2 to obtain individual or mixed tocopherols. For antioxidant applications, extracted tocopherols (usually diluted in a vegetable oil), and synergistic mixtures composed of extracted tocopherols, ascorbyl palmitate, or other antioxidants, synergists, e.g., lecithin, citric acid, and other carriers, are marketed in oily form. Commercially, colorless and odorless mixed tocopherols of 50 and 70% strength, respectively, as Tenox GT-1 and Tenox GT-2 is marketed by Eastman Chemicals, Kingsport, Tennessee. Similarly, Henkel Corporation, United States, has two commercial products called Covi-ox T50 and T70 having similar concentrations of tocopherols. They represent the largest group of commercial natural antioxidants currently being marketed [12].

During the degumming of crude edible oils, lecithin is isolated from crude oil. Lecithin or its concentrates may also be used as a food additive with an antioxidant activity [10].

Spice and Herbal Extracts as Antioxidants

Spices and herbs are rich in antioxidant. They are used not only to enhance flavor but also the shelf-life of various foods in their natural form for their antioxidant characteristics. If they are applied to foods, they do not need to be declared as antioxidants. However, these are aromatic and pungent; therefore, their direct use as antioxidants is limited to foods that are usually seasoned. This propels extraction as a prerequisite for general use. Spices like clove, ginger, garlic, mace, nutmeg, etc., and labiatae herbs like rosemary, sage, thyme, and oregano are currently used for extracting natural antioxidants commercially. Oregano is found to be the most effective for lipid-containing foods. The extraction of antioxidants from rosemary and sage with edible vegetable oil has been patented [2].

Labex™, a commercial antioxidant oleoresin fraction from rosemary and sage, shows excellent performance in food preservation. The usage levels are sufficiently low for Labex™ antioxidants and accordingly there is no change in the original aroma and flavor of

the base food products. Labex™ has also been found to protect the color of paprika oleoresin during extended heating by preventing the oxidation of carotenoid pigments. It is GRAS and can be used at any level for any food applications. An evaluation of the antioxidant activity for a number of herbal extracts from Labiatae plants reveals that they are fully effective at a level of 200 to 300 ppm and are at least as strong as synthetic antioxidants BHA + BHT (1:1) mixture. Rosemary Deodorized™ is also marketed by Cal-Pfizer.

Commercial antioxidants from spice oleoresins, e.g., Spicer Extract AR™ marketed by Nestle, are normally in the form of fine powders. Depending on their content of active substances, it is recommended to use them at levels between 200 to 1000 mg/kg of finished product to be stabilized [2].

ANTIOXIDANT IN HUMAN HEALTH

Antioxidants in human health include:

- 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.
- Antioxidant enzymes, e.g., superoxide dismutase, glutathione peroxidase, and glutathione reductase, which catalyze free radical quenching reactions.
- Metal binding proteins, such as ferritin, lactoferrin, albumin, and ceruloplasmin that sequester free iron and copper ions that are capable of catalyzing oxidative reactions.
- Numerous other antioxidant phytonutrients present in a wide variety of plant foods. For instance, oligomeric proanthocyanidins present in grape seed extracts evidenced by clinical trials revealed fifty times more potential than vitamin C in regards with bioavailability and tissue repair mechanism [13].

EXTRACTION AND ISOLATION

The increasing preference for natural foods has obliged the food industry to include natural antioxidants in various products to delay oxidative degradation of lipids, improve quality and nutritional value of foods, and replace synthetic antioxidants. To be considered as an antioxidant, a compound must be free of any toxicological or physiological effect, no impairment of any strong odor or flavor to base food, and must have considerable antioxidant potential even at low concentration. To fulfill these pertaining aims, one has to extract and isolate these antioxidants reasonably from the natural sources. The aim of extraction and isolation is to concentrate the antioxidant components of the raw material, apart from inert substances, so that the product of the extraction could be added to the food in smaller quantities. Currently, extraction is being carried out using traditional methods including Soxhlet extraction, solid-liquid and liquid-liquid extraction. These methods have been associated with high solvent consumption, longer extraction times and an increased risk of thermal degradation of labile components. Soxhlet extraction has been the most regarded amongst all the conventional extraction methods even though it requires lengthy extraction times (up to 24 h or more) and high energy consumption. Additionally, the extended extraction times severely decreases efficiency, which is a tremendous liability in terms of commercial applicability. Alternative novel extraction procedures are now being practiced and studied to reduce extraction time and solvent consumption, increase extraction efficiency and improve antioxidant recovery. Some of these novel methods include supercritical fluid extraction (SFE), accelerated solvent extraction (ASE) (ASE is a new extraction technique that is similar in principle to Soxhlet extraction, but the use of elevated temperature and pressure with ASE allows the extraction to be completed within a short time and with a small quantity of solvent), ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE). Optimization and standardization of the extraction process is desperately needed to combat the increasing trend of antioxidant demand and usage.

DETERMINATION OF ANTIOXIDANT POTENTIAL

To satisfy the intended use in fetching benefits in health or food preservation, it is essential to assess antioxidant potential of the pertaining compound. Antioxidant capacity of the compound or extract could be determined *in-vitro* as well as *in-vivo.*

In-Vitro Estimation of Antioxidant Activity

Depending on the mechanism, the *in-vitro* estimations of antioxidants are designed on following principles:

- Estimation by free radical scavenging capacity
- Estimation by reduction of metal ions
- Estimation by inhibition of lipid peroxidation in plasma
- Estimation using cultured cell against oxidative stress

Estimation by Free Radical Scavenging Capacity

The primary mode of action of an antioxidant is scavenging free radicals either by hydrogen atom or single electron transfer. On the basis of this mechanism, antioxidant assays have been designed and utilized to assess potentiality of compound as an antioxidant. In these assays free radicals are added/generated and the potency is calculated on the basis of decrease in concentration of free radicals. The scavenging activity is either conducted versus stable radical like 2,2-diphenyl-1-picrylhydrazyl (DPPH) or done on comparison basis with other standard antioxidants like trolox, BHT, tocopheroal, gallic acid etc. Table 2 explains few of the assays with the stated approach to evaluate antioxidant potential.

Table 2: *Methods and Approaches to Measure Antioxidant Activity Based on Different Free Radicals.*

Methods	Principle of measurement	Methodology	References
DPPH scavenging assay	Ability to scavenge DPPH radicals generated in a model system	The reaction is set up between DPPH radicals and test compound, incubated for 30 min in dark followed by measuring absorbance at 517 nm.	[14]
Hydroxyl radical scavenging assay	Ability to scavenge hydroxyl radicals generated in a model system. H_2O_2 is the source for hydroxyl radicals	The test compound is mixed with reaction buffer and incubated for 1 h at 37°C. To the mixture, trichloroacetic acid and thiobarbituric acid are mixed and kept in boiling water bath for 10 min, followed by cooling to room temperature and measure the absorbance at 532 nm. The reaction mixture prepared by $FeCl_3$, ascorbic acid, ethylenediamine tetra acetate (EDTA) and deoxyribose in phosphate buffer (pH 7.4), and H_2O_2 in phosphate buffer. Each of these solutions were mixed well and used as reaction buffer for analysis.	[15]
Superoxide radical scavenging assay (SOSA)	Ability to scavenge superoxide radicals generated in a model system. Phenazine methosulphate is the source of superoxide radicals generated in a model system.	The reaction was started by adding Phenazine methosulphate (PMS) to the mixture of nitro blue tetrazolium (NBT), reduced Nicotinamide Adenine Dinucleotide (NADH) and sample. Then the reaction mixture is incubated at 25°C for 5 min and absorbance of reaction was measured at 560 nm against blank. The decrease in absorbance indicates increase in SOSA.	[16]
Oxygen radical absorption capacity (ORAC)	Ability to scavenge oxygen radical (peroxy radicals). Oxygen radicals are generated using 2,2'-azobis(2-amidino-propane) dihydrochloride.	In ORAC, fluorescent probe (like Fluorescein) and test sample are incubated in fluorescent tubes for 30 min at 37°C. Followed by addition of 2,2'-azobis(2-amidino-propane) dihydrochloride and reading fluorescence at $excitation_{485nm}$ and $Emission_{520nm}$.	[17]
Total radical trapping antioxidant parameter (TRAP)	Measure length of time that oxygen uptake needed for oxidation induced by thermal decomposition of 2,2'-Azobis(2-	TRAP measurement is carried out on organic substrate may be lipid or plasma. In this, the control (without test sample), experiment and standard	[18, 19]

	aminopropane) hydrochloride (AAPH)	samples are incubated with AAPH at 37°C. After adding AAPH the time duration for oxygen uptake by oxidizable substrate is measured. The time is regulated using oxygen electrode. The activity is expressed in terms of trolox. However, there is another approach for TRAP in which the rate of peroxidation induced by AAPH is monitored through the loss of fluorescence of the protein R phycoerythrin (R-PE). In the TRAP assay the lag-phase induced by plasma is compared with that induced by Trolox in the same plasma sample.	
Trolox equivalent antioxidant capacity (TEAC)	Based on neutralization of 2,2′-Azinobis(3-ethylbenzthiazoline)-6- sulfonic acid (ABTS) radical cation and mM concentration of a Trolox solution having antioxidant capacity equivalent to 1.0-mM solution of test substance.	The reaction is set up using generated ABTS radicals and test compound, incubated for 30 min in dark followed by measuring absorbance at 734 nm.	[14]
Total oxyradical scavenging capacity (TOSC)	Measures inhibition of ethylene production from oxidation of α-keto-γ-methiolbutyric acid (KMBA) by oxy radicals generated from thermal homolysis of 2,2′-azobis-amidinopropane (ABAP).	Test compound, ABAP and KMBA are added in gas-tight vials. After stipulated time interval ethylene production is measured by Gas Chromatography with flame ionization detector. The result can be expressed as TOSC = 100 – (ethylene production in test/ethylene production in control X 100)	[20]

Estimation by Reduction of Metal Ions

Transition metals act as catalyst in free radical chain reaction or autoxidation. Antioxidants, particularly phenolic compounds are capable of chelating metal ions and non-phenolic are capable of reducing metal ions by single electron transfer mechanism. Based on action mechanism against metal ions, two popular assays known as Ferric Reducing/Antioxidant Power (FRAP) and Cupric Ion Reducing *Antioxidant* Capacity (CUPRAC) have been established and utilized in assessment of antioxidant activity. In these the potency of the compound to reduce ferric [Fe(III)] and cupric [Cu(II)] to their respective lower valency states. In FRAP, the potency is measures through reaction between FRAP reagent [mixture of 2,4,6-TPTZ [2,4,6-tri (2-pyridyl)-1,3,5-triazine] solution in hydrochloric acid + ferric chloride + acetate buffer (pH 3.6)] and compound. The blue color developed is measured at 593 nm in spectrophotometer [21]. Ironically, Fe(II) and Cu(I) are more reactive ions than Fe(III) and Cu(II) in decomposing hydrogen peroxide and hydroperoxides, resultant will be prooxidant effect of antioxidants showing reducing action on ferric and cupric ions.

Estimation by Inhibition of Lipid Peroxidation in Plasma

This method, using plasma as reaction medium, has biological relevance with respect to evaluation of potency of hydrophilic and lipophilic antioxidants and their interaction in biological fluid. The assessment is done either by adding antioxidant to separated blood plasma of test animal or by first administering antioxidant to test animal and then separating plasma for further analysis. The testing parameters include cholesteryl linoleate hydroperoxide, a marker of lipid peroxidation in human plasma. Niki [22] revealed that the free radical scavenging capacity of an antioxidant does not necessarily correlate with the lipid peroxidation inhibition capacity.

Estimation using Cultured Cell against Oxidative Stress

Cultured cells have often been used as a substrate to elucidate the underlying mechanisms of oxidative stress and also to evaluate the protective effects of antioxidants against various oxidative stressors. The estimation provides the functional aspects of the antioxidant in human body for the suppression of Reactive oxygen species (ROS)

formation, oxidation of lipids, proteins and DNA, and cell death. In this method, the antioxidants are added to the culture medium simultaneously with the stressor or pre-incubated to incorporate in the cells [22].

The advantage of using cultured cells is that various different stressors and cell types for some specific disease can be used for evaluation of the antioxidant effects. It may also be noted that cultured cells may overcome the difficulty involved in procuring experimental animals in the future.

In-Vivo Estimation of Antioxidant Activity

In concern with the health benefits of the antioxidant, it is necessary to demonstrate the role and capacity of antioxidants *in-vivo*. These studies are necessary for elucidation, understanding and evaluation of antioxidant action, capacity and efficacy in human system. These studies are carried out by analysis of biological fluids and tissues of humans and experimental animals after intake of antioxidants. The studies are advantageous to judge the bioavailability of various antioxidants and effect of its dosage and duration to show the health beneficial effectuates. Oxidative stress has been associated with the origin of many diseases; it is of prime requirement of an antioxidant to regulate the stress level in biological system. The capacity and efficacy of antioxidant *in-vivo* may be assessed most accurately by the effect of antioxidant compounds on the level of oxidation in biological fluids and tissues. Generally following are used as biomarkers for estimation of antioxidant potential:

- Plasma, erythrocytes, urine, cerebrospinal fluids, saliva, and tears [22]
- Oxidative products of lipids including ratio of *cis,trans/ trans,trans* fatty acids (as free radicals induces isomerisation of unsaturated lipids from *cis* to *trans* form) [23]
- Oxidative modified proteins and sugars [24]
- Strand breaks of DNA and oxidative products of DNA bases [25]
- Levels of oxidation products of antioxidants like tocopheryl quinine, 5-nitro-γ-tocopherol, allantoin, and biopyrrin, antioxidant enzymes including superoxide dismutase, catalase, glutathione reductase, etc., and ratio of its oxidized/reduced forms, i.e., glutathione (GSSG/GSH), tocopheryl quinine/tocopheryl hydroquinone and ubiquinone/ubiquinol [22].

It has been reported that with increasing oxidative stress the ratios and amount of biomarkers increases [22].Considerations for antioxidant capacity determinations

The following drawbacks are usually associated with the estimation of antioxidants:

- Assays are sensitive to experimental details, i.e., different labs might measure different outcomes for same compound or biomarker.
- There is weak correlation between different assays of same compound. For instance, Cao and Prior [26] reported weak but significant correlation between serum ORAC and FRAP, but no correlation either between ORAC and TEAC or between FRAP and TEAC.
- Each antioxidant assay is a function of external interfering factors including time, temperature, and light intensity during reaction period, extract composition.
- High variations in expression of antioxidant activity units.

Because of various mechanisms between lipid and antioxidant interactions, complex heterogeneous biological and food-related systems, and inconsistency among the analytical results pertaining to antioxidant capacity, it is important to use a variety of methods for assessment of antioxidant capacity. As proposed by Becker et al. [27], evaluation of antioxidant capacity should be standardised on a four-step procedure as follows: (1) quantification and possible identification of phenolic compounds; (2) quantification of radical scavenging capacity and reduction potential; (3) evaluation of the ability to inhibit or halt lipid oxidation in model biological and food systems; and (4) storage studies using actual antioxidants incorporated in the food product, or human intervention studies using relevant biomarkers.

Moreover, when targeted for food processing, thermal stability of antioxidant as well as synergistic action, in presence of more than one, should also be evaluated.

TOXICOLOGICAL ASPECTS

In limelight, antioxidants are popular for suppressing oxidative stress and its related diseases, however, abusive and careless application may result in toxicological effects. Researchers have marked the delusive safety of natural antioxidants with risk of toxicity and array of side effects [28]. Conceptually, every antioxidant acts as pro-oxidant after certain concentration. To assure the function of antioxidant, it is necessary to study the effect of the antioxidant with respect to dosage, pro-oxidant action, side effects, bioavailability, and interaction with other nutrients.

Different studies have depicted pros and cons of antioxidants. Jakeman and Maxwell [29] found that vitamin C supplementation prior to the exercise resulted in a faster recovery of muscle strength, however, Urso and Clarkson [30] reported that antioxidant supplements could have a negative effect on recovery from muscle damaging exercise. The pro-oxidant effects of vitamins C and E have also been reported [28]. As pro-oxidants these vitamins creates transition metal ions.

The pro-oxidant effects of vitamin E supplements have been reported to cause fatal myocardial infarctions [28, 31] and inhibit glutathione S-transferase P 1-1 (GST P 1-1) [32]. Moreover, high levels of vitamin E have been reported to exacerbate impaired blood coagulation [33]. Bast and Haenen [28] reported some toxic metabolites of vitamin E like quinines which are highly cytotoxic. However, another metabolite 2,7,8-trimethyl-2-(β-carboxyethyl)-6-hydroxychroman has been reported to possess a strong nutraceutical effect [28,34]. γ-Tocopherol is an effective inhibitor of the cyclooxygenase enzyme (COX-1) [28] which is associated with significant damage to the gastrointestinal tract.

β-Carotene has been reported to act as an antioxidant at low oxidative stress, whereas under high stress conditions it stimulates lipid peroxidation [35]. Also, the unstable oxidised metabolites of β-carotene facilitate carcinogenesis. Similar to vitamin C and E, dihydrolipoic acid, a metabolic product of lipoic acid, can also function as a pro-oxidant [28]. Caffeic acid, a widely used antioxidant, may also act as a pro-oxidant under thermal treatment. In fact, highly reactive cations were generated during the early phases of caffeic acid degradation, affecting both the oxidative status and the reaction pathway of the system [36].

The fact list of repercussions related to abusive use of antioxidants relay the need of detailed toxicological studies, and specific standards to rule out toxicological effects. It is necessary to enlighten the general public regarding toxicological as well as beneficial effects of antioxidant in a balanced manner.

CONCLUSION

Because of various mechanisms between lipid and antioxidant interactions, complex heterogeneous biological and food-related systems, and inconsistency among the analytical results pertaining to antioxidant potency, it is important to use a variety of methods for assessment of antioxidant capacity. Additionally, antioxidants have been shown to possess toxicity and pro-oxidant action which calls for more research for better understanding and development of regulatory norms.

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