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Biological Activity Estimation and Chemical Analysis of Natural and Synthetic Antioxidants

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Abstract Antioxidants are an inhibitor of the process of oxidation, even at relatively small concentration and thus have diverse physiological role in the body. This review is a comprehensive article, which reviews the classification and sources of antioxidants with their biological activities including the mechanism of action. In addition, it gives the most trusted common methods to estimate the activities of antioxidants which are chosen from huge number of methods. The article also reviews the chemical analysis of the most common antioxidant.

Keywords Antioxidants, Synthetic, Natural, Classification, Sources, Biological Activity, Activity Estimation, Chemical Analysis

1. Introduction

Antioxidants are synthetic or natural molecules capable of delaying or preventing the oxidation of other molecules. They may protect cells from damage caused by free radicals. Antioxidants terminate these chain reactions by removing free radical intermediates. Antioxidants are often reducing agents such as thiols or polyphenols. They were thought to play a role in preventing the development of such chronic diseases as cancer, heart disease, stroke, Alzheimer's disease, Rheumatoid arthritis, and cataracts. In addition, it is believed that they provide excellent support for the immune system, protecting cells against premature, abnormal ageing, and help fight age-related macular degeneration [1,2].

In biochemistry and medicine, antioxidants are enzymes or other organic substances that can counteract the damaging effects of oxidation in animal tissues which is essential to most living organisms for production of energy and biological processes such as metabolic regulations, metabolic energy control, signal transduction, activation/inactivation of biomolecules, gene expression [3, 4].

The activity of antioxidant enzymes and non-enzyme defenses equate to the continuous production of free radicals. Under physiological conditions, oxidizing agents and antioxidant defenses are in balance. Living cells can either produce or take in anti-oxidative defense molecules, which include enzymes such as catalase, superoxide dismutase, glutathione peroxidase, and non-enzymatic antioxidants such as glutathione, and vitamins C and E. However, if the production of free radicals exceeds the antioxidant capacity of a living system, these reactive oxygen and nitrogen species can react with lipids, proteins, and DNA causing structural and/or functional harm to the cell's enzymes and genetic material [5]. The predominance of oxidants and their consequent damage is called oxidative stress. In chemistry, Free radicals are highly reactive molecules, which are electrically charged molecules, i.e., they have an unpaired electron, which causes them to capture electrons from other substances to neutralize themselves and cause

stability. As a result, another free radical is formed causing a chain reaction to occur. Several free radical reactions can occur within the short time of the initial reaction [6]. Antioxidants promote beneficial oxidation that produces energy and kill bacterial invaders. If free radicals are at reasonable levels, the human body produces antioxidants to resist them and is helpful in immune system and anti-bacterial cell activity. Antioxidants are capable of stabilizing, or deactivating, free radicals before they attack cells. Antioxidants are critical for maintaining optimal cellular and systemic health and well-being. Free radicals attack us from many different sources every day. They are tobacco, smoked and barbecued foods, harmful Chemicals and pesticides, and food additives…etc. When the production of damaging free radicals exceeds the capacity of the body's antioxidant defenses to detoxify them, a condition known as oxidative stress occurs. The cellular injury caused by oxidative stress has been linked to over 200 clinical disorders [7]. Table 1 shows some antioxidants in our food.

Table 1: Antioxidants in common food [8].

Source	Antioxidant
Ginger	Gingerd
Berries	Flavanols, hyroxycinamic acid, hydroxyl cinamic acids, anthocyanins
Citrus fruit	Flavanones, flavonols, phenolic acid
Green and black tea	Flava3ols, flavonols
Cherries	Hydroxycinamic acid, anthocyanins
Cider	Hydroxycinamic acid
Kiwi, plums, pears, apple	Hydroxycinamic acid, catechins
Parsley	Flavones
Spinach	Flavonoids, *p*- coumaric acid
Beans	Flavanols
Sweet potato	Flavonols, flavones
Wheat, rice	Caffeic, ferulic acid
Chocolate	Flavanols
Thyme	Thymol, caravacol, flavonoids, lubeolin
Rosemary	Caranosic acid, carnosol, rosmarinic acid, lateolin, rosmanol
Sage	Caranosic acid, carnosol, rosmarinic acid, lateolin, rosmanul

2. Medical Role of Antioxidants Against Diseases

The human body has a complex system of natural enzymatic and non-enzymatic antioxidant defenses, which counteract the harmful effects of free radicals and other oxidants. Free radicals are responsible for causing many diseases including cancer, cardiovascular disease, Parkinson's disease, Alzheimer's disease, mild cognitive impairment, neural disorders, ulcerative colitis and atherosclerosis [6]. Protection against free radicals can be enhanced by redundant intake of dietary antioxidants. Some evidence indicates that foods containing antioxidants and possibly in particular the antioxidant nutrients may be important in disease prevention [4]. There is, however, a growing consensus among scientists that a combination of antioxidants, rather than single entities, may be more effective over the long term. Antioxidants may be of great benefit in improving the quality of life by preventing or postponing the onset of degenerative diseases. In addition, they have a potential for substantial savings in the cost of health care delivery.

The nutritional supplies containing antioxidants play an important role in prevention of the consequences of free radicals' activity in the organism. It is considered that a suitable level of antioxidants supplied with diet induces immunological processes and increases defensive abilities of cell [9]. It is considered that tocopherols (vitamin E), ascorbic acid (vitamin C), vitamin A, and its provitamin β-carotene as well as selenium and phenolic compounds among antioxidants. Properties and significance of vitamins A, C and E were studied for a long time as valuable nutritional compounds [10]. Recently, attention has been paid to antioxidative properties of other compounds belonging to carotenoids, particularly to lycopene and lutein. It has been shown that lycopene is the most effective compound removing singlet oxygen, and its high consumption is correlated with prevention or protection of some

types of cancer [11]. Lutein plays a role in protection of retina against harmful effect of free radicals and in prevention of atherosclerosis [12].

3. Classification of Antioxidant Compounds and Their Structures

Antioxidants may be broadly grouped according to their mechanism of action, activity effect, the chemical nature, or the sources.

3.1 According to Mechanism

3.1.1 Preventive Antioxidant

These are enzymes as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GTX), glutathione reductase and some minerals like Se, Mn, Cu etc. SOD mainly acts by quenching of superoxide (O_2), catalase by catalyzing the decomposition of hydrogen peroxide (H_2O_2) to water and oxygen. GTX catalyzes the reduction of H_2O_2 and lipid peroxide generated during lipid peroxidation to water using reduced glutathione as substrate [13].

3.1.2 Radical Scavenging Antioxidant

They are glutathione, vitamin C, uric acid, albumin, bilirubin, vitamin E, carotenoids, flavonoids. β-carotene is an excellent scavenger of singlet oxygen. Vitamin C works as scavenger of oxidizing free radicals and oxygen-derived species, such as hydroxyl radical, hydrogen peroxide (H_2O_2), and singlet oxygen. GSH is a good scavenger of many free radicals and various lipid hydroperoxides and may help to detoxify many inhaled oxidizing air pollutants like ozone [14].

3.1.3 Repairing Enzymes

These are a complex group of enzymes for repair of damaged DNA, protein, oxidized lipids and peroxides and also to stop chain propagation of peroxyl lipid radical. These enzymes repair the damage to biomolecules and reconstitute the damaged cell membrane [2]. Reports of glutathione transferases and peroxidases are also involved for repairing oxidized DNA. There are some publications that showed that several prokaryotic and eukaryotic enzymes repair oxidized DNA by both direct and excision repair mechanisms [15-17].

3.2 According to Activity

3.2.1. Primary Antioxidants

Primary antioxidants are referred to as type I or chain-breaking antioxidants. Because of the chemical nature of these molecules, they can act as free radical acceptors and delay or inhibit the initiation step or interrupt or stop the propagation step of autoxidation [18, 19]. Table 2 shows some primary antioxidants.

Table 2: Some common primary antioxidants

Compound	Source	Usage
Carotenoids	Natural	Therapeutic use
Flavonoids	Natural	Medical use
Phenolic compounds	Natural	Food industries
Tocopherols	Natural	Medical use
Propyl galate (PG)	Synthetic	Lipid preservative
Butylated hydroxyanisole (BHA)	Synthetic	Lipid preservative
Butylated hydroxyl toluene (BHT)	Synthetic	Lipid preservative
Tertiary butylhydroxyquinone (TBHQ)	Synthetic	Food preservative

3.2.2 Secondary Antioxidants

Secondary antioxidants are classified as preventive or class II antioxidants. They offer their antioxidant effect through several mechanisms to slow the rate of oxidation process. The main difference with primary antioxidants is that the secondary antioxidants do not convert free radicals into stable molecules. They act as chelators for prooxidant or catalyst metal ions, provide H to primary antioxidants, decompose hydroperoxide to nonradical

species and consequently, deactivate singlet oxygen. They absorb ultraviolet radiation, and /or act as oxygen scavengers. They often enhance the antioxidant activity of primary antioxidants. Table 3 provides examples of some of these compounds that exhibit secondary antioxidant activity [19].

Table 3: Some common secondary antioxidants and their activities mode

Compounds	Activity
EDTA, citric acid, Succinic acid, tartaric acid,	Metal chelation
Ascorbic acid, Erythrobic acid, Sulfites, Ascorbyl palmitate	Oxygen scavenging and reducing agents
Carotenoids	Singlet oxygen quenching

3.2.3 Synergistic Effect

Synergism is the cooperative effect of antioxidants or an antioxidant with other compounds to produce enhanced activity than the sum of activities of the individual component when used separately [19]. Two types of synergism are known. One contains primary antioxidants only and the other contains a combination of primary antioxidants with metal chelators or peroxyl scavengers.

3.3 According to the chemical nature

Antioxidants can be divided according to the nature of the compounds such as high or low molecular weight compounds, enzymes, vitamins, minerals …etc. This classification is shown in table 4 [19, 20].

Table 4: Classification of antioxidants according to chemical nature

Non-Enzymatic antioxidant		Enzymatic antioxidant	
Compound class	**Examples**	**Compound class**	**Examples**
Polyphynolic compounds • Xanthones • Flavonoids • Flavanols • Flavanones • Flavones • Isoflavanoids • Anthocyanidins • Hydroxycinnamic acids • Hydroxybenzoic acid • Gingerol • Curcumin	Mangostin, Quercein, Kaempferol, Catechin, EGCG, Hesperitin, Chrysin, Genistein, Cyanidin, Pelagonidin, Ferulic, *p*-coumaric, Gallic acid, Ellagic acid	Primary antioxidants	SOD, Catalase, Glutathione peroxidase
Carotenoids	Carotene, Lycopene, Lutein, Zeaxanthin	Secondary enzymes	Glutathione reductase, Gluose 6-phosphate dehydrogenase
Antioxidant cofactors	Coenzyme O_{10}		
Organosulfur compounds	Allium, Allyl sulfide, indoles		
Low molecular weight Antioxidants:	glutathione, uric acid		
Vitamins	Vitamin A, Vitamin C, Vitamin E, Vitamin F		
Minerals	Zinc, Selenium		

3.4 According to Sources

They are classified into two categories: natural or synthetic compounds.

3.4.1. Natural Antioxidant

The natural antioxidants are phenolic compounds (flavonoids, tocopherols, and phenolic acids), nitrogen compounds (amino acids, alkaloids, amino acids, chlorophyll derivatives, and amines), carotenoids or ascorbic acid. Figure 1 represents some of the most common natural antioxidants. Natural antioxidants are present mainly in plants, and this explains that the sources of these compounds are plant-derived products. Antioxidant vitamins are soluble in lipids

and selenium occur also in food derived from animals (milk and eggs) but in small amounts. The richest sources of antioxidants are fruits, vegetables, cereals and legumes, tea, and spices [19, 21].

Vitamin C (Ascorbic Acid)

Ascorbic acid (vitamin C) is known for its antioxidant activity and is consequently used in cosmetics and degenerative disease treatments. Vitamin C has many physiological functions, among them a highly antioxidant power to recycle vitamin E in membrane and lipoprotein lipid peroxidation. On the other hand, vitamin C is also capable of pro-oxidant activity, in vitro. It is known that the combination of ascorbate and ferrous ions generates hydroxyl radicals, which induces lipid peroxidation [22]. Vitamin C is a potent antioxidant for hydrophilic radicals, but poor against lipophilic radicals.

Vitamin E (Tocopherols)

Tocopherols are widely distributed in nature. Vitamin E is the common name given to a group of lipid-soluble compounds of which α-tocopherol is the most common. It is found in lipoproteins and membranes and acts to block the chain reaction of lipid peroxidation by scavenging intermediate peroxyl radicals being generated. The highly steric hindered tocopheryl radical is much less reactive to attack fatty acid side chains and consequently breaks the chain reaction [22].

Carotenoids

Carotenoids protect lipids against peroxidative damage by inactivating singlet oxygen reacting with hydroxyl, superoxide, and peroxyl radicals. Carotenoids are not particularly good quenchers of peroxyl radicals, but they are exceptional at quenching singlet oxygen, at which most other phenolics and antioxidants are relatively ineffective. The antioxidant activity of carotenoids is due to the ability to delocalize unpaired electrons through their structure of conjugated double bonds. Some forms of cancer and cardiovascular disease appear to be lower in populations with large relative intakes of antioxidant nutrients such as vitamins C, and E, and the various carotenoids [4,5]. β-carotene is the most abundant of the carotenoids and widely used in therapies. Many studies have shown that β-carotene inhibits lipid auto-oxidation in biological tissues and food [23].

Phenolic Compounds

Phenolic compounds are commonly found in plants, and they have been reported to have antioxidant activity. Crude extracts of plant materials rich in phenolic compounds are widely used in the food industry because they retard oxidative degradation of lipids and improve the nutritional value of food. The importance of antioxidant constituents of plants is increased due to their health effects to protect from coronary heart disease, and cancer [24]. Phenolic compounds derived from phenylalanine and tyrosine occur ubiquitously in plants and are much diversified [25].

Flavonoids

Flavonoids naturally occur in plants, and they have potent antioxidant activities (e.g., Quercetin). Studies on flavonoidic derivatives have shown a wide range of antibacterial, antiviral, anti-inflammatory, anticancer, and anti-allergic activities [26, 27]. With their biological activity, flavonoids are important components in the human diet. Sources of flavonoids are foods, beverages, different herbal drugs 27. The antioxidant activity of flavonoids was the first study of the mechanism of action of the antioxidant as protective compounds against cardiovascular diseases. Flavonoids exhibit a highly effective scavenging activity of the most oxidizing molecules, including singlet oxygen, and various free radicals responsible for several diseases [28].

Essential Oils

Essential oils (volatile oils) are mixture of several constituents in the form of oily liquids obtained from different plant parts, and widely used as food flavors. Chemically, they are derived from terpenes, and their oxygenated compounds. Monoterpenes are primary components of essential oils, and the effects of many medicinal herbs have been attributed to them. Among various monoterpenes that have antioxidant activity are carvacrol, thymol, and γ-terpinene [29]. Essential oils have been useful in food preservation and aromatherapy [30]. In nature, essential oils have an important role in protecting plants. They serve as antibacterial agents, antivirals, antifungals, and insecticides. In addition, it works against the action of herbivores [30].

Selenium

The bioactivity of selenium was proved, in practice, in the second part of last century. The presence of selenium in glutathione peroxidase (GSHPx), which plays main protective role against oxidation of cell membranes lipids and involved in hydrogen peroxide (H2O2) and lipids' hydroxyperoxides metabolism. Selenium plays the role like vitamin E, and sometimes can substitute vitamin E in its function. It acts as an antioxidant and protects cell membranes against free radicals' generation and consequently the risk of cancer and cardiovascular diseases decreases [31].

Vitamin C | Vitamin E

β-Carotene | Thymol

γ-terpinene | Carvacrol | Quercetin

Tannic acid

Figure 1: Some Examples of common natural antioxidants

3.4.2 Synthetic Antioxidants

Synthetic antioxidants are phenolic compounds with various degrees of alkyl substitution 32. The structures of the most common synthetic antioxidants are shown in figure 6. Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and propyl gallate are well known antioxidants used for long time to inhibit lipid oxidation. Synthetic antioxidants are commonly used to preserve food. Restrictions on the use of such compounds are considered because of their participation in harmful effects on human health. Thus, the importance of natural antioxidants has increased considerably 32. Consumption of fruit and vegetables containing natural antioxidants is the main cause of decreasing risks for diseases such as cancer and cardiovascular diseases [33].

In the food industry, synthetic antioxidants such as BHT have been widely used as additives to preserve and stabilize foods and animal feed products for freshness, nutritive value, flavor, and color. A study has shown BHT to be toxic, especially in high doses and with long-term dietary intake [34]. Recently, the toxicity of synthetic chemical antioxidants has been considered and some studies have begun to investigate the potential of plant products such as phenolics, flavonoids, tannins, proanthocyanidins to serve as antioxidants for protection against free radicals. Synthetic antioxidants Trolox, and Tertiary butylhdroxyquinone (TBHQ) are widely used. TBHQ is a highly effective antioxidant used in foods as a preservative for unsaturated vegetable oils and many edible animal fats.

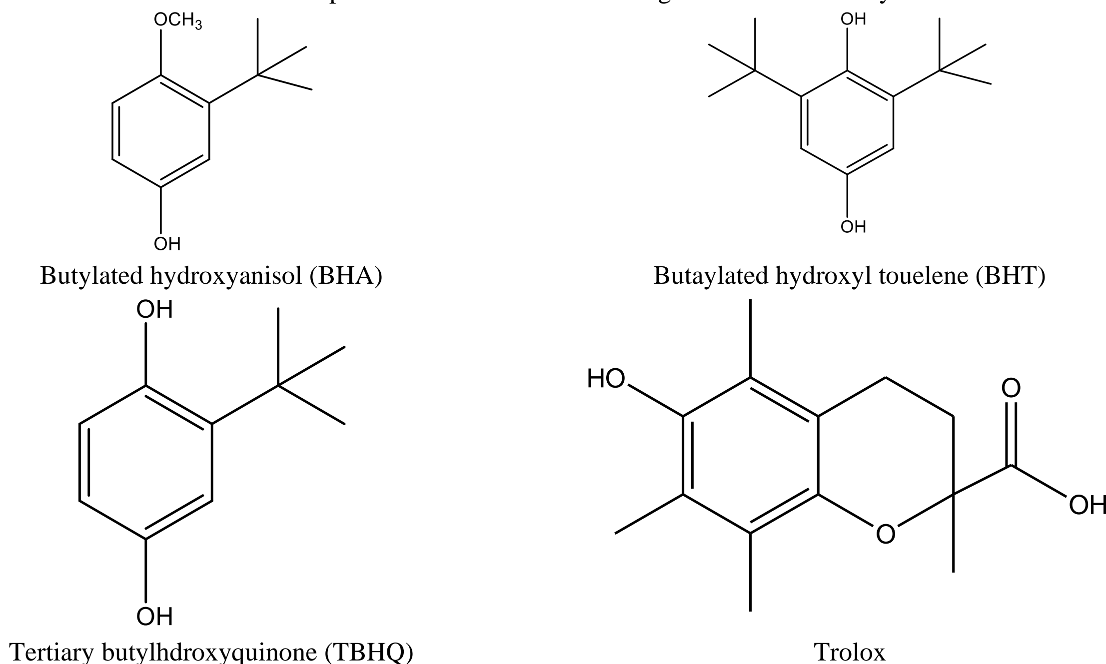

Figure 2: The structures of the common synthetic antioxidants

4. Mechanism of Antioxidant Effect

Many reports [35-37] discussed the role of antioxidants in body against the excess of reactive oxygen species (ROS). Oxidative stress may be classified into three categories. The first one is the preventive mechanism; proteins which have a coordinated nucleus of iron or copper with the capacity to bind (albumin, myoglobin, metallothionein, ceruloplasmin, ferritin, transferrin,), which prevents the overproduction of HO●. The second one is the repairing mechanism; enzymes which repair or eliminate damaged biomolecules by ROS, like glutathione peroxidase, glutathione reductase, and methionine-sulfoxide reductase. The third one is the scavenger mechanism; enzymes with capacity to scavenge excess ROS like superoxide dismutase, glutathione peroxidase, catalase, other metalloenzymes, and chemical entities with scavenging capacity like polyunsaturated fatty acids, vitamins C and E, uric acid, bilirubin, carotenoids, and flavonoids.

The antioxidant enzymes inside cells serve as primary line of defense against free radicals. The main enzymatic scavengers responsible for the prevention of ROS formation and oxidation are superoxide dismutase (SOD), catalase, and glutathione. SOD is found in virtually every oxygen-based organism, and its major function is to catalyze the dismutation of superoxide to hydrogen peroxide. This reaction is generally considered to be the body's primary antioxidant defense because it prevents further generation of free radicals. In humans, the highest levels of SOD are found in the liver, adrenal gland, kidney, and spleen. Catalase and glutathione peroxidase work to detoxify oxygen-reactive radicals by catalyzing the formation of H2O2 derived from superoxide. The liver, kidney, and red blood cells possess high levels of catalase which helps to detoxify chemicals in the body. Glutathione also plays an important role in a variety of detoxification processes. Glutathione readily interacts with free radicals, especially the hydroxyl radical, by donating a hydrogen atom. This reaction provides protection by neutralizing reactive hydroxyl radicals that are a major source of free radical pathology, including cancer [38].

5. Estimation of Antioxidant Activity

Studies on free radicals and the development of new methods for estimation of antioxidant activity have increased recently. The harmful effect of free radicals on cells has encouraged the seeking for new substances that can prevent or minimize oxidative damage. Because of the different types of free radicals, a single, simple, and accurate universal method will not be developed to measure antioxidant activity. It was reported that a single method is not enough to evaluate the antioxidant capacity of most of the complex natural products [39]. Searching for faster and more efficient testing has generated many methods [23]. Several methods have been used to estimate the antioxidant activity in vitro to allow rapid screening of substances of interest.

5.1. In Vitro Methods

It is difficult to compare one method to another one. To some extent comparison among different in vitro methods has been done by Badarinath [40]. For example, DPPH method is furthermore rapid, simple, and inexpensive in comparison to other test methods. On the other hand, ABTS assay is applicable for both hydrophilic and lipophilic antioxidants. The following in vitro methods are the frequently used methods in literature.

5.1.1 DPPH Scavenging Activity [41, 42]

DPPH (1,1-diphenyl-2-picrylhydrazyl) was described by Blois in 1958 39. It is a stable free radical, so that the molecule does not dimerize, as would be the case with most other free radicals. It is characterized by an absorption band in ethanol solution at about 517 nm. DPPH solution loses its violet color when it is mixed with molecules that can donate a hydrogen atom. DPPH reactivity is one popular method of screening for free radical-scavenging activity. The degree of the change of color is proportional to the concentration and potency of the antioxidants. The method is affected by the solvent and the pH of the reactions. The sample extract (0.2 mL) is diluted with methanol and 2 mL of DPPH solution (0.5 mM) is added. The absorbance is measured at 517 nm after 30 min. The percentage of the DPPH radical scavenging is calculated in terms of Vitamin C, BHA, BHT or Trolox activities.

5.1.2 ABTS Method [42]

ABTS, (2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonic acid), is radical scavenging method which is extensively used in plant samples. It is based on the activation of metmyoglobin with hydrogen peroxide in the presence of ABTS.+ to produce a radical cation. The method generates a blue/green ABTS.+ chromophore via the reaction of ABTS and potassium persulfate. The ABTS radical cation is generated by the oxidation of ABTS with potassium persulfate, its reduction in the presence of hydrogen-donating antioxidants is measured at 734 nm. This assay measures the total antioxidant capacity in both lipophilic and hydrophilic substances. Trolox is used as a positive control. The activity is calculated in terms of Trolox-equivalent antioxidant capacity for the extract or substance (TEAC/mg).

5.1.3 Superoxide Radical Scavenging Activity [43]

Superoxide anion radicals are produced in 3.0 mL of Tris–HCl buffer (16 mM, pH 8.0), involving 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. The reaction is initiated by adding 0.5 mL phenazine methosulfate solution (0.12 mM) to the mixture. Then, it incubated at 25 °C for 5 min. The absorbance is measured at 560 nm against a blank sample.

5.1.4 Oxygen Radical Absorbance Capacity (ORAC) Assay [42]

This assay is based on generation of free radical using AAPH (2,2-azobis 2-amidopropane dihydrochloride) and measurement of decrease in fluorescence in the presence of free radical scavengers. In this assay b-phycoerythrin (β-PE) or fluorescein was used as target free radical effect, AAPH as a peroxyl radical generator and Trolox as a standard control. In 96-well polypropylene fluorescence plates with a final volume of 200 µL. Assays are performed at pH 7.0 using Trolox (6.25, 12.5, 25, and 50 µmol/L for lipophilic assays; 12.5, 25, 50 and 100 µmol/L hydrophilic assays) as the standard. Phosphate buffer (75 mM/L) is used as the blank. After the addition of AAPH, the plate is placed immediately in a multilabel counter preheated to 37 C. The plate is shaken for 10 s and the fluorescence is read at 1 min intervals for 35 min at the excitation wavelength of 485 nm and emission wavelength of 520 nm at 37°C. Area-under-the-curve is calculated for each sample. Final calculations of results are made by taking the difference of areas-under-the-decay curves between blank and sample and/or standard (Trolox) and expressing this in µM of Trolox equivalents (TE) per g dry weight of sample (µM TE/g).

5.1.5 Thiobarbituric Acid (TBA) Method [44]

In TBA method, the final sample concentration of 0.02% w/v was used. Two mL of 20% trichloroacetic acid and 2 mL of 0.67% of thiobarbituric acid were added to 1 mL of sample solution. The mixture was heated in a boiling water bath for 10 min and then centrifuged after cooling at 3000 rpm for 20 min. The absorbance activity of the supernatant was calculated at 552 nm and recorded after it has reached its maximum.

5.1.6 DMPD Method [45]

This method has been developed for the measurement of the antioxidant activity in food and biological samples. It is based on the reduction of buffered solution of colored DMPD (N, N-dimethyl-p-phenylene diamine dihydrochloride) in acetate buffer and ferric chloride. The procedure relay on the decrease in absorbance of DMPD in the presence of scavengers at 505 nm. The activity was expressed as percentage reduction of DMPD. In this method, 1 mL of DMPD solution (200 mM), 0.4 mL of ferric chloride (0.05 M), and 100 mL of sodium acetate buffer solution (0.1 M), adjusting the pH to 5.25. The reactive mixture must be kept in darkness at 4 °C. The reaction takes place when 50 µL of the sample (a dilution of 1:10 in water) is added to 950 µL of the DMPD solution. Absorbance is measured after 10 min of continuous stirring. The results are quantified in terms of Trolox.

5.1.7 β-carotene Bleaching Test [23]

β-carotene/linoleic acid oxidation method evaluates the inhibitory activity of free radicals generated during the peroxidation of linoleic acid. This method is used for plant samples. β-carotene bleaching method is based on the loss of β-carotene's yellow color due to its reaction with radicals formed by linoleic acid oxidation. The rate of β-carotene bleaching can be inhibited in the presence of antioxidants. The reaction is measured spectrophotometrically at 470 nm for 2 h. The results are calculated as IC50 (µg/ml). The results are expressed in terms of BHA, BHT and Trolox, and quercetin.

5.2. In vivo Models

In vivo methods the samples under investigation are usually administered to the testing animals at a definite dosage regimen as described by the method. After a specified period of time, the animals are usually sacrificed and their blood or their tissues are used for the assay.

5.2.1 Reduced Glutathione (GSH) Estimation [46]

In this method, the tissue homogenate (in 0.1 M phosphate buffer pH 7.4) is taken and added with equal volume of 20% trichloroacetic acid (TCA) involving 1 mM EDTA to precipitate the tissue proteins. The mixture is let to stand for 5 min before centrifugation for 10 min. The supernatant (200 µL) is then transferred to a new set of test tubes and 1.8 mL of the Ellman's reagent (5,50-dithiobis-2-nitrobenzoic acid (0.1 mM) prepared in 0.3 M phosphate buffer with 1% of sodium citrate solution) is added. Test tubes are made up to the volume of 2 mL. After completion of the total reaction, the solution is measured at 412 nm against blank. The activity was calculated with a standard curve generated from known GSH.

5.2.2 Superoxide Dismutase (SOD) Method [47]

This method can be applied for determination of antioxidant activity. It is estimated in the erythrocyte lysate prepared from the 5% RBC suspension. Seventy-five mM of Tris–HCl buffer (pH 8.2), 30 mM EDTA and 2 mM of

pyrogallol are added to 50 μL of the lysate. The increase in absorbance is measured at 420 nm for 3 min. One unit of enzyme activity is 50% inhibition of the rate of autooxidation of pyrogallol. It is determined by change in absorbance/min at 420 nm. The activity of SOD is expressed as units/mg protein.

5.2.3. Catalase (CAT) [48]

In this method, fifty μL of the lysate is added to 2 mL of phosphate buffer (pH 7.0) and 1 mL of 30 mM H2O2. Catalase activity is measured at 240 nm for 1 min. The molar extinction coefficient of H2O2, 43.6M cm −1 was used for determination of catalase activity. One unit of activity is equal to 1 mmol of H2O2 degraded per minute and is expressed in units/ mg of protein.

5.2.4 Ferric Reducing Ability of Plasma [49]

It is a rapid test and very useful for routine analysis. The method is based on the use of blood samples that are collected from the rat retroorbital venous plexus into heparinized glass tubes at 0, 7 and 14 days of treatment. The antioxidant activity is estimated by measuring the increase in absorbance developed by the formation of ferrous ions from FRAP [1 mL (10 mM) of 2,4,6 tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl + 1 mL 20 mM (FeCl2.6-H2O) + 10 mL of 0.3 M acetate buffer (pH 3.6)]. The absorbance is measured at 593 nm. Three mL of freshly prepared FRAP reagent, at 37 °C, is mixed with 0.375 mL distilled water and 0.025 mL of test samples. The absorbance of produced color in organic layer is measured at 593 nm at 37 °C. The readings are recorded at 180 s.

6. Chemical analysis of common antioxidants

6.1 Determination of Total Phenol Contents:

Folins Ciocalteu's Reagents [50, 51]

One mL of the solution of the material under investigation is mixed with Folin Ciocalteu's reagent (0.1 mL, 1N) and allowed to stand for 15 min. Then, 5 mL of saturated Na2CO3 is added. The mixtures are let to stand for 30 min at room temperature. The total phenols are determined at 760 nm. Gallic acid is used as a standard reference. Total phenol is determined in terms of gallic acid equivalent (mg/ g of investigated compound).

6.2 Determination of Flavonoid Contents

6.2.1. Chang Method [52]

Two mL of the material under investigation, in methanol, is mixed with 0.5 mL of 1.2% of AlCl3 and 0.5 mL of 120 mM of potassium acetate. The mixture is let to stand for 30 min at room temperature. The absorbance is measured at 415 nm. Quercetin or rutin is used as standard reference. Flavonoid content is determined in terms of quercetin or rutin equivalent (mg/g of investigated compound).

6.2.2. Aluminum Chloride Method [53]

Several dilutions of standard solution of catechin are added to a ten mL volumetric flask containing 4 mL of water. Then, 0.3 mL of 5 % NaNO2 is added. After 5 minutes, 0.3 mL of 10% AlCl3 is added. After 6 min, 2 mL of 1 M of NaOH is added. The total volume is completed to 10 mL with distilled water. Then, the solution is mixed well, and the absorbance is measured against a freshly prepared reagent blank at 510 nm. The total flavonoid content of the extracts is expressed as percentage of catechin equivalent/ 100 g dry weight of sample.

6.2.3. Dinitrophenylhydrazine Colorimetric Method [54]

One mL of various concentrations of standard solutions of naringenin is treated with 2 mL of 2,4-dinitrophenylhydrazine (1%) and 2 mL methanol. The mixture is warmed at 50 °C for 50 minutes. After cooling at room temperature, the mixture is treated with 5 mL solution potassium hydroxide (1%) in methanol and kept at room temperature for 2 minutes. 1 mL of mixture is treated with 5 mL methanol and centrifuged. The supernatant is completed to 25 mL. The absorbance of the supernatant is recorded at 495 nm.

6.3 Determination of Vitamin C [55]

One mL of the analyzed liquid is added into a test-tub and 1 ml of phosphotungstate reagent (sodium tungstate + sodium hydrogen phosphate in deionized water) is added. The solution is mixed thoroughly and is left in a room

temperature for 30 minutes. Then the solution is centrifuged, and the supernatant is collected and measured at 700 nm spectrophotometrically.

6.4 Determination of Vitamin E [56]

In this, the sample is mixed with anhydrous ethanol, and it is shaken for 1 min. Xylene is added, followed by shaking for 1 min and centrifuging to collect the extract. Then, a solution of batophenanthroline is added to the upper layer. Then, FeCl3 solution is added and followed by H3PO4 solution with shaking after each addition. This solution is measured at 539 nm spectrophotometrically.

6.5 Determination of Vitamin A [57]

One mL of KOH solution is added to the analyzed liquid and is shaken vigorously for 1 minute. The solution is heated at 60 °C for 20 min. Then, it cooled down. One mL of Xylene is added and is shaken vigorously again for 1 min. then the mixture is centrifuged and the whole of the separated extract (upper layer) is collected and transferred to the test tube of "soft" (sodium) glass. The absorbance is measured at 335 nm against xylene. The solution extract is irradiated with UV light for 30 minutes, and then the absorbance is measured. The concentration of vitamin A is calculated in µM in the analyzed liquid.

6.6 Determination of Total Carotenoids

6.6.1. Lichtenthaler Method [58]

This method depends on determination of total carotenoids after subtracting the concentration of chlorophyll A and B, using wavelengths 661.6 and 644.8 nm, respectively, and using the corresponding absorption coefficients at which carotenoids do not absorb.

6.6.2. Hornero-Méndez and Mínguez Mosquera Method [59]

This method is used to determine total carotenoid content in paprika and red pepper oleoresins, which can be divided into red and yellow isochromic fractions. The red fraction is mainly composed of capsanthin and capsorubin and the yellow fraction contains zeaxanthin and beta-carotene. This method can be developed to determine carotenoid concentrations in vegetables or fruits after removing potentially interfering chlorophylls. The absorbance values at 472 (yellow) and 508 (red) nm and corresponding absorption coefficients are used for the isochromic fractions.

6.7 Determination of Selenium [60]

This method is used to determine selenium in cosmetic and pharmaceutical products. In this method a mixture of selenium solution is mixed with Triton X-100, dithizone, and HCl, then it was kept for 15 min to complete the color development. When 0.5% (w/v) of the phenol was used for inducing the cloud point, the mixture was placed in a water bath at 40°C for 10 min. The mixture was centrifuged, and the aqueous phase was decanted, and the surfactant-rich phase was made up to 5 mL by methanol. The absorbance of two methanolic solutions was measured at 424 and 592 nm, against a blank (without Se and dithizone). The absorption maxima of the complex (424 nm) and dithizone (434 nm) were overlapping. Hence, the corrected absorbance was used to overcome the problem using the absorbance at 124 and 434 nm.

7. Conclusion

Oxidative stress is involved in the development of various diseases and their symptoms including diabetes, cardiovascular diseases, inflammatory and cancer. Antioxidants offer resistance against oxidative stress, which increases their importance in health care. Thus, many antioxidants are widely used in the food industry, medicines, and health care products. Scientific knowledge of the antioxidant activity in vivo and in vitro testing methods for evaluation has been increasing over time. They have become important tools in the search for bioactive substances, and for raw material choice as well. Finally, the increase of dietary antioxidants may maintain the normal physiological functions of living systems. It was expected that antioxidants will be considered as a basic nutritional constituent as protein, fats, minerals in the near future.

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References

[1]. Poli, G., Leonarduzzi, G., Biasi, F., & Chiarpotto, E. (2004), Oxidative stress and cell signalling. Curr. Med. Chem., 11, 1163-1182.
[2]. Halliwell, B. (2002). Antioxidants in human health and disease. Ann. Rev. Nutr., 16, 33-50.
[3]. Droge, W. (2002). Free radicals in the physiological control of cell function. Review. Physiol. Rev., 82, 47-49.
[4]. Willcox, J. K., Ash, S. L. & Catignani, G. L. (2004). Antioxidants and prevention of chronic disease. Review. Crit. Rev. Food Sci. Nutr., 44, 275-295.
[5]. Kalia, K., and Flora, S. J. S. (2005). Strategies for Safe and Effective Treatment for Chronic Arsenic and Lead Poisoning. J. Occup. Health, 47, 1-21.
[6]. Gutteridge, J. M., & Halliwell, B. (1989). Iron toxicity and oxygen radicals. Baillieres Clin. Haematol., 2, 195-256.
[7]. Kohen, R., & Nyska, A. (2002). Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification, Toxicol. Pathol., 30, 620-650.
[8]. Dimitrios, B. (2006). Sources of natural phenolic antioxidants. Trends Food Sci. Tech., 17, 505-512.
[9]. Prior, R.L. (2005). Fruits and vegetables in the prevention of cellular oxidative damage. Am. J. Clin. Nutr., 78, 570S-578S.
[10]. Scheibmeir, D., Christensen, K., Whitaker, S. H., Jegaethesan, J., Clancy, R., Janet, D., & Pierce, J. D. (2005). A review of free radicals and antioxidants for critical care nurses Heath. Intensive and Critical Care Nursing, 21, 24-28.
[11]. Omoni, A. O., & Aluko, R. E. (2005). The anti-carcinogenic and anti-atherogenic effects of lycopene: a review. Trends Food Sci. Techn., 16, 344-350.
[12]. Boban, T. (2002), The role of lutein in the prevention of atherosclerosis. J. Am. Coll. Cardiol., 40, 835-836.
[13]. Kuhn, M. A. (2003). Oxygen free radicals & antioxidants. Am. J. Nurs., 103, 58-62.
[14]. McDermott, J. H. (2000). Antioxidant nutrients: current dietary recommendations and research update. J. Am. Pharmacol. Assoc., 40, 785-799.
[15]. Demple, B., Halbrook, J. (1993). Inducible repair of oxidative DNA damage in Escherichia coli, Nature, 304, 466-468.
[16]. Keyse, S. M. (2004). Stress Response, Methods and Protocols. Methods in Molecular Biology, Humana Press, Totowa, New Jersey, 99-108.
[17]. Wang, D., Kreutzer, D. A., & Essigmann, J. M. (1998), Mutagenicity and repair of oxidative DNA damage: insightsfromstudies using defined lesions. Mutat. Res., 400, 99-115.
[18]. Buettner, O. R. (1993). The pecking order of free radicals and antioxidants: Lipid peroxidation, α-tocopherol, and ascorbate, Arch. Biochem. Biophys., 300, 535-543.
[19]. Wanasundara, P. K. J. P. D., & Shahidi, F. (2005). Antioxidant: Science, technology, and Applications, In bailey's industrial oil and fat products, John Wiley & sons Inc., U.K., 341-490.
[20]. Gupta, V. K., & Sharma, S. K. (2006). Natural antioxidant from plants, Nat. Prod. Rad., 5, 326-334.
[21]. Shikora, E., Cieślik, E., & Topolska, K. (2008). The sources of natural antioxidants. Acta. Sci. Pol.,7, 5-17.
[22]. Haslam, E. (1996). Natural Polyphenols (Vegetable Tannins) as Drugs: Possible Modes of Action, J. Nat. Prod., 59, 205-215.
[23]. Alves, C. Q., David, J. M., David, J. P., Bahia, M.V., & Aguiar, R. M. (2010). Methods for determination of in vitro antioxidant activity for extracts and organic compounds. Química Nova, 33,2202-2210.
[24]. Kähkönen, M.P., Hopia, A. I., Vuorela, H. J., Rauha, J. P., Pihlaja, K., Kujala, T. S., & Heinonen, M. (1999). Antioxidant activity of plant extracts containing phenolic compounds. J. Agric. Food Chem., 47,

3954-3962.

[25]. Lourenço, S. C., Martins, M. M., & Alves, V. D. (2019). Antioxidants of Natural Plant Origins: From Sources to Food Industry Applications. Molecules, 24, 4132-4156.

[26]. Di Carlo, G., Mascolo, N., Izzo, A. A., & Capasso, F. (1999). Flavonoids: old and new aspects of a class of natural therapeutic drugs. Life Sci., 65, 337-353.

[27]. Montoro, P., Braca, A., Pizza, C., & De Tommasi, N. (2005). Structure–antioxidant activity relationships of flavonoids isolated from different plant species. Food Chem., 92, 349-355.

[28]. Bravo, L. (1998). Polyphenols: chemistry, dietary sources, metabolism and nutritional significance, Nutrition Reviews, 56, 317-333.

[29]. Bakkali, F., Averbeck, S., Averbeck, D., & Idaomar, M., Biological effects of essential oils – a review. Food Chem. Toxicol., 46, 446-475.

[30]. Dewick, P.M. (2009). The mevalonate pathway: terpenoids and steroids. In: Dewick, P.M. Medicinal Natural Products: a biosynthetic approach (Jonh Wiley & Sons Inc., U.K.). 187-310.

[31]. Tinggi, U. (2008). Selenium: its role as antioxidant in human health. Environ. Health Prev., 13, 102-108.

[32]. Velioglu, Y.S., Mazza, G., Gao L., & Oomah, B. D. (1998). Antioxidant activity and total phenolics in selected fruits, vegetables and grain products. J. Agric. Food Chem., 46, 4113-4117.

[33]. Jang, I. C., Jo, E. K., Bae, M. S., Lee, H. J., Jeon, G. I., Park, E., Yuk, H. G., Ahn, G. H., & Lee, S. C. (2010). Antioxidant and antigenotoxic activities of different parts of persimmon (Diospyros kaki cv. Fuyu) fruit. J. Med. Plants Res., 4, 155-160.

[34]. Oliveira, A. C., Valentim, I. B., Silva C. A., Bechara, E. J. H., Barros, M. P., Mano, C. M., & Goulart, M. O. F. (2009). Total phenolic content and free radical scavenging activities of methanolic extract powders of tropical fruit residues. Food Chem., 115, 469-475.

[35]. Young, I., & Woodside, J. (2001). Antioxidants in health and disease, J. Clin. Pathol., 54, 176-186.

[36]. Lü, J., Lin, P. H., Yao, Q., & Chen, C. (2010), Chemical and molecular mechanisms of antioxidants: experimental approaches and model systems. J. Cell Mol. Med., 14, 840-860.

[37]. Pham-Huy, L. A., He, H., & Pham-Huy, C. (2004). Free Radicals, Antioxidants in Disease and Health. Int. J. Biomed.. Sci., 4, 89-96.

[38]. Clark, S. F. (2002). The biochemistry of antioxidants revisited. Nutr. Clin. Pract., 17, 5-17.

[39]. Reese, R. N, Tulio, A. Z., Scheerens, J. C., & Miller A. R. (2006), Modified 2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic Acid (ABTS) method to measure antioxidant capacity of selected small fruits and comparison to ferric reducing antioxidant power (FRAP) and 2,2'-Diphenyl-1-picrylhydrazyl (DPPH) methods. J. Agric. Food Chem., 54,1151-1157.

[40]. Badarinath, A.V., Rao, K. M., Chetty, C. M. S., Ramkanth, V., Rajan, T. V. S., & Gnanaprakash K. (2010). A review on in-vitro antioxidant methods: comparisons, correlations and considerations. Int. J. Pharm. Tech. Res., 2, 1276-1285.

[41]. Blois, M.S. (1958). Antioxidant determinations by the use of a stable free radical. Nature, 181, 1199-1200.

[42]. Krishnaiah, D., Sarbatly, R., & Nithyanandam R. A. (2011). Review of the antioxidant potential of medicinal plant species. Food Bioprod. Process, 89, 217-233.

[43]. Robak, J., & Gryglewski, R. J. (1988). Flavonoids are scavengers of superoxide anions. Biochem. Pharmacol., 37, 837-841.

[44]. Ottolenghi A., Interaction of ascorbic acid and mitochondria lipids, Arch. Biochem. Biophys., 79, 355-365(1959).

[45]. Fogliano, V., Verde, V., Randazzo, G., & Ritieni A. (1999). Method for measuring antioxidant activity and its application to monitoring antioxidant capacity of wines. J. Agric. Food Chem., 47, 1035-1040.

[46]. Ellman, G.L. (1959). Tissue sulfhydryl groups. Arch. Biochem. Biophys., 82, 70-77.

[47]. McCord, J., Fridovich, I. (1969). Superoxide dismutase, an enzymic function for erythrocuprin. J. Biol. Chem., 244, 6049-6655.

[48]. Aebi, H. (1984). Catalase. Methods Enzymol., 105, 121-126.

[49]. Benzie, I. F. F., & Strain J. J. (1999). Ferric reducing antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods Enzymol., 299, 15-57.

[50]. Singleton, V. L., & Rossi, J.A. (1954). Colourimetry of total phenolics with phosphomolibdic phosphotungstic acid reagent. Am. J. Enol. Vitic., 16, 144-158.

[51]. McDonald, S., Prenzler, P. D., Autolovich M., & Robards, K. (2001), Phenolic content and antioxidant activity of olive extracts. Food Chem., 73, 73-84.

[52]. Chang, C., Yang, M., Wen, H., & Chem, J. (2002), Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J Food Drug Anal., 10, 178-182.

[53]. Zhishen, J., Mengcheng, T., & Jianming, W. (1999). The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem., 64, 555-559.

[54]. Nagy, M., & Grancai, D. (1996). Colorimetric determination of flavanones in Propolis. Pharmazie, 51, 100-101.

[55]. Rutkowski, M., Grzegorczyk, K., Klupińska, G. A. (1999). Rapid method for the determination of vitamin C (ascorbic acid) in gastric juice. Adv Clin Exp Med, 8, 139-145.

[56]. Rutkowski, M., Grzegorczyk, K., Paradowski, M. T. (2005). Colorimetric method of blood plasma total vitamin E determination – the own modification of Tsen method, Diagn. Lab, 41, 375-385.

[57]. Rutkowski, M., & Grzegorczyk, K. (2007). Modification of spectrophotometric methods for antioxidative vitamins determination convenient in analytic practice, Acta Sci Pol, Technol Aliment, 6, 17-28.

[58]. Landrum, J. T., Bone, R. A., Kilburn, M. D. (1997). The macular pigment: a possible role in protection from age-related macular degeneration. Adv Pharmacol, 38, 537-556.

[59]. Hornero-Mendez, D., Mínguez-Mosquera, M. I. (2010), Rapid spectrophotometric determination of red and yellow isochromic fractions in paprika and red pepper oleoresins. J Agric Food Chem, 49, 3584-3588.

[60]. Soruraddin, M. H., Heydari, R., Puladvand. M., Zahedi. M. M. (2011). A New Spectrophotometric Method for Determination of Selenium in Cosmetic and Pharmaceutical Preparations after Preconcentration with Cloud Point Extraction, Int J Anal Chem, 2011, 1-8.