Critical Reviews in Analytical Chemistry

ISSN: 1040-8347 (Print) 1547-6510 (Online) Journal homepage: http://www.tandfonline.com/loi/batc20

Critical Review on the Analytical Mechanistic Steps in the Evaluation of Antioxidant Activity

Anantharaman Shivakumar & M. S. Yogendra Kumar

To cite this article: Anantharaman Shivakumar & M. S. Yogendra Kumar (2018) Critical Review on the Analytical Mechanistic Steps in the Evaluation of Antioxidant Activity, Critical Reviews in Analytical Chemistry, 48:3, 214-236, DOI: 10.1080/10408347.2017.1400423

To link to this article: https://doi.org/10.1080/10408347.2017.1400423

Published online: 16 Jan 2018.

CRITICAL REVIEWS IN ANALYTICAL CHEMISTRY
2018, VOL. 48, NO. 3, 214–236
https://doi.org/10.1080/10408347.2017.1400423

Taylor & Francis
Taylor & Francis Group

Critical Review on the Analytical Mechanistic Steps in the Evaluation of Antioxidant Activity

Anantharaman Shivakumar [a] and M. S. Yogendra Kumar [b]

[a]PG Department of Chemistry, St. Philomena's College, Mysore, Karnataka, India; [b]Defence Bio-Engineering and Electromedical Laboratory, Defense Research and Development Organization, Bengaluru, Karnataka, India

ABSTRACT

Electrophysiological systems are prone to release free radicals for functioning of biological system with proper balancing of antioxidant–prooxidant ratio for establishing a healthy living system. The biostress condition releases different reactive oxygen species, such as hydroxyl, alkoxyl, superoxide, hydrogen peroxide, hydroperoxyl, ozone, singlet oxygen, hypochlorus acid, thiyl radical, etc. This review tries to discuss the general aspects of the antioxidant assay methodologies that are currently used for the detection of antioxidant property. The entire review has been divided into three different sections. The first deals with the release of free radical by mitochondrial dysfuctioning and its curbing action by local antioxidants. The second and third sections discuss the general procedure adopted and reaction mechanism involved in the assay procedure along with the limitations and advantages.

KEYWORDS

Antioxidant assay methodology; free radicals; oxidative implication; reactive oxygen species

Introduction

Redox is an essential metabolic reaction in living system, wherein the electrons are transferred from one species to another. These redox processes are the main reactions in any biological system, whereby the chain of chemical reactions takes place in the living system that uses oxygen from air for oxidation and to provide energy in the form of ATP.[1] In a normal cell, balance persists between pro-oxidants and antioxidants. Any imbalance in the release of electron and the oxygen generates short-lived reactive species like superoxide, hydroxyl, peroxyl, alkoxyl, and other nitrogen species. It has been estimated that 1–3% of oxygen inhaled by human get converted into superoxide radical anion.[2] When the equilibrium gets shifted toward pro-oxidants, the free-radical reactive species generation is enhanced greatly, leading to oxidative stress condition. These free radicals being highly reactive in nature, combines with macromolecules to produce, cross-linking and lipid peroxidation. The controlled and periodical production of derivatives of reactive oxygen species (ROS) during the respiratory burst are essential for the defense mechanism in killing microorganisms without causing significant loss in the function of the associated tissues in the biological counterpart.[3]

The instability of these reactive species is a serious threat to nearby cells and biomolecular system and damage caused by them is an inevitable process and should be dealt by the repairing processes. Reactive species been implied in aging and more than 100 diseases, including heart disease, diabetes, and cancer[4,5] and neurodegenerative disease. The cause of the initiation of these diseases is attributed by damaging of crucial biomolecules such as DNA, RNA, lipids, proteins, polyunsaturated fatty acids, carbohydrates, etc. The oxidant inhaled during the cigarette smoke, industrial gases, ozone, and vehicular gases are reactive species that can initiate degradation of biomolecules.[6]

Reductant and oxidant are chemical terms, whereas antioxidants and pro-oxidants have the meaning in the context of a biological system. Pro-oxidants and antioxidants initiate and inhibit the oxidation process, respectively. An antioxidant is a reductant, but a reductant is not necessarily an antioxidant. Antioxidants inhibit or delay oxidative damage of a target molecule by the process of neutralization of free radicals by donating one of the available electrons of its own by free radical stealing or by scavenging mechanism. Hence, high antioxidant food content intake is necessary for controlling the release of oxidants during different biological stress conditions, such as inflammation, cardiac disease, aging, cataract, autism, Alzheimer's, and cancer.

Based on the nature of mechanism of interaction, antioxidant can be classified as primary, secondary, endogenous, exogenous, enzymatic, and non-enzymatic. Generally antioxidants are the molecules that contain reactive hydroxyl groups, which may be phenolic or non-phenolic that includes ascorbic acid, tocopherol, polyphenols, and flavanoids. This definition is not rigid as there deviation in classical definition, for example, ecdysteroids which although do not contain active hydroxyl groups but still possess antioxidant property and free-radical-scavenging tendency.[7]

Antioxidant activity and antioxidant capacity are two terms, the former corresponds to rate constant of single antioxidant against a given free radical, whereas the latter is the number of

CONTACT Anantharaman Shivakumar, Assistant Professor shivakem77@yahoo.co.in St. Philomena's College, PG Department of Chemistry, Bangalore-Mysore Road, Bannimantap, Mysore 570 015, Karnataka, India.

Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/batc.

moles of a given free radical scavenged by the test solution independently by one antioxidant in the mixture.[8] The antioxidant assay can be either distinct assay (e.g., Ascorbic acid, Vitamin E, uric acid, etc) or total antioxidant potency that is assayed by the combined reducing activities of the given substance. Indeed, the assay of individual antioxidant is not of much use compared to total antioxidant assay capacity. The individual antioxidant assay methods are especially useful in understanding the structure–activity relationship of pure antioxidant compounds in the determination of antioxidant contribution to specific dietary components and in the study of decrease in plasma antioxidant activity in individuals under oxidative stress in specific disease states.[9] In the case of plasma being a heterogeneous mixture, the antioxidant status can be better reflected by capacity rather than activity. This capacity is the total redox chain antioxidants, which includes thiol-bearing antioxidants and uric acid. The strategic path in antioxidant assay method depends upon the source and scavenging ability of antioxidants. Antioxidant assay includes direct and indirect methods; one direct method relies on the capacity of the free radical to oxidize or reduce the reaction probe and the indirect one adopts the inhibition of the antioxidant for the formation of products. A same sample of heterogeneous mixture of antioxidants is analyzed by different methodology, such as Ferric reducing antioxidant parameter (FRAP), Copper reducing antioxidant capacity (CUPRAC), 2,2'-azinobis (3-ethylbenzothiazolline-6-sulfonic acid) (ABTS), 2,2-Diphenyl-1-picrylhydrazyl (DPPH) to evaluate single or mixed mechanisms of Electron transfer (ET) or Hydrogen atom transfer (HAT). Nevertheless, the activity will contribute to different values due to varied experimental conditions and thus enabling full antioxidant capacity of the sample extract.

Chemical diversity of antioxidants makes it difficult to separate and determine individual components from the matrix. All antioxidant assay mechanisms cannot be considered under one roof of determination or detection due to uniqueness in mechanistic steps, redox potential, pH, and solvent dependencies. The coupling reactions are not consistent in its antioxidant capacity assays due to their variable absorption maximum. The problem associated with true biological consequences is the formation of short-lived ROS, which are very difficult to quantify *in vivo*. However, several trapping methods have been developed for use *in vitro*. The total antioxidants can be assayed by different methodologies that include luminol-enhanced chemiluminiscence, β-phycoerythrin bleaching, FRAP, CUPRAC, Cerric reducing antioxidant capacity (CERAC), lipid peroxides generation, and Crocin bleaching assay (CBA).

The aim of the present review is to provide comprehensive chemical investigation methods available for the assay of antioxidants. There are quite number of research articles pertaining to the assay of antioxidant methodology. But, few articles are concerned on the analytical reaction mechanistic steps involved. In this route, authors have made a modest attempting in fulfilling the gap between the reaction mechanism and methodology involved. Authors acknowledge the review carried out by other researchers.[1,10] Amorati research group[11] highlights the advantages and limitations of different antioxidant assay procedures. However, there is no discussion on the core part in the process of the reaction such as mechanistic steps involved. The present review discusses these aspects in a logical frame work that include implication of ROS in the mitochondrial dysfunctioning, sites of ROS production, and related self-defense mechanisms, structure and activity relationship of different antioxidants and their relation to the chemistry of antioxidant assays.

Reactive species: A free radical and its implication

Free radical, also known as reactive oxygen or nitrogen or carbon that contains one or more unpaired electrons in the outermost orbital is an unstable species that exists independently. A contributing factor for this unstability is lack of octet configuration. Reactive species of oxygen and nitrogen have characteristics such as short life, generation of new vulnerable ROS, and damage to various tissues.

The four-step sequential univalent reduction of oxygen can be presented as follows:

$$O_2 \xrightarrow{e^-} O_2^{\cdot} \xrightarrow{e^-} O_2^{2-} \xrightarrow{e^-} O\dot{H} \xrightarrow{e^-} H_2O$$

Oxygen is reduced from a variety of sources, including either the formation of superoxide or conversion into singlet oxygen or formation of hydrogen peroxide. Most of the oxygen consumed in the body is fully reduced to water in a single stage at the terminal *cytochrome* of the oxygen transport chain in the mitochondria. However, under certain conditions, while reduced oxygen passes through two intermediate stages in the formation of hydrogen peroxide, is accelerated by the intracellular superoxide dismutase, widely distributed as a defense mechanism against superoxide toxicity. The damage by ROS is created by the partial reduction of oxygen. An imbalance between the reduction of oxygen and electron transport chain creates the havoc of ROS, which is modulated by the rate of electron flow through respiratory chain complexes. Hydrogen peroxide itself breaks down into water and oxygen under the influence of CAT and various peroxidase enzymes. Indeed most ROS are produced at low levels by normal aerobic metabolism and play important role in the redox-dependent regulation of many signaling processes.

Production of reactive species by complexes in electron transport chain

The complex IV is surrounded by hydrophilic arm from *Thermus thermophiles*, which is unlikely to react with molecular oxygen at a significant rate. The oxygen accesses only the live parts of the complex at each end of the flavin moiety or the quinone binding site. This complex produces minimum ROS under normal condition, but increases significantly by the addition of inhibitors such as rotenone,[12] cyanide, carbon monoxide, nitric oxide, hydrogen sulphide,[13] azide,[14] chloropromazine,[15] carboxin,[16] and antimycin.[17] Electrons escaped from complex I and III reacts with molecular oxygen to produce superoxide species. The superoxide radical species in the presence of SOD gets converted into hydrogen peroxide. If hydrogen peroxide is not eliminated, it can generate the highly reactive hydroxyl radical

by Fenton reaction.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^-$$

Short-lived hydroxyl radicals unspecifically attack the biomolecules in a diffusion limited reaction and thus able to crack polysaccharides, proteins, and nucleic acids located within a few nanometers from their site of generation.[18,19]

Sites of ROS production and its self-defense organization

Mitochondria has two layers one inner and another outer layer both composed of phospholipid bilayer. The inner membrane has more surface area compared to outer layer due to folding shelf like called "cristae," which is filled with matrix. It maintains high proton gradient created by the mitochondrial complexes, which is necessary in the ATP synthesis whereas outer layer is permeable to small molecules. Mitochondria constitute a major critical region of site for ROS production. The factor responsible for oxidative stress is increased respiration that initiates chances of ROS generation which in turn causes ATP depletion, mitochondrial permeability, and transition programmed cell death. Most of the oxygen inhaled by the body gets converted into water at the terminal cytochrome in the mitochondrion. But under stress conditions this consumed oxygen passes through two intermediate stages namely, formation of superoxide and hydrogen peroxide. Formation of superoxide ion is favorable due to its standard reduction potential that is −160 mV at pH 7.0 under standard state 1 M O_2, which is practically an irreversible process involving kinetically controlled reaction. The Nernst equation for both the species in equilibrium is given as

$$E_h = -160 + 61.5\log\frac{[O_2]}{[O_2^-]}$$

where E_h is the Nernst reduction potential.

Hydrogen peroxide can also be produced by several oxidase enzymes such as amino acid oxidase and xanthine oxidase. If this hydrogen peroxide comes in contact with the Iron complex or copper complex (myoglobin, hemoglobin, and cytochromes) in the vicinity, it further gets mobilized to hydroxyl radical.[20] Hydroxyl radical can also be formed by the reaction of superoxide radical with hydrogen peroxide by Haber–Weiss reaction.[21] Majority of the ROS in the mitochondrion gets involved in the damage of the tissue such as sulphydryl, deoxyribonucleic acid, and lipids which may involve lipid peroxidation.[22] The different species that can initiate the formation of superoxide radical include NAD(P)H oxidase, phagocytic cells,[3] PQ,[23] hyperoxic, and hypoxic conditions,[24] enzymes such as xanthine oxidase[25] and aldehyde oxidase,[26] drugs used in cancer medication such as bleomycin[27] and anthracyclines[28] (Figure 1). Apart from this mineral dust particles such as asbestos, silica and air pollutant ozone, X-rays, cigarette smoking, and industrial chemicals are shown to generate free radicals, which are direct inducers of lipid

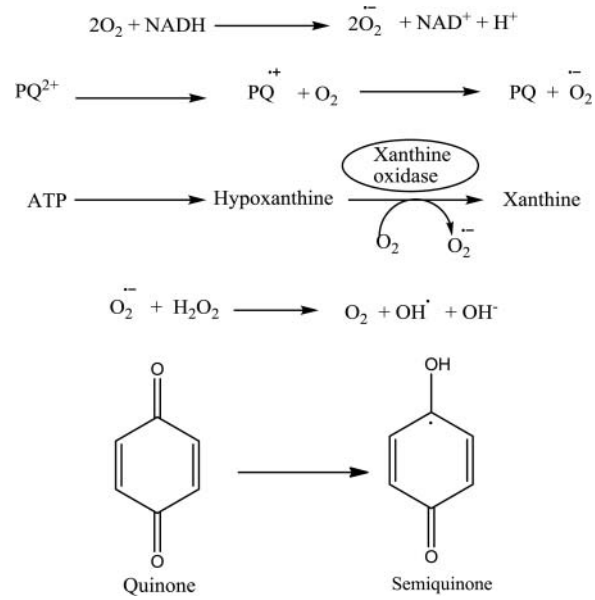

Figure 1. Molecular species responsible for initiation in the formation of ROS.

peroxidation.[22] It was also proposed two decades ago that the reaction between mitochondrial aconitase and superoxide plays a major role in the production of hydroxy radical.[29] The free radical release takes place through the reaction of iron released from mitochondrial aconitase with the hydrogen peroxide.[30] NADH produces superoxide ions by the process of respiratory burst during phagocytosis. The phagocytic cells produce increased levels of ROS by exposure to increased levels of toxins. The exposure of living organism to ionizing radiation homolytically splits water molecule into hydrogen and hydroxyl radical and further into hydroperoxyl radical, superoxide, hydrogen peroxide, and further forms.[22]

Human cells have SOD, CAT, and GPx as enzymatic antioxidant defense and non-enzymatic counterpart as α-tocopherol, ascorbic acid, flavonoids, lipoic acid, glutathione, L-arginine, co-enzyme Q10, melatonin, and uric acid which are either nutrient or metabolic antioxidants. SOD is classified as MnSOD and CuZnSOD. The former is present in large concentration in the mitochondria, while the latter is largely present in the cytosolic region. The main function of the SOD is to accelerate the conversion of superoxide into hydrogen peroxide. CAT converts hydrogen peroxide into water and oxygen. GPx, a selenium-dependent enzyme removes hydrogen peroxide by using it to oxidize the reduced glutathione to oxidized glutathione (Figure 2d). A healthy human body requires an appropriate balance between the production of antioxidants and its defenses. The primary function of the antioxidant is not only the prevention of formation of ROS but also to develop a conducive environment for repairing.

Structure and activity relationship of antioxidants

The most important among natural antioxidants are tocopherols, flavanoids, and phenolics acids. Ascorbic acid, a hydroxyl containing antioxidant, is one of the simple, powerful, and least toxic and water soluble natural antioxidants found in many

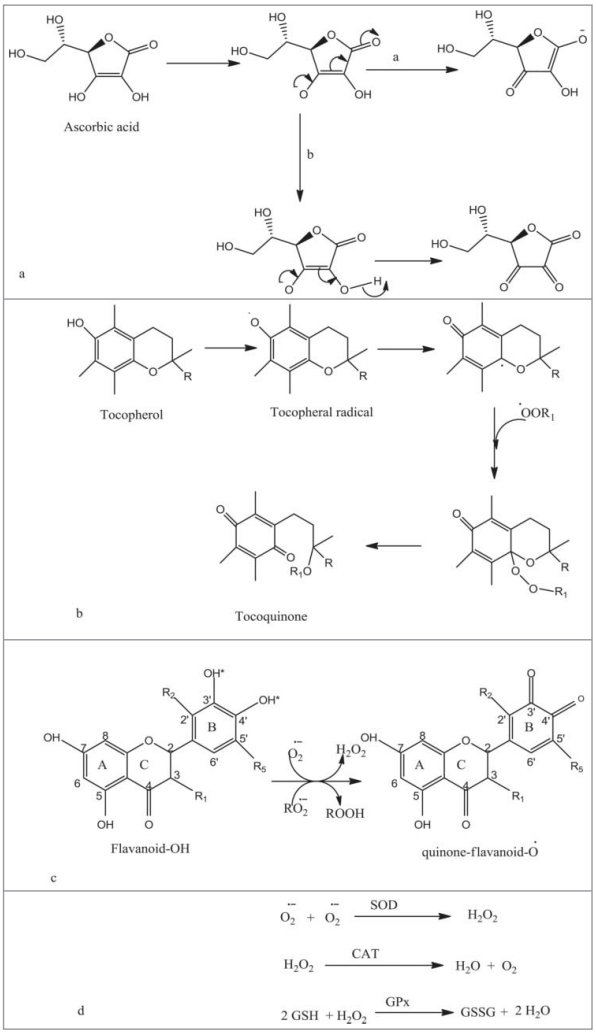

Figure 2. Mechanistic steps on antioxidant action (a) ascorbic acid (b) Flavanoids (c) Tocopherols (d) enzymatic antioxidants.

dietary food and plants. Oxidation of ascorbic acid produces two moles of electron along with its quinone analogue. But some researchers claim single electron change through the formation of vinylagous carboxylic acid where the double bond, oxygen with a lone electron pair, and carbonyl group form a conjugated system.[1] The hydroxyl group of ascorbic acid is much more acidic than the alcohols due to the stabilization of the deprotonated enolate form. Ascorbic is good scavenger of superoxide radical anion, hydrogen peroxide, hydroxyl radical, and singlet oxygen. In human system, ascorbic acid is converted into dehydroascorbic acid which gets reversed to ascorbic acid by the enzyme dehydroascorbate reductase. Ascorbic is capable of regenerating tocopherol from tocopheroxyl radical (Figure 2a).

Tocopherols, a highly lipophillic and operative in lipo membranes, can be classified into two main classes namely tocopherols and tocotrienols, within this each is further classified into four group; α, β, γ, and δ making a total of eight tocopherol isomers. The hydrogen donating capacity is in the order $\delta > \beta = \gamma > \alpha$.[31] α-tocopherol shows protection against Parkinson's disease, Spinocerebellar ataxia, and Myopathies. The common sharing in the structure is that all these contain chromanol ring with a hydroxyl group that can donate hydrogen atom to reduce free/lipid peroxyl radicals with hydrophobicity for penetration into the biological membranes.[32] The tocopheryl radical is stabilized by the presence of delocalized electron over the aromatic ring system (Figure 2b).

Flavanoids are polyphenolic compounds which are very effective antioxidants that serve against cardiovascular disease. Flavanoids include flavones, flavanols, isoflavones, flavanones, and chalones are seen in higher plant tissues. Flavanoids act as scavenger of superoxide anion radicals, lipid peroxyl radicals hydroxyl radicals, singlet oxygen and possess metal chelation ability. Their various functions include protection against oxidative diseases, modulation of enzyme activity and interaction with specific receptors. Flavanoids are formed in plants from the aromatic amino acids like phenylalanine, and tyrosine and malonate. They consist of 15 carbon atoms arranged in three rings, which are labeled as A, B, and C. The presence of double bonds, carbonyl group and hydroxyl group in the pyranyl group serves as a base for classification into main and sub-classes. Flavanoid has metal chelating potential which depends on the arrangement of hydroxyl group and carbonyl group and the presence of electron donating or withdrawing substituents and its ability to delocalize the electron for the formation of stable phenoxyl radical. The major contribution to metal chelating ability is primarily due to the presence of catechol moiety as demonstrated by the copper chelation to keampferol and quercetin.[33] The generally accepted mechanisms for phenolics antioxidant action include HAT, single electron transfer-proton transfer, and sequential proton loss electron transfer. The antioxidant activity of flavanoids is due to catechol moieties in B ring, the oxo group in conjugation with the double bond in the C ring and presence of hydroxyl groups at the 3 and 5 positions (Figures 2c and 3a–d).

Trolox [6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid or vitamin E], serves as a positive control that inhibits the formation of radical cation. The antioxidant activity present in the biological fluids, cells, tissues, and natural extracts is optimized by trolox as a standard. Trolox is used as standard in the well-known assays like TEAC and oxygen radical absorbance capacity (ORAC). The total antioxidant assay or trolox equivalent antioxidant capacity measures the concentration of trolox solution with reference to the concentration of standard antioxidant solution under investigation. The TEAC is defined as the equivalent concentration of trolox solution to 1 mM concentration tested or investigated antioxidant with a similar antioxidant potential. The decay curves involve the dose-dependent lag phase inhibition of trolox. Linoleic acid can be evaluated by the relative degree of oxidation by measuring the ferric thiocyanate values.[34] The TEAC is consolidated in the following discussions in terms of structure–activity relationship[35] by antioxidant capacity by DPPH and ABTS assays. The scavenging activity of antioxidant is a factor that can be related to the number and position of hydroxylation, methoxylation, and glycosylation position in the mother compound. Generally, the radical scavenging activity decreases in the following order: tannins, flavonoids, stilbenes, curcuminoids,

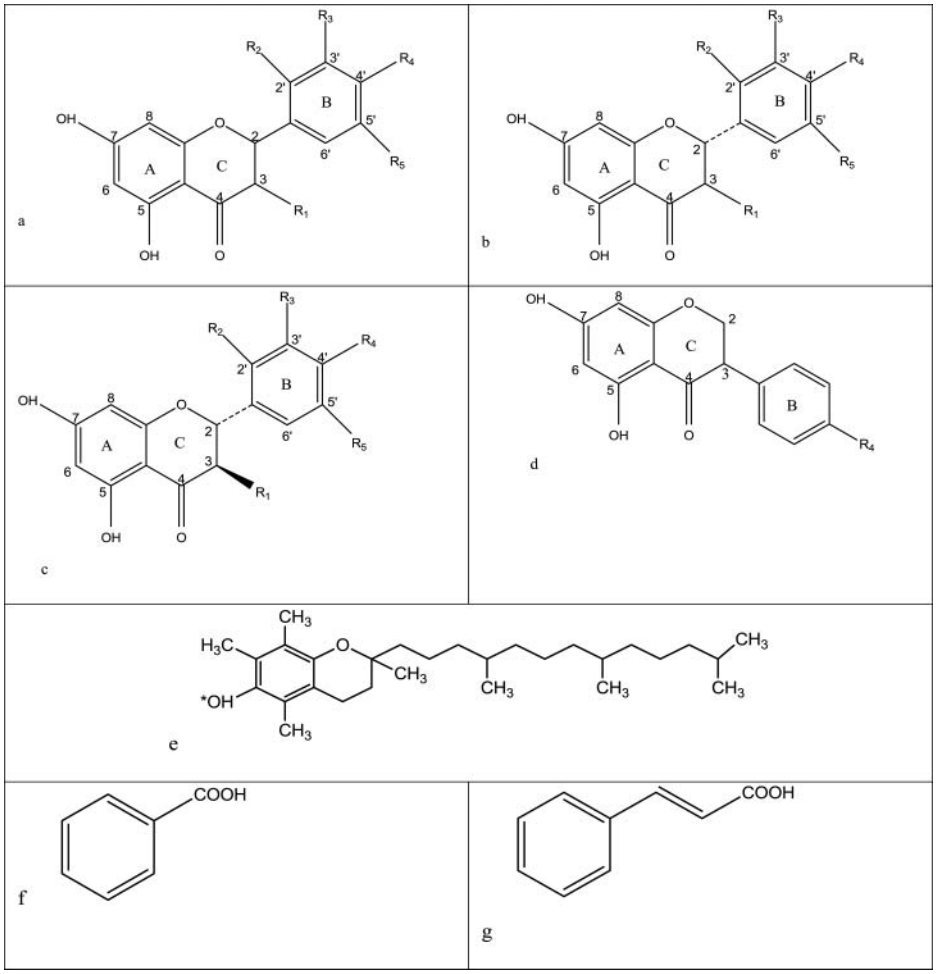

Figure 3. Chemical structure of selected antioxidants (a) Flavanoids (b) Flavanones (c) Flavanonol (d) Isoflavones (e) Tocopherols (f) Benzoic acid (g) Cinnamic acid.

phenolic acids, coumarins, lignans, and quinones with some variation in the class of compounds due to radical scavenging activity. The structural activity differences that can be attributed include number and location of hydroxylation, glycosylation, and methoxylation. Overall, it can be seen that a general trend cannot be adopted in relating structure and activity (Figure 3e).

Phenolic acids are hydroxyl derivatives of aromatic carboxylic acids. They include benzoic acid and cinnamic acid derivatives. Cinnamic acid is more effective as an antioxidant than the corresponding benzoic acid. Structure activity relationship claims the presence of hydroxyl group in the ortho or para position and carbonyl functional moieties, such as acid, ester, or lactone markedly enhances the antioxidant activity.[36] The activities are controlled by a number of hydroxyl groups in the molecule. To complement these observations it is known that L-dopa has higher antioxidant and radical scavenging activity than that of L-tyrosine[37,38] (Figure 3f, g).

Carotenoids are a class of natural fat-soluble compounds in which their chemical properties are closely related to the presence of extended conjugated double bonds. In human system, such carotenoids as β-carotene, lycopene, lutein, β-cryptoxanthin, zeaxanthin, and asthaxanthin are greatly associated in mitigating cardiovascular diseases.[39] In plants, carotenoids play a major role in protecting them against photo-oxidative process and acts as scavengers of singlet molecular oxygen and peroxyl radicals. The structures of some antioxidants are shown in Figure 3.

In vitro and *in vivo* assays of antioxidants

The formation of ROS can be monitored by a variety of instrumental methods which include fluorometric, spectrophotometric, chemiluminiscence, and electron spin resonance. The inactivation mechanism involved in the antioxidant capacity assay methods can be generally classified into two categories namely, HAT and ET reactions. The HAT- and ET-based assays rely on the hydrogen transfer and electron transfer and are primarily redox type reactions. The HAT- and ET-based reactions are pH dependent; particularly ET is also controlled by ionization potential. The ET-based reactions are relatively slow as compared to HAT-based assays due to stabilization of the charged species in the solvent environment.[40] The feasibility of the reaction depends upon addressing different factors such as chemical properties of the probes, chemical reactivity, and physical properties such as homogeneity, biological distribution, absorption, fluorescence spectra, lifetime in the exited state and susceptibility to bleaching capacity. The antioxidant

capacity assays rely on the suppression or expression in the photoluminescence or chemiluminiscence capacity of the probe in the presence or absence of antioxidants. The extent of change in the intensity is taken as a measure of antioxidant efficiency. It is practically impossible to assay all the available antioxidants by either ET or HAT, hence it is expressed in standards such as ascorbic acid or trolox or gallic acid equivalents. If possible, it is recommended to use more than one assay methodology to identify ROS. The choice of the method depends upon the source of ROS under study. Further antioxidant capacity assay are classified as under

- Ferric reducing antioxidant parameter
- Cupric reducing antioxidant power
- Ceric antioxidant capacity assays

} Metal reducing antioxidant power

- Hydroxyl radical averting capacity
- Oxygen radical absorbance capacity assays
- Total radical antioxidant parameter
- Crocin-bleaching assay
- Total phenol assay – Folin Ciocalteau Reagent assay
- Trolox equivalent antioxidant capacity assays – DPPH radical scavenging and ABTS scavenging antioxidant capacity assays
- Total oxidant scavenging capacity assays
- Chemiluminiscence assay

Table 1[41–64] and flow chart (Figure 4) provide quick look of the chemistry behind the assay procedures.

Hydroxyl radical averting capacity (HORAC)

HORAC provides the direct measurement for antioxidant against chain breaking hydrophillic hydroxyl radical. The

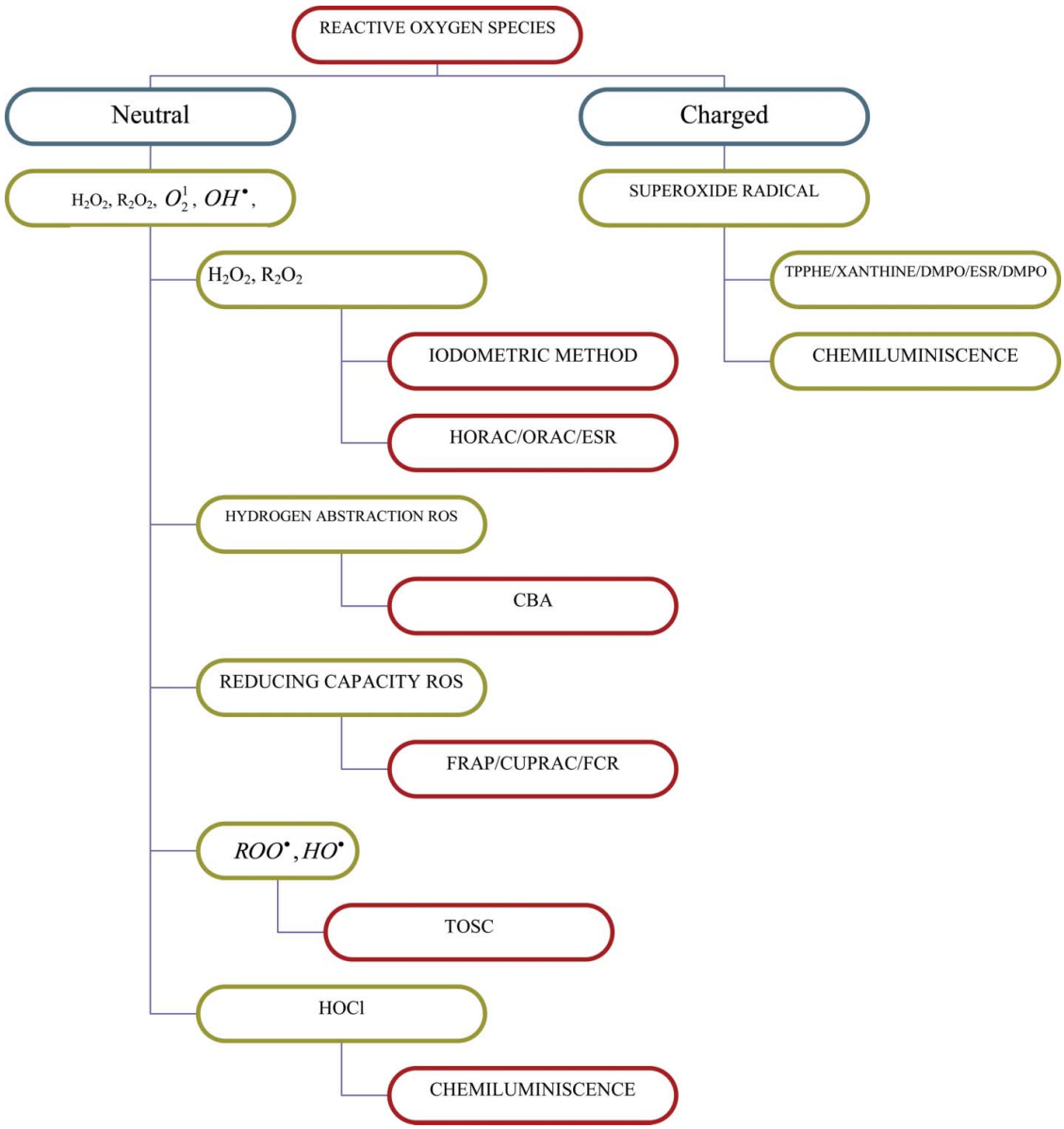

Figure 4. Flow chart for antioxidant capacity assays.

Table 1. Chemistry behind the antioxidant capacity assays.

Antioxidant assay procedure	Entities tested	assay units	Chemistry of the assay methods	Advantages	Disadvantages	applications
ORAC/HORAC	Peroxyl radical-ORAC Hydroxyl radical-HORAC	Area under the curve	Peroxyl radical generated by decomposition of AAPH or hydroxyl generated from the metal hydrogen peroxide reaction	These allow the absolute rate constants and the stoichiometry of the reaction between free radical and antioxidants.	Autocatalysis by antioxidant-metal mixture	[41–43]
CBA	Flux of free radicals	Rates of Crocin bleaching in the presence and absence of antioxidants	Decrease in the inhibition of Crocin bleaching by peroxy radicals in the presence antioxidants	High molar extinction co-efficient, probe for both hydrophilic and hydrophilic antioxidants	It provides the antioxidant reactivity rather than the antioxidant activity	[44–46]
FCR	Polyphenols	Beer's law	Transfer of electrons in the alkaline medium from antioxidants to form reduced molybdenum blue	Affordable titration method in basic analytical laboratory	It is not specific for only polyphenols, reducing agents will also respond. It cannot be used for assay of Total antioxidant capacity assays of biological fluids	[47–49]
CERAC	Antioxidants equivalents	Beer's law	The cerric reducing activity of antioxidant determined by decrease in the intensity of cerium absorption.	—	Cerium, a powerful oxidant can oxidize the reductants which can contribute to overestimation.	[50,51]
TEAC	Trolox	Activity is defined as the amount of $ABTS^{+\cdot}$ quenched for fixed time	Measured as the ability of the antioxidant to decrease the color of ABTS radical cation, expressed in terms TROLOX equivalents	It can react by either ET and HAT mechanism	This does not distinguish between the kinetics of radical trapping and stoichiometry. Outcome depends on the time chosen for analysis	[52–54]
DPPH	An antioxidant having reactive hydrogen group	EC_{50}/IC_{50}	Reducing ability of antioxidant is evaluated by ESR or decrease in absorbance at 520 nm	Simple investigation by UV-Vis spectrophotometry. ET and HAT mechanisms	Results indicate the radical trapping power or reducing rather than the real antioxidant activity. EC_{50}/IC_{50} cannot suggest actual activity	[55–57]
CUPRAC/FRAP	Antioxidants equivalents	Beer's law	Relative power of antioxidants to reduce metal to lower oxidation state further getting complexed with heteroaromatic compound (TPTZ/1,10-phenanthroline)	—	The antioxidant power is arbitrarily time dependent. It out shows the reducing power rather than the antioxidant activity. Fe (II) in the presence of hydrogen peroxide is pro-oxidant	[58–60]
Chemiluminiscence	Peroxyl radicals	Time lag in the antioxidant presence	Ability of the luminol related compounds for luminescence with the attack of free radicals	Time lag is directly proportional to the total concentration of antioxidants	After time lag the luminescence property is regained	[61,62]
ESR	Peroxyl radicals	Semi-quantitative competitive data in the presence and absence of antioxidants	Peroxyl radical reacting with a probe forms a stable nitroxide, quantified by ESR at a fixed time	This method have no interference from the sample color and turbidity as it is sensitive to the unpaired electron	This does not distinguish between the kinetics and stoichiometry. The life time of most of the spin adducts are very limited	[63]
TOSC	Peroxyl radicals	Area under the curve	Inhibition of the oxidation of α-keto-γ-methiobutyric acid to methylene by peroxyl radicals generated by AAPH by antioxidants.	—	Use of area under curve, long reaction time and multiple gas chromatographic analysis	[64]

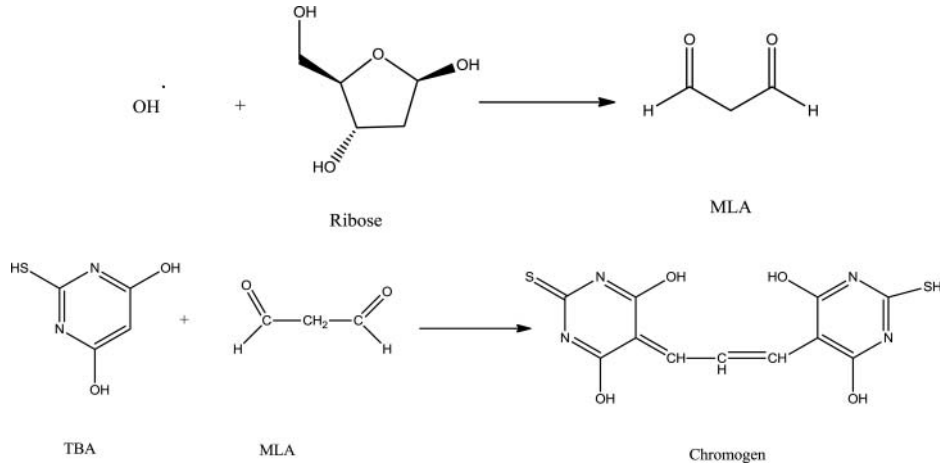

Figure 5. Mechanistic steps in the TBA-MLA antioxidant assay.

recent developments in HORAC have been surveyed by classical titrimetric, luminescence, fluorescence, and chromatographic methods. Numerous hydroxyl radical capacity assays are available for the study of scavenging capacity that include 2-deoxyribose/TBA, DNA, riboflavin/methionine, phenolic acids, DMPO derivatives, Fluoroscence probes, β-phycoerythrin, coumarin and its derivatives and chemiluminiscent probes such as luminal and its derivatives. Fluorimetric hydroxylation probing system includes fluorescein and its derivatives, scoloptein, homovanillic acid, DCFH, dihydrorhodamine-123, folic acid, and 2′,7′-dichloroflorofluorescein diacetate. The methods that are available for the evaluation of hydroxylated product are HPLC in conjunction with electrochemical and UV detectors, ESR,[65] HPLC-electrochemical/UV methods,[66] trapping methods by the formation of hydroxylated products,[67,68] molecular absorbance, fluorescence, GLC, MS, capillary electrophoresis, and chemiluminiscence, or photochemiluminiscence.

Iodometric procedures for peroxides[69–71] scavenging assay have been proposed by two different research groups using potassium iodide. The liberated iodine is titrated with sodium thiosulphate using starch as an indicator. The results are reported as peroxide value. The peroxide value is milliequivalents of peroxide oxygen per kilogram of the sample. Limitations of this method include poor sensitivity and selectivity, which may lead to erroneous results. Also addition of iodide across the unsaturated double bond may lead to lowering of the value and the presence of dissolved oxygen may induce an excess in its value. This method is not specific, because any compound that reacts with iodide and iodine may interfere.

The deoxyribose method is the most commonly used in testing hydroxyl radical scavenging antioxidants in the aqueous phase.[72–74] It adopts classical Fenton reaction in the liberation of hydroxyl radical species to degrade 2-deoxyribose like compounds to MLA products, the product formed is chromogenic with thiobarbituric acid having absorption maximum at 532 nm. The indirect TBA–MLA has distinct steps involving the generation of free radical from copper, iron, or ABAP. The free radical released generates a coupled product of TBA and MLA. The formation of the coupled product is shown in Figure 5.[75] The inhibitory effect of antioxidant in the coupling process is taken as a measure of antioxidant capacity. It involves the reduction in the absorbance of the solution by increase in the concentration of antioxidant. This assay has been adopted in the study of different antioxidants such as uric acid, trolox, and mannitol as standards.[76,77] This method cannot distinguish between the kinetics and stoichiometry of the reaction. It also suffers from serious limitations such as reaction of TBA with other compounds not related to lipid peroxidation and the formation of Schiff's bases between malondialdehyde and amines, leading to over estimation of the antioxidant protection.

High reactivity of hydroxyl radical permits the formation of hydroxycyclohexadienyl radical.[66] Prevention of hydroxyl radical formation takes place either by deactivating metal ions through chelation or converting hydrogen peroxide into harmless product. Interception of hydroxyl radical at the immediate juncture of the cell is required for HORAC assay.[78] Among the available oxidants, hydroxyl radical constitutes the chemically most ROS primarily responsible for the cytotoxic effects of oxygen, for organisms living in an oxygenic environment.[79] There is a dose-dependent assay of antioxidant involving the suppression of the formation of dihydroxybenzoic acid by the reaction of salicylic acid and hydroxyl radicals.[80] The induced formation of hydroxyl radical is indicated by the trapping procedure involving 4-hydroxybenzoic acid that extends to the pico level concentration. Addition of DMSO resulted in a small decrease in adduct formation because of the scavenging property.[81]

A simple method has been adopted by Botchway et al. (2007) in the detection and determination of hydroxyl radical, involving DMSO that results in the formation of formaldehyde. The formaldehyde reacts with ammonia and 1, 3-cyclohexanedione at pH 4.5 with a characteristic fluorescence having excitation and emission wavelengths at 400.4 nm and 452.3 nm, respectively. The major demerit of this procedure is that the assay is performed at 95°C, which is unsuitable for biological species and released free radical.[82]

Hydroxyl radical scavenging activity was carried out for the chickpea protein hydrosylate by using 1,10-phenanthroline in the presence of ferrous sulphate and hydrogen peroxide.[83] The

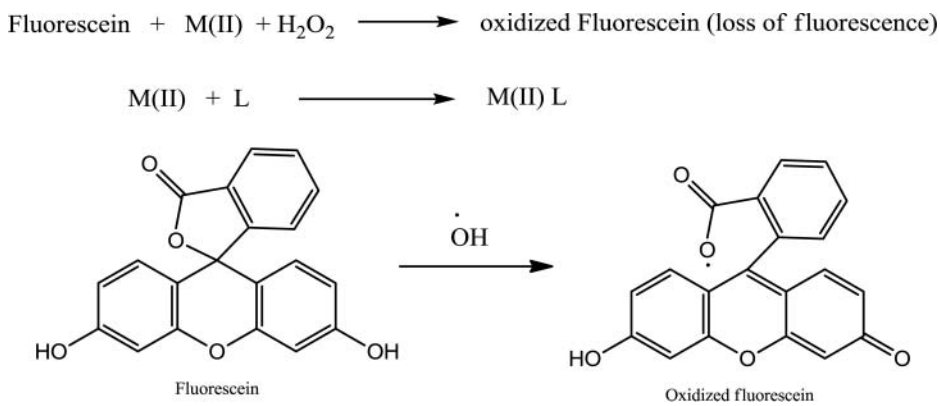

Figure 6. Reaction scheme for Fluorescein-based assay in the loss of fluorescence property.

inhibition in the formation of Fe (II)-1,10-phenanthroline by hydroxyl radical is taken as quantitative measure of scavenging activity.

The hydroxyl radical can be quantified by preventive antioxidant or scavenging procedure in which the metal gets deactivated due to its co-ordination with the antioxidant (Figure 6). The fluorescence decay curve of fluorescein is monitored in the presence and absence of antioxidant. The area under the curve (AUC) is integrated and net area gives the hydroxyl radical prevention activity. Standards tested included gallic acids, flavonoids, and phenolic acid. Non-benzenoid antioxidants cannot be assayed by this procedure due to their lack of complexation with the metallic form. The formation of hydroxyl radical was confirmed by p-hydroxybenzoic acid as a trapper.[66] Studies have confirmed that the electron transfer reaction between metal and hydrogen peroxide follows an inner sphere mechanism.[84] The loss in fluorescence is due to lack of structural rigidity. The advantageous feature of this reaction procedure is that the SOD enzyme has minimum effect on the oxidation of fluorescein in the generation of superoxide enzymatically.[85,86]

The decrease in the fluorescence intensity either by scopoletin or homovanillic acid which is non-fluorescent in the presence of peroxidase and hydrogen peroxide has been studied by two different research groups.[87,88] Hydroxyl radical was detected by ESR spin trapping using 5-di-ethoxyphosphoryl-5-methylpyrroline N-oxide.[30]

The fluorogenic compound DCFH-DA has been extensively used as a marker for oxidative stress, which reflects the overall oxidative status of the cell. But one of the limitations in its use is the presence of intracellular esterase activity that results in the formation of DCFH, a non-fluorescent, which emits fluorescence when oxidized to DCF. Different researchers claim that the formation of DCF of high or low sensitivity for superoxide, hydrogen peroxide, and hydroxyl radical.[89]

A novel hydroxyl radical scavenger was designed by Hiroshi yoshida et al.[90] involving edaravone, which inhibits hydroxyl dependent/independent lipid peroxidation and tyrosine nitration induced by peroxynitrite. Electron donation from edaravone to free radicals produces peroxyl anion and edaravone radical, which get transformed to 4,5-dione. 2-Oxo- 3-(phenylhydrazono)-butanoic acid is generated by hydrolysis reaction of the 4,5-dione (Figure 7).

The mechanism of HRP-catalyzed oxidation of DCFH to DCF involves the formation of HRP-compound I. This oxidizes DCFH to DCF semiquinone free radical, which is oxidized to the fluorescent compound DCF by oxygen by the production of superoxide. ESR in combination with spin trapping methods has demonstrated the formation of superoxide and hydroxyl radical adducts of DMPO and DEPMPO in the system containing DCFH, hydrogen peroxide, and HRP.

A cationic derivative of dihydroethidium, TPPHE is designed for highly selective detection of superoxide in the mitochondria of live cells. Oxidation of TPPHE by superoxide results in hydroxylation at 2-position (2-hydroxyethidium) derivative that exhibits a strong fluorescence excitation peak at 400 nm. The absence of excitation spectrum of ethidium oxidation product generated by ROS other than superoxide clearly claims its specificity toward superoxide radical. The mechanism of TPPHE interaction with lysosomes and DNA has been described in detail by Horobin research group[91] (Figure 8).

The assay of superoxide generated by xanthine/xanthine oxidase system is generally based on the inhibition capacity of the probes such as autoxidation pyrogallol,[92] NBT-MTT,[93,94] and ferricytochrome C.[95] The NBT and ferricytochrome C are not applicable to non-enzymatic antioxidants. Upon reduction, NBT-MTT water soluble colorless compounds form uncharged, brightly colored formazans (Figure 9), which are applicable in cytotoxicity assays.

The formation of adduct of superoxide (DMPO-OOH), which spontaneously gets decomposed to DMPO-OH, was analyzed by ESR using of manganese dioxide as an internal standard.[96]

The transformation of a non-fluorescent amplex red reagent to a fluorescent resorufin can be carried out by peroxidase catalyzed reaction (Figure 10).

Saturated group getting converted into conjugated organic molecules by lipid peroxidation can be used in end point determination for antioxidant activity of the sample.[97] The lipid peroxidation can be initiated by the addition of copper, iron, ABAP, and thermolysis. Initially, lipid abstracts hydrogen from the methylene group, which is further stabilized by the molecular rearrangement to form a conjugated diene. Furthermore, the quantification of antioxidant can be performed by the increase in the absorbance per sample at a fixed time. The presence of

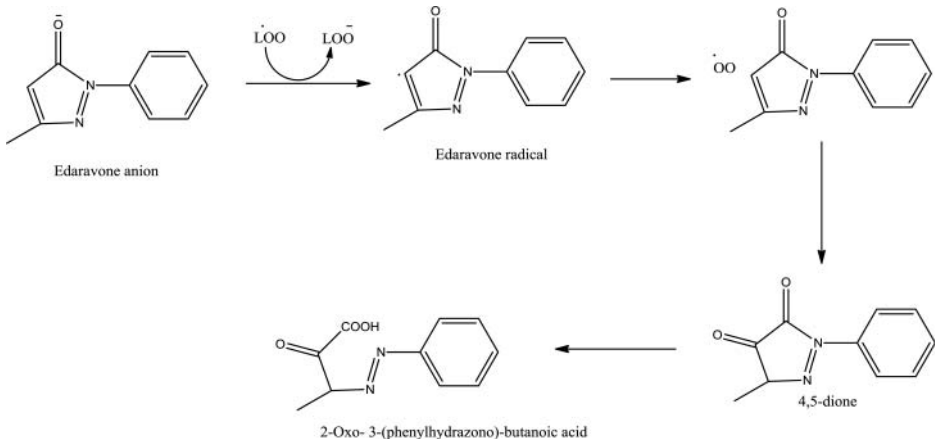

Figure 7. Hydroxyl-dependent assay procedure of edaravone.

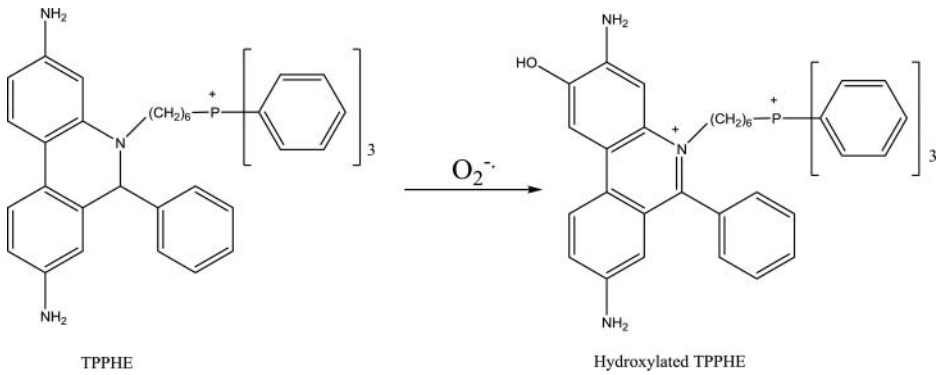

Figure 8. Oxidation of TPPHE in the superoxide assay methodology.

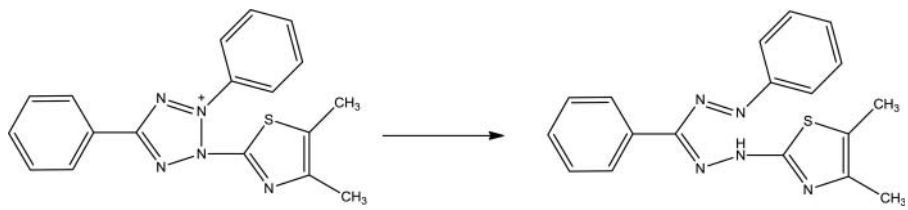

Figure 9. NBT-MTT cytotoxicity assays.

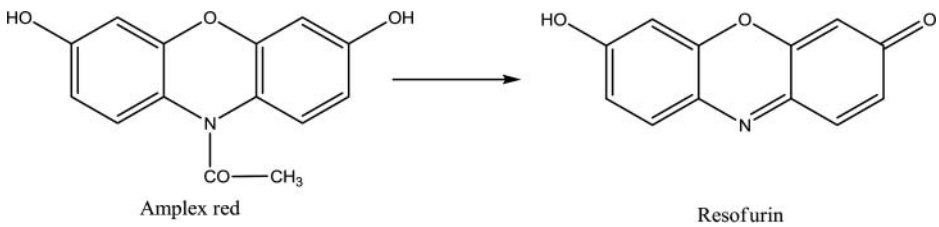

Figure 10. Principle of the dual enzymatic systems in the use of amplex red as a peroxidative probe. Oxidation of oxidase enzyme produces hydrogen peroxide, which is coupled to the conversion of amplex red to fluorescent resofurin by HRP.

distinct peak at 233 nm clearly indicates the presence of diene conjugated moiety. This conjugated diene moiety can be extended to the triene system, which shows three signature absorption bands. The main peak will be observed at 268 nm with a secondary peak at 278 nm. The increase in the absorption maximum is due to the stabilization of the LUMO and destabilization of the

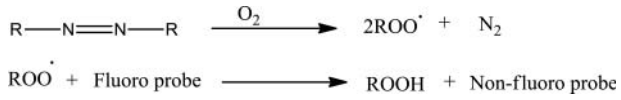

Figure 11. ORAC assay methodology in the antioxidant detection.

HOMO orbitals of the polyenes. The concentration of the conjugated dienes can be calculated by the relation

$$[CD] = \frac{A}{\varepsilon \times l}$$

A = Absorbance at 233 nm, ε = molar absorptivity of linoleic acid hydroperoxide, l = optical path length = 1 cm and the conjugated diene value as,

$$CD_{value} = \frac{[CD]2.5 \times 10^4}{m}$$

that includes the correction factor 2.5×10^4 and m the mass of the sample.

Oxygen radical absorbance capacity

ORAC could provide a great value in the prevention of oxidation reaction in food processing and storage in dietery industry. These are based on the AUC technique, which combines both inhibition time and degree of inhibition of free radical action by an antioxidant. The basis of the assay is inhibition of the intensity of the fluorescent molecule, β-phycoerythrin by peroxyl radical generated by thermal decomposition of azo compounds such as ABAP in aqueous buffer. Initially, β-phycoerythrin, a protein isolated from porphyridium cruentum is used as a fluorescent probe that reacts with peroxyl radical to form a non-fluorescent product (Figure 11).

But later on the shortcoming of β-phycoerythrin, which included large variability in reactivity to peroxyl radicals, photo bleaching, and interaction with polyphenols by non-specific binding, paved the way for the search for new fluorescent probes. To overcome these limitations, fluoro probes that were tested included 3′,6′ -dihydroxyspiro[isobenzofuran-1[3H],9′ [9H]-xanthen]-3-one[98] and dichlorofluorescein.[99] ORAC can be adapted to detect hydrophilic and hydrophobic antioxidants by varying the radical source and solvent.[66] In the samples that contain slow and fast reacting antioxidants, ORAC is especially beneficial as it measures the samples with and without antioxidant samples. Fluorescent markers though sensitive require expensive instrumentation that may not be available in the routine analytical laboratories. The major criticism is the time of analysis for the assay, which can be overcome by the high throughput assay methodology.[100] Several modified and higher techniques are adopted, including the measurement of decrease in the radical concentration of ABAP by EPR.[101] The limitation of ORAC is use of AUC as a parameter of study and low reactivity of peroxyl radical. An improvement was suggested by López-Alarcón and lissi[102] which involved the use of pyrogallol red as an alternative for competitive antioxidant probe.

Metal reducing antioxidant parameter

The metals that are used in the study of antioxidants are iron, copper, and cerium. These can be classified under electron transfer mechanism. The essential requisite is that the metal should either be stable in the reduced state or it gets complexes with the ligand in which oxidized and reduced forms have large differences in the absorption maximum. The metal reducing antioxidant parameter quantifies the total concentration of the metal rather than the antioxidant activity. Metal reducing antioxidant parameter is defined as the reduction of 1 mol of metal ion.

FRAP, originally applied to assay of plasma later on extended to other biological fluids, foods, plant extracts, juices, etc., which is based on the ability of phenolic compounds to reduce yellow [Fe (III)-TPTZ] to intensely blue colored [Fe(II)-TPTZ] in acidic medium (Figure 12a). Frankel et al.[103] claims that the measured reducing capacity does not necessarily reflect antioxidant activity, it provides useful "total antioxidant concentration" or the summation of concentration of all antioxidants involved. The redox potential of Fe (700 mV) is comparable with that of the ABTS (680 mV). An acidic pH of 3.6 should be maintained for essential solubility of iron.

The copper reducing ability is measured by complexation of Cu (I) with bathocuproine (2, 9-dimethyl-4,7-diphenyl-1,10-phenanthroline) and neocuproine at 490 nm and 450 nm, respectively (Figure 12b). FRAP and CUPRAC have comparable values with TEAC values with some exceptions because of similar redox potential probes used in the assay because both assay methodology are based on the ability of the antioxidant to reduce metal complexes.[104] The factors responsible under the reaction conditions for antioxidant capacity assays include pH, temperature, and stability constant.

Cerric reducing antioxidant capacity determination is based on the redox reaction between antioxidant, amaranth[105]/indigo carmine dye,[106] and Ce (IV) at room temperature and measurement of the unreacted dye (Figure 13). The quantification is directly proportional to the concentration of antioxidants.

Fe (II) is a pro-oxidant, which in the presence of hydrogen peroxide produces hydroxyl radical. A species with redox potential less than the Fe (III)/Fe (II) system can also contribute to antioxidant parameter, which adds to false high values. Another potential problem with the FRAP system is that some of the chelators in the food extract can bind to Fe system, thus resulting in low values. FRAP system is not applicable to thiols, glutathione, and proteins that contain –SH groups. CUPRAC method can be adopted for both hydrophilic and lipophillic antioxidants. Slow reacting antioxidants should be heated in order to increase the rate of reaction with CUPRAC complexing ligand. CUPRAC overcomes the oxidization of thiol-type antioxidants that cannot be assayed by FRAP procedure. Moreover, it is selective due to its lower redox potential than that of redox couples like Ce (IV)/Ce(III) and Fe (III)/Fe (II).[107]

Spectrophotometric radical probing system

DPPH relies on hydrogen transfer and ABTS on electron transfer mechanism. When DPPH reacts with an antioxidant the

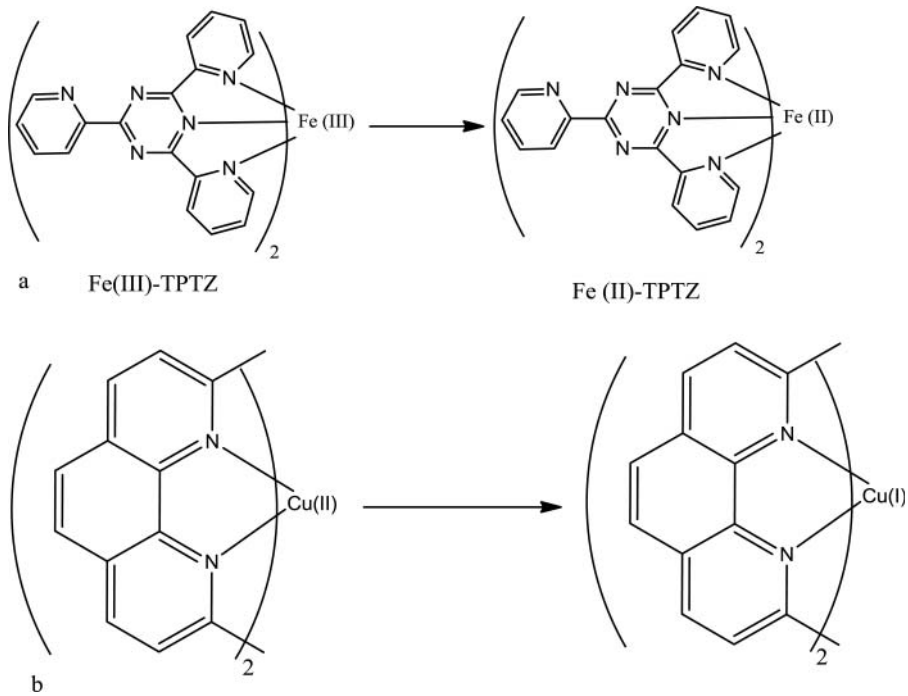

Figure 12. (a) FRAP and (b) CUPRAC in the total reducing parameter assay methodology.

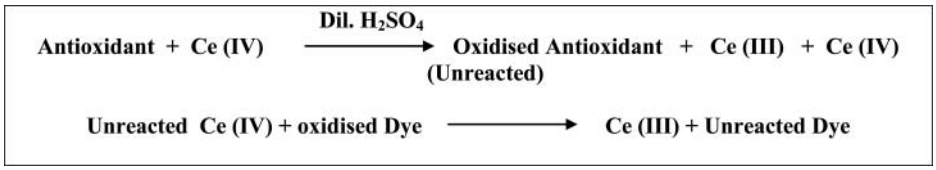

Figure 13. Dye-based antioxidant parameter assay involving cerium.

free radical property of DPPH is lost due to chain breakage or hydrogen transfer or radical transfer process (Figure 14). The molar absorptivity on its reaction reduces to 1640 from 9660.

DPPH has deep blue color and it is a long-lived nitrogen radical species, due to its inability to undergo dimerization. It is an extensively used antioxidant parameter molecule with wide applicability. The reduced form of DPPH is generated by the antioxidant, which is accompanied by the disappearance of the violet color. The decrease in intensity of the color due to photons is also contributing factor during the assay that requires careful control of the experimental parameters. During the assay procedure, Trolox is used as an internal antioxidant standard.[108,109] This DPPH can be used to study anticoagulant, antitumor, antiviral, anti-inflammatory, antioxidants, antimicrobial, and enzyme inhibition properties of variety of drugs. The reducing ability is usually studied by the decrease in the intensity of color at wavelength range of 515–528 nm or by ESR monitoring technique.

ABTS radical cation has absorption maxima at 414, 734, and 815 nm in aqueous medium and at 414, 730, and 873 nm in ethanolic medium. The hydrogen transfer TEAC assay is the hydrogen donating ability of the antioxidants to scavenge ABTS radical cation at 734, 645, and 815 nm by comparing with trolox, a water-soluble vitamin E analog.[110] Measuring at the absorption maximum of 734 nm, eliminates the color and turbidity interferences therefore, it is suitable for antioxidant assay of plant extracts.[111] The ABTS radical cation can be generated either enzymatically or non-enzymatically (Figure 15a). Addition of exogenous antioxidants reduces back the ABTS radical cation to neutral ABTS molecule. The extent of

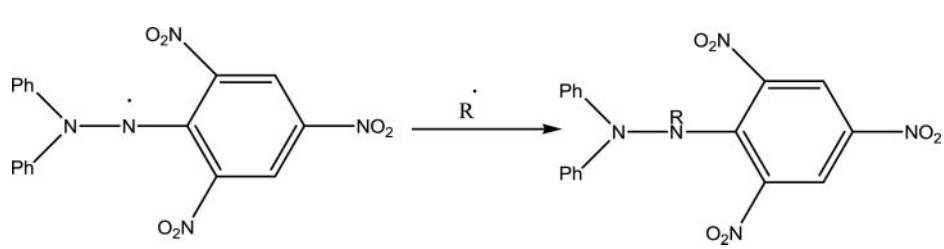

Figure 14. Decolorization assay methodology of DPPH.

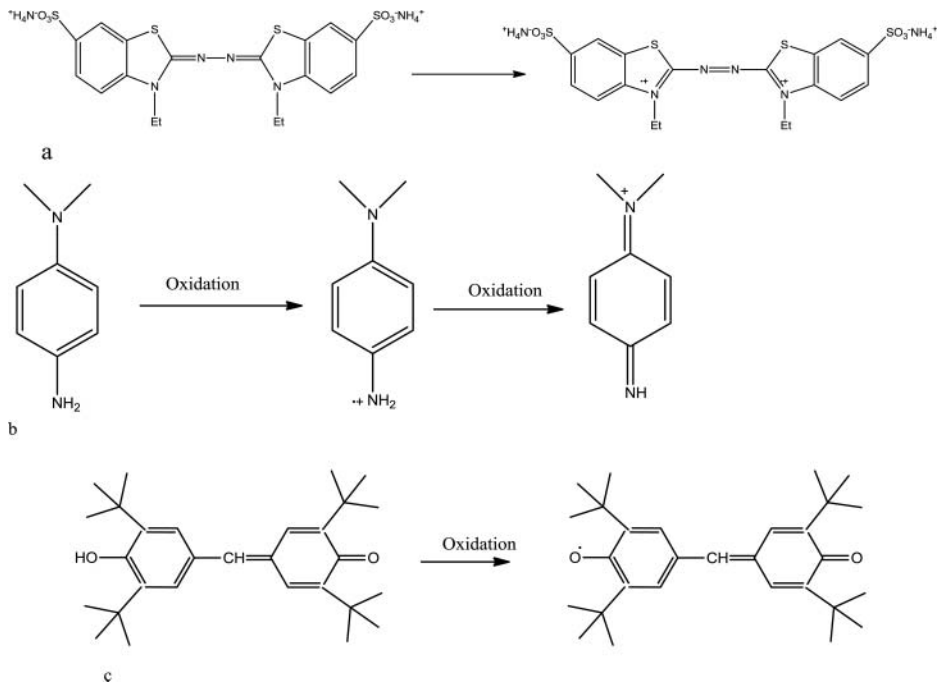

Figure 15. Generation of free radical/cations by ABTS, DMPD, and Galvinoxyl.

decolorization is determined as a function of concentration and time, which is normally calculated as Trolox equivalents under similar experimental conditions

Peroxidase and myoglobin are used in enzymatic generation, whereas manganese dioxide, potassium persulfate, ABAP, and phosphate buffer saline are used in non-enzymatic generation. The non-enzymatic chemical reactions require longer time (upto 16 h for potassium persulphate) or high temperature (60°C for ABAP) for generation. The generation of ABTS radical cation by persulphate system involves the scission of the peroxodisulphate after the transfer of electron.[1]

$$S_2O_8^{2-} + \mathrm{ABST} \rightarrow SO_4^{2-} + SO_4^{\cdot -} + \mathrm{ABTS}^{\cdot +}$$
$$SO_4^{\cdot -} + 2\mathrm{ABTS}^{\cdot +} \rightarrow SO_4^{2-} + 2\mathrm{ABTS}^{\cdot +}$$

that leads to overall reaction represented as

$$S_2O_8^{2-} + 3\mathrm{ABTS} \rightarrow 2SO_4^{2-} + 3\mathrm{ABTS}^{\cdot +}$$

The enzymatic generation is faster and requires mild experimental condition. The generation of ABTS radical cation can also be carried out electrochemically. Thermodynamically, an antioxidant with low redox potential can react.[112] TEAC is often taken as a measure to rank antioxidants and to construct the structural activity relationship[113] as it measures the ability of antioxidants to quench a radical cation ($ABTS^+$) in both lypophillic and hydrophilic environments.[114]

DMPD radical cation is an unstable species that can be generated *in situ* by oxidation with potassium persulphate and ferric chloride. Galvinoxyl radical is commercially available due to high stability (Figure 15b, c). The DMPD radical-based assays are particularly suitable for hydrophilic antioxidants as compared to hydrophobic bioactive compounds. Hence the standards like tocopherol and butylated hydroxyl toluene are avoided in the anti-radical assay.[115] The formation of radical cation is very slow which results in the continuous increase in the absorbance with better reaction with ferric chloride.[116] The principle involved in the DMPD is similar to that of ABTS, which shows a maximum absorption at 505 nm involving the transfer of hydrogen atom to DMPD radical cation for quenching the color in decolorization process. The galvinoxyl radical exhibits a strong UV absorption at 429 nm. DMPD and ABTS are involved in single electron reduction process in the antioxidant quantification. The exact stoichiometry of the antiradical action is not known and the end point is taken as a measure of antioxidant efficiency.

CPH is a drug used in psychopharmacology that forms a colored cation through partial oxidation, which is stable in both aqueous and non-aqueous medium. Our research group[117] proposed an antioxidant activity study by CPH. The mechanistic approach involves the generation of a pink colored CPH radical with an absorption maximum at 530 nm by Cr (VI) in 1:1 *O*-phosphoric acid and methanol mixture. Under this reaction condition, CPH loses one electron upon oxidation in the presence of Cr (VI) forming an electrophillic species. The radical cationic nature of CPH was confirmed by Dowex-50 cationic exchange resin column and ESR. On addition of antioxidant solution, the radical decolorizes (Figure 16). The main advantage of this method is that the pink colored radical cation turns colorless upon addition of antioxidant and it is economical as it uses metal during the oxidation process. The standard antioxidants that were tested include trolox, ascorbic acid, gallic acid, quercetin, Rutin, eugenol, catechin, TBHQ, and BHA.

The parameters adopted in the assay of radical scavenging activity are EC_{50} and antiradical efficiency. EC_{50} is the concentration of antioxidant that causes a decrease in the initial

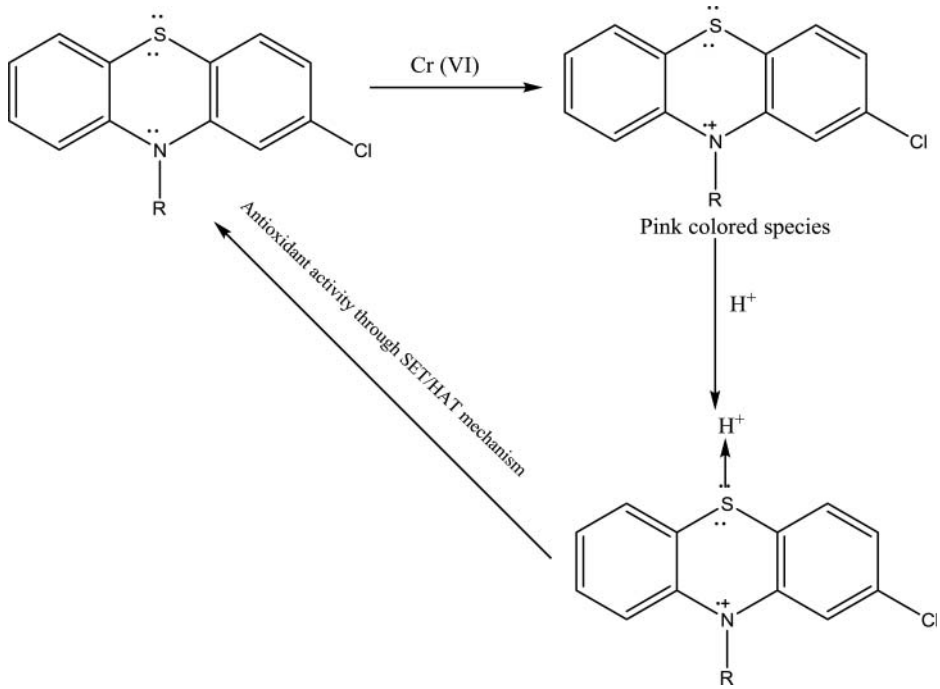

Figure 16. CPH antiradical action by antioxidant assay procedure.

concentration by 50%. TEC_{50} is the time needed to reach the steady state with EC_{50}. Based on the fastness of the reaction it can be classified as rapid, intermediate, or slow.[11]

The antioxidant capacity was measured by the ability of the antioxidant to reduce potassium ferricyanide to ferrocyanide, which reacts with ferric chloride yielding a blue colored ferri-ferrocyanide complex with absorption maximum at 700 nm (Figure 17). Further addition of sodium dodecylsulphate overcomes the precipitation and pH of 1.7 was maintained for redox activity of Fe (III) with prevention for hydrolytic activity by Isil Berker et al.[118] The order of trolox equivalents are in the order quercetin, rosmarinic acid, gallic acid, ferulic acid, catechin, caffeic acid, rutin, ascorbic acid, and Trolox, which was non-responsive to simple sugars and citric acid and responsive to biologically important thiols which did not get oxidized by other ferric-based antioxidant assays.[119]

Linoleic acid oxidation generates its corresponding hydroperoxide, which in turn decomposes the secondary oxidation products. The Ferric-thiocyanate method was taken as a measure to quantify the peroxide level during the initial level of oxidation. Peroxides formed during the oxidation of linoleic acid oxidation react with ferrous, to get oxidized to ferric. The ferric ion gets complexed with thiocyanate to form Fe (III)-thiocyanate complex with maximum absorbance at 500 nm. In the presence of antioxidants, oxidation of linoleic acid will be suppressed influencing the intensity of color formation of the complex.[120]

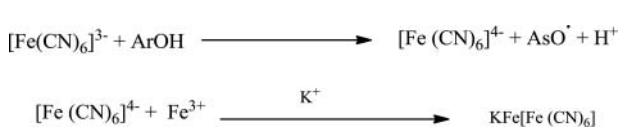

Figure 17. Reaction scheme for ferro-ferricyanide assay of antioxidants.

In case of either DPPH, ABTS, DMPD, or gavinoxyl radical-based assay method, the absorption decreases proportionally with the increase in the concentration of antioxidant that involves either electron transfer or hydrogen atom transfer mechanism. In fact, DPPH, ABTS, and CPH are nitrogen centered and hinder-free radical where the size the incoming probe plays an important role rather than the chemical reaction. Although, DPPH and ABTS methods belong to ET and HAT mechanisms, the kinetics of reaction depends upon the solvent ratio.[107] In addition, none of these methods have strong confirmation to show that they have antiradical action. DPPH and gavinoxyl are commercially available due to stable nature whereas ABTS and DMPD cationic radical should be generated *in situ* by oxidation with potassium persulphate and ferric chloride, respectively. One of the advantages is that ABTS radical cation assay can be performed under wide range of pH values as it is not affected by the ionic strength and is soluble in water and organic solvents enabling the determination of antioxidant activity of both lypophillic and hydrophilic compounds. Single point measurement of absorbance decay of DPPH after a fixed time varies from lab to lab which tells lack of reproducibility. Also DPPH assay procedure leads to color interference with those samples that contain anthocyanins leading to underestimation of antioxidant activity.

ESR methodology

ESR is the only available analytical technique in establishing the free-radical nature of some ROS released during the auto-oxidation and related processes. One of the major disadvantages is the insensitivity to detect transient species. To overcome this, various cross-routes have been adopted such as spin trapping methods to convert into stable free-radical derivative with increased life time, pulse radiolysis, plasma initiated UV

photolysis, and continuous flow systems. Spin trapping method involves the reaction of unstable free radicals with the probe resulting in free radical adduct with considerably longer life time which can be detected by ESR without any difficulty. The intensity of spin adduct is taken as a quantitative marker of free-radical concentration. The decrease in the intensity of signal is a dose dependent in the presence of antioxidant. The spin trapping probe involves nitroso or nitrones, which include AC-TMPO, proxyl fluorescamine, DEPMPO, 5, 5-dimethyl-1-pyrroline-N-oxide, and 4-((9-acridinecarbonyl)amino-2,2,6,6-tetramethylpiperidin. Each molecule containing nitroxide moiety is non-fluorescent but restores its fluorescence property on reacting with hydroxyl radical or superoxide radical thus destroying the electron spin resonance signal. Thus probes can be used in the assay either by fluorescence or ESR spectroscopy. AC-TMPO and proxyl fluorescamine can also be used to detect glutathionyl,[121] methyl[122] and hydroxyl radicals,[123] respectively. Since the ESR selectively responds to the unpaired electron which do not distinguish between kinetics and stoichiometry, it may be influenced by the possible antioxidant action of the nitroxides.[124]

TMPD reacts with singlet oxygen to form the radical adduct TAN, which can be measured by ESR. This method is highly specific to singlet oxygen. The advantage of this reaction is that superoxide and hydroxyl radicals cannot convert TMPD to TAN.[125] The reaction of KMBA with XOD/xanthine has been used by GC for detecting superoxide.[126] A 5-ethoxycarbonyl-5-methyl-pyrroline-N-oxide was adopted by Calliste research group as a superoxide trapper.[127] The formation of carbon centered free radicals such as α-hydroxyethyl and hydroxymethyl involving the proton abstraction from the solvent such as ethanol, methanol, and DMSO by the reaction of hydroxyl radical with DMPO/solvent system was confirmed by ESR spectroscopy.[122]

Crocin bleaching or β-carotene assay

Isolation of crocin was carried out from saffron by water/methanol extraction after repeated extractions with diethylether as described by Lussignoli research group.[128] Crocin is highly conjugated structure with molar extinction coefficient of 8.9×10^5 M^{-1} cm^{-1}. Crocin is considered as probe in hydrophilic and related radical scavengers but when lipophillic compounds are to be tested, canthaxanthin is selected as a probe.

CBA provides a reliable and straight forward method for peroxyl radical scavenging activity.[129] This was first suggested by Bors et al.[130] for radical scavenging activity. In this assay abstraction of hydrogen or addition of radical to polyene structure of Crocin resulted in the destruction of conjugated system that accounted for bleaching assay. CBA can be performed to test antioxidant activity of variety of samples such as plasma, plant extracts, and serum. This assay is based on simple competitive reaction between the colored probe, Crocin and the test antioxidant compound for scavenging of peroxyl radicals generated by thermolysis or photolysis of hydrophilic azo initiator. The azo initiator selected for CBA assay is ABAP. The inhibition of β-carotene bleaching is caused by the addition of sample of antioxidant. Decrease in rate is spectrophotometrically

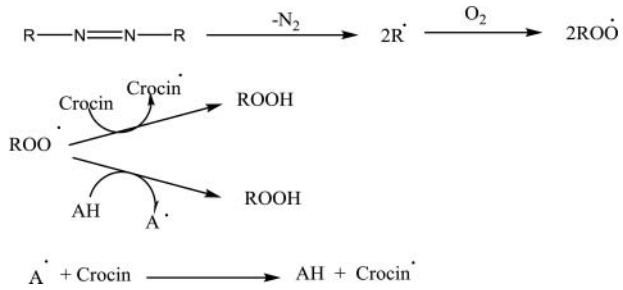

Figure 18. Reaction scheme for CBA by competitive methodology.

followed at 443 nm by antioxidants with reference to the blank, containing Crocin and peroxyl radicals (Figure 18).

The mechanism involves the following steps:

Peroxyl radical is formed by the unimolecular thermal degradation of an initiator in the presence of oxygen in air. The Peroxyl radical formed can either bleach the Crocin or abstract a proton from radical scavengers. The radical derived from radical scavengers can also bleach Crocin. The bleaching of Crocin can either be abstraction of hydrogen atom or addition of radical to polyene system.[131] The hydroxyl and azyl radicals cannot be assayed because of their high reactivity toward organic compounds. Superoxide and alkyl radicals are not found suitable due to least reactivity.

The antioxidant activity can be calculated by the following relation:

$$\frac{Abs_o - Abs_{sample}}{Abs_o} \times 100$$

where Abs_o and Abs_{sample} are the absorbances in the absence and presence of samples, respectively.

Total phenolic and total flavonoids assay techniques

So far, about 8000 species of aromatic hydroxyl nucleus have been identified in nature. The polyphenolics acts as a reducing agent, hydrogen atoms donor, scavenger of singlet oxygen, and some phenolics antioxidants act as chelators of metal that otherwise react with hydrogen peroxide to liberate hydroxyl radical.

FCR is the modification of FDR, which was applied to indirect total determination of tyrosine and tryptophan with higher sensitivity and good reproducibility. The basic principle of each method relies on the reaction between FCR, an oxidizing agent with either tryptophan or tyrosine resulting in the formation of reduced molybdenum blue proportional to the concentration of the protein. The basic difference between FCR and FDR lies in the molybdate concentration used in the preparation. The amount of molybdate added is more in FCR to avoid the white precipitate formed during the assay involving FDR.

The FCR can be prepared by dissolving 100 g of sodium tungstate with 25 g of sodium molybdate in 700 ml distilled water followed by acidification with 50 ml of concentrated HCl and 50 ml of 85% orthophosphoric acid. The acidified solution is boiled for 10 h and 150 g of lithium sulphate is added for intense yellow solution called FCR.[122]

The oxidizing mixture comprises molybdophosphotungstic heteropolyacid ($3H_2O$–P_2O_5–$13WO_3$–5 MoO_3–10 H_2O

(heteropoly anion: $P_2Mo_5W_{13}O_{62}^{6-}$) with hypothetical Mo (VI) as the action center and thus is still in experimental stage. The exact nature of the reduced blue color species that can spectrophotometrically monitored at 750–765 nm is still not known due to the complexity of reaction. However, it may involve one or two electron process of molybdate because of ease of reduction. The addition of sodium carbonate facilitates the formation of phenolate ion which is capable of reducing FCR. Phenolics are generally oxidized in basic medium resulting in the formation of superoxide, which inturn reacts with molybdate with the formation of molybdenum oxide (MoO^{4+}) with a strong absorption in the region of 750 nm. The absorption maximum is independent of the nature of the phenolics group that overrules the formation of the complex between the metal and phenolics antioxidants.

Among the reducing species that can interfere with FCR are ascorbic acid and sugars like glucose and fructose due to the alcoholic groups in the oxidized form. Under acidic condition near to pH 3.0, presence of ascorbic acid glucose and fructose hampers the accuracy due to the formation of blue color by the reaction with polyphosphotungstate. This can be taken as a measure to qualify the presence of ascorbic acid, dehydroascorbic acid, or other reducing compounds. However, it is also claimed that the oxidation of ascorbic acid takes place prior to the addition of alkali and subsequently the oxidation of phenolics.[132] Methods that are adopted in the purification step include: (i) Partial purification by using SPE cartridges (ii). Calculation of the corrected total phenolics content by subtracting the ascorbic acid reducing activity. (iii). Treatment of phenolics extract with oxidative agents prior to FCR assay procedure.

It is common to use total phenolic assay capacity applicable to all hydrophilic reducing substances but not to lypophillic antioxidants. The standards that are being used as reference compounds from phenolics and flavonoids are gallic acid, pyrocatechol, catechin, tannic acid, chlorogenic acid, caffeic acid, protocatechuic acid, ferulic acid, vanillic acid, hydroxycinnamic acid, flavanols, and anthocyanins. The advantage of the FCR total phenolic assay being the excellent correlation among DPPH, FRAP, TEAC, and ORAC methodology in terms of total phenolic profiles and antioxidant activity.[133] In addition, the main advantage of FCR is its simplicity, reproducibility, and the commercial availability of reagents. The absorption at the higher absorption maximum reduces the interference from sample matrix. The disadvantage of this assay is that it is not only specific to phenolic compounds but other non-phenolic compound also gets reduced. Earlier it was believed that FCR method is useful only for hydrophilic antioxidants, but very recently a modified procedure involving NaOH–isobutanol–water was developed for the quantification of lypophillic antioxidants.[134]

A modified CERAC procedure by tuning the redox potential of Ce (IV) by a mixture of sodium sulphate and sulphuric acid was carried out by Ozyurt research group for oxidizing true antioxidants but not citric acid, reducing sugars, and other pharmaceutical ingredients in which TEAC followed an order: quercetin, rutin, gallic acid, catechin, caffeic acid, ferulic acid, naringenin, naringin, trolox, and ascorbic acid.[50] Furthermore, Cerric metric procedure based on the oxidation of antioxidants with Ce (IV) in acidic medium involves the measurement of Ce (III), a fluorescent product at 360 nm.[135] A colorimetric technique was adopted by Zhishen research group[136] for the quantification of total flavonoids involving sodium nitrite, and aluminum in sodium hydroxide medium.

Total oxidant scavenging capacity assays

Total radical antioxidant assays refers to the integrated parameter present in a complex mixture, which is often more meaningful to evaluate the beneficial health effects due to cooperative action of antioxidants.[107] These were originally developed to study marine organisms such as Mediterranean *demosponge petrosia ficiformis*, scallops, mussels, penguins, and red mullet for their susceptibility to oxidative stress. TOSC assay was later extended to measure antioxidant activity of food samples such as tomatoes and fruit such as strawberries. The assay is based on the formation of ethylene by ROS with α-keto-γ-methiobutyric acid that can be monitored by gas chromatographic technique.[137,138] The time course of the ethylene formation during TOSC assay is monitored by repeated GC analysis of aliquots from the head space reaction vessels. The assay conditions lead to equivalent ethylene yield by all the applied ROS such as peroxyl radicals, peroxynitrite, and hydroxyl radicals. Peroxyl radicals can be generated by thermal homolysis of 2,2′-azobis-(2-methylpropionamide)dichloride, whereas Peroxy nitrite by decomposition of 3-morpholinosynnonime-N-ethylcarbamide.[139] Hydroxyl radicals by iron-ascorbate solution[140] (Figure 19).

In TOSC assay, an antioxidant is characterized by its capacity to inhibit the production of ethylene by respective ROS as compared to an uninhibited control reaction. A kinetic curve that fits the experimental GC data for ethylene production and AUC is calculated mathematically. TOSC values are quantified by comparing AUC for sample and control in accordance with the formula

$$\mathrm{TOSC}_{\%} = 100 - \frac{\int \mathrm{Sample}}{\int \mathrm{control}} \times 100$$

A compound that suppresses the formation of ethylene completely possess a 100% TOSC activity. Antioxidant and pro-oxidant achieve positive and negative values, respectively, because AUC is lesser and greater, respectively, than that of control reaction. Results are displayed by two different general approaches such as relative TOSC and comparative TOSC.

Apart from usual ROS (peroxyl, peroxynitrite, and hydroxyl radicals) hypochlorous acid, alkoxy radicals, and ascorbate radicals can also be detected in the lower μM range. This is applicable to lipophillic, hydrophilic antioxidants, pro-oxidants, and complex biological fluids and tissues. The limitations include long-time requirement and GC expert labor consumption, which makes it unsuitable for common analytical laboratories. The test solution should be prepared whenever in need as the shelf life is of very short duration. This involves complex and time-consuming calculations and limited possibility of displaying result. The main limitation of this technique is long-reaction time and necessity of multiple chromatographic analyses for each experiment.

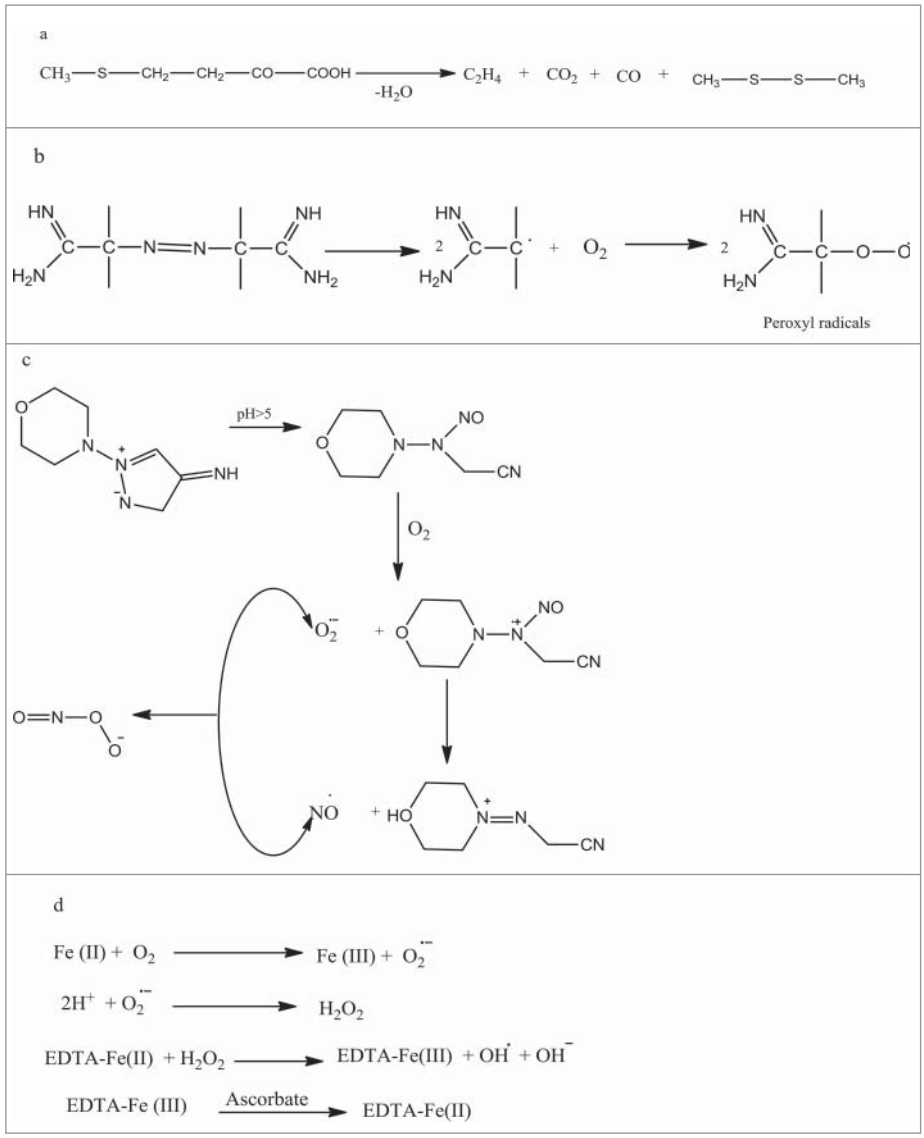

Figure 19. Assay methodology of (a) TOSC assay. Generation of (b) peroxyl radicals (c) peroxy nitrite (d) superoxide and hydroxyl radical during TOSC assays.

Chemiluminiscence

Chemiluminscence take place by the transfer of energy to chemical compound with compatible energy background, which then becomes an emitter of the light either by direct or sensitized procedure. These light producing reactions can be adopted in the quantitative feature of the concentration in terms of emission intensity measurement.[141]

These assays are based on the attack of ROS with chemiluminiscence active compounds. This can be a sensitive detection assay method involving the production of radical. But one of the serious disadvantage is that this process produces low-intensity light emission that may decay rapidly; addition of *p*-iodophenol gives a prolonged and stable emission. The light emission will be restored when all the added antioxidants get depleted.

ROS formation was measured by chemiluminescence assay using luminol (5 mM, 5-amino-2, 3-dihydro-1, 4-phthalazinedione.[142] Luminol is sensitive toward HOCl, while lucigenin is oxidized by superoxide.[89] Some authors suggest that the hydroxyl radical or superoxide may oxidize lucigenin and luminal.[143] The specificity of the latter is questioned since the probe itself may act as a source of superoxide generation.[144] When used alone, luminol can detect the oxidative damage in cells with sufficient peroxidases, granulaocytes, and spermatozoa.

Antioxidative and pro-oxidative properties of many biologically important compounds were evaluated by the formation high luminescent superoxide radical from potassium superoxide and 18-crown-6 in dimethylsulphoxide medium.[145]

Antioxidants which are phenolic and polyphenolic in nature have received greater attention by permangnometric chemilumi-

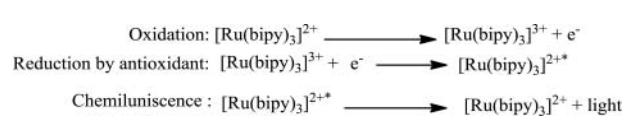

Figure 20. Chemiluminiscence property of Ruthenium–bipyridyl complex.

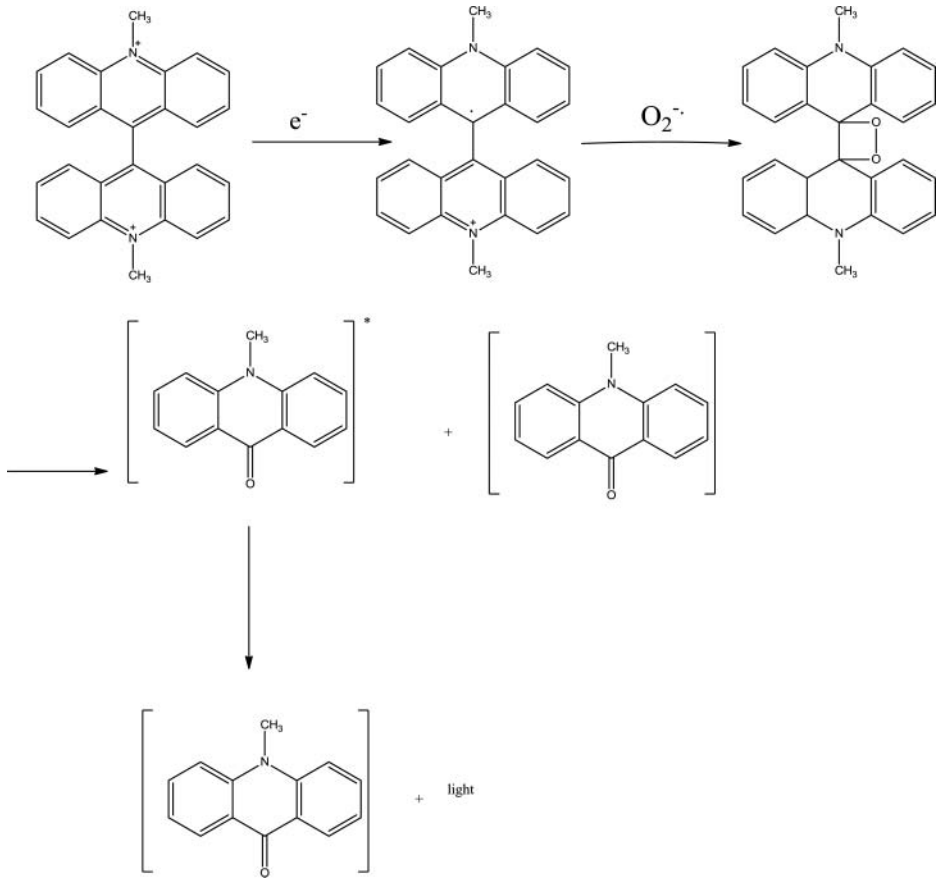

Figure 21. Chemiluminiscent lucigenin in the assay of superoxide.

niscence assay methodology,[146] which was later extended to flow injection based analysis, considered as a good technique in the analysis of individual antioxidants.[147–149] These include antioxidants like ascorbic acid, quercetin, catechin, melatonin, caffeic acid, resveratrol, rosmarinic acid with detection limits ranging from 1 to 10 nM. This method is ineffective without chromatographic method of separation as it monitors the total antioxidant capacity in terms of phenolic content. A good correlation between DPPH and ABTS commercial assay kit method was observed by Francis et al along with the rapidity.[150]

Potassium permanganate exhibits characteristic red chemiluminiscence emission property at wavelength of 689 nm due to the relaxation of Mn (II) species from an excited species. The presence of polyphosphates increases the intensity proportionately by 50 times as demonstrated by two different research groups.[146,151] The exact mechanism of the chemiluminiscence property of potassium permanganate was elucidated recently.[152] A radical intermediate generates by the oxidation of antioxidant by Mn (IV). This transient radical species reacts with Mn (III) previously present in the solution to produce Mn (II)* emission source. The enhancement in the chemiluminiscence intensity is due to the prevention of disproportionate Mn (III) intermediate in which polyphosphates form a cage like structure around Mn (II)*, limiting non-radioactive pathways.

The chemiluminiscence property of $[Ru(bipy)_3]^{2+}$ relies on oxidation to produce $[Ru(bipy)_3]^{3+}$ which in turn gets reduced by an antioxidant leaving behind Ruthenium (II) complex in the electronically exited state which subsequently returns to the ground state by emission of photon.[153–156] The significant property is its inherent stability in the aqueous solution (due to its ability to oxidize water) that makes analytical utilization of $[Ru(bipy)_3]^{2+}$ chemiluminiscence reagent. The instability was overcome by electrochemical oxidation process involving *in situ* production of the exited state species.[155] The production of chemilumiscent $[Ru(bipy)_3]^{2+}$ is shown in Figure 20.

As $[Ru(bipy)_3]^{2+}$ chemiluminescence is primarily employed in the determination of compounds containing amine moieties such as alkaloids, amino acids, organic acids, pesticides, and proteins.[157] Further Nana research group extends this chemiluminiscence property of $[Ru(bipy)_3]^{2+}$ for the determination of amine containing antioxidants such as GSH.[158]

The commonly used Manganous dioxide is of limited application due to its minimum solubility in most of the solvents. Permanganate can be reduced to non-colloidal brown solution of Mn (IV) by sodium formate in 3 M phosphoric acid with agitation. Recently soluble Mn (IV) with broad spectral distribution at 730 ± 5 nm was adopted in the determination of opiate alkaloids, indoles, and analytes of forensic interest.[159] The emission of light between 725 and 740 nm claims the characteristic Mn (II)*. The intensity of light can be enhanced by reagents such as formaldehyde.

The chemiluminiscent lucigenin assay involves several steps that gives rise to the formation of dioxetane. The dioxetane decomposes into two molecules of N-methylacridone, one

among this is in an electronically excited state, which emits a photon on its de-exitation to the ground state [160] (Figure 21).

Conclusion

Biological stress is a condition that results in elevated risk for different types of disease states such as inflammation, cardiac disease, aging, cataract, autism, Alzheimer's, and cancer. Antioxidant has definite roles to play such as that it may directly react with the free radicals released or may decrease the formation of free radical by inhibiting the activities of free radical releasing enzymes or enhancing the activities of antioxidant releasing enzymes.

Many research efforts are devoted toward the assay of antioxidants with the objective of studying the mechanism of their action and also to explore new naturally available antioxidants. Some of the assay methodologies are concentrated toward specific ROS, while others tabulate the total antioxidant activity. A standardized antioxidant assay procedure which meets the maximum underlying requirements such as; simple and within run and between day reproducible with less adamant experimental features, defined end point and chemically relevant mechanism, biologically relevant species as a source of free radical, adaptability for both hydrophilic and lypophillic antioxidants, high through analysis with good quality control, easy instrumentation, and less hazardous chemicals is the need of the time. However, all these assessment may be considered and appear exaggerated for screening purpose and it is hard to get an antioxidant assay procedure that satisfies the all criteria. The methods cannot assess all the available reactive species due to the nature of the probe selected. For a complete antioxidant profile, a difficult process can be created by the selection of the probe for assay. Hence several procedures are required to evaluate the total antioxidant activity.

Abbreviations

ABAP	2,2-azobis-(2-amidinopropane) dihydrochloride
ABTS	2,2′-azinobis(3-ethylbenzothiazolline-6-sulfonic acid
ATP	Adenosine triphosphate
CAT	Catalase
CBA	Crocin bleaching assay
CPH	Chloropromazine hydrochloride
CERAC	Cerric reducing antioxidant capacity
CUPRAC	Copper reducing antioxidant capacity
DEPMPO	5-(diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide
DMPD	N,N-dimethyl-p-phenylenediaminedihydrochloride
DMPO	5,5-dimethyl-1-pyrroline-N-oxide
DMSO	Dimethylsulphoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
ESR	Electron spin resonance
ET	Electron transfer
FCR	Folin-ciocalteu reagent
FDR	Folin-Denis reagent
FRAP	Ferric reducing antioxidant parameter
GPx	Glutathione peroxidase
GSH	Reduced glutathione
HAT	Hydrogen atom transfer
HRP	Horseradish Peroxidase
MLA	Malondialdehyde
NBT-MTT	Nitroblue tetrazolium-3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ORAC	Oxygen radical absorbance capacity
PQ	Paraquat
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TAN	2,2,6,6-Tetramethyl-4-piperidone-N-oxyl
TBA	Thiobarbituric acid
TEAC	Trolox equivalents antioxidant capacity
AC-TMPO	4-((9-Acridinecarbonyl)amino)-2,2,6,6-tetramethyl piperidine-1-oxyl
TMPD	2,2,6,6-Tetramethyl-4-piperidone
TOSC	Total oxidant scavenging capacity
TPPHE	Triphenylphosphonium dihydroethidium
TPTZ	Tripyridyltriazine

Acknowledgments

We thank St. Philomena's College for providing facilities to carry out the research investigation.

Funding

One of the authors (A.S.K.) expresses gratitude for the generous support by the University Grants Commission, New Delhi, India (2166-MRP/15-16/KAMY006/UGC-SWRO).

Conflict of interest

We confirm that any aspect of the work covered in this manuscript has not involved either experimental animals or human patients.

We understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). He is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. We confirm that we have provided a current, correct email address, which is accessible by the Corresponding Author and which has been configured to accept email from shivakem77@yahoo.co.in and shivakem77@gmail.com.

ORCID

Anantharaman Shivakumar http://orcid.org/0000-0003-1132-7087

References

[1] Gülçin, İ. Antioxidant Activity of Food Constituents: An Overview. *Arch. Toxicol.* **2011**, *86*, 345.
[2] Halliwell, B. Commentary Oxidative Stress, Nutrition and Health. Experimental Strategies for Optimization of Nutritional Antioxidant Intake in Humans. *Free Radical Res.* **1996**, *25*, 57.
[3] Lü, J.-M.; Lin, P. H.; Yao, Q.; Chen, C. Chemical and Molecular Mechanisms of Antioxidants: Experimental Approaches and Model Systems. *J. Cell. Mol. Med.* **2010**, *14*, 840.
[4] Alho, H.; Leinonen, J. Total Antioxidant Activity Measured by Chemiluminescence Methods. In Methods in Enzymology, Academic Press, **1999**, *299*, 3.
[5] Duh, P.-D. Antioxidant Activity of Burdock (Arctium Lappa Linné): Its Scavenging Effect on Free-Radical and Active Oxygen. *J. Am. Oil Chem. Soc.* **1998**, *75*, 455.

[6] Diplock, A. T.; Charuleux, J.-L.; Crozier-Willi, G.; Kok, F. J.; Rice-Evans, C.; Roberfroid, M.; Stahl, W.; Viña-Ribes, J. Functional Food Science and Defence Against Reactive Oxidative Species. *Br. J. Nutr.* **1998**, *80*, S77.

[7] Cai, Y.-J.; Dai, J.-Q.; Fang, J.-G.; Ma, L.-P.; Hou, L.-F.; Yang, L.; Liu, Z.-L. Antioxidative and Free Radical Scavenging Effects of Ecdysteroids from Serratula Strangulata. *Can. J. Physiol. Pharmacol.* **2002**, *80*, 1187.

[8] Amarowicz, R.; Pegg, R. B.; Rahimi-Moghaddam, P.; Barl, B.; Weil, J. A. Free-Radical Scavenging Capacity and Antioxidant Activity of Selected Plant Species from the Canadian Prairies. *Food Chem.* **2004**, *84*, 551.

[9] Rice-Evans, C.; Miller, N. Measurement of the Antioxidant Status of Dietary Constituents, Low Density Lipoproteins and Plasma. *Prostaglandins, Leukot. Essent. Fatty Acids* **1997**, *57*, 499.

[10] Alam, M. N.; Bristi, N. J.; Rafiquzzaman, M. Review on In Vivo and In Vitro Methods Evaluation of Antioxidant Activity. *Saudi Pharm. J.* **2013**, *21*, 143.

[11] Amorati, R.; Valgimigli, L. Advantages and Limitations of Common Testing Methods for Antioxidants. *Free Radical Res.* **2015**, *49*, 633.

[12] Xiong, N.; Long, X.; Xiong, J.; Jia, M.; Chen, C.; Huang, J.; Ghoorah, D.; Kong, X.; Lin, Z.; Wang, T. Mitochondrial Complex I Inhibitor Rotenone-Induced Toxicity and Its Potential Mechanisms in Parkinson's Disease Models. *Crit. Rev. Toxicol.* **2012**, *42*, 613.

[13] Cooper, C. E.; Brown, G. C. The Inhibition of Mitochondrial Cytochrome Oxidase by the Gases Carbon Monoxide, Nitric Oxide, Hydrogen Cyanide and Hydrogen Sulfide: Chemical Mechanism and Physiological Significance. *J. Bioenerg. Biomembr.* **2008**, *40*, 533.

[14] Starkov, A. A. Measurement of Mitochondrial ROS Production. In Protein Misfolding and Cellular Stress in Disease and Aging: Concepts and Protocols, P. Bross, N. Gregersen, Eds.; Humana Press: Totowa, NJ, **2010**; 245.

[15] Anthérieu, S.; Azzi, P. B.-E.; Dumont, J.; Abdel-Razzak, Z.; Guguen-Guillouzo, C.; Fromenty, B.; Robin, M.-A.; Guillouzo, A. Oxidative Stress Plays a Major Role in Chlorpromazine-Induced Cholestasis in Human HepaRG Cells. *Hepatol.* **2013**, *57*, 1518.

[16] Quinlan, C. L.; Orr, A. L.; Perevoshchikova, I. V.; Treberg, J. R.; Ackrell, B. A.; Brand, M. D. Mitochondrial Complex II Can Generate Reactive Oxygen Species at High Rates in Both the Forward and Reverse Reactions. *J. Biol. Chem.* **2012**, *287*, 27255.

[17] Turrens, J. F.; Alexandre, A.; Lehninger, A. L. Ubisemiquinone is the Electron Donor for Superoxide Formation by Complex III of Heart Mitochondria. *Arch. Biochem. Biophys.* **1985**, 237, 408.

[18] Mandavilli, B. S.; Santos, J. H.; Van Houten, B. Mitochondrial DNA Repair and Aging. *Mutat. Res./Fundam. Mol. Mech. Mutagen.* **2002**, *509*, 127.

[19] Susanna Hipelli; Elstner, E. F. OH-Radical-Type Reactive Oxygen Species: A Short Review on the Mechanisms of OH-Radical and Peroxynitrite Toxicity. *Z- Naturforsch* **1997**, *52c*, 555.

[20] Lipinski, B.; Pretorius, E. Hydroxyl Radical-Modified Fibrinogen as a Marker of Thrombosis: The Role of Iron. *Hematol.* **2012**, *17*, 241.

[21] Kanti Das, T.; Wati, M. R.; Fatima-Shad, K. Oxidative Stress Gated by Fenton and Haber Weiss Reactions and Its Association With Alzheimer's Disease. *Arch Neurosci.* **2015**, *2*, e20078.

[22] Lobo, V.; Patil, A.; Phatak, A.; Chandra, N. Free Radicals, Antioxidants and Functional Foods: Impact on Human Health. *Pharmacognosy Rev.* **2010**, *4*, 118.

[23] Morán, J. M.; Ortiz-Ortiz, M. A.; Ruiz-Mesa, L. M.; Fuentes, J. M. Nitric Oxide in Paraquat-Mediated Toxicity: A Review. *J. Biochem. Mol. Toxicol.* **2010**, *24*, 402.

[24] Schülke, S.; Dreidax, D.; Malik, A.; Burmester, T.; Nevo, E.; Band, M.; Avivi, A.; Hankeln, T., Living with Stress: Regulation of Antioxidant Defense Genes in the Subterranean, Hypoxia-Tolerant Mole Rat, Spalax. *Gene* **2012**, *500*, 199.

[25] Kelley, E. E.; Khoo, N. K. H.; Hundley, N. J.; Malik, U. Z.; Freeman, B. A.; Tarpey, M. M. Hydrogen Peroxide is the Major Oxidant Product of Xanthine Oxidase. *Free Radical Biol. Med.* **2010**, *48*, 493.

[26] Buonocore, G.; Perrone, S.; Tataranno, M. L. Oxygen Toxicity: Chemistry and Biology of Reactive Oxygen Species. *Semin. Fetal Neonatal Med.* **2010**, *15*, 186.

[27] Froudarakis, M.; Hatzimichael, E.; Kyriazopoulou, L.; Lagos, K.; Pappas, P.; Tzakos, A. G.; Karavasilis, V.; Daliani, D.; Papandreou, C.; Briasoulis, E. Revisiting Bleomycin from Pathophysiology to Safe Clinical Use. *Crit. Rev. Oncol./Hematol.* **2013**, *87*, 90.

[28] Jung, K.; Reszka, R. Mitochondria as Subcellular Targets for Clinically Useful Anthracyclines. *Adv. Drug Deliv. Rev.* **2001**, *49*, 87.

[29] Liochev, S. I. Commentary: The Role of Iron-Sulfur Clusters in In Vivo Hydroxyl Radical Production. *Free Radical Res.* **1996**, *25*, 369.

[30] Vásquez-Vivar, J.; Kalyanaraman, B.; Kennedy, M. C. Mitochondrial Aconitase Is a Source of Hydroxyl Radical: An electron spin resoance investigation. *J. Biol. Chem.* **2000**, *275*, 14064.

[31] Timmermann, F. Tocopherole — Antioxidative Wirkung bei Fetten und Ölen. *Lipid/Fett* **1990**, *92*, 201.

[32] Burton, G. W.; Ingold, K. U. Autoxidation of Biological Molecules. 1. Antioxidant Activity of Vitamin E and Related Chain-Breaking Phenolic Antioxidants In Vitro. *J. Am. Chem. Soc.* **1981**, *103*, 6472.

[33] Andjelković, M.; Van Camp, J.; De Meulenaer, B.; Depaemelaere, G.; Socaciu, C.; Verloo, M.; Verhe, R., Iron-Chelation Properties of Phenolic Acids Bearing Catechol and Galloyl Groups. *Food Chem.* **2006**, *98*, 23.

[34] Mitsuda, H.; Yasumoto, K.; Iwami, K. Antioxidative Action of Indole Compounds during the Autoxidation of Linoleic Acid. *Eiyo To Shokuryo* **1966**, *19*, 210.

[35] Cai, Y.-Z.; Mei, S.; Jie, X.; Luo, Q.; Corke, H. Structure–Radical Scavenging Activity Relationships of Phenolic Compounds From Traditional Chinese Medicinal Plants. *Life Sci.* **2006**, *78*, 2872.

[36] İlhami, G.; Mshvildadze, V.; Gepdiremen, A.; Elias, R. The Antioxidant Activity of a Triterpenoid Glycoside Isolated from the Berries of Hedera Colchica: 3-O-(β-d-Glucopyranosyl)-Hederagenin. *Phytother. Res.* **2006**, *20*, 130.

[37] Gülçin, İ.; Elmastaş, M.; Aboul-Enein, H. Y. Determination of Antioxidant and Radical Scavenging Activity of Basil (Ocimum Basilicum L. Family Lamiaceae) Assayed by Different Methodologies. *Phytother. Res.* **2007**, *21*, 354.

[38] Gülçin, İ. Comparison of In Vitro Antioxidant and Antiradical Activities of L-Tyrosine and L-Dopa. *Amino Acids* **2006**, *32*, 431.

[39] Lichtenstein, A. H. Nutrient Supplements and Cardiovascular Disease: A Heartbreaking Story. *J. Lipid Res.* **2009**, *50*, S429.

[40] Ou, B.; Huang, D.; Hampsch-Woodill, M.; Flanagan, J. A.; Deemer, E. K. Analysis of Antioxidant Activities of Common Vegetables Employing Oxygen Radical Absorbance Capacity (ORAC) and Ferric Reducing Antioxidant Power (FRAP) Assays: A Comparative Study. *J. Agricul. Food Chem.* **2002**, *50*, 3122.

[41] Roy, Molay K; Koide, M.; Rao, Theertham P.; Okubo, T.; Ogasawara, Y.; Juneja, Lekh R. ORAC and DPPH Assay Comparison to Assess Antioxidant Capacity of Tea Infusions: Relationship Between Total Polyphenol and Individual Catechin Content. *Int. J. Food Sci. Nutr.* **2010**, *61*, 109.

[42] Číž, M.; Čížová, H.; Denev, P.; Kratchanova, M.; Slavov, A.; Lojek, A. Different Methods for Control and Comparison of the Antioxidant Properties of Vegetables. *Food Cont.* **2010**, *21*, 518.

[43] Grajeda-Iglesias, C.; Salas, E.; Barouh, N.; Baréa, B.; Panya, A.; Figueroa-Espinoza, M. C. Antioxidant Activity of Protocatechuates Evaluated by DPPH, ORAC, and CAT Methods. *Food Chem.* **2016**, *194*, 749.

[44] Prieto, M. A.; Vázquez, J. A.; Murado, M. A. Crocin Bleaching Antioxidant Assay Revisited: Application to Microplate to Analyse Antioxidant and Pro-Oxidant Activities. *Food Chem.* **2015**, *167*, 299.

[45] Lage, M. Á. P.; García, M. A. M.; Álvarez, J. A. V.; Anders, Y.; Curran, T. P. A New Microplate Procedure for Simultaneous Assessment of Lipophilic and Hydrophilic Antioxidants and Pro-Oxidants, Using Crocin and β-Carotene Bleaching Methods in a Single Combined Assay: Tea Extracts as a Case Study. *Food Res. Int.* **2013**, *53*, 836.

[46] Zou, T.-B.; He, T.-P.; Li, H.-B.; Tang, H.-W.; Xia, E.-Q. The Structure-Activity Relationship of the Antioxidant Peptides from Natural Proteins. *Mol.* **2016**, *21*, 72.

[47] Alvarez-Jubete, L.; Wijngaard, H.; Arendt, E. K.; Gallagher, E. Polyphenol Composition and In Vitro Antioxidant Activity of

Amaranth, Quinoa Buckwheat and Wheat as Affected by Sprouting and Baking. *Food Chem.* **2010**, *119*, 770.
[48] Gülçin, İ. Antioxidant Activity of Food Constituents: An Overview. *Arch. Toxicol.* **2012**, *86*, 345.
[49] Apak, R.; Özyürek, M.; Güçlü, K.; Çapanoğlu, E. Antioxidant Activity/Capacity Measurement. 1. Classification, Physicochemical Principles, Mechanisms, and Electron Transfer (ET)-Based Assays. *J. Agricul. Food Chem.* **2016**, *64*, 997.
[50] Ozyurt, D.; Demirata, B.; Apak, R. Modified Cerium(IV)-Based Antioxidant Capacity (CERAC) Assay with Selectivity Over Citric Acid and Simple Sugars. *J. Food Compos. Anal.* **2010**, *23*, 282.
[51] Meng, Q.; Li, G.; Luo, B.; Wang, L.; Lu, Y.; Liu, W. Screening and Isolation of Natural Antioxidants from Ziziphora Clinopodioides Lam. With High Performance Liquid Chromatography Coupled to a Post-Column Ce(iv) Reduction Capacity Assay. *RSC Adv.* **2016**, *6*, 62378.
[52] Fu, L.; Xu, B.-T.; Xu, X.-R.; Gan, R.-Y.; Zhang, Y.; Xia, E.-Q.; Li, H.-B. Antioxidant Capacities and Total Phenolic Contents of 62 Fruits. *Food Chem.* **2011**, *129*, 345.
[53] Yang, Z.; Zhai, W., Identification and Antioxidant Activity of Anthocyanins Extracted from the Seed and Cob of Purple Corn (Zea mays L.). *Innovative Food Sci. Emerg. Technol.* **2010**, *11*, 169.
[54] Mocan, A.; Schafberg, M.; Crişan, G.; Rohn, S., Determination of Lignans and Phenolic Components of Schisandra Chinensis (Turcz.) Baill. Using HPLC-ESI-ToF-MS and HPLC-Online TEAC: Contribution of Individual Components to Overall Antioxidant Activity and Comparison with Traditional Antioxidant Assays. *J. Funct. Foods* **2016**, *24*, 579.
[55] Mishra, K.; Ojha, H.; Chaudhury, N. K. Estimation of Antiradical Properties of Antioxidants Using DPPH Assay: A Critical Review and Results. *Food Chem.* **2012**, *130*, 1036.
[56] Carmona-Jiménez, Y.; García-Moreno, M. V.; Igartuburu, J. M.; Garcia Barroso, C. Simplification of the DPPH Assay for Estimating the Antioxidant Activity of Wine and Wine by-Products. *Food Chem.* **2014**, *165*, 198.
[57] Wollinger, A.; Perrin, É.; Chahboun, J.; Jeannot, V.; Touraud, D.; Kunz, W. Antioxidant Activity of Hydro Distillation Water Residues from Rosmarinus Officinalis L. Leaves Determined by DPPH Assays. *Comp Rendus Chim.* **2016**, *19*, 754.
[58] Fung, A.; Hamid, N.; Lu, J., Fucoxanthin Content and Antioxidant Properties of Undaria Pinnatifida. *Food Chem.* **2013**, *136*, 1055.
[59] Martins, A. C.; Bukman, L.; Vargas, A. M. M.; Barizão, É. O.; Moraes, J. C. G.; Visentainer, J. V.; Almeida, V. C. The Antioxidant Activity of Teas Measured by the FRAP Method Adapted to the FIA System: Optimising the Conditions Using the Response Surface Methodology. *Food Chem.* **2013**, *138*, 574.
[60] Karadirek, Ş.; Kanmaz, N.; Balta, Z.; Demirçivi, P.; Üzer, A.; Hızal, J.; Apak, R. Determination of Total Antioxidant Capacity of Humic Acids Using CUPRAC, Folin–Ciocalteu, Noble Metal Nanoparticle- and Solid–Liquid Extraction-Based Methods. *Talanta* **2016**, *153*, 120.
[61] Muller, C. H.; Lee, T. K. Y.; Montaño, M. A. Improved Chemiluminescence Assay for Measuring Antioxidant Capacity of Seminal Plasma. In Spermatogenesis: Methods and *Protocols*, D. T. Carrell, K. I. Aston, Eds.; Humana Press: Totowa, NJ, **2013**; 363.
[62] Oliveira, S. d.; d. Souza, G. A.; Eckert, C. R.; Silva, T. A.; Sobral, E. S.; Fávero, O. A.; Ferreira, M. J. P.; Romoff, P.; Baader, W. J., Evaluation of Antiradical Assays Used in Determining the Antioxidant Capacity of Pure Compounds and Plant Extracts. *Química Nova* **2014**, *37*, 497.
[63] Tabart, J.; Franck, T.; Kevers, C.; Pincemail, J.; Serteyn, D.; Defraigne, J.-O.; Dommes, J. Antioxidant and Anti-Inflammatory Activities of Ribes Nigrum Extracts. *Food Chem.* **2012**, *131*, 1116.
[64] Gorbi, S.; Avio, G. C.; Benedetti, M.; Totti, C.; Accoroni, S.; Pichierri, S.; Bacchiocchi, S.; Orletti, R.; Graziosi, T.; Regoli, F. Effects of Harmful Dinoflagellate Ostreopsis cf. Ovata Exposure on Immunological, Histological and Oxidative Responses of Mussels Mytilus Galloprovincialis. *Fish & Shellfish Immunol.* **2013**, *35*, 941.
[65] Moore, J.; Yin, J.-J.; Yu, L. Novel Fluorometric Assay for Hydroxyl Radical Scavenging Capacity (HOSC) Estimation. *J. Agricul. Food Chem.* **2006**, *54*, 617.
[66] Ou, B.; Hampsch-Woodill, M.; Flanagan, J.; Deemer, E. K.; Prior, R. L.; Huang, D. Novel Fluorometric Assay for Hydroxyl Radical Prevention Capacity Using Fluorescein as the Probe. *J. Agricul. Food Chem.* **2002**, *50*, 2772.
[67] Coudray, C.; Favier, A. Determination of Salicylate Hydroxylation Products as an In Vivo Oxidative Stress Marker. *Free Radical Biol. Med.* **2000**, *29*, 1064.
[68] Thomas, C.; Mackey, M. M.; Diaz, A. A.; Cox, D. P. Hydroxyl Radical is Produced Via the Fenton Reaction in Submitochondrial Particles Under Oxidative Stress: Implications for Diseases Associated with Iron Accumulation. *Redox Rep.* **2009**, *14*, 102.
[69] Zhao, G.-R.; Xiang, Z.-J.; Ye, T.-X.; Yuan, Y.-J.; Guo, Z.-X. Antioxidant Activities of Salvia Miltiorrhiza and Panax Notoginseng. *Food Chem.* **2006**, *99*, 767.
[70] Tatzber, F.; Griebenow, S.; Wonisch, W.; Winkler, R. Dual Method for the Determination of Peroxidase Activity and Total Peroxides-Iodide Leads to a Significant Increase of Peroxidase Activity in Human Sera. *Anal. Biochem.* **2003**, *316*, 147.
[71] Shahidi, F.; Kamil, J.; Jeon, Y.-J.; Kim, S.-K. Antioxidant Role of Chitosan in a Cooked COD (Gadus morhua) Model System. *J. Food Lipids* **2002**, *9*, 57.
[72] Dorman, H. J. D.; Bachmayer, O.; Kosar, M.; Hiltunen, R. Antioxidant Properties of Aqueous Extracts from Selected Lamiaceae Species Grown in Turkey. *J. Agricul. Food Chem.* **2004**, *52*, 762.
[73] Daglia, M.; Racchi, M.; Papetti, A.; Lanni, C.; Govoni, S.; Gazzani, G. In Vitro and Ex Vivo Antihydroxyl Radical Activity of Green and Roasted Coffee. *J. Agricul.Food Chem.* **2004**, *52*, 1700.
[74] Zou, Y.; Lu, Y.; Wei, D. Antioxidant Activity of a Flavonoid-Rich Extract of Hypericum perforatum L. In Vitro. *J. Agricul. Food Chem.* **2004**, *52*, 5032.
[75] Papastergiadis, A.; Mubiru, E.; Van Langenhove, H.; De Meulenaer, B. Malondialdehyde Measurement in Oxidized Foods: Evaluation of the Spectrophotometric Thiobarbituric Acid Reactive Substances (TBARS) Test in Various Foods. *J. Agricul. Food Chem.* **2012**, *60*, 9589.
[76] Floriano-Sánchez, E.; Villanueva, C.; Noel Medina-Campos, O.; Rocha, D.; Javier Sánchez-González, D.; Cárdenas-Rodríguez, N.; Pedraza-Chaverrí, J. Nordihydroguaiaretic Acid is a Potent In Vitro Scavenger of Peroxynitrite, Singlet Oxygen, Hydroxyl Radical, Superoxide Anion and Hypochlorous acid and Prevents In Vivo Ozone-Induced Tyrosine Nitration in Lungs. *Free Radical Res.* **2006**, *40*, 523.
[77] Haces, M. L.; Hernández-Fonseca, K.; Medina-Campos, O. N.; Montiel, T.; Pedraza-Chaverri, J.; Massieu, L. Antioxidant Capacity Contributes to Protection of Ketone Bodies Against Oxidative Damage Induced During Hypoglycemic Conditions. *Exp. Neurol.* **2008**, *211*, 85.
[78] Wardman, P. Fluorescent and Luminescent Probes for Measurement of Oxidative and Nitrosative Species in Cells and Tissues: Progress, Pitfalls, and Prospects. *Free Radical Biol. Med.* **2007**, *43*, 995.
[79] Halliwell, B.; Gutteridge, J. M. C. Free Radicals in Biology and Medicine, OUP Oxford: Oxford, **2015**.
[80] Akashi, K.; Nishimura, N.; Ishida, Y.; Yokota, A. Potent Hydroxyl Radical-Scavenging Activity of Drought-Induced Type-2 Metallothionein in Wild Watermelon. *Biochem. Biophys. Res. Commun.* **2004**, *323*, 72.
[81] Myhre, O.; Vestad, T. A.; Sagstuen, E.; Aarnes, H.; Fonnum, F. The Effects of Aliphatic (n-Nonane), Naphtenic (1,2,4-Trimethylcyclohexane), and Aromatic (1,2,4-Trimethylbenzene) Hydrocarbons on Respiratory Burst in Human Neutrophil Granulocytes. *Toxicol. Appl. Pharmacol.* **2000**, *167*, 222.
[82] Botchway, S. W.; Crisostomo, A. G.; Parker, A. W.; Bisby, R. H. Near Infrared Multiphoton-Induced Generation and Detection of Hydroxyl Radicals in a Biochemical System. *Arch. Biochem. Biophys.* **2007**, *464*, 314.
[83] de Avellar, I. G. J.; Magalhães, M. M. M.; Silva, A. B.; Souza, L. L.; Leitão, A. C.; Hermes-Lima, M. Reevaluating the Role of 1,10-

Phenanthroline in Oxidative Reactions Involving Ferrous Ions and DNA Damage. *Biochim. et Biophys. Acta (BBA) – Gen. Sub.* **2004**, *1675*, 46.

[84] Goldstein, S.; Meyerstein, D.; Czapski, G. The Fenton Reagents. *Free Radical Biol. Med.* **1993**, *15*, 435.

[85] LeBel, C. P.; Ischiropoulos, H.; Bondy, S. C. Evaluation of the Probe 2',7'-Dichlorofluorescin as an Indicator of Reactive Oxygen Species Formation and Oxidative Stress. *Chem. Res. Toxicol.* **1992**, *5*, 227.

[86] Zhu, H.; Bannenberg, G. L.; Moldéus, P.; Shertzer, H. G. Oxidation Pathways for the Intracellular Probe 2′,7′-Dichlorofluorescin. *Arch. Toxicol.*, **1994**, *68*, 582.

[87] Schopfer, P.; Plachy, C.; Frahry, G. Release of Reactive Oxygen Intermediates (Superoxide Radicals, Hydrogen Peroxide, and Hydroxyl Radicals) and Peroxidase in Germinating Radish Seeds Controlled by Light, Gibberellin, and Abscisic Acid. *Plant Physiol.* **2001**, *125*, 1591.

[88] Paździoch-Czochra, M.; Wideńska, A. Spectrofluorimetric Determination of Hydrogen Peroxide Scavenging Activity. *Anal. Chim. Acta* **2002**, *452*, 177.

[89] Myhre, O.; Andersen, J. M.; Aarnes, H.; Fonnum, F. Evaluation of the Probes 2′,7′-Dichlorofluorescin Diacetate, Luminol, and Lucigenin as Indicators of Reactive Species Formation. *Biochem. Pharmacol.* **2003**, *65*, 1575.

[90] Yoshida, H.; Yanai, H.; Namiki, Y.; Fukatsu-Sasaki, K.; Furutani, N.; Tada, N. Neuroprotective Effects of Edaravone: A Novel Free Radical Scavenger in Cerebrovascular Injury. *CNS Drug Rev.* **2006**, *12*, 9.

[91] Horobin, R. W.; Rashid, F. Interactions of Molecular Probes with Living Cells and Tissues. Part 1. Some General Mechanistic Proposals, Making Use of a Simplistic Chinese Box Model. *Histochemistry*, *94*:205.

[92] Li, Y.; Jiang, B.; Zhang, T.; Mu, W.; Liu, J. Antioxidant and Free Radical-Scavenging Activities of Chickpea Protein Hydrolysate (CPH). *Food Chem.* **2008**, *106*, 444.

[93] Okado-Matsumoto, A.; Fridovich, I. Assay of Superoxide Dismutase: Cautions Relevant to the Use of Cytochrome c, a Sulfonated Tetrazolium, and Cyanide. *Anal. Biochem.* **2001**, *298*, 337.

[94] Aleryani, S.; Milo, E.; Rose, Y.; Kostka, P. Superoxide-Mediated Decomposition of BiologicalS-Nitrosothiols. *J. Biol. Chem.* **1998**, *273*, 6041.

[95] Aruoma, O. I.; Murcia, A.; Butler, J.; Halliwell, B. Evaluation of the Antioxidant and Prooxidant Actions of Gallic Acid and Its Derivatives. *J. Agric. Food Chem.* **1993**, *41*, 1880.

[96] Lavelli, V.; Hippeli, S.; Peri, C.; Elstner, E. F. Evaluation of Radical Scavenging Activity of Fresh and Air-Dried Tomatoes by Three Model Reactions. *J. Agric. Food Chem.* **1999**, 47, 3826.

[97] Foti, M.; Ruberto, G. Kinetic Solvent Effects on Phenolic Antioxidants Determined by Spectrophotometric Measurements. *J. Agric. Food Chem.* **2001**, *49*, 342.

[98] Ou, B.; Hampsch-Woodill, M.; Prior, R. L. Development and Validation of an Improved Oxygen Radical Absorbance Capacity Assay Using Fluorescein as the Fluorescent Probe. *J. Agric. Food Chem.* **2001**, *49*, 4619.

[99] Wolfe, K. L.; Liu, R. H. Cellular Antioxidant Activity (CAA) Assay for Assessing Antioxidants, Foods, and Dietary Supplements. *J. Agric. Food Chem.* **2007**, *55*, 8896.

[100] Huang, D.; Ou, B.; Hampsch-Woodill, M.; Flanagan, J. A.; Prior, R. L. High-Throughput Assay of Oxygen Radical Absorbance Capacity (ORAC) Using a Multichannel Liquid Handling System Coupled with a Microplate Fluorescence Reader in 96-Well Format. *J. Agric. Food Chem.* **2002**, *50*, 4437.

[101] Kohri, S.; Fujii, H.; Oowada, S.; Endoh, N.; Sueishi, Y.; Kusakabe, M.; Shimmei, M.; Kotake, Y. An Oxygen Radical Absorbance Capacity-Like Assay that Directly Quantifies the Antioxidant's Scavenging Capacity Against AAPH-Derived Free Radicals. *Anal. Biochem.* **2009**, *386*, 167.

[102] López-Alarcón, C.; Lissi, E. A Novel and Simple ORAC Methodology Based on the Interaction of Pyrogallol Red with Peroxyl Radicals. *Free Radical Res.* **2006**, *40*, 979.

[103] Frankel, E. N.; Meyer, A. S. The Problems of Using One-Dimensional Methods to Evaluate Multifunctional Food and Biological Antioxidants. *J. Sci. Food Agric.* **2000**, *80*, 1925.

[104] Rubio, C. P.; Hernández-Ruiz, J.; Martinez-Subiela, S.; Tvarijonaviciute, A.; Ceron, J. J. Spectrophotometric Assays for Total Antioxidant Capacity (TAC) in Dog Serum: An Update. *BMC Vet. Res.* **2016**, *12*, 166.

[105] Nagaraja, P.; Aradhana, N.; Suma, A.; Chamaraja, N. A.; Shivakumar, A.; Ramya, K. V. Amaranth Dye in the Evaluation of Bleaching of Cerium (IV) by Antioxidants: Application in Food and Medicinal Plants. *Spectrochim. Acta Part A: Mol. Biomol. Spectros.* **2012**, *95*, 505.

[106] Nagaraja, P.; Suma, A.; Aradhana, N.; Shivakumar, A.; Avinash, K.; Krishna, H. Quantification of Antioxidants in Medicinal Plants and Foodstuffs Using Ce(IV) with Indigo Carmine as Chromogenic Probe. *Food Anal. Methods* **2011**, *5*, 909.

[107] Apak, R.; Güçlü, K.; Demirata, B.; Özyürek, M.; Çelik, S.; Bektaşoğlu, B.; Berker, K.; Özyurt, D. Comparative Evaluation of Various Total Antioxidant Capacity Assays Applied to Phenolic Compounds with the CUPRAC Assay. *Molecules* **2007**, *12*, 1496.

[108] Pisoschi, A. M.; Cheregi, M. C.; Danet, A. F. Total Antioxidant Capacity of Some Commercial Fruit Juices: Electrochemical and Spectrophotometrical Approaches. *Molecules* **2009**, *14*, 480.

[109] Thaipong, K.; Boonprakob, U.; Crosby, K.; Cisneros-Zevallos, L.; Hawkins Byrne, D. Comparison of ABTS, DPPH, FRAP, and ORAC Assays for Estimating Antioxidant Activity from Guava Fruit Extracts. *J. Food Comp. Anal.* **2006**, *19*, 669.

[110] Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure-Antioxidant Activity Relationships of Flavonoids and Phenolic Acids. *Free Radical Biol. Med.* **1996**, *20*, 933.

[111] Arnao, M. B. Some Methodological Problems in the Determination of Antioxidant Activity Using Chromogen Radicals: A Practical Case. *Trends Food Sci. Technol.* **2000**, *11*, 419.

[112] Magalhães, L. M.; Segundo, M. A.; Reis, S.; Lima, J. L. F. C. Methodological Aspects About In Vitro Evaluation of Antioxidant Properties. *Anal. Chim. Acta* **2008**, *613*, 1.

[113] Arts, M. J. T. J.; Haenen, G. R. M. M.; Voss, H.-P.; Bast, A. Antioxidant Capacity of Reaction Products Limits the Applicability of the Trolox Equivalent Antioxidant Capacity (TEAC) Assay. *Food Chem. Toxicol.* **2004**, *42*, 45.

[114] Pellegrini, N.; Serafini, M.; Colombi, B.; Del Rio, D.; Salvatore, S.; Bianchi, M.; Brighenti, F. Total Antioxidant Capacity of Plant Foods, Beverages and Oils Consumed in Italy Assessed by Three Different In Vitro Assays. *J. Nutr.* **2003**, *133*, 2812.

[115] Sánchez-Moreno, C. Review: Methods Used to Evaluate the Free Radical Scavenging Activity in Foods and Biological Systems. *Food Sci. Technol. Int.* **2002**, *8*, 121.

[116] Fernández-Pachón, M. S.; Villaño, D.; García-Parrilla, M. C.; Troncoso, A. M. Antioxidant Activity of Wines and Relation with Their Polyphenolic Composition. *Anal. Chim. Acta* **2004**, *513*, 113.

[117] Nagaraja, P.; Aradhana, N.; Suma, A.; Shivakumar, A.; Chamaraja, N. A. Quantification of Antioxidants by Using Chlorpromazine Hydrochloride: Application of the Method to Food and Medicinal Plant Samples. *Anal. Sci.* **2014**, *30*, 251.

[118] Işıl Berker, K.; Güçlü, K.; Tor, İ.; Demirata, B.; Apak, R. Total Antioxidant Capacity Assay Using Optimized Ferricyanide/Prussian Blue Method. *Food Anal. Methods* **2010**, *3*, 154.

[119] Berker, K.; Güçlü, K.; Tor, İ.; Demirata, B.; Apak, R. Total Antioxidant Capacity Assay Using Optimized Ferricyanide/Prussian Blue Method. *Food Anal. Methods* **2009**, *3*, 154.

[120] Gülçin, İ.; Oktay, M.; Küfrevioğlu, Ö. İ.; Aslan, A. Determination of Antioxidant Activity of Lichen Cetraria Islandica (L) Ach. *J. Ethnopharmacol.* **2002**, *79*, 325.

[121] Borisenko, G. G.; Martin, I.; Zhao, Q.; Amoscato, A. A.; Tyurina, Y. Y.; Kagan, V. E. Glutathione Propagates Oxidative Stress Triggered by Myeloperoxidase in HL-60 Cells: Evidence for Glutathionyl Radical-Induced Peroxidation of Phospholipids and Cytotoxicity. *J. Biol. Chem.* **2004**, *279*, 23453.

[122] Huang, D.; Ou, B.; Prior, R. L. The Chemistry behind Antioxidant Capacity Assays. *J. Agric. Food Chem.* **2005**, *53*, 1841.

[123] Li, B.; Gutierrez, P. L.; Blough, N. V. Trace Determination of Hydroxyl Radical in Biological Systems. *Anal. Chem.* **1997**, *69*, 4295.
[124] Vladimirov, Y. A.; Proskurnina, E. V. Free Radicals and Cell Chemiluminescence. *Biochemistry* **2010**, *74*, 1545.
[125] Aradhana, N. *Development of Analytical Methods for the Assay of Antioxidants in Food and Medicinal Samples*. University of Mysore: Mysore, **2014**; Vol. 1.
[126] Aboul-Enein, H. Y.; Kładna, A.; Kruk, I.; Lichszteld, K.; Michalska, T.; Olgen, S. Scavenging of Reactive Oxygen Species by Novel Indolin-2-One and Indoline-2-Thione Derivatives. *Biopolymers* **2005**, *78*, 171.
[127] Calliste, C.-A.; Trouillas, P.; Allais, D.-P.; Simon, A.; Duroux, J.-L. Free Radical Scavenging Activities Measured by Electron Spin Resonance Spectroscopy and B16 Cell Antiproliferative Behaviors of Seven Plants. *J. Agric. Food Chem.* **2001**, *49*, 3321.
[128] Lussignoli, S.; Fraccaroli, M.; Andrioli, G.; Brocco, G.; Bellavite, P. A Microplate-Based Colorimetric Assay of the Total Peroxyl Radical Trapping Capability of Human Plasma. *Anal. Biochem.* **1999**, *269*, 38.
[129] Ordoudi, S. A.; Tsimidou, M. Z. Crocin Bleaching Assay Step by Step: Observations and Suggestions for an Alternative Validated Protocol. *J. Agric. Food Chem.* **2006**, *54*, 1663.
[130] Bors, W.; Michel, C.; Saran, M. Inhibition of the Bleaching of the Carotenoid Crocin a Rapid Test for Quantifying Antioxidant Activity. *Biochim. et Biophys. Acta (BBA) – Lipids Lipid Metabol.* **1984**, *796*, 312.
[131] Liebler, D. C.; McClure, T. D. Antioxidant Reactions of β-Carotene: Identification of Carotenoid—Radical Adducts. *Chem. Res. Toxicol.* **1996**, *9*, 8.
[132] Singleton, V. L.; Orthofer, R.; R. M. Lamuela-Raventós, Analysis of Total Phenols and Other Oxidation Substrates and Antioxidants by Means of Folin-Ciocalteu Reagent. In Methods in Enzymology, Academic Press, **1999**, *299*, pp 152.
[133] Shahidi, F.; Liyana-Pathirana, C. M.; Wall, D. S. Antioxidant Activity of White and Black Sesame Seeds and Their Hull Fractions. *Food Chem.* **2006**, *99*, 478.
[134] Berker, K. I.; Ozdemir Olgun, F. A.; Ozyurt, D.; Demirata, B.; Apak, R. Modified Folin–Ciocalteu Antioxidant Capacity Assay for Measuring Lipophilic Antioxidants. *J. Agric. Food Chem.* **2013**, *61*, 4783.
[135] Ozyurt, D.; Demirata, B.; Apak, R. Determination of Total Antioxidant Capacity by a New Spectrofluorometric Method Based on Ce (IV) Reduction: Ce(III) Fluorescence Probe for CERAC Assay. *J. Fluoresc.* **2011**, *21*, 2069.
[136] Zhishen, J.; Mengcheng, T.; Jianming, W. The Determination of Flavonoid Contents in Mulberry and Their Scavenging Effects on Superoxide Radicals. *Food Chem.* **1999**, *64*, 555.
[137] Regoli, F.; Winston, G. W. Quantification of Total Oxidant Scavenging Capacity of Antioxidants for Peroxynitrite, Peroxyl Radicals, and Hydroxyl Radicals. *Toxicol. Appl. Pharmacol.* **1999**, *156*, 96.
[138] Winston, G. W.; Regoli, F.; Dugas, A. J.; Jr; Fong, J. H.; Blanchard, K. A. A Rapid Gas Chromatographic Assay for Determining Oxyradical Scavenging Capacity of Antioxidants and Biological Fluids. *Free Radical Biol. Med.* **1998**, *24*, 480.
[139] Deshpande, S. R.; Satyanarayana, K.; Rao, M. N. A.; Pai, K. V. Nitric Oxide Modulators: An Emerging Class of Medicinal Agents. *Indian J. Pharmaceut. Sci.* **2012**, *74*, 487.
[140] Gutteridge, J. M. C.; Maidt, L.; Poyer, L. Superoxide Dismutase and Fenton Chemistry. Reaction of Ferric-EDTA Complex and Ferric-Bipyridyl Complex with Hydrogen Peroxide Without the Apparent Formation of Iron(II). *Biochem. J.* **1990**, *269*, 169.
[141] Garcia-campana, A. M.; Beayens, W. R. G. Chemiluminiscence in *Analytical Chemistry*, Marcel Dekker: New york, **2001**.
[142] Kobayashi, H.; Gil-Guzman, E.; Mahran, A. M.; Sharma, R. K.; Nelson, D. R.; Jr, A. J. T.; Agarwal, A. Quality Control of Reactive Oxygen Species Measurement by Luminol-Dependent Chemiluminescence Assay. *J. Androl.* **2001**, *22*, 568.
[143] Oldenburg, B.; van Kats-Renaud, H.; Koningsberger, J. C.; van Berge Henegouwen, G. P.; van Asbeck, B. S. Chemiluminescence in Inflammatory Bowel Disease Patients: A Parameter of Inflammatory Activity. *Clin. Chim. Acta* **2001**, *310*, 151.
[144] Liochev, S. I.; Fridovich, I. Lucigenin (Bis-N-methylacridinium) as a Mediator of Superoxide Anion Production. *Arch. Biochem. Biophys.* **1997**, *337*, 115.
[145] Ma, Z.; Zhao, B.; Yuan, Z. Application of Electrochemical and Spin Trapping Techniques in the Investigation of Hydroxyl radicals. *Anal. Chim. Acta* **1999**, *389*, 213.
[146] Adcock, J. L.; Francis, P. S.; Barnett, N. W. Acidic Potassium Permanganate as a Chemiluminescence Reagent—A Review. *Anal. Chim. Acta* **2007**, *601*, 36.
[147] Anastos, N.; Barnett, N. W.; Hindson, B. J.; Lenehan, C. E.; Lewis, S. W. Comparison of Soluble Manganese(IV) and Acidic Potassium Permanganate Chemiluminescence Detection Using Flow Injection and Sequential Injection Analysis for the Determination of Ascorbic Acid in Vitamin C Tablets. *Talanta* **2004**, *64*, 130.
[148] Gómez-Taylor Corominas, B.; Catalá Icardo, M.; Lahuerta Zamora, L.; García Mateo, J. V.; Martínez Calatayud, J. A Tandem-Flow Assembly for the Chemiluminometric Determination of Hydroquinone. *Talanta* **2004**, *64*, 618.
[149] Costin, J. W.; Barnett, N. W.; Lewis, S. W.; McGillivery, D. J. Monitoring the Total Phenolic/Antioxidant Levels in Wine Using Flow Injection Analysis with Acidic Potassium Permanganate Chemiluminescence Detection. *Anal. Chim. Acta* **2003**, *499*, 47.
[150] Francis, P. S.; Costin, J. W.; Conlan, X. A.; Bellomarino, S. A.; Barnett, J. A.; Barnett, N. W. A Rapid Antioxidant Assay Based on Acidic Potassium Permanganate Chemiluminescence. *Food Chem.* **2010**, *122*, 926.
[151] Hindson, B. J.; Barnett, N. W. Analytical Applications of Acidic Potassium Permanganate as a Chemiluminescence Reagent. *Anal. Chim. Acta* **2001**, *445*, 1.
[152] Hindson, C. M.; Francis, P. S.; Hanson, G. R.; Adcock, J. L.; Barnett, N. W. Mechanism of Permanganate Chemiluminescence. *Anal. Chem.* **2010**, *82*, 4174.
[153] Yin, X.-B.; Dong, S.; Wang, E. Analytical Applications of the Electrochemiluminescence of Tris (2,2′-Bipyridyl) Ruthenium and Its Derivatives. *TrAC Trends Anal. Chem.* **2004**, *23*, 432.
[154] Richter, M. M. Electrochemiluminescence (ECL). *Chem. Rev.* **2004**, *104*, 3003.
[155] Gorman, B. A.; Francis, P. S.; Barnett, N. W. Tris(2,2'-Bipyridyl) Ruthenium (ii) Chemiluminescence. *Analyst* **2006**, *131*, 616.
[156] Miao, W. Electrogenerated Chemiluminescence and Its Biorelated Applications. *Chem. Rev.* **2008**, *108*, 2506.
[157] Gámiz-Gracia, L.; García-Campaña, A. M.; Huertas-Pérez, J. F.; Lara, F. J. Chemiluminescence Detection in Liquid Chromatography: Applications to Clinical, Pharmaceutical, Environmental and Food Analysis—A Review. *Anal. Chim. Acta* **2009**, *640*, 7.
[158] Nana, C. G.; Jian, W.; Xi, C.; Pinga, D. J.; Feng, Z. Z.; Qing, C. H. The Enhanced Electrogenerated Chemiluminescence of Ru(bpy) by Glutathione on a Glassy Carbon Electrode Modified with Some Porphine Compounds. *Analyst* **2000**, *125*, 2294.
[159] Brown, A. J.; Lenehan, C. E.; Francis, P. S.; Dunstan, D. E.; Barnett, N. W. Soluble Manganese(IV) as a Chemiluminescence Reagent for the Determination of Opiate Alkaloids, Indoles and Analytes of Forensic Interest. *Talanta* **2007**, *71*, 1951.
[160] Spasojević, I.; Liochev, S. I.; Fridovich, I. Lucigenin: Redox Potential in Aqueous Media and Redox Cycling with O^{2-} Production1. *Arch. Biochem. Biophys.* **2000**, *373*, 447.