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Vol. 7(10), pp. 528-539, 15 Marvh, 2013
DOI 10.5897/AJPP2013. 3474
ISSN 1996-0816 ©2013 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Review

Antioxidant compounds, assays of determination and mode of action

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Accepted 18 March, 2013

The human body uses an antioxidant defense system to neutralize the excessive levels of reactive oxygen species. This system consists of enzymatic and non enzymatic antioxidants, catalase, peroxidase, superoxide dismutase and gluatathion s-transferease as major defense enzymes. However, ascorbic acid, tocopherol, and phenolic compounds are considered as examples for non-enzymatic antioxidants. Increasing research on natural antioxidants in foods and development of new assays has prompted critical reflection on the field. It has been common practice to identify health benefits from antioxidant activity on the cellular level with antioxidant capacity of food measured *in vitro.* The use of antioxidants and their positive effects on food quality has been demonstrated in a large variety of foods and beverages using various methods for detection of lipid and protein oxidation or various assays based on electron transfer or hydrogen-atom transfer. There is a need for screening studies in order to identify the mode of action of different antioxidant compounds (enzymatic and non-enzymatic in addition, comparing between synthetic and natural antioxidant compounds) by different assays, in addition to highlighting the advantage and disadvantage of it. Some of these assays depend on hydrogen atom transfer methods or electron transfer methods in addition, metal chelating compounds and free radical scavenging activity.

Key words: Synthetic and natural antioxidant compounds, assays, mechanism.

INTRODUCTION

Natural and synthetic antioxidants are widely used in modern medicine. Some of them proved to be efficient geroprotectors, that is they extend the life span of laboratory animals when added to food or drinking water on a regular basis. *In vitro*, antioxidants inhibit free radical chain oxidation reactions, resulting in oxidation of fatty acids, edible fats, etc. However, their efficiency as scavengers of oxygen free radicals in cells and tissues is negligible as compared with natural antioxidant enzymes (Koltover, 2010). An antioxidant is a chemical that prevents the oxidation of other chemicals. They protect the key cell components by neutralizing the damaging effects of free radicals (Figure 1), which are natural by-products of cell metabolism (Miller et al., 2000). The oxidative stress (OS) induced by reactive oxygen species (ROS) can be described as a dynamic imbalance between the amounts of free radicals generated in the body and levels of antioxidants to quench and/or scavenge them and protect the body against their deleterious effects (Shirwaikar et al., 2006).

Excessive amounts of ROS may be harmful because they can initiated bimolecular oxidations which lead to cell injury and death, and create oxidative stress which results to numerous diseases and disorders such

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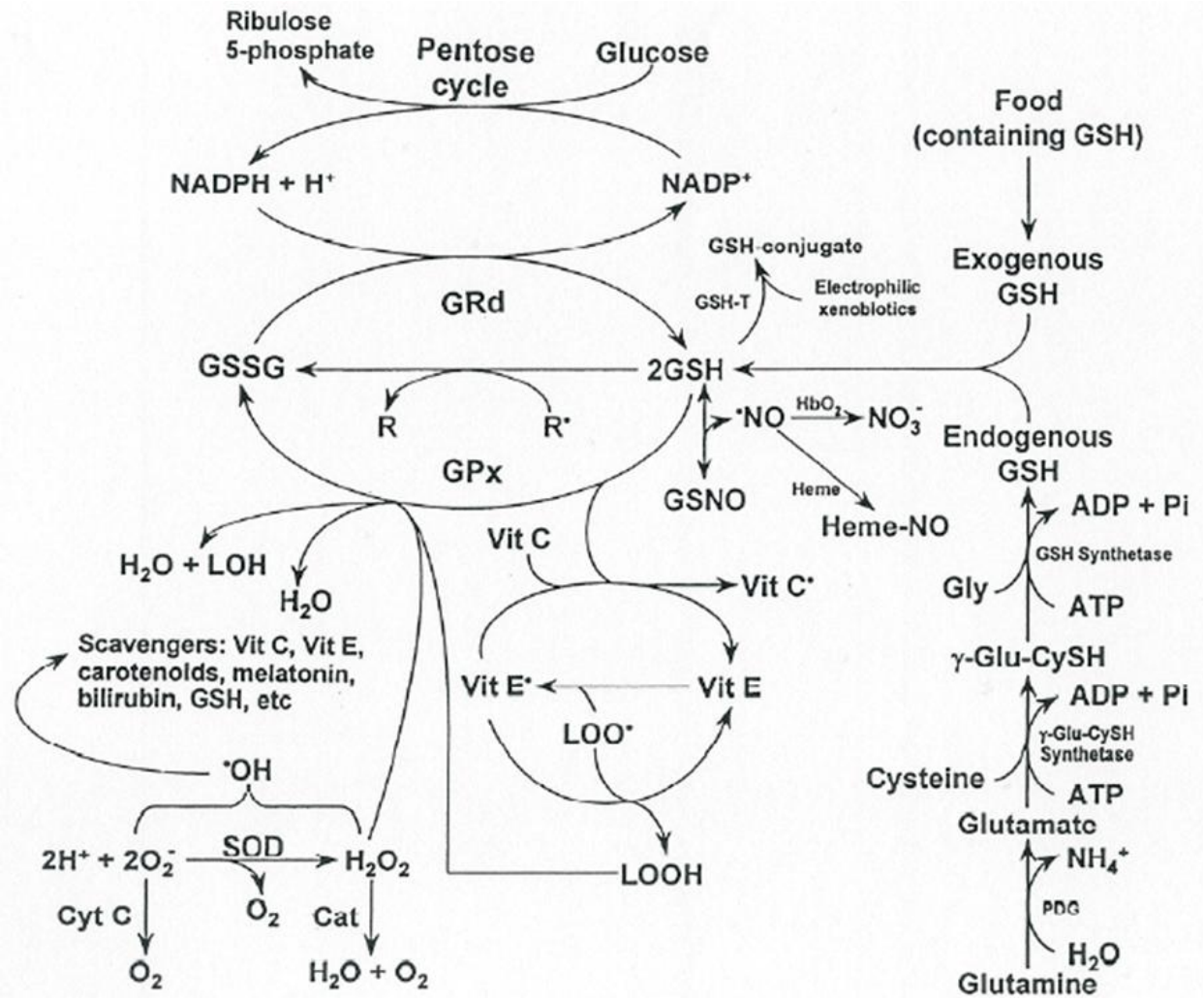

Figure 1. Free radicals and other reactants are enzymatically removed from cells by a series of antioxidative enzymes. Source: Reiter et al. (2003).

as aging, cancer therosclerosis, cirrhosis and cataracts (Halliwell and Gutteridge,2000). There has been a growing considerable interest to identify new sources of safe and inexpensive antioxidant and antimicrobial potential of natural origin (Anwar et al., 2009). Free radicals are formed when oxygen is metabolized or formed in the body and are chemical species which posses an unpaired electron in the outer (valance) shell of the molecule. This is the reason why the free radicals are highly reactive and can react with proteins, lipids, carbohydrates and DNA.

These free radicals attack the nearest stable molecules, stealing its electron. When the attacked molecule loses its electron, it becomes a free radical itself, beginning a chain reaction, finally resulting in the description of a living cell (Prior et al., 1998; Cao et al., 1995). Free radicals may be either oxygen derived (ROS, reactive oxygen species) or nitrogen derived (RNS, reactive nitrogen species). The oxygen derived molecules are O^{-2} (superoxide), $HO^{\cdot}$ (hydroxyl), HO_2 (hydroperoxyl), $ROO^{\cdot}$ (peroxyl), $RO^{\cdot}$ (alkoxyl) as free radicals and H_2O_2 oxygen as non-radical. Nitrogen derived oxidant species are mainly $NO^{\cdot\,-}$ (nitric oxide), $ONOO^{\cdot\,-}$ (peroxy nitrate), NO_2 (nitrogen dioxide) and N_2O_3 (dinitrogen trioxide) as shown in Figure 2.

In a normal cell, there is appropriate oxidant: antioxidant balance. However, this balance can be shifted, when production of oxygen species is increased or when levels of antioxidants are diminished. This stage is called oxidative stress (Vinson et al., 1998; Cuvelier et al., 1992). Oxidative stress results in the damage of biopolymers including nucleic acids, proteins, poly-

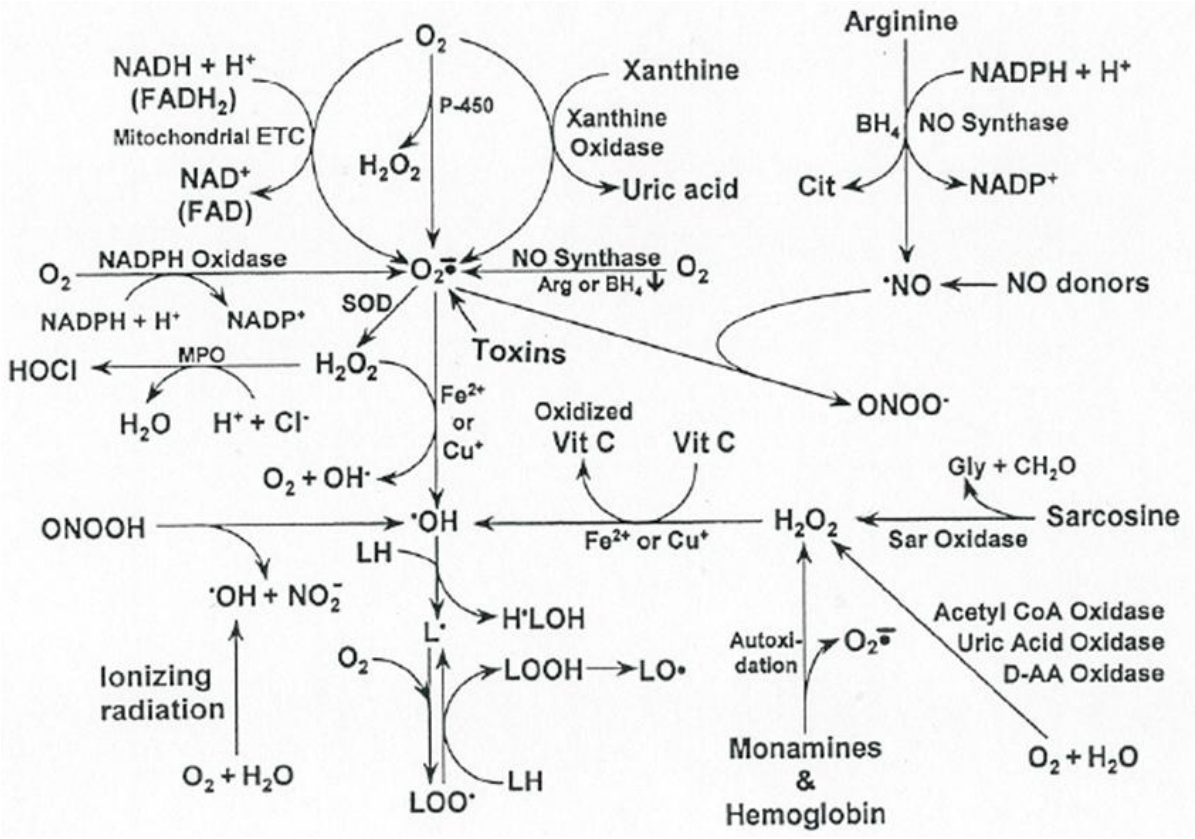

Figure 2. Oxygen and nitrogen-based free radicals and associated reactants that are generated in cells by various processes. Source: Reiter et al. (2003).

unsaturated fatty acids and carbohydrates.

Lipid peroxidation is an oxidative deterioration of polyunsaturated lipids and it involves ROS and transition metal ions. It is a molecular mechanism of cell injury leading to the yielding of a wide range of cytotoxic products, most of which are aldehydes, like malon-dialdehyde (MDA), 4- hydroxynonrnal (HNE). Oxidative stress causes serious cell damage leading to a variety of human diseases like Alzheimer's disease, Parkinson's disease, atherosclerosis, cancer, arthritis, immunological incompetence and neurodegenerative disorders, etc. Nutritional antioxidant deficiency also leads to oxidative stress which signifies the identification of natural anti-oxidative agents present in die consumed by human population (Hogg et al., 1961; Cao et al., 1966). Plant extracts rich in phenol acids exhibit strong antioxidant and antiradical activity *in vitro* (Mary et al., 2003) and *in vivo* (Rajlakshmi et al., 2003).

Among the compounds with strong antiradical features, one could mention tannins, flavonoids, and phenolic acids. Tannins are high-molecular compounds with complicated and variable structure, exhibiting usually strong antiradical and antioxidant activity. An interesting group of compounds are derivatives of catechins and gallic acid, the so-called gallocatechins, which are present in green tea (Polovka et al., 2003). Research has shown that these compounds have strong antioxidant activity as well as some anticancer features. The present work aims to make comparison between synthetic and natural antioxidant in addition to antioxidant determination assays and its mode of actions.

ANTIOXIDANT COMPOUNDS

The chemical compounds which can delay the start or slow the rate of lipid oxidation reaction in different biological systems are known as antioxidant compounds.

Types of antioxidant compounds

The human body uses an antioxidant defense system to neutralize the excessive levels of reactive oxygen

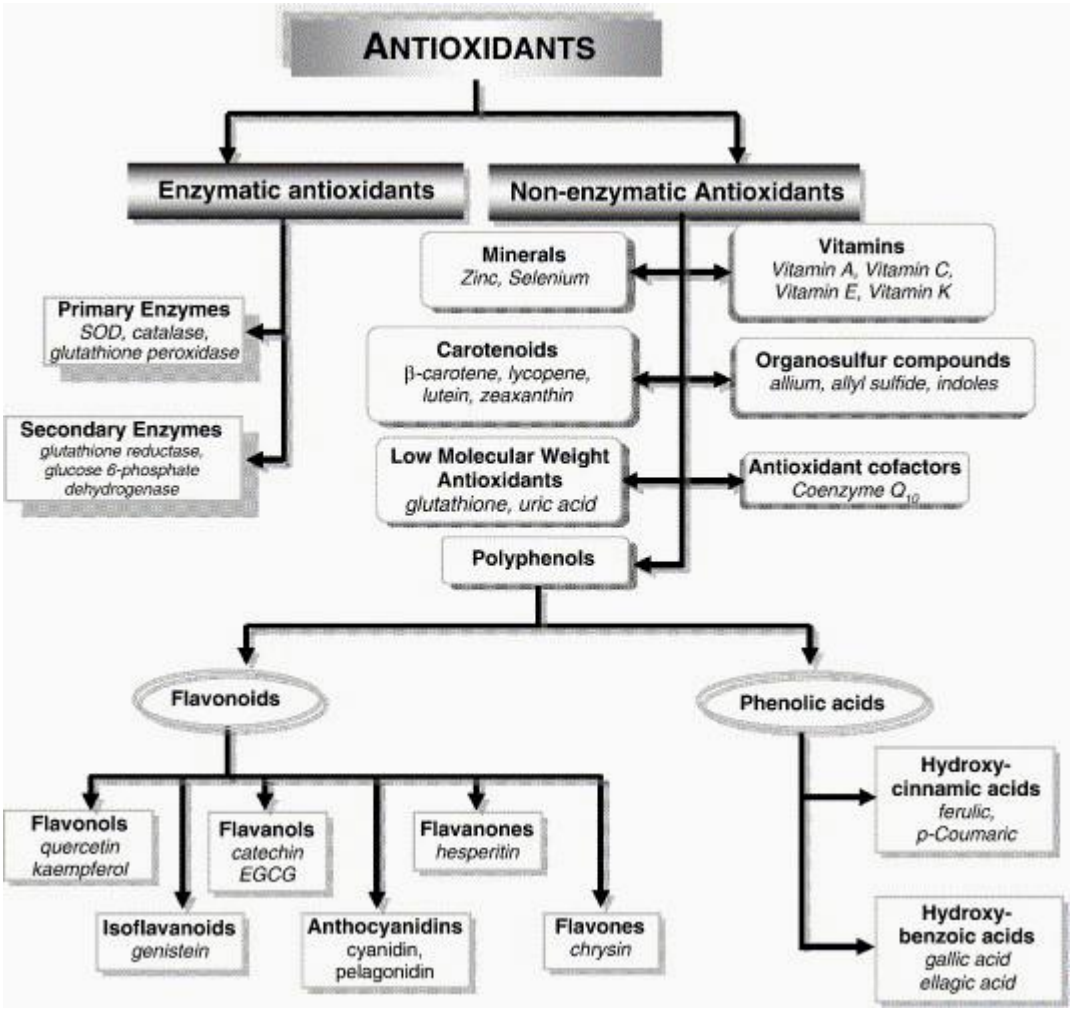

Figure 3. Classification of antioxidants.

species. This system consists of enzymatic and non enzymatic antioxidants. Some of the antioxidant enzymes that are found to provide a protection against ROS are superoxide dismutases, catalases, and glutathione peroxidases, in addition to numerous non-enzymatic small molecules distributed widely in the biological system and capable of scavenging free radicals (Figure 3). These non enzymatic molecules include glutathione, tocopherol (vitamin E), vitamin C, β-carotene, and selenium. The antioxidants may be natural or synthetic ones. Polyhydroxy flavones, flavanones, flavanols, isoflavones, chalcones and many members are of these groups of natural substances which proved to have a high degree of antioxidant activity and they are found to be widely spread in plant material (Rajani, 2004). The antioxidant characteristics of plant derived materials can be attributed to their content of polyphenols (Andrea et al., 2003).

Natural and synthetic antioxidants

Natural antioxidants: Natural antioxidants are the cell's defense mechanisms that scavenge reactive species, and they can be classified into different groups according to their properties: endogenous antioxidants, and natural antioxidants. Endogenous antioxidants include glutathione, alpha-lipoic acid, coenzyme Q, ferritin, uric acid, bilirubin, metallothionein, l-carnitine, melatonin, enzymatic superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPXs), thioredoxins (TRX) and peroxiredoxins (PRXs). PRXs are a ubiquitous family of antioxidant enzymes (PRX I-VI) that also control cytokine-induced peroxide levels and mediate signal transduction in mammalian cells (Yoshida et al., 2003). For example, PRX III scavenges up to 90% of H_2O_2, and PRX V behaves more effectively as a scavenger of peroxynitrite. Natural antioxidants coexist in a delicate

balance with oxidative inputs. Other antioxidants can be obtained from the diet, such as ascorbic acid (Vitamin C), tocopherol (Figure 4a), β-carotene (Vitamin A), lipoic acid, uric acid, glutathione and polyphenol metabolites.

Synthetic antioxidants: Butylated hydroxy anisole (BHA); butylated hydroxy toluene (BHT); Propyl Gallate (PG); and tertiary butyl hydroquinone (TBHQ) all fall into this class. In 1975, a research scientist who worked on a project commissioned by the Food and Drug Administration (FDA) to investigate the safety/side effects of BHT/BHA observed that the laboratory rats used in this study developed cancerous tumors at an alarming rate. BHA and BHT are laboratory synthesized phenolic compounds (Figure 4b) that are often added to foods to preserve fats. However, the same chemical properties which make BHA and BHT excellent preservatives may also be implicating in health effects. The oxidative characteristics and/or metabolites of BHA and BHT may contribute to carcinogenicity or tumorigenicity. Extensive research has shown high doses of this ingredient to cause significant damage to the lungs, liver and kidneys. Oral consumption of this ingredient has also been shown to have toxic effects on the body's blood coagulation system. Evidences are also available to prove that metabolizing activity can be difficult with BHA and BHT, resulting in health and behavior changes. The Feingold Association maintains that BHA/BHT promotes lung cancer. They also found that it may be the causing agent of developmental neurobehavioral toxicity in their experiments. Kate Murphy, in an article entitled "Do Food Additives Subtract From Health?" for Business Week, noted that:

"Repeated studies have shown that BHA and BHT increase the risk of cancer as well as its accumulation in body tissue, cause liver enlargement, and retard the rate of DNA synthesis and thus, cell development" (Koltover, 2010; U.S. National Library of Medicine, 2010; Baur et al., 2001).

These substances are petroleum derived and created as a by-product in the petroleum refining process. The International Agency for Research on Cancer, and part of the World Health Organization, considers BHA to be possibly carcinogenic to humans, and the State of California has listed it as a carcinogen. Studies showed the same cancer causing possibilities for BHT. The compound has been banned for use in food in Japan, Romania, Sweden, and Australia. The US has barred it only from infant foods.

Despite all these dangers, butylated hydroxyanisole (BHA; tert-butyl-4-hydroxyanisole) is perhaps the most extensively used antioxidant in the food industry. BHA is used in fats and oils, fat-containing foods, confectioneries, essential oils, food-coating materials, and waxes. Butylated hydroxytoluene (BHT; 2,6-di-*tert*-butyl-p-cresol) on the other hand, is one of the antioxidants used extensively in the food industry. It is used in low-fat foods, fish products, packaging materials, paraffin, and mineral oils. BHT is also widely used in combination with other antioxidants such as BHA, propyl gallate, and citric acid for the stabilization of oils and high-fat foods (Shalaby et al., 2010; Shanab et al., 2010).

Some non-enzymatic antioxidants like uric acid, vitamin E, glutathione and CoQ_{10} are synthesized in the human body and they can also be derived from dietary sources. Polyphenols are the major class of antioxidants which are derived from diet (Venkat Ratnam et al., 2006) (http://dx.doi.org/10.1016/j.jconrel.2006.04.015).

Identifying the antioxidant mechanism

The different types of methods published in the literature for the determinations of antioxidant activity of different biological systems involve more than one mechanism (Tables 1 and 2). So, when you determine the antioxidant activity you must use more than one method for comparing the mode of action of crude or pure compounds.

TLC autography technique: The antiradical screening by thin layer chromatography (TLC) autography technique provides an easy, effective and rapid way to study plant extract profiles. No sample purification is needed as this technique provides a simultaneous separation and radical scavenging activity measurement of antioxidative compounds in plant extract. Qualitative as well as semi quantitative analysis of antioxidants can be done by this technique for example, dot blot assay of the crude and various fractions of *Phellinus merrillii* on a silica sheet stained with a 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution in methanol (Chang et al., 2007; Wang et al., 1996) as shown in Figure 5.

Electron spin resonance (ESR) method: These analytical methods measure the radical scavenging activity of antioxidants against free radicals like the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, the superoxide anion radical (O_2), the hydroxyl radical (OH), or the peroxyl radical (ROO). The various methods used to measure antioxidant activity of food products can give varying results depending on the specific free radical being used as a reactant. There are other methods which determine the resistance of lipid or lipid emulsions to oxidation in the presence of the antioxidant being tested.

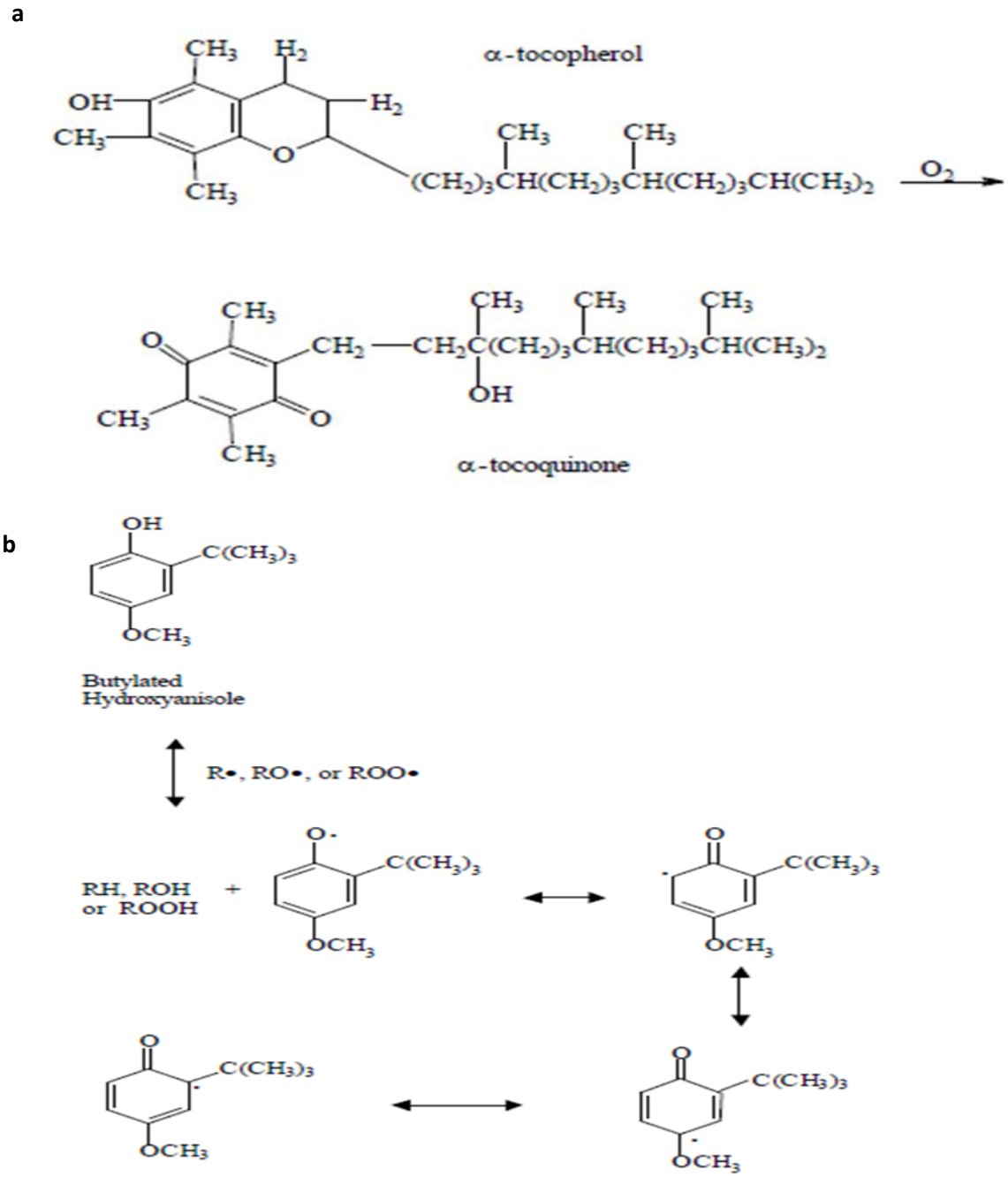

Figure 4. (a) Natural antioxidant (Tocopherol), (b) synthetic antioxidant (BHA).

Enhanced chemiluminescence (ECL): ECL has been used to measure antioxidant capacity in biological fluids. The assay involves the chemiluminescent substrate luminal. Light emission occurs when the luminal is oxidized by hydrogen peroxide that is generated in a reaction catalyzed by horseradish peroxidase (HRP). This method can quantify the antioxidant capacity of a substance which is sensitive to radical scavenging antioxidants that reduce the light output. A method of assay of the antioxidant activity of biological sample suspected of having such activity, is under patent and this method comprises the steps of: (a) initiating a chemiluminescent reaction and allowing reaction to progress, thereby to generate a level of luminescence,

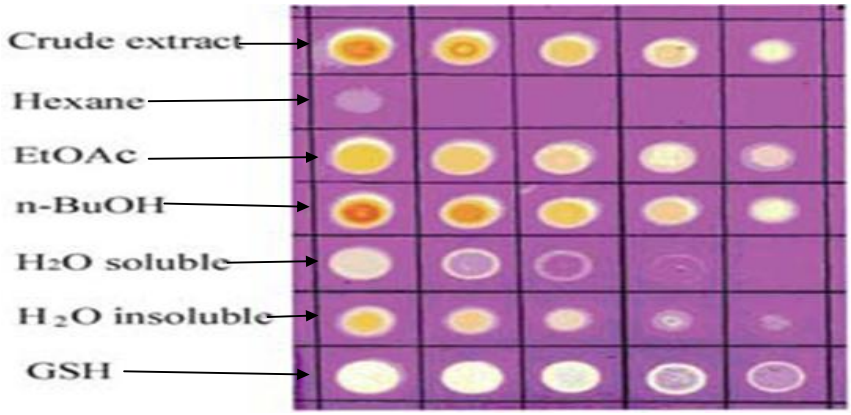

Figure 5. Dot blot assay of the crude and various fractions of *Phellinus merrillii* on a silica sheet stained with DPPH solution in methanol. The crude and fractions of *P. merrillii* (2, 1, 0.5, 0.25, and 0.125 mg/ml) were applied from left to right in sample row; GSH (2, 1, 0.5, 0.25, and 0.125 mg/ml) were applied from left to right in control row.

Table 1. List of *in vitro* antioxidant methods.

S/No	Name of the method
I	**Hydrogen atom transfer methods (HAT)**
1	Oxygen radical absorbance capacity (ORAC) method
2	Lipid peroxidation inhibition capacity (LPIC) assay
3	Total radical trapping antioxidant parameter (TRAP)
4	Inhibited oxygen uptake (IOC)
5	Crocin bleaching Nitric oxide radical inhibition activity
6	Hydroxyl radical scavenging activity by p-NDA (p-butrisidunethyl aniline)
7	Scavenging of H_2O_2 radical
8	ABTS radical scavenging method
9	Scavenging of super oxide radical formation by alkaline SASA
II	**Electron transfer methods (ET)**
1	Trolox equivalent antioxidant capacity (TEAC) decolourization
2	Ferric reducing antioxidant power (FRAP)
3	DPPH zree radical scavenging assay
4	Copper (II) reduction capacity
5	Total phenols by Folin-Ciocalteu
6	N,N-dimethyl-p-Phenylenediamine (DMPD) assay
III	**Other assays**
1	Total oxidant scavenging capacity (TOSC)
2	Inhibition of Briggs – Rauscher oscillation reaction
3	Chemiluminescence
4	Electrochemiluminescence
5	Fluorometric Analysis
6	Enhanced chemiluminescence (ECL)
7	TLC bioautography
8	Cellular antioxidant activity (CAA) assay
9	Dye-substrate oxidation method

Table 2. Comparison between the advantage and disadvantage of different antioxidant methods

Methods	Advantage	Disadvantage
2,2-Diphenyl-1-picrylhydrazyl (DPPH)	This technique is easy, effective, and rapid way to study plant extract profiles. No sample separation is needed. Potency of sample can be known	Time consuming, costly
Cellular antioxidant activity (CAA) assay	More accurate guage of antioxidant power of whole foods and individual antioxidant nutrients and compounds. This approach is more biologically relevant as it accounts for uptake, metabolism, distribution	Time consuming, costly
Dye-substrate oxidation method	It provides fro-concurrent multisample analysis with automated data storage, regression analyses, and calculation of oxidation inhibition rates. For screening crude extracts and typical assay results are presented	Nil
Cupric Ion Reducing antioxidant capacity (CUPRAC)	It requires sophisticated instrumentation. As a more convenient and less costly alternative. The developed method is less lengthy, more specific and of a higher yield than the classical TBARS assay	Sophisticated instruments are required which are more expensive
Cellular antioxidant activity	Antioxidant capacity of number of non-refined seed oils is compared with that of refined oils by using this simple technique	Nil
Enhanced chemiluminescence (ECL)	ECL has been used to measure antioxidant capacity of biological fluids. This method can quantify the antioxidant capacity of a substance which is sensitive to radical scavenging antioxidants that reduce the light output	This method is cumbersome and time-consuming because fresh signaling reagent solution must be prepared. Also, expensive instrumentation is needed to measure the chemiluminescence (for example, Luminometer)
Ferric-reducing antioxidant power (FRAP) assay	It is simple, speedy, inexpensive, and robust does not required specialized equipment. It can be performed using automated, semiautomated, or manual methods	FRAP cannot detect species that act by radical quenching (H transfer), particularly SH group containing antioxidants like thiols, such as glutathione and proteins.
Total radical trapping antioxidant parameter (TRAP)	Used for measurements of *in-vivo* antioxidant capacity in serum or plasma because it measures nonenzymatic antioxidants such as glutathione, ascorbic acid.24	Many different end points have been used, so comparisons between laboratories are difficult. It is relatively complex and time consuming. It also requires a high degree of expertise and experience
Oxygen radical absorbing capacity (ORAC) assay	The advantage of the AUC approach is that it implies equally well for both antioxidants that exhibit distinct lag phase and those that have no lag phases. ORAC assay has been broadly applied in academy and in the food and dietary supplement industries as a method of choice to quantify AOC	ORAC is limited to measurement of hydrophilic chain but ignores lipophilic antioxidants. It requires fluorometers, which may not be routinely available in analytical laboratories.Temperature control decreases reproducibility
Trolox equivalent antioxidant capacity (TEAC), ABTS.+	The ABTS method has the extra flexibility in that it can be used at different pH levels (unlike DPPH, which is sensitive to acidic pH) and thus is useful when studying the effect of pH on antioxidant activity of various compounds. It is also useful for measuring antioxidant activity of samples extracted in acidic solvents. Additionally, ABTS is soluble in aqueous and organic solvents and is thus useful in assessing antioxidant activity of samples in different media and is currently most commonly used in simulated serum ionic potential solution (pH 7.4 phosphate buffer solution containing 150 mM NaCl) (PBS). Another advantage of ABTS+ method was that samples reacted rapidly with ABTS in the aqueous buffer solution (PBS) reaching a steady state within 30 min	The price of ABTS reagent is high

Table 3. Simplicity, instrument required, biological relevance, mechanism and time required for different antioxidant assays. Source: Reiter et al. (2003).

Antioxidant assays	Simplicity	Instrumentation required	Biological relevance	Mechanism	Time required
ORAC	++	+	+++	HAT	++
TRAP	+++	--	--	HAT	+++
FRAD	+++	+++	--	SET	--
TEAC	++	++	-	SET	-
F-C	+++	-	-	SET	+
TLC autography	+++	+	---	HAT, SET	---
CAA assay	-	-	+++	HAT	+++
Dye substrate oxidation method	+	++	++	HAT	+
CUPRIC	+++	+++	-	HAT	+
Flourometric analysis	++	++	+	HAT	+
ECL	---	+++	+++	HAT	+++
ABTS	+	+	+	HAT	+

+, ++, +++ = Desirable to highly desirable characteristic. -, --, --- = Less desirable to highly undesirable characteristic.

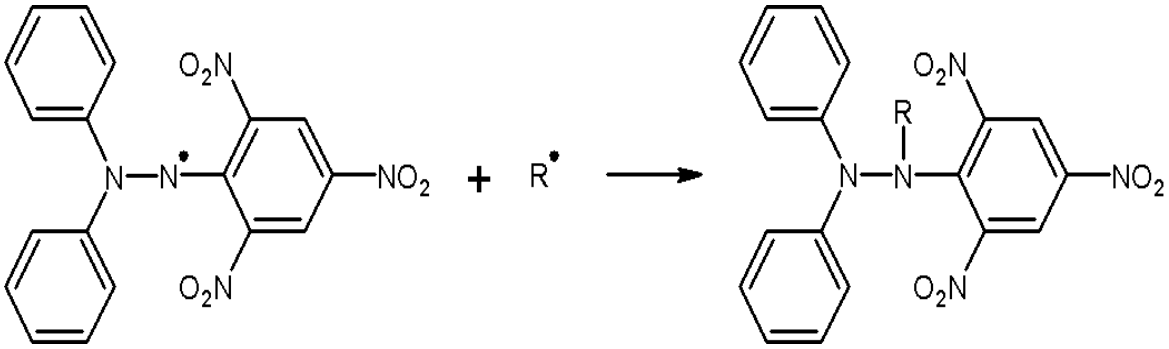

Figure 6. Mode of action of DPPH radical with antiradical compound (RH).

the level being selected from the group consisting of (i) A rising level between 90 to 100 % of maximum; (ii) the maximum; (iii) a postmaximum substantially constant plateau level, (b) adding sample to progressing chemiluminescent reaction, sample causing the level of luminescence generated by the reaction to change when the sample has antioxidant activity, (c) monitoring the change in the level of luminescence, and (d) determining the antioxidant activity of said sample assayed by reference to that of samples of known antioxidant activity subjected to steps (a) to (c). The principle behind the enhanced chemiluminescent assay for total antioxidant capacity (TAC) measurement is best described in the work by Whitehead et al. (1992). To perform the enhanced chemiluminescence assay, a signal reagent (luminal plus para-iodophenol), which is a source of chemiluminescence, is mixed with horseradish peroxidase (HRP)-linked immunoglobulin to produce ROS, which in turn is mixed with a substrate, hydrogen peroxide (H_2O_2).

4-2,2-Diphenyl-1-picrylhydrazyl (DPPH): A rapid, simple and inexpensive method to measure antioxidant capacity of food involves the use of the free radical 2,2-Diphenyl-1-picrylhydrazyl (DPPH). DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors (Figure 6), and to evaluate antioxidant activity of foods. It has also been used to quantify antioxidants in complex biological systems in recent years. The DPPH method can be used for solid or liquid samples and is not specific to any particular antioxidant component, but applies to the overall antioxidant capacity of the sample. A measure of total antioxidant capacity helps understand the functional properties of foods.

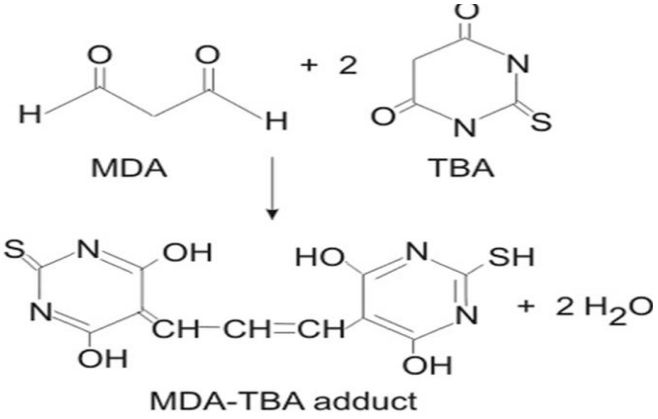

Figure 7. MDA-TBA reaction.

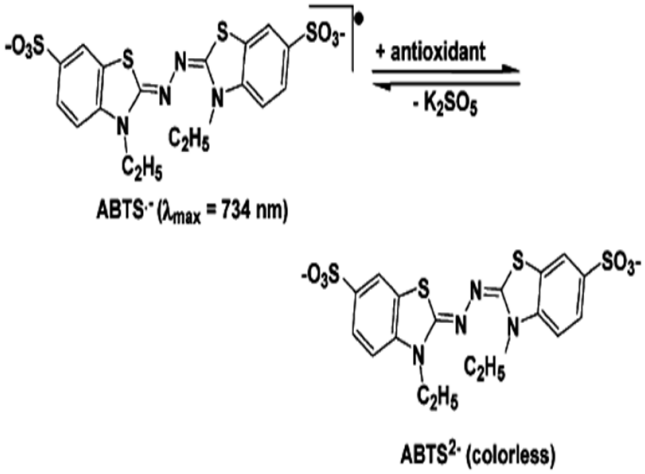

Figure 8. Reaction between ABTS radical and antioxidant compound.

$$Cu(II) + AOH \rightarrow Cu(I) + AO^{\bullet} + H^{+}$$

$$AO^{\bullet} + L{-}H \rightarrow AOH + L^{\bullet}$$

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$$

$$LOO^{\bullet} + L{-}H \rightarrow LOOH + L^{\bullet}$$

$$Cu(I) + LOOH \rightarrow Cu(II) + LO^{\bullet} + HO^{-}$$

Figure 9. The reaction of cupric ion with antioxidant compound (AOH).

The malondialdehyde (MDA) or thiobarbituric acid-reactive-substances (TBARS): These assays have been used extensively since the 1950's to estimate the peroxidation of lipids in membrane and biological systems. These methods can be time consuming because they depend on the oxidation of a substrate which is influenced by temperature, pressure, matrix etc. and may not be practical when large numbers of samples are involved. Antioxidant activity methods using free radical traps are relatively straightforward to perform (Figure 7).

The ABTS [2,2'- azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation: This has been used to screen the relative radical-scavenging abilities of flavonoids and phenolics. ABTS is a better choice than DPPH and more sensitive than DPPH. The ABTS method has the extra flexibility in that it can be used at different pH levels (unlike DPPH, which is sensitive to acidic pH) and thus is useful when studying the effect of pH on antioxidant activity of various compounds. It is also useful for measuring antioxidant activity of samples extracted in acidic solvents. Additionally, ABTS is soluble in aqueous and organic solvents and is thus useful in assessing antioxidant activity of samples in different media and is currently most commonly used in simulated serum ionic potential solution (pH 7.4 phosphate buffer solution containing 150 mM NaCl) (PBS). Another advantage of ABTS+ method was that samples reacted rapidly with ABTS in the aqueous buffer solution (PBS), reaching a steady state within 30 min. The DPPH reacted very slowly with the samples, approaching but not reaching steady state after 8 h. This slow reaction was also observed when ABTS was reacted with samples in alcohol (Figure 8).

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The Oxygen Radical Absorbance Capacity (ORAC): This procedure is used to determine antioxidant capacities of fruits and vegetables. In the ORAC method, a sample is added to the peroxyl radical generator, 2,2'-azobis(2-amidinopropane)dihyd rochloride (AAPH) and inhibition of the free radical action is measured (Cao et al., 1995) using the fluorescent compound, B-phycoery-thrin or R-phycoerythrin. Phenolic and polyphenolic compounds constitute the main class of natural antioxidants present in plants, foods, and beverages and are usually quantified employing Folin's reagent. Vinson et al. (1998) have measured phenolics in fruits and vege-tables colorimetrically using the Folin-Ciocalteu reagent and determined the fruit and vegetables antioxidant capacity by inhibition of low density lipoprotein oxidation mediated by cupric ions.

Cupric assay: Cu (II) may act as a catalyst in the presence of excessive antioxidants, and the antioxidants

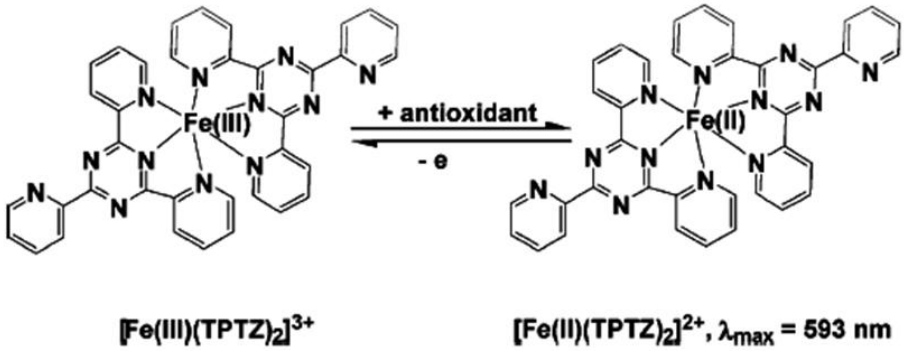

Figure 10. Antioxidant reaction with ferric salt, Fe(III)(TPTZ)2Cl3 (TPTZ) 2,4,6-tripyridyls-triazine.

may act as pro-oxidants. Thus, Cu (II) is a questionable initiator for assaying the radical chain-breaking capacity of antioxidants (Figure 9).

Ferric reducing antioxidant power (FRAP): FRAP assay also takes advantage of electron-transfer reactions. Here, a ferric salt, Fe (III) (TPTZ)2Cl3 (TPTZ) 2,4,6-tripyridyls-triazine), is used as an oxidant. The redox potential of Fe (III) salt (-0.70 V) is comparable to that of ABTS¥- (0.68 V). Therefore, essentially, there is no much difference between TEAC assay and the FRAP assay except that TEAC is carried out at neutral pH and FRAP assay under acidic (pH 3.6) conditions (Figure 10).

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