Chapter 15

Assay Guided Comparison for Enzymatic and Non-Enzymatic Antioxidant Activities with Special Reference to Medicinal Plants

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Additional information is available at the end of the chapter

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1. Introduction

Recently there has been an increasing interest in free radicals in biological systems and their implied role as causative agents in a variety of pathological physiologies. Free radicals can be described as any species, which is capable of independent existence and contained one or more unpaired electrons, which makes them highly reactive. They promote beneficial oxidation to generate energy and kill microbial invaders. But in excess they cause harmful oxidation that can damage cell membrane and even cell death. Antioxidant nutrients have the ability to scavenge free radicals in the system and neutralize them before they do any damage to body cells. Most plants have protective biochemical functions of naturally occurring antioxidants in the cells. Many secondary compounds and enzymes of higher plants have been demonstrated with *in vitro* experiments to protect against oxidative damage by inhibiting or quenching free radicals and reactive oxygen species. Naturally occurring antioxidants in plant cells include i) enzymatic and peptide defence mechanisms (catalases, peroxidases, superoxide dismutases, glutathione and other proteins), ii. Non-enzymatic mechanisms, phenolic defence compounds (vitamin E, flavonoids, phenolic acids and other phenols); nitrogen compounds (alkaloids, amino acids and amines), cartenoids and chlorophyll derivatives. Both the enzymatic and non-enzymatic antioxidants have been playing an important role as natural antioxidant. Ascorbate oxidase is a member of the multicopper oxidase family which catalyzes the one-electron oxidation of ascorbate with the concomitant four-electron reduction of dioxygen to water. Catalase is a tetrahedral protein, constituted by four heme groups which catalyze the dismutation of hydrogen peroxide in water and oxygen. Peroxidases refer to heme containing enzymes which are able to oxidise organic and inorganic compounds using hydrogen peroxide as co-substrate. Ascorbate peroxidase functions as hydrogen peroxide detoxification and gluthathione regeneration via

ascorbate-gluthathione pathway. Ascorbate peroxidase is able to scavenge hydrogen peroxide produced by superoxide dismutase using ascorbate as an electron donor. Since plants provide protection against free radicals, much attention has been drawn to the antioxidant activity of plant extracts. As plants have to themselves counteract stress caused by oxygen, they present a potential source of natural antioxidants. Hence, screening of medicinal plants for their antioxidant potential is essential.

Plants play a significant role in the development of new drugs and in many developing countries attention has been paid to explore natural substances as substitutes for synthetic compounds. The commonly used anti-oxidants, butylated hydroxyanisol and butylated hydroxytolune are synthetic chemicals and the possible toxicity of these anti-oxidants has resulted in their reduced usage [1]. Due to health concerns, natural anti-oxidants have been extensively employed in recent years [2]. Plants and other natural products contain hundreds of compounds those act as natural antioxidant. Therefore, several methods have been developed to quantify these compounds individually. The techniques are different in terms of mechanism of reaction, effectiveness and sensitivity [3,4,5]. Methods that are widely used to measure the antioxidant activity level in herbal sample, fruits and vegetables, and their products are thiobarbituric acid reactive species (TBARS) [6], oxygen radical absorbance capacity (ORAC) [7,8,9], β-carotene bleaching test (BCBT) [10], ABTS radical-cation [11,12], DPPH titration [13], Folin Ciocalteu [14], as well as FTC and FRAP. Therefore, an attempt has been made to review different *in vitro* models for estimating antioxidant properties (both enzymatic and non-enzymatic) from medicinal plants. In the present chapter, various models are described along with the different standards that can be used for estimation. Result comparability is largely dependent upon the techniques employed in the investigations and conclusive results can only be obtained if methods are standardized and universal.

2. Free radicals, reactive oxygen and nitrogen species

A free radical may be defined as a molecule or molecular fragment containing one or more unpaired electrons in its outermost atomic or molecular orbital and is capable of independent existence. Reactive oxygen species (ROS) is a collective term for oxygen derived species namely oxygen radicals and reactive nitrogen species (RNS) are certain non-radical reactive derivatives that are oxidizing agents and/ or are easily converted into radicals. The reactivity of radicals is generally stronger than non-radical species though radicals are less [15], ROS and RNS includes radicals such as superoxide ($O_2^{\bullet -}$), hydroxyl (OH^-), peroxyl ($RO_2^{\bullet}$), hydroperoxyl ($HO_2^{\bullet}$), alkoxyl ($RO^{\bullet}$), peroxyl ($ROO^{\bullet}$), nitric oxide ($NO^{\bullet}$), nitrogen dioxide ($NO_2^{\bullet}$) and lipid peroxyl ($LOO^{\bullet}$) and non radicals like hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3), singlet oxygen ($^1\Delta g$), peroxynitrate (ONOO-), nitrous acid (HNO_2), dinitrogen trioxide (N_2O_3), lipid peroxide (LOOH) [15], Biological systems get exposed to ROS either from endogenous or exogenous. They may be generated *in vivo* by enzymes (XO, NADPH oxidase etc) or by auto oxidation (e.g. adrenaline, dopamine etc.), by leakage of electrons from the mitochondrial electron

transport chain (ETC), by the use of certain chemicals (e.g. doxorubicin, cigarettes etc.), by the catalytic action of free transition metals (e.g. Fe^{++}, Cu^{+} etc.) and by radiation from the environment (e.g. radon, UV, etc.) [16]. $O_2^{\bullet -}$ radical is responsible for lipid peroxidation and to decrease the activity of antioxidant defense system enzymes such as catalase (CAT) and glutathione peroxidasee (GPx). It also causes damage to the ribonucleotide which is required for DNA synthesis. The protonated form of $O_2^{\bullet -}$ ($HO_2^{\bullet}$), is more reactive and able to cross the membrane and causes damage to tissue. $OH^{\bullet}$ radical in most reactive chemical species act as a potent cytotoxic agent and damage almost every molecule found in living tissue. H_2O_2 is not a radical but it produces toxicity to cell by causing DNA damage, membrane disruption and releases Ca^{+} within cell, resulting iactivation of calcium dependent proteolytic enzyme. HOCl is produced by the enzyme myeloperoxidase in activated neutrophils and initiates the deactivation of antiproteases and activation of latent proteases leading to tissue damage [17].

3. Oxidative stress and human health

Active oxygen molecules such as superoxide (O_2, $OOH^{\bullet}$), hydroxyl ($OH^{\bullet}$) and peroxyl ($ROOH^{\bullet}$) radicals play an important role in oxidative stress related to the pathogenesis of different diseases [18]. These free radicals and other related compounds are generated in (a) mitochondria (superoxide radical and hydrogen peroxide); (b) phagocytes (generators of nitric oxide and hydrogen peroxide during the 'respiratory burst' that takes place in activated phagocytic cells in order to kill bacteria after phagocytosis); (c) peroxisomes or microbodies (degrade fatty acids and other substances yielding hydrogen peroxide); and (d) cytochrome P^{450} enzymes, responsible for many oxidation reactions of endogenous substrates [19].

4. Antioxidants

Antioxidants are defined as compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. They are also called as oxidation inhibitor [20]. At any point of time, one antioxidant molecule can react with single free radical and is capable to neutralize free radical(s) by donating one of their own electrons, ending the carbon-stealing reaction. Antioxidants prevent cell and tissue damage as they act as scavenger. A variety of components act against free radicals to neutralize them from both endogenous and exogenous origin [21]. These include-endogenous enzymatic antioxidants; non enzymatic, metabolic and nutrient antioxidants; metal binding proteins like ferritin, lactoferrin, albumin, ceruloplasmin; phytoconstituents and phytonutrients [21]. Antioxidant can be classified as (i) primary antioxidant (terminate the free-radical chain reaction by donating hydrogen or electrons to free radicals and converting them to more stable products), (ii) secondary antioxidant (oxygen scavengers or chelating agent). Antioxidants play an important role as inhibitors of lipid peroxidation in living cell against oxidative damage [22]. It is well established that lipid peroxidation reaction is caused by the formation of free radicals in cell and tissues. Antioxidants also can

be classified into three main types: first line defence antioxidants, second line defence antioxidants and third line defence antioxidants.

4.1. Mechanism of enzymatic and non-enzymatic antioxidant activity

Antioxidants help to prevent the occurrence of oxidative damage to biological macromolecules caused by reactive oxygen species [23]. All aerobic organisms possess an antioxidant defense system to protect against ROS, which are constantly generated *in vivo*, both by accidents of chemistry and for specific purposes [24]. The human antioxidant defence system consists of both enzymatic and non-enzymatic systems. Enzymatic system includes enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GSHPx), catalase etc. SOD catalyses the dismutation of $O_2^{\bullet-}$ at a rate ten times higher than that for spontaneous dismutation at pH 7.4 [25].

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

Human cells have a Mn containing SOD in the mitochondria where as Cu and Zn bearing SOD present in the cytosol [25]. Enzyme catalase located in the peroxisomes converts H_2O_2 into H_2O and O_2 [26]. Another group of Se containing enzymes called glutathione peroxidase uses H_2O_2 as an oxidant to convert reduced glutathione (GSH) to oxidized glutathione (GSSG) [26].

$$2H_2O_2 \xrightarrow{catalase} 2H_2O + O_2$$

$$2GSH + 2H_2O_2 \xrightarrow{GSHPx} GSSG + 2H_2O$$

SOD, CAT, GTx, glutathione reductase and some minerals viz. Se, Mn, Cu and Zn are known as the first line defence antioxidants. As discussed earlier, SOD mainly act by quenching of superoxide (O_2^-), catalase by catalyzing the decomposition of hydrogen peroxide (H_2O_2) to water and oxygen. Glutathione peroxidase is a selenium containing enzyme which catalyses the reduction of H_2O_2 and lipid hydroperoxide, generated during lipid peroxidation, to water using reduced glutathione as substrate. Selenium and vitamin E act as scavengers of peroxides from cytosol and cell membrane, respectively. Cu exerts its antioxidant activity through the cytosolic superoxide dismutase. Second line defence antioxidants are glutathione (GSH), vitamin C, uric acid, albumin, bilirubin, vitamin E (α-tocopherol), carotenoids and flavonoid. β-carotene is an excellent scavenger of singlet oxygen. Vitamin C interacts directly with radicals like O_2^-, HO (hydroxyl). GSH is a good scavenger of many free radicals like O_2^-, HO and various lipid hydroperoxides and may help to detoxify many inhaled oxidizing air pollutants like ozone, NO_2 and free radicals in cigarette smoke in the respiratory tract. Vitamin E scavenges peroxyl radical intermediates in lipid peroxidation and is responsible for protecting poly unsaturated fatty acid present in cell membrane and low density lipoprotein (LDL) against lipid peroxidation. Flavonoids are phenolic compounds, present in several plants, inhibit lipid peroxidation and lipoxygenases. The most important chain breaking antioxidant is α-

tocopherol, present in human membranes. Vitamin C and α-tocopherol both help to minimize the consequences of lipid peroxidation in membranes. Third line antioxidants are a complex group of enzymes for repair of damaged DNA, damaged 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, e.g. lipase, proteases, DNA repair enzymes, transferase, methionine sulphoxide reductase etc. Non-enzymatic antioxidants can also be divided into metabolic antioxidants and nutrient antioxidants. Metabolic antioxidants are the endogenous antioxidants, which produced by metabolism in the body like lipoid acid, glutathione, L-ariginine, coenzyme Q10, melatonin, uric acid, bilirubin and metal-chelating proteins. While nutrient antioxidants belonging to exogenous antioxidants, which cannot be produced in the body but provided through diet or supplements viz. trace metals (selenium, manganese, zinc), flavonoids, omega-3 and omega-6 fatty acids etc. Vitamin E and C are the non enzymatic antioxidants exist within normal cells as well as they can be supplied through diet. Primary antioxidants, for example phenolic compounds react with peroxyl radicals and unsaturated lipid molecules and convert them to more stable products. Whereas, secondary antioxidants or preventives are compounds that retard the rate of chain initiation by various mechanism. This antioxidant reduce the rate of auto-oxidation of lipids by such processes as binding metal ions, scavenging oxygen and decomposing hydroperoxides to non radical products [27]. Secondary may function as electron or hydrogen donors to primary antioxidant radicals, thereby regenerating the primary antioxidant. Chelating agents remove prooxidant metals and prevent metal catalyzed oxidations. The oxygen scavenger such as ascorbic acid is able to scavenge oxygen and prevent oxidation of foods, regenerate phenolic or fat soluble antioxidant, maintain sulphohydryl groups in -SH form and act synergistically with chelating agents [28]. Metal chelating is an example of secondary antioxidant mechanism by which many natural antioxidants can influence the oxidation process. Metal chelators can stabilize the oxide forms of metals that have reduced redox potential, thus preventing metals from promoting oxidation.

4.2. Assessments of antioxidant properties with special reference to plants

A number of methods are available for determination of antioxidant activity of plant extracts. These assays differ from each other in terms of reagents, substrates, experimental condition, reaction medium, and standard analytical evaluation methods. Evaluation of natural and synthetic antioxidants requires antioxidant assays. The exact comparison and selection of the best method are practically impossible due to the variability of experimental conditions and difference in the physical and chemical properties of oxidisable substrates. However, the assay can be described in two systems (i) Antioxidant assays in aqueous system (DPPH, ABTS, DNA protection etc.) and (ii) Antioxidant assays in lipid system (TBARS). Also based on their involvement of chemical reaction they, can be divided into two basic categories-(i) hydrogen atom transfer reaction (HAT) and (ii) single electron transfer (ET) reaction based system.

4.2.1. HAT based assay

These assay are based on hydrogen atom donating capacity. Commonly a synthetic free radical generator, an oxidisable molecular probe and an antioxidant are involved in such assays. The antioxidant competes with probe for free radicals as a result inhibiting the oxidation of probe. This type of assays includes oxygen radical absorbance capacity, total radical trapping parameter assay etc.

4.2.1.1. Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay uses a peroxyl radical induced oxidation reaction to measure the antioxidants chain breaking ability. It uses beta-phycoerythrin (PE) as an oxidizable protein substrate and 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) as a peroxyl radical generator or Cu^{2+}. H_2O_2 as a hydroxyl radical generator. It is the only method that takes free radical action to completion and uses an area under curve (AUC) technique for quantitation. It combines both inhibition percentage and the length of inhibition time of the free radical action by antioxidants into a single quantity. The capacity of a compound to scavenge peroxyl radicals, generated by spontaneous decomposition of 2,2′-azo-bis, 2- amidinopropane dihydrochloride (AAPH), was estimated in terms of standard equivalents, using the ORAC assay [29]. The reaction mixture (4.0 ml) consists of 0.5 ml extract in phosphate buffer (75 mM, pH 7.2) and 3.0 ml of fluorescein solution (both are mixed and pre incubated for 10 min at 37°C). Then, 0.5 ml of AAPH solution is added and immediately the loss of fluorescence (FL) is observed at 1 min intervals for 35 min. The final results are calculated using the differences of areas under the FL decay curves between the blank and a sample and are expressed as micromole trolox equivalents per gram (μmol TE/g).

4.2.1.2. Total radical trapping parameter (TRAP) assay

TRAP is the most widely used *in vivo* method for measuring total antioxidant capacity of plasma or serum during the last decade. The TRAP assay uses peroxyl radicals generated from AAPH and peroxidizable materials contained in plasma or other biological fluids. After adding AAPH to the plasma, the oxidation of the oxidizable materials is monitored by measuring the oxygen consumed during the reaction. During an induction period, this oxidation is inhibited by the antioxidants in the plasma. The length of the induction period (lag phase) is compared to that of an internal standard, Trolox (6-hydroxyl-2,5,7,8,-tetramethylchroman-2-carboxylic acid), and then quantitatively related to the antioxidant capacity of the plasma. Although TRAP is a useful assay for antioxidant measurement activity, the precision and reliability of the method is problematic due to the fact that antioxidant activity can continue after the lag phase.

4.2.1.3. Dichlorofluorescin-diacetate (DCFH-DA) based assay

TRAP can also be measured spectrophotometrically by using dichlorofluorescin diacetate (DCFH-DA) [30]. This assay uses AAPH to generate peroxyl radicals and DCFH-DA as the oxidisable substrate for the peroxyl radicals. The oxidation of DCFH-DA by peroxyl radicals

converts DCFH-DA to dichlorofluorescein (DCF). DCF is highly fluorescent having an absorbance at 504 nm. Therefore, the produced DCF can be monitored either fluorometrically or spectrophotometrically.

4.2.2. ET based assays

These assay are based on the involvement of transfer of electron i.e. a probe (oxidant) is reduced by transfer of electron from an antioxidant (oxidised). The degree of color change of the probe by oxidation is proportional to the amount of antioxidants. These types of assay are questionable to work in *in vivo* systems. So these are basically based on assumption that antioxidant capacity is equal to its reducing capacity. Commonly these types of assay are used in preliminary screening and speed up the experiments. It involves total phenolic content, ferric ion reducing power, ABTS and DPPH.

4.2.2.1. Total phenolic content

The amount of total phenolic content can be determined by Folin-Ciocalteau reagent (FCR) method [31-36]. Commonly 0.5 ml of extract and 0.1 ml of Folin-Ciocalteu reagent (0.5 N) are mixed and incubated at room temperature for 15 min. Then 2.5 ml of saturated sodium carbonate is added and further incubated for 30 min at room temperature and absorbance measured at 760 nm. Gallic acid [34], tannic acid [37], quercetin [31], or guaicol [38], can be used as positive controls. The total phenolic content is expressed in terms of standard equivalent (mg/g of extracted compound).

4.2.2.2. Total flavonoid content

The antioxidative properties of flavonoids are due to several different mechanisms, such as scavenging of free radicals, chelation of metal ions, and inhibition of enzymes responsible for free radical generation [39]. Depending on their structure, flavonoids are able to scavenge practically all known ROS. The amount of total flavonoid content can be determined by aluminium chloride method [40]. The reaction mixture (3.0 ml) comprised of 1.0 ml of extract, 0.5 ml of aluminium chloride (1.2%) and 0.5 ml of potassium acetate (120 mM) is incubated at room temperature for 30 min and absorbance measured at 415 nm. Quercetin [41] or catechin [42] can be used as a positive control. The flavonoid content is expressed in terms of standard equivalent (mg/g of extracted compound).

4.2.2.3. Reducing power

Reducing power showcase the major antioxidant activity of different plant samples [43]. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation process. The reducing power can be determined by the method of Athukorala [44]. 1.0 ml extract is mixed with 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of potassium ferricyanide (30 mM) and incubated at 50°C for 20 min. Thereafter, 2.5 ml of trichloroacetic acid (600 mM) is added to the reaction mixture, centrifuged for 10 min at 3000 rpm. The upper layer of solution (2.5 ml) is mixed with 2.5 ml of distilled

water and 0.5 ml of $FeCl_3$ (6 mM) and absorbance is measured at 700 nm. Ascorbic acid, butylated hydroxyanisole (BHA), a-tocopherol, trolox can be used as positive control.

4.2.2.4. Ferric ion reducing antioxidant power (FRAP)

The FRAP assay measures the reduction of a ferric salt to a blue colored ferrous complex by antioxidants under acidic condition (pH 3.6). The FRAP unit is defined as the reduction of one mole of Fe (III) to Fe (II). Ferric reducing ability of plasma (FRAP) determines the total antioxidant power as the reducing capability. The increase in absorbance (ΔA) at 593 nm is measured and compared with ΔA of a Fe (II) standard solution. The results were expressed as micromole Trolox equivalents (TE) per gram on dried basis. 0.2 ml of the extract is added to 3.8 ml of FRAP reagent (10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10 mM TPTZ solution and 1 part of 20 mM $FeCl_3.6H_2O$ solution) and the reaction mixture is incubated at 37°C for 30 min and the increase in absorbance at 593 nm is measured. $FeSO_4$ solution is used for calibration. The antioxidant capacity based on the ability to reduce ferric ions of sample is calculated from the linear calibration curve and expressed as mmol $FeSO_4$ equivalents per gram of sample. BHT, BHA, ascorbic acid, quercetin, catechin or trolox [45] can be used as a positive control. The FRAP assay is a simple, economic and reducible method which can be applied to both plasma and plant extracts. This method has the advantage of determining the antioxidant activity directly in whole plasma, it is not dependent on enzymatic and non-enzymatic methods to generate free radicals prior to the valuation of antiradical efficiency of the plasma.

4.2.2.5. DPPH method

This method uses a stable chrogen radical, DPPH in methanol, which give deep purple color. By addition of DPPH, the color of the solution fades and the reduction is monitored by the decrease in the absorbance at 515 nm. When a solution of DPPH is mixed with a substance that can donate a hydrogen atom, the reduced form of the radical is generated accompanied by loss of color. This delocalization is also responsible for the deep violet color, characterized by an absorption band at about 515 nm. The reaction mixture (3.0 ml) consists of 1.0 ml of DPPH in methanol (0.3 mM), 1.0 ml of the extract and 1.0 ml of methanol. It is incubated for 10 min in dark, and then the absorbance is measured at 520 nm. In this assay, the positive controls can be ascorbic acid, gallic acid [46] and BHT [47]. The percentage of inhibition can be calculated using the formula:

$$\text{Inhibition}\,(\%) = (A0 - A1 \,/\, A0) \times 100$$

where

A0 is the absorbance of control and A1 is the absorbance of test.

This assay is simple and widely used. However, it has some disadvantages i.e. unlike reactive peroxyl radicals DPPH reacts slowly. The reaction kinetics between the DPPH and antioxidants are not linear as a result EC_{50} measurement is problematic for DPPH assay.

4.2.2.6. ABTS or TEAC assay

TEAC assay is a decolorisation assay applicable to both lipophillic and hydrophilic antioxidants. The TEAC assay is based on the inhibition by antioxidants of the absorbance of the radical cation of 2,2'-azinobis (3-ethylbenzothiazoline 6-sulfonate) (ABTS), which has a characteristic long-wavelength absorption spectrum showing maxima at 660, 734 and 820 nm. Generation of the ABTS radical cation forms the basis of one of the spectrophotometric methods that have been applied to the measurement of the total antioxidant activity. The experiments are carried out using a decolourisation assay, which involves the generation of the ABTS chromophore by the oxidation of ABTS with potassium persulphate. The ABTS free radical-scavenging activity of plants samples is determined by the method of Stratil et al. [48]. The radical cation $ABTS^{+}$ is generated by persulfate oxidation of ABTS. A mixture (1:1, v/v) of ABTS (7.0 mM) and potassium persulfate (4.95 mM) is allowed to stand overnight at room temperature in dark to form radical cation $ABTS^{+}$. A working solution is diluted with phosphate buffer solution to absorbance values between 1.0 and 1.5 at 734 nm. An aliquot (0.1 ml) of each sample is mixed with the working solution (3.9 ml) and the decrease of absorbance is measured at 734 nm after 10 min at 37°C in the dark. Aqueous phosphate buffer solution (3.9 ml, without $ABTS^{+}$ solution) is used as a control. The $ABTS^{+}$ scavenging rate is calculated. The reaction is pH - independent. A decrease of the $ABTS^{+}$ concentration is linearly dependent on the antioxidant concentration. Trolox, BHT, rutin [49], ascorbic acid [50] or gallic acid [51] can be used as a positive control. The only problem with ABTS does not resemble the radical found in the biological system. However, this assay is widely used because of its simplicity and automation.

4.2.2.7. Assay of superoxide radical (O_2^-) scavenging activity

Superoxide anion generates powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative stress [52]. In the PMS/NADH-NBT system, the superoxide anion derived from dissolved oxygen from PMS/NADH coupling reaction reduces NBT. The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. The superoxide anion scavenging activity is measured as described by Robak and Gryglewski [53]. The superoxide anion radicals are generated in 3.0 ml of Tris-HCl buffer (16 mM, pH 8.0), containing 0.5 ml of NBT (0.3 mM), 0.5 ml NADH (0.936 mM) solution and 1.0 ml extract. The reaction is started by adding 0.5 ml PMS solution (0.12 mM) to the mixture, incubated at 25°C for 5 min and then the absorbance is measured at 560 nm. Later, Dasgupta and De [55] modified this method using riboflavin-light-NBT system. Each 3 ml mixture contains 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 μM riboflavin, 100 μM EDTA, NBT (75μM) and 1 ml sample solution. Gallic acid [53], BHA, ascorbic acid, a-tocopherol, curcumin [56] can be used as a positive control.

4.2.2.8. Assay of hydroxyl radical (-OH) scavenging activity

Plant extracts have ability to inhibit non-specific hydroxyl radical (hydroxyl radical reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cell [57, 58]. The model used is ascorbic acid-iron-EDTA model of OH generating

system, in which ascorbic acid, iron and EDTA work together with each other to generate hydroxyl radicals. The reaction mixture (1.0 ml) consist of 100 µl of 2-deoxy-D-ribose (28 mM in 20 mM KH_2PO_4-KOH buffer, pH 7.4), 500 µl of the extract, 200 µl EDTA (1.04 mM) and 200 µM $FeCl_3$ (1:1 v/v), 100 µl of H_2O_2 (1.0 mM) and 100 µl ascorbic acid (1.0 mM) which is incubated at 37°C for 1 hour. 1.0 ml of thiobarbituric acid (1%) and 1.0 ml of trichloroacetic acid (2.8%) are added and incubated at 100°C for 20 min. After cooling, absorbance is measured at 532 nm, against a blank. Gallic acid, catechin [59], vitamin E [60] can be used as a positive control. Later, this method was modified by Dasgupta and De [55] based on benzoic acid hydroxylation using spectroflurometer. The reaction mixtures (2 ml) consist of 200 µl each of sodium benzoate (10mM), $FeSO_4.7H_2O$ (10mM) and EDTA (10mM). The solution mixtures are volume makeup to 1.8 ml by adding phosphate buffer (pH 7.4, 0.1 M). Finally 0.2 ml of H_2O_2 (10mM) is added and incubated at 37 ^oC for 2 hours. The fluorescens are measured at 407 nm emission (Em) and excitation (Ex) at 305 nm.

4.2.2.9. Hydrogen peroxide radical scavenging assay

Hydrogen peroxide occurs naturally at low concentration levels in the air, water, human body, plants, microorganisms and food. Hydrogen peroxide enters the human body through inhalation of vapor or mist and through eye or skin contact. In the body, H_2O_2 is rapidly decomposed into oxygen and water and this may produce hydroxyl radicals (OH_y) that can initiate lipid peroxidation and cause DNA damage. The ability of plant extracts to scavenge hydrogen peroxide is determined according to the method of Ruch et al. [61]. A solution of hydrogen peroxide (40 mM) is prepared in phosphate buffer (50 mM, pH 7.4). Extract concentration (20-50 g/ml) aqueous is added to hydrogen peroxide and absorbance at 230 nm after 10 min. incubation against a blank solution (phosphate buffer without hydrogen peroxide). The percentage of hydrogen peroxide scavenging is calculated as follows:

$$\% \text{ Scavenged } \left(H_2O_2\right) = \left(A0 - A1 \;/\; A0\right) \times 100$$

where

A0 is the absorbance of control and A1 is the absorbance of test. Ascorbic acid, rutin, BHA [62] can be used as a positive control.

4.2.2.10. Nitric oxide radical scavenging assay

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which were measured using the Griess reaction reagent (1% sulphanilamide, 0.1% naphthyethylene diamine dihydrochloride in 2% H_3PO_3) [63]. 3.0 ml of 10 mM sodium nitroprusside in phosphate buffer is added to 2.0 ml of extract and reference compound in different concentrations (20-100 µg/ml). The resulting solutions are then incubated at 25°C for 60 min. A similar procedure is repeated with methanol as blank, which serves as control. To 5.0 ml of the incubated sample, 5.0 ml of Griess reagent is added and absorbance is measured at 540 nm. Percent inhibition of the nitrite oxide generated is measured by comparing the absorbance values of control and test. Curcumin, caffeic acid, sodium nitrite [64], BHA, ascorbic acid, rutin [55] can be used as a positive control.

4.2.3. Xanthine oxidase assay

To determine superoxide anion-scavenging activity, two different assays can be used: the enzymatic method with cytochrome C [65] and nonenzymatic method with nitroblue tetrazolium (NBT) [66]. With cytochrome C method, superoxide anions can be generated by xanthine and xanthine oxidase system. The extract (500 μl of 0.1 mg/ml) and allopurinol (100 μg/ml) (in methanol) is mixed with 1.3 ml phosphate buffer (0.05M, pH 7.5) and 0.2 ml of 0.2 units/ml xanthine oxidase solution. After 10 min of Incubation at 25°C, 1.5 ml of 0.15 M xanthine substrate solution is added to this mixture. The mixture is re-incubated for 30 min at 25°C and then the absorbance is taken at 293 nm using a spectrophotometer against blank (0.5 ml methanol, 1.3 ml phosphate buffer, 0.2 ml xanthine oxidase). BHT [67] can be used as a positive control. Percentage of inhibition was calculated using the formula:

$$\text{Inhibition}\ (\%) = \left[1 - (\text{As} / \text{Ac})\right] \times 100$$

where

As and Ac are the absorbance values of the test sample and control, respectively.

4.2.4. Metal chelating activity

Ferrozine can chelate with Fe^{++} and form a complex with a red color which can be quantified. This reaction is limited in the presence of other chelating agents and results in a decrease of the red color of the ferrozine-Fe^{++} complexes. Measurement of the color reduction estimates the chelating activity to compete with ferrozine for the ferrous ions [68]. The ferrous ions chelating activity can be measured by the decrease in absorbance at 562nm of iron (II)-ferrozine complex [69]. 1 ml of the extract is added to a solution of 1 ml of ferrous sulphate (0.125 mM). The reaction is initiated by the addition of 1 ml of ferrozine (0.3125 mM) and incubated at room temperature for 10 min and then the absorbance is measured at 562 nm. EDTA or citric acid [69] can be used as a positive control. The ability of sample to chelate ferrous was calculated relative to the control using formula

$$\text{Chelating effect}\ (\%) = (\text{Ac} - \text{As} / \text{Ac}) \times 100$$

where

Ac-Absorbance of control, As-Absorbance of sample

4.2.5. Lipid peroxidation

The oxidation of linoleic acid generates peroxyl free radicals due to the abstraction of hydrogen atoms from diallylic methylene groups of linoleic acid. These free radicals later oxidize the highly unsaturated beta carotene (orange colour disappear) and the results can be

monitored spectrophotometrically. The antioxidant activity is determined by the conjugated diene method [70]. Different concentration of extracts (0.1-20 mg/ml) in water or ethanol (100 μl) is mixed with 2.0 ml of 10 mM linoleic acid emulsion in 0.2 M sodium phosphate buffer (pH 6.6) and kept in dark at 37°C. After incubation for 15 h, 0.1 ml from each tube is mixed with 7.0 ml of 80% methanol in deionized water and the absorbance of the mixture is measured at 234 nm against a blank in a spectrophotometer. Later, this method was replaced by using thiocyanate. 0.5 ml of each extract sample with different concentration is mix up with linoleic acid emulision (2.5 ml 40 mM, pH 7.0). The final volume was adjusted to 5 ml by adding with 40 mM phosphate buffer, pH 7.0. After incubation for 72 hours at 37° C in dark, 0.1 ml aliquot is mixed with 4.7 ml of ethanol (75%), 0.1 ml $FeCl_2$ (20mM) and 0.1 ml ammonium thiocyanate (30%). The absorbance of mixture is measured at 500 nm in spectrophotometer. Ascorbic acid, BHT, gallic acid, α-tocopherol [70] can be used as a positive control.

The antioxidant activity is calculated as follows:

$$\text{Antioxidant activity } (\%) = (\text{Ac} - \text{As} / \text{Ac}) \times 100,$$

where

Ac-Absorbance of control, As-Absorbance of sample

4.2.6. Cyclic voltammetry method

The cyclic voltammetry procedure evaluates the overall reducing power of low molecular weight antioxidants. The sample is introduced into a well in which three electrodes are placed: the working electrode (e.g., glassy carbon), the reference electrode (Ag/AgCl), and the auxiliary electrode (platinum wire). The potential is applied to the working electrode at a constant rate (100 mV/s) either toward the positive potential (evaluation of reducing equivalent) or toward the negative potential (evaluation of oxidizing species). During operation of the cyclic voltammetry, a potential current curve is recorded (cyclic voltammogram). Recently quantitative determination of the phenolic antioxidants using voltammetric techniques was described by by Raymundo et al. [71] and Chatterjee et al. [72].

4.2.7. Photochemiluminescence (PCL) assay

PCL assay was initially used by [73, 74] to determine water-soluble and lipid-soluble antioxidants. The photochemiluminescence measures the antioxidant capacity, towards the superoxide radical, in lipidic and water phase. This method allows the quantification of the antioxidant capacity of both the hydrophilic and/or lipophilic substances, either as pure compounds of complex matrix from different origin. The PCL method is based on an acceleration of the oxidative reactions *in vitro.* The PCL is a very quick and sensitive measurement method (1000 times faster than the normal conditions). Wang et al. [75] determined antioxidant property in marigold flowers using this technique.

5. Preparations of enzyme extracts

For determination of antioxidant enzymes activities, enzyme extraction can be prepared according to methods of Nayar and Gupta [76], Hakiman and Maziah [77]. Each plant material (0.5 g) was ground with 8 ml solution containing 50 mM potassium phosphate buffer (pH 7.0) and 1% polyvinylpolypyrolidone. The homogenate was centrifuged at 15000 rpm for 30 min and supernatant was collected for enzymes assays (ascorbate oxidase, peroxidase, catalase, ascorbate peroxidase, glutathione s-transferase and superoxide dismutase).

5.1. Ascorbate oxidase activity

Ascorbate oxidase activity can be measured with the method of Diallinas et al. [78]. 1.0 ml of reaction mixture contained 20 mM potassium phosphate buffer (pH 7.0) and 2.5 mM ascorbic acid. The reaction was initiated with the addition of 10 μl enzyme extract. The decrease in absorbance was observed for 3 min at 265 nm due to ascorbate oxidation and calculated using extinction coefficient, $mM^{-1}cm^{-1}$.

5.2. Peroxidase activity

Peroxidase activity was determined using the guaicol oxidation method [79, 80]. The 3 ml reaction mixture contains 10 mM potassium phosphate buffer (pH 7.0), 8 mM guaicol and 100 μl enzyme extract. The reaction was initiated by adding 0.5 ml of 1% H_2O_2. The increase in absorbance was recorded within 30 s at 430/470 nm. The unit of peroxidase activity was expressed as the change in absorbance per min and specific activity as enzyme units per mg soluble protein (extinction coefficient 6.39 $mM^{-1}cm^{-1}$).

5.3. Catalase activity

Catalase activity can be determined following the methods of Aebi [81] and Luck [82]. The reaction mixture (1ml) contain potassium phosphate buffer (pH 7.0), 250 μl of enzyme extract and 60 mM H_2O_2 to initiate the reaction. The reaction was measured at 240 nm for 3 min and H_2O_2 consumption was calculated using extinction coefficient, 39.4 $mM^{-1}cm^{-1}$.

5.4. Ascorbate peroxidase activity

The reaction mixture for ascorbate peroxidase activity includes 100 mM tris-acetate buffer at pH 7.0, 2 mM ascorbic acid, enzyme extracts and 2 mM of H_2O_2 to initiate the reaction. The decrease in absorbance at 290 nm was measured and monitored for 100 s. The reaction was calculated using extinction coefficient, 2.8 $mM^{-1}cm^{-1}$[83].

5.5. Glutathione S-transferase activity

This assay can be performed according to the method of Habig [84]. The assay mixture containing 100 μl of GSH, 100 μl of CDNB and phosphate buffer 2.7 ml. The reaction was

started by the addition of 100 µl enzyme extract to this mixture and absorbance was recorded against blank for three minutes. The complete assay mixture without the enzyme served as the control to monitor non-specific binding of the substrates. One unit of GST activity is defined as the nmoles of CDNB conjugated per minute.

5.6. Polyphenol oxidase (PPO) activity

The activity of polyphenol oxidase, comprising of catechol oxidase and laccase, can be simultaneously assayed by the spectrophotometric method proposed by Esterbauer [85]. Plant samples (5g) were homogenized in about 20 ml medium containing 50 mM Tris HCl, pH 7.2, 0.4 M sorbitol and 10 mM NaCl. The homogenate was centrifuged at 2000 rpm for 10 minutes and the supernatant was used for the assay. The assay mixture contained 2.5ml of 0.1M phosphate buffer and 0.3 ml of catechol solution (0.01 M). The spectrophotometer was set at 495 nm. The enzyme extract (0.2 ml) was added to the same cuvette and the change in absorbance was recorded every 30 seconds up to 5 minutes. One unit of either catechol oxidase or laccase is defined as the amount of enzyme that transforms 1 µmole of dihydrophenol to 1 µmole of quinine per minute under the assay conditions. Activity of PPO is calculated using the formula KxΔA/min where K for catechol=0.272 and K for laccase=0.242

5.7. Assay of superoxide dismutase (SOD)

The activity of superoxide dismutase was assayed spectrophotometrically by the method of Misra and Fridovich [86]. The incubation medium contained, in a final volume of 3.0 ml, 50 mM potassium phosphate buffer (pH 7.8), 45 µM methionine, 5.3 mM riboflavin, 84 µM NBT and 20 µM potassium cyanide. The amount of homogenate added to this medium was kept below one unit of enzyme to ensure sufficient accuracy. The tubes were placed in an aluminium foil-lined box maintained at 25°C and equipped with 15W fluorescent lamps. After exposure to light for 10 minutes, the reduced NBT was measured spectrophotometrically at 600nm. The maximum reduction was observed in the absence of the enzyme. One unit of enzyme activity was defined as the amount of enzyme giving a 50% inhibition of the reduction of NBT. The values were calculated as units/mg protein.

6. Conclusion

Currently there has been an increased global interest to identify antioxidant compounds from plant sources which are pharmacologically potent and have low or no side effects. Increased use of different chemicals, pesticides, pollutant, smoking and alcohol intake and even some of synthetic medicine enhances the chance of free radicals based diseases. Plants produces large amount of antioxidants to prevent the oxidative stress, they represent a potential source of new compounds with antioxidant activity. Increasing knowledge of antioxidant phytoconstituents and their inclusions can give sufficient support to human body to fight against those diseases. Phytoconstituents and herbal

medicines are also important to manage pathological conditions of those diseases caused by free radicals. Therefore, it is time, to explore and identify our traditional therapeutic knowledge and plant sources and interpret it according to the recent advancements to fight against oxidative stress, in order to give it a deserving place. The present review is a compilation of different *in vitro* assay methods used in determining the antioxidant activity of different plant extracts. Free radicals are often generated as byproducts of biological reactions or from exogenous factors. The involvement of free radicals in the pathogenesis of a large number of diseases is well documented [24]. A potent scavenger of free radicals may serve as a possible spering intervention for the diseases. Although *in vitro* antioxidant assays have been carried out for a number of medicinal plants, there is lack of information on *in vivo* studies. Consequently, there is a need for more detailed studies to elucidate the mechanism of the pro-oxidant effect and to determine its relevance *in vivo*. Active compounds of many plant extracts possessing antioxidant activity are yet to be identified. Currently scores of techniques are used in testing antioxidant properties are highly specialized and the results depend often on the applied techniques. Therefore, there is need for collaborative studies to standardize these methods. In most of the studies the purity of the phytochemicals is not mentioned, this can mask their activity. Also several articles represent the extract are not readily water soluble, therefore dissolved in organic solvents viz. DMSO, ethanol, chloroform etc those are powerful $OH^{\bullet}$ scavengers. Also many publications show the extract concentration in molar and millimolar concentration, while these concentrations are not relevant, because such concentration never obtained in plasma level. Simultaneously, phytochemicals exhibit not only antioxidant properties but also other biological properties. Hence, a complete study could be useful in future for treatment of various diseases due to their combined activities.

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