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METHODS FOR DETERMINATION OF ANTIOXIDANT CAPACITY: A REVIEW

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ABSTRACT: Antioxidants have become a vital part of our lives today. Antioxidants help neutralize or destroy "Reactive Oxygen Species" (ROS) or free radicals before they can damage cells. This paper focuses on types of damaging free radicals generated in metabolic processes and also gives an insight of mechanistic aspect of various in-vitro methods for evaluation of antioxidant capacity of plant metabolites and dietary supplements. The various HAT based, ET based assays and cellular antioxidant capacity assay (CAA) are discussed here. The oxidation induced by Reactive oxygen species (ROS) may result in cell membrane disintegration, membrane protein damage and DNA mutations which play an important role in aging and can further initiate or propagate the development of many diseases, such as arteriosclerosis, cancer, diabetes mellitus, liver injury, inflammation, skin damages, coronary heart diseases and arthritis.

INTRODUCTION: The chemical compounds which can delay the start or slow the rate of lipid oxidation reaction in food systems are called Antioxidants. By definition, a substance that opposes oxidation or inhibits reactions promoted by oxygen or peroxides, many of these substances being used as preservatives in various products are antioxidants. A more biologically relevant definition of antioxidant is "synthetic or natural substances added to products to prevent or delay their deterioration by action of oxygen in air. In biochemistry and medicine, antioxidants are enzymes or other organic substances, such as vitamin E or β-carotene that are capable of counteracting the damaging effects of oxidation in animal tissues."

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In a chemical industry, antioxidants often refer to compounds that retard autoxidation of chemical products such as rubber and plastics. The autoxidation is caused primarily by radical chain reactions between oxygen and the substrates. Effective antioxidants like sterically hindered phenols and amines are radical scavengers that break down radical chain reactions.

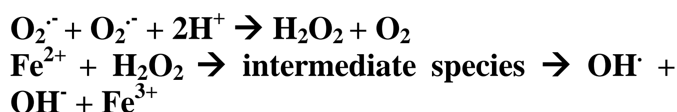
In food chemistry, antioxidants include components that prevent rancidity of fat, a substance that significantly decreases the adverse effects of reactive oxygen species on the normal physiological function of human being. A dietary antioxidant can (sacrificially) scavenge reaction oxygen/ nitrogen species (ROS/RNS) to stop radical chain reactions, or it can inhibit the reactive oxidants from being formed in the first place (preventive). Biological antioxidants include enzymatic antioxidants (e.g., Superoxide dismutase, catalase and glutathione peroxidase) and nonenzymatic antioxidants such as oxidative

enzyme (e.g., cycloxygenase) inhibitors, antioxidant enzyme cofactors (Se, Coenzyme Q10), ROS/RNS scavengers (Vitamin C and E), and transition metal chelators [1]. The oxidation induced by Reactive oxygen species (ROS) may result in cell membrane disintegration, membrane protein damage and DNA mutations which play an important role in aging and can further initiate or propagate the development of many diseases, such as arteriosclerosis, cancer, diabetes mellitus, liver injury, inflammation, skin damages, coronary heart diseases and arthritis [2].

Types of Free Radicals

The damaging free radicals are broadly divided into two types: Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS). ROS includes both oxygen radicals and certain radicals that are oxidizing agents or can easily converted into radicals. RNS is also a collective term including nitric oxide and nitrogen dioxide radicals as well as non radicals like nitrous acid, N_2O_3, $ONOO^-$ are also included.

Superoxide anion ($O_2^{\cdot -}$): An oxygen molecule with an extra electron that can damage mitochondria, DNA and other molecules. Superoxide generated both in vivo and in foods can undergo several reactions, including dismutation to give H2O2.



$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$$

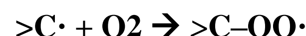
$$Fe^{2+} + H_2O_2 \rightarrow \text{intermediate species} \rightarrow OH^{\cdot} + OH^- + Fe^{3+}$$

Hydroxyl radical ($^{\cdot}OH$): A highly reactive molecule formed by the reduction of an oxygen molecule, capable of damaging almost any organic molecule in its vicinity, including carbohydrates, lipids, proteins, and DNA. $OH^{\cdot}$ cannot be eliminated by an enzymatic reaction.

Singlet oxygen: Formed by our immune system, singlet oxygen causes oxidation of LDL.

Hydrogen peroxide (H_2O_2): Not a free radical itself, but easily converts to free radicals like OH, which then do the damage. Hydrogen peroxide is neutralized by peroxidase (an enzymatic antioxidant).

Peroxyl radical ($ROO^{\cdot}$): Formation of peroxyl radicals ($RO_2\cdot$) is the major chain-propagating step in lipid peroxidation and in nonlipid systems, such as proteins.(3) Decomposition of both lipid and protein peroxides on heating or by addition of transition metal ions can generate peroxyl and alkoxyl (RO•) radicals. Peroxyl radicals can easily be generated by allowing O2 to add to carbon-centered radicals



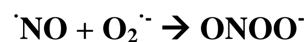
$$>C\cdot + O2 \rightarrow >C{-}OO\cdot$$

Peroxyl radical is very important in biological systems, including lipid peroxidation, DNA cleavage, protein backbone modification and also involved in food spoilage.

Alkoxy radical ($RO^{\cdot}$): The oxidative deterioration of lipids or lipid peroxidation produces alkoxyl radicals non enzymatically via a Fenton reaction, a one electron reduction, or the combination between two peroxyl radicals. Alkoxyl radicals are highly oxidizing and can cause DNA mutations and apoptosis.

Reactive Nitrogen Species (RNS): Nitrogen is present in foods as nitrates, amines, nitrites, peptides, proteins, and amino acids, and its metabolites in vivo include nitric oxide, higher oxides of nitrogen, and peroxynitrite [3, 4]. These reactive nitrogen species may cause risk for cancer development in hepatitis or other chronic inflammatory processes [4, 5].

Dinitrogen trioxide (N2O3), nitrous acid (HNO2), and peroxynitrite ($ONOO^-$) can lead to deamination and nitration of DNA. Peroxynitrite anion ($ONOO^-$) is stable at highly alkaline pH, but undergoes reaction with CO2, protonation, isomerization, and decomposition at physiological pH to give noxious products that deplete antioxidants and oxidize and nitrate lipids, proteins, DNA and have a potential to cause changes in catalytic activity of enzymes, altered cytoskeletal organization and impaired cell signal transduction [4, 6]. These noxious products may include $NO_2\cdot$, NO^{2+}, and OH•. Peroxynitrite is a cytotoxic species that can be generated in several ways, most usually by the rapid addition of superoxide and nitric oxide radicals [3].



$$^{\cdot}NO + O_2^{\cdot -} \rightarrow ONOO^-$$

These various free radical species can damage DNA in different ways. They can disrupt duplication of DNA, interfere with DNA maintenance, break open the molecule or alter the structure by reacting with the DNA bases. Cancer, atherosclerosis, Parkinson's, Alzheimer's disease, and cataracts are examples of diseases thought to result from free radical damage.

Lipids in cell membranes are quite prone to oxidative damage because free radicals tend to collect in cell membranes, known as "lipid per oxidation." (The lipid peroxide radical is sometimes abbreviated as $LOO^{\cdot}$) When a cell membrane becomes oxidized by an ROS, it becomes brittle and leaky. Eventually, the cell falls apart and dies [7].

Role of Antioxidants

An antioxidant is a molecule capable of inhibiting the oxidation of another molecule. Antioxidants break the free radical chain of reactions by sacrificing their own electrons to feed free radicals, without becoming free radicals themselves.

Antioxidants are nature's way of defending your cells against attack by reactive oxygen species (ROS). Your body naturally circulates a variety of nutrients for their antioxidant properties and manufactures antioxidant enzymes in order to control these destructive chain reactions. For example, vitamin C, vitamin E, carotenes, and lipoic acid are well-known and well-researched antioxidant nutrients.

Oxidative stress can be defined as the state in which the free radicals in our body outnumber our antioxidant defenses. They can also serve to shorten the telomere length of chromosome, which many experts believe to be the most accurate biological clock we have.

Classification of Antioxidants

Enzymatic and Non-Enzymatic Antioxidants

Antioxidants can be categorized into two types:

Non-enzymatic antioxidants work by interrupting free radical chain reactions. For example, vitamin E may interrupt a chain of free radical activity after only five reactions. Non-enzymatic antioxidants include vitamin C, vitamin E, plant polyphenols, carotenoids, Se and glutathione (GSH).

Glutathione (cysteine containing natural antioxidant) has been called the "master antioxidant" and is found in every single cell of your body, maximizing the activity of all the other antioxidants. Glutathione (GSH) is a tripeptide with a gamma peptide linkage between the amine group of cysteine (which is attached by normal peptide linkage to a glycine) and the carboxyl group of the glutamate side-chain [8].

Glutathione exists in both reduced (GSH) and oxidized (GSSG) states. In the reduced state, the thiol group of cysteine is able to donate a reducing equivalent (H^{+}+ e^{-}) to other unstable molecules, such as reactive oxygen species. In donating an electron, glutathione itself becomes reactive, but readily reacts with another reactive glutathione to form glutathione disulfide (GSSG). Such a reaction is probable due to the relatively high concentration of glutathione in cells (up to 5 mM in the liver).

GSH can be regenerated from GSSG by the enzyme *glutathione reductase* (GSR) [9]. In healthy cells and tissue, more than 90% of the total glutathione pool is in the reduced form (GSH) and less than 10% exists in the disulfide form (GSSG). An increased GSSG-to-GSH ratio is considered indicative of oxidative stress.

Enzymatic antioxidants work by *breaking down and removing free radicals*. In general, these antioxidant enzymes flush out dangerous oxidative products by converting them into hydrogen peroxide, then into water, in a multi-step process that requires a number of trace metal cofactors (copper, zinc, manganese and iron). These enzymatic antioxidants cannot be supplemented orally but must be produced in our body.

The principle enzymatic antioxidants are the following:

Superoxide dismutase (SOD): Assisted by copper, zinc, manganese and iron, SOD breaks down superoxide (which plays a major role in lipid per oxidation) into oxygen and hydrogen peroxide. SOD is present in nearly all aerobic cells and extracellular fluids.

Catalase (CAT): Converts hydrogen peroxide into water and oxygen (using iron and manganese cofactors), hence finishing up the detoxification process that SOD started.

Glutathione peroxidase (GSHpx) and glutathione reductase: These selenium-containing enzymes help break down hydrogen peroxide and organic peroxides into alcohols, and are particularly abundant in your liver. **Selenium** is an essential trace element having fundamental importance to human health as it is a constituent of the small group of selenocysteine containing selenoproteins (over 25 different proteins) which is important for structural and enzymatic functions. Selenoproteins include several forms of the enzymes glutathione peroxidase (GPx), thioredoxin reductase and iodothyronine deiodinase. Selenium glutathione peroxidases catalyze the elimination of hydrogen peroxide as well as organic peroxides (R-O-OH) by the oxidation of GSH [10].

Water-Soluble (Hydrophilic) and Lipid-Soluble (Lipophilic) Antioxidants

Another categorization of antioxidants is based on whether they are soluble in water (hydrophilic) or in lipids (hydrophobic). The interior of our cells and the fluid between them are composed mainly of water but cell membranes are made largely made of lipids.

The lipid-soluble antioxidants (such as vitamins E and A, carotenoids, and lipoic acid) are primarily located in the cell membranes, whereas the water-soluble antioxidants (such as vitamin C, polyphenols and glutathione) are present in aqueous body fluids, such as blood and the fluids within and around the cells (the cytosol, or cytoplasmic matrix). Free radicals can strike the watery cell contents or the fatty cellular membrane, so the cell needs defenses for both. The lipid-soluble antioxidants are the ones that protect the cell membranes from lipid peroxidation [3].

Natural and Artificial Antioxidants: Antioxidants are divided into two groups according to their origin as ‘natural antioxidants’ and ‘synthetic antioxidants’. Most of the synthetic antioxidants are of the phenolic type. The differences in their antioxidant activities are related to their chemical structures, which also influence their physical properties such as volatility, solubility and thermal stability [11]. The commercially available and currently used synthetic antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butyl hydroquinone (TBHQ) as shown in **Figure 1**.

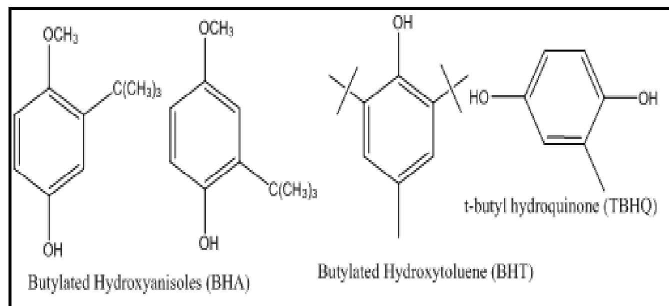


FIG.1: SYNTHETIC ANTIOXIDANTS

In recent years, there is an increasing interest in natural antioxidants and subsequently looking through the literature it is recognized that the replacement of synthetic antioxidants by natural ones may have several benefits and much of the research on natural antioxidants has focused on phenolic compounds, in particular flavonoids as potential sources of natural antioxidants [12, 13, 14].

Numbers of naturally existing antioxidant compounds present in fruits, vegetables and dietary supplements are ascorbic acid, α-tocopherol, phenolic acids (Benzoic acid, trans-cinnamic acid and hydroxycinnamic acid), coumarins, lignans, stilbenes (in glycosylated form), flavonoids, isoflavonoids and phenolic polymers (tannins) [15].

FLAVONOIDS AS ANTIOXIDANTS:

Flavonoids are secondary plant products recognized as the characteristic red, blue and purple anthocyanin pigments of plant tissues. Apart from their physiological roles in the plants, flavonoids as important components in human diet but never considered as nutrient [16]. The basic structure of flavonoid is a phenylated benzopyrone consists of 3 rings A, B and C as shown in **Figure 2a.**

The various classes of flavonoids differ in the level of oxidation and pattern of substitution of the C ring. Among the various classes of flavonoids, the important ones are flavones, flavanones, isoflavones, flavonols, flavanol (catechin), flavanonols, flavan-3-ols and anthocyanidins.

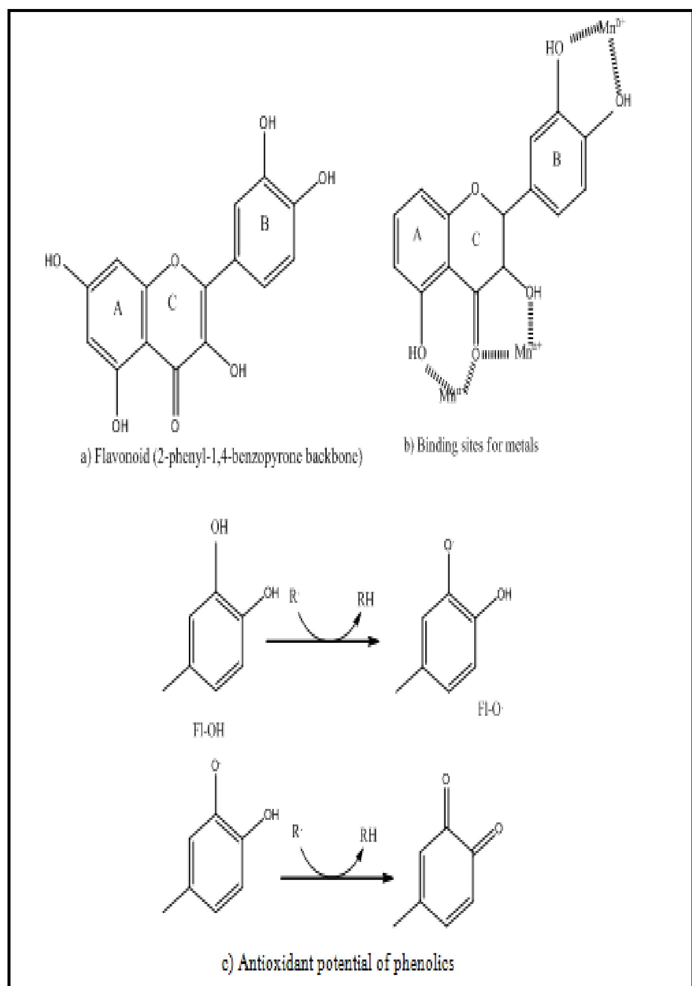
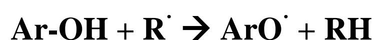


FIG. 2: A) STRUCTURE OF QUERCETIN B) BINDING SITES OF A FLAVONOID C) REACTIONS SHOWING RADICAL SCAVENGING ACTIVITY OF PHENOLICS

The proposed binding sites for trace metals to flavonoids as highlighted in **figure 2b** are the catechol moiety in ring B, the 3-hydroxyl, 4-oxo groups in the heterocyclic ring and the 4-oxo, 5-hydroxyl groups between the heterocyclic and the A rings.

The major contribution to metal chelation activity of flavonoids is due to the catechol moiety. Due to their lower redox potentials (0.23-0.75 V), flavonoids are thermodynamically able to reduce highly oxidizing free radicals with redox potentials in the range of 2.13-1.0 V, such as superoxide, peroxyl, alkoxyl, and hydroxyl radicals by hydrogen atom donation [17].



$$\mathbf{Ar\text{-}OH + R^{\cdot} \rightarrow ArO^{\cdot} + RH}$$

The aroxyl radical $ArO^{\cdot}$ may react with a second radical, acquiring a stable quinone structure as indicated in **Figure 2c**. Structural features and nature of substitutions on rings B and C determine the antioxidant activity of flavonoids. This can be summarized as follows:

- The degree of hydroxylation and the positions of the –OH groups in the B ring, in particular an ortho-dihydroxyl structure of ring B (catechol group) results in higher activity as it confers higher stability to the aroxyl radical by electron delocalisation or acts as the preferred binding site for trace metals.
- The presence of hydroxyl groups at the 3'-, 4'-, and 5'-positions of ring B (a pyrogallol group) has been reported to enhance the antioxidant activity of flavonoids compared to those that have a single hydroxyl group. However, under some conditions, such compounds may act as pro-oxidants, thus counteracting the antioxidant effect. This is consistent with the observation of Seeram and Nair who reported that the conservation of the 3',4'-dihydroxyphenyl to 3',4',5'-trihydroxylphenyl increases the antioxidant activity for anthocyanidins but decreases the activity for catechins.
- A double bond between C-2 and C-3, conjugated with the 4-oxo group in ring C enhances the radical scavenging capacity of flavonoids.
- A double bond between C-2 and C-3, combined with a 3-OH, in ring C, also enhances the active radical scavenging capacity of flavonoids, as seen in the case of kaempferol. Substitution of the 3-OH results in increase in torsion angle and loss of coplanarity and subsequently reduced antioxidant activity.
- Substitution of hydroxyl groups in ring B by methoxyl groups alters the redox potential, which affects the radical scavenging capacity of flavonoids.

Quercetin (**Fig. 2a**), the most abundant dietary flavonol, is a potent antioxidant because it has all the right structural features for free radical scavenging activity. Xanthohumol (a chalcone) and isoxanthohumol and 6-prenylnaringenin (flavanones) are the major prenyl-flavonoids found in beer. Xanthohumol is a more powerful antioxidant than vitamin E or genistein but less potent than quercetin. The prenyl group plays an

important role in the antioxidant activity of certain flavonoids.

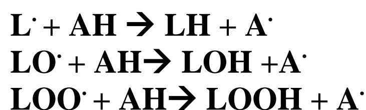
A flavonoid chalcone (chalconaringenin) and a flavanone (naringenin) with no prenyl groups act as pro-oxidants, i.e. they promote rather than limit the oxidation of LDL by copper. Genistein, an isoflavone in soy also has high antioxidant potential [18].

Antioxidant Capacity

The total antioxidant capacity or antioxidant activity is a meaningless term without the context of specific reaction conditions such as temperature, pressure, reaction medium, reference points, chemical reactivity etc. We must refer to an oxidant specific terms like "peroxy radical scavenging capacity", "superoxide scavenging capacity", "ferric ion reducing capacity" etc.

Antioxidants have been traditionally divided into two classes; primary or chain-breaking antioxidants, and secondary or preventative antioxidants [11].

The chain breaking mechanisms are represented by:

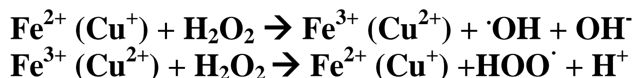


$$L^{\cdot} + AH \rightarrow LH + A^{\cdot}$$
$$LO^{\cdot} + AH \rightarrow LOH + A^{\cdot}$$
$$LOO^{\cdot} + AH \rightarrow LOOH + A^{\cdot}$$

Here, $L^{\cdot}$ stands for lipid radical and AH stands for an antioxidant.

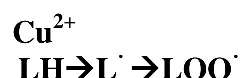
The secondary or preventive antioxidants retard the rate of oxidation. Redox active metals like iron (Fe), copper (Cu), chromium (Cr), cobalt (Co) and other metals undergo redox cycling reactions and possess the ability to produce reactive radicals such as superoxide anion radical and nitric oxide in biological systems [19].

These metal ions are essential for many physiological functions, as the constituents of hemoproteins and cofactors of different enzymes (like Fe for catalase, Cu for ceruloplasmin and Cu, Zn-superoxide dismutase) in the antioxidant defense. Typical Fenton type reaction generating free radicals involves the oxidation of ferrous ions to ferric ions by hydrogen peroxide to generate a hydroxyl radical and a hydroxyl anion. Iron (III) is then reduced back to iron (II), a superoxide radical, and a proton by the same hydrogen peroxide. The free radicals generated by this process get involved in number of secondary reactions.



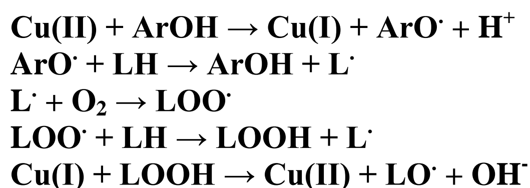
$$Fe^{2+}\ (Cu^{+}) + H_2O_2 \rightarrow Fe^{3+}\ (Cu^{2+}) + {}^{\cdot}OH + OH^{-}$$
$$Fe^{3+}\ (Cu^{2+}) + H_2O_2 \rightarrow Fe^{2+}\ (Cu^{+}) + HOO^{\cdot} + H^{+}$$

The formation of free radicals may be inhibited by reducing the hydroperoxides and hydrogen peroxide and by sequestering metal ions through complexation/chelation reactions [20]. A number of flavonoids (polyphenolic compounds) efficiently chelate trace metals like copper and iron which are potential enhancers of ROS. Copper can also oxidation of low density lipoproteins (LDL) represented as LH.



$$LH \xrightarrow{Cu^{2+}} L^{\cdot} \rightarrow LOO^{\cdot}$$

The prooxidative effect of phenolic antioxidants (ArOH), generally induced by transition metal ions like Cu(II) in the presence of dissolved oxygen, gives rise to oxidative damage to lipids as shown by the reactions below. The prooxidant activity of flavonoids generally depends on concentration as well as number and position of –OH substituents in its back bone structure [21].

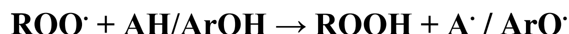


$$Cu(II) + ArOH \rightarrow Cu(I) + ArO^{\cdot} + H^{+}$$
$$ArO^{\cdot} + LH \rightarrow ArOH + L^{\cdot}$$
$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot}$$
$$LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot}$$
$$Cu(I) + LOOH \rightarrow Cu(II) + LO^{\cdot} + OH^{-}$$

On the basis of the chemical reactions involved, major antioxidant capacity assays are divided into hydrogen atom transfer (HAT) reactions based assays and single electron transfer (ET) reactions based assays. The ET based assays involve one redox reaction with the oxidant as indicator of the reaction endpoint. Most HAT-based assays monitor competitive reaction kinetics, and the quantification is derived from the kinetic curves. HAT-based methods generally are composed of a synthetic free radical generator, an oxidizable molecular probe, and an antioxidant. HAT- and ET-based assays are intended to measure the radical (or oxidant) scavenging capacity, instead of the preventive antioxidant capacity of a sample [1].

HAT−based assays measure the capability of an antioxidant to quench free radicals (generally peroxyl radicals) by H-atom donation. The HAT

mechanism of antioxidant action in which the hydrogen atom ($H^·$) of a phenol (Ar-OH) is transferred to an $ROO^·$ radical, can be summarized by the reaction:



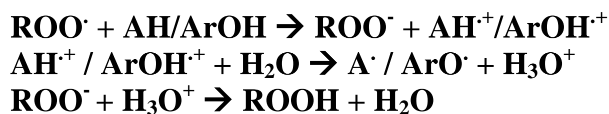
$ROO^· + AH/ArOH \rightarrow ROOH + A^· / ArO^·$

where the aryloxy radical ($ArO^·$) formed from the reaction of antioxidant phenol with peroxyl radical is stabilized by resonance. The AH and ArOH species denote the protected biomolecules and antioxidants, respectively. Effective phenolic antioxidants need to react faster than biomolecules with free radicals to protect the latter from oxidation.

Since in HAT–based antioxidant assays, both the fluorescent probe and antioxidants react with $ROO^·$, the antioxidant activity can be determined from competition kinetics by measuring the fluorescence decay curve of the probe in the absence and presence of antioxidants, and integrating the area under these curves [1, 22]. As an example of HAT−based assays, oxygen radical absorbance capacity (ORAC) assay [23], total radical trapping antioxidant parameter (TRAP) assay, β-carotene bleaching assay and crocin bleaching assay.

ET based assays

The ET mechanism of antioxidant action is based on the reactions:



$ROO^· + AH/ArOH \rightarrow ROO^- + AH^{·+}/ArOH^{·+}$
$AH^{·+} / ArOH^{·+} + H_2O \rightarrow A^· / ArO^· + H_3O^+$
$ROO^- + H_3O^+ \rightarrow ROOH + H_2O$

Where the reactions are relatively slower than those of HAT– based assays, and are solvent– and pH–dependent. The aryloxy radical (ArO.) is subsequently oxidized to the corresponding quinone (Ar=O). The more stabilized the aryloxy radical is, the easier will be the oxidation from ArOH to Ar=O due to reduced redox potential. In fact, in most ET–based assays, the antioxidant action is simulated with a suitable redox-potential probe, i.e., the antioxidants react with a fluorescent or colored probe (oxidizing agent) instead of peroxyl radicals. Spectrophotometric ET−based assays measure the capacity of an antioxidant in the reduction of an oxidant, which changes colour when reduced.

The degree of colour change (either an increase or decrease of absorbance at a given wavelength) is correlated to the concentration of antioxidants in the sample. ABTS/TEAC (Trolox equivalent antioxidant capacity) and DPPH are decolorization assays, whereas in Folin total phenols assay, FRAP (ferric reducing antioxidant power) and CUPRAC (cupric reducing antioxidant capacity), there is an increase in absorbance at a pre specified wavelength as the antioxidant reacts with the chromogenic reagent (i.e., in the latter two methods, the lower valencies of iron and copper, namely Fe(II) and Cu(I), form charge-transfer complexes with the ligands, respectively [24].

Various Antioxidant Capacity Assays

A number of protocols have been proposed to determine the antioxidant capacity. Few use radicals and some use metal ions as the oxidizing agents. The wavelength at which measurement is done in the various protocols is tabulated in **Table1.**

TABLE 1: TYPE OF ASSAY WITH OPERATING PRINCIPLE

Assay	Radical/Chromophore	Wavelength of Measurement	pH of measure-ment	Mode of assay	HAT / ET based
ORAC	$AAPH^·$ (Fluoresein)	λ_{ex}=485 nm and λ_{em}=538 nm	pH 7.4	Fluorescence decay measurement	HAT based assay
Total Peroxyl Radical-Trapping Antioxidant Parameter (TRAP)	$AAPH^·$ (R-phycoerythrin/Luminol)	λ_{ex}=495 nm and λ_{em}=575 nm	pH 7.5	Fluorescence decay measurement	HAT based assay
B-Carotene Bleaching Assay	Peroxyl radicals, $ROO^·$	470 nm	pH 5.5 – 7.5	Absorbance measurement	HAT based assay
Crocin Bleaching Assay	Peroxyl radicals, $ROO^·$	440 nm	pH 7.0-7.5	Absorbance measurement	HAT based assay

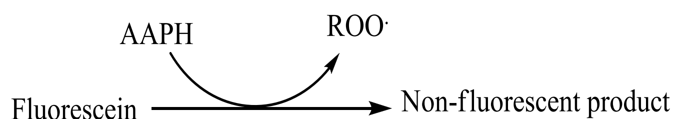
Total Phenolic Content	Mo^{6+}(yellow)→Mo^{5+}(blue)	765 nm	pH 10	Absorbance measurement	ET based assay
Ferric ion Reducing Antioxidant Power assay (FRAP)	Chelated Fe^{3+} ions	595 nm	pH 3.6	Absorbance measurement	ET based assay
DPPH	$DPPH^{\cdot}$	515 nm	pH 7.0-7.4	Absorbance measurement	ET based assay
Trolox equivalent Antioxidant capacity (TEAC)	$ABTS^{\cdot+}$	734 nm	pH 7.4 (using PBS)	Absorbance measurement	ET based assay
CUPRAC	Cu^{2+}→Cu^{+} (complexed with neocuproine)	450 nm	Acidic/ Neutral/ alkaline	Absorbance measurement	ET based assay
CERAC	Ce^{4+}→Ce^{3+}	λ_{ex}=256 nm and λ_{em}=360 nm	Acidic (0.3 M H_2SO_4)	Fluorescence decay measurement	ET based assay
Lipid Peroxidation Inhibition Assay	N-methyl-2-phenylindole	586 nm	pH 7.4	Absorbance measurement	HAT based assay
Hydroxyl radical averting Capacity (HORAC assay)	$HO^{\cdot}$ (p-hydroxybenzoic acid) fluorescein	λ_{ex}=488 nm and λ_{em}=515 nm	Phosphate buffer	Fluorescence decay measurement	HAT based assay
Fe^{2+} ions chelating Assay	Ferrozine-Fe^{2+} complex	562 nm	pH 4-10	Absorbance measurement	ET based assay
Nitric oxide free radical scavenging activity	Griess reagent	546 nm	pH7.2	Absorbance measurement	ET based assay
Potassium Ferricyanide Reducing Power	Fe^{3+}→Fe^{2+}	700 nm	pH 6.6	Absorbance measurement	ET based assay
Thiobarbituric acid reactive substances (TBARS)	MDA-TBA Adduct	532 nm	pH 2	Absorbance measurement	ET based assay
N,N-dimethyl-p-phenylenediamine DMPD	$DMPD^{\cdot+}$(Purple)	505 nm	pH 5.25	Absorbance measurement	Fenton type ET based reaction
Photochemiluminescence Assay	$O_2^{\cdot-}$ (Using Luminol)	360 nm (blue luminescence)	pH 10.5	Chemiluminescence	HAT reaction

ORAC Assay (Oxygen radical absorbance capacity): One of the standardized methods for determining antioxidant capacity is ORAC assay [25]. It is based upon the inhibition of peroxyl radical induced oxidation initiated by thermal decomposition of azo compounds such as AAPH [26].

The assay measures the loss of fluorescein (**Fig. 3a** givesthe structure of fluorescein) fluorescence over time due to peroxyl-radical formation by the breakdown of a bis azide initiator, AAPH (2, 2'-azobis – 2 – methyl - propanimidamide, dihydrochloride) at 37 °C. The reduction in fluorescence is followed optically, and antioxidant activity is determined by slowing of loss in fluorescence in presence of antioxidant.

Trolox [6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid], a water soluble vitamin E analog, serves as a positive control inhibiting fluorescein decay in a dose dependent manner.

The peroxyl radical can oxidize fluorescein (3', 6'-dihydroxy-spiro [isobenzofuran-1[3H], 9' [9H]-xanthen]-3-one) to generate a product without fluorescence. Antioxidants suppress this reaction by a hydrogen atom transfer mechanism, inhibiting the oxidative degradation of the fluorescein signal. The fluorescence signal is measured over 30 minutes by excitation at 485 nm, emission at 538 nm, and cutoff 530 nm. The concentration of antioxidant in the test sample is proportional to the fluorescence intensity through the course of the assay and is assessed by comparing the net area under the curve to that of a known antioxidant, trolox [27].



Antioxidants inhibit the oxidation of fluorescein by hydrogen atom transfer. The original ORAC assay was developed using the fluorescent protein β-phycoerythrin as the radical target which was later replaced by fluorescein due to its inconsistency, light sensitivity and problems with binding of polyphenols.

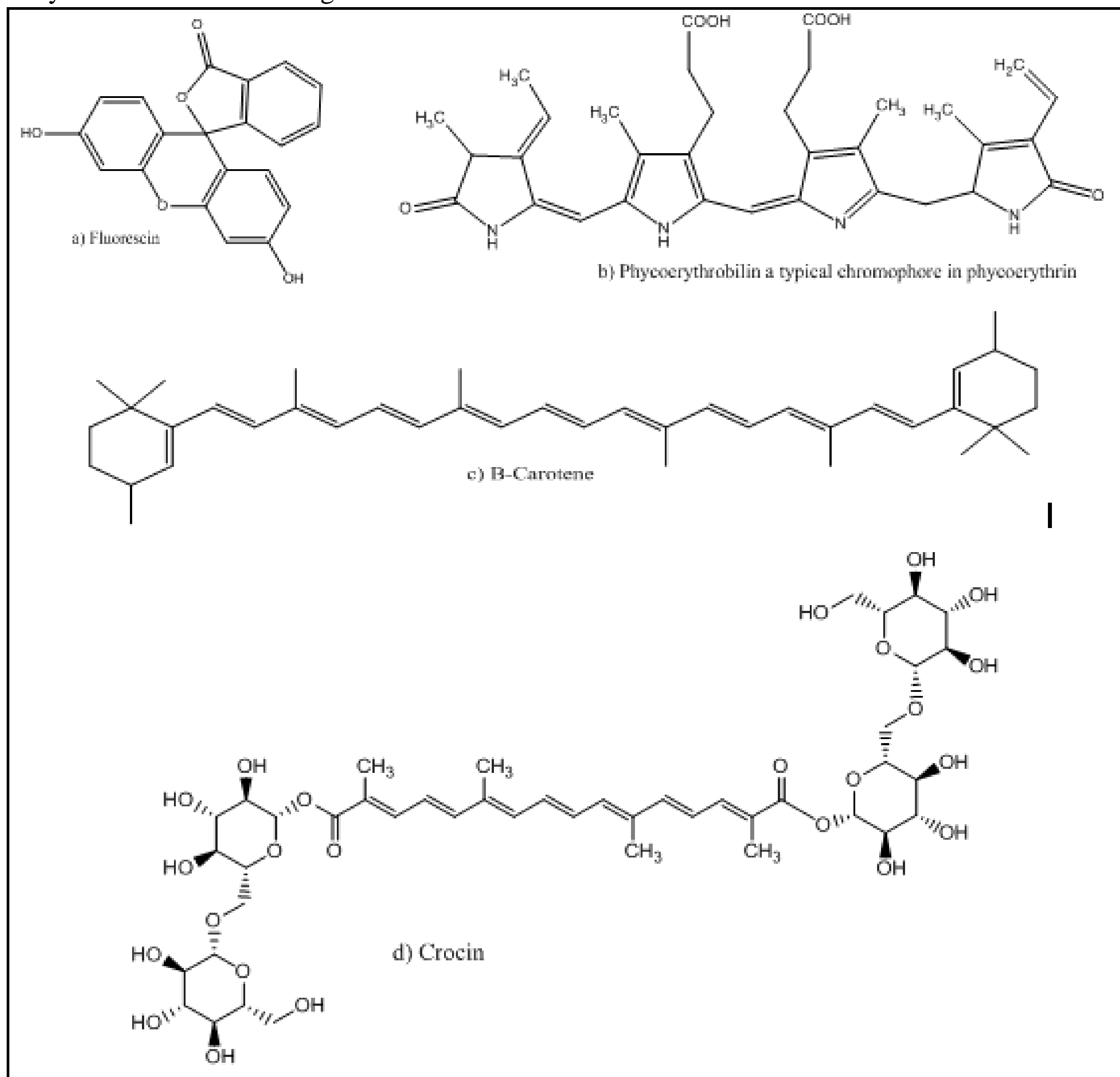


FIG. 3: STRUCTURES OF VARIOUS CHROMOPHORES USED IN DIFFERENT ASSAYS

TRAP Assay (Total Peroxyl radical trapping antioxidant Parameter) This method uses R-phycoerythrin (R-PE) as the fluorescent probe. The structure of R-phycoerythrin is given in **Figure 3b.** The reaction progress of R-PE with AAPH was monitored fluorometrically (λ_{ex}=495 nm and λ_{em}=575 nm). R-PR is the brightest fluorescent dye ever identified and is originally isolated from red algae *Gracilaria*.

TRAP values are calculated from the length of the lag phase caused by the antioxidant compared to that of Trolox. Luminol can also be used as the chemiluminescent substance [28].

β-carotene bleaching assay: This method is suitable for estimation of antioxidant potential of essential oils and non plar antioxidants. The β-carotene bleaching method is based on the loss of the yellow colour of β-carotene (**in Figure 3c)** due to its reaction with radicals which are formed by linolenic acid oxidation (peroxyl radicals; $ROO^{\cdot}$) in an emulsion. The rate of β-carotene bleaching can be slowed down in the presence of antioxidants

(AH) which donate a hydrogen atom to quench the free radical what results in antioxidant radical ($A^{\cdot}$) and lipid derivative (ROOH) formation. The antioxidants compete with β-carotene for peroxyl radicals as shown below:

$ROO^{\cdot}$ + β carotene → Bleaching

$ROO^{\cdot}$ + AH → $A^{\cdot}$ + ROOH

For a typical assay, 1 mL of *β*-carotene solution, 0.2 mg/mL in chloroform, is added to round-bottom flasks (50 mL) containing 0.02 mL of linoleic acid and 0.2 mL of Tween 20). Each mixture is then treated with 0.2 mL of 80% MeOH (as control) or corresponding plant extract or standard. After evaporation to dryness under vacuum at room temperature, oxygenated distilled water (50 mL) is added and the mixture is shaken to form a liposome solution. The samples are then subjected to thermal autoxidation at 50 °C for 2 h. The absorbance of the solution at 470 nm is monitored on a spectrophotometer by taking measurements at 10 min intervals, and the rate of bleaching of β-carotene is calculated by fitting linear regression to data over time [29].

The antioxidant activity coefficient, C_{AA} was calculated as:

$$C_{AA} = 1-[A_{s\ 470nm(t=0)} - A_{s\ 470\ nm(t=80)}/A_{c\ 470\ nm\ (t=0)} - A_{c\ 470\ nm\ (t=80)}];$$

Where $A_{s\ 470nm(t=0)}$ is the initial absorbance of the sample containing antioxidant, $A_{c\ 470\ nm\ (t=0)}$ is the initial absorbance of the control, $A_{s\ 470nm(t=80)}$ is the absorbance of the sample at t= 80 min and $A_{c\ 470nm(t=80)}$ is the absorbance of the control at t= 80 min. [30]

Crocin bleaching Assay: Crocin ($C_{44}H_{64}O_{24}$) Fig. 3d, is a naturally occurring carotenoid obtained from dries stigma of culinary spice *Crocus sativus* L. (Saffron).

The "crocin bleaching assay" (CBA) was suggested by Bors et al [31] as suitable for screening radical scavenging activity. Originally, inhibition of crocin bleaching by a range of substances was monitored by competition kinetics in the presence of photolytically produced alkoxyl radicals. In CBA, abstraction of hydrogen atoms and/ or addition of the radical to the polyene structure of crocin results in a disruption of the conjugated system accounting for crocin bleaching. The latter is recorded as reduction of absorbance at 440 nm in the presence or absence of radical scavengers. The pro oxidant activity was taken as a ratio of decrease in crocin absorbance at 5 min and the relevant oxidant concentration [32, 33]. Later on, Bors and co-workers [34] found that the absolute rate of crocin bleaching depends heavily on the sort of radical attacking the polyene structure.

In the latter, peroxyl radical formation was achieved by using azo-initiators (hydrophilic or lipophilic) [35]. In this way, Tubaro and co-workers [36] made an effort to average antioxidant and pro-oxidant effects of the constituents of complex natural mixtures. Results were expressed with reference to α-tocopherol (for lipophilic molecules) or Trolox (for hydrophilic ones).

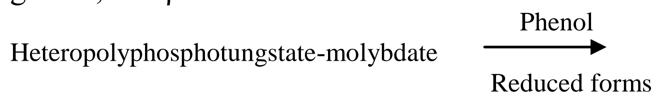
Total Phenolic Assay by Folin-Ciocalteau reagent: Folin-Ciocalteau (FC) colorimetry is based on early work of Singleton and Rossi's method of chemical reduction of the reagent, a mixture of tungsten and molybdenum oxides. This method was originally intended for the analysis of proteins like tyrosine [37] containing a phenolic group but later extended by Singleton et al for analyzing the total phenolic content in wine. This method is sensitive, quantitative and relatively independent of the degree of polymerization of phenols but correction for proteins, nucleic acids and ascorbic acid may be required for their interfering action.

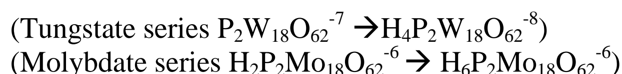
The products of metal oxide reduction have a blue colour that exhibits a broad light absorption with a maximum at 765 nm (750-770nm). Since most phenolic compounds are in dissociated form (as conjugate bases or phenolate anions) at the working pH of the assay (pH~10), they can be more easily oxidized with the FC reagent.

The molybdenum centre in the complex reagent is reduced from Mo (VI) to Mo (V) with an e^- donated by an antioxidant to produce a blue colour. The intensity of light absorption at that wavelength is proportional to the concentration of phenols and results are expressed in Gallic acid equivalents (GAE). Phenols stoichiometrically reduce

phosphomolybdic/phosphotungstic acid [38]. The FC chromophore which is a multivalent charged phospho-tungsto- molybdate (V) having a great affinity for water was found to be incapable of measuring lipophilic antioxidants but the reagent was modified and standardized to enable simultaneous measurements of lipophilic and hydrophilic antioxidants in NaOH added isobutanol-water medium by R. Apak et al [39].

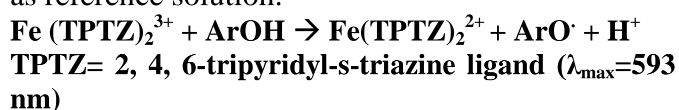
The modified procedure was successfully applied to the total antioxidant capacity assay of trolox, quercetin, ascorbic acid, gallic acid, catechin, caffeic acid, ferulic acid, rosmarinic acid, glutathione, and cysteine, aswell as of lipophilic antioxidants such as α-tocopherol (Vitamin E), butylated hydroxyanisole, butylated hydroxytoluene, tertiary butylhydroquinone, lauryl gallate, and β-carotene.


Heteropolyphosphotungstate-molybdate $\xrightarrow[\text{Reduced forms}]{\text{Phenol}}$


(Tungstate series $P_2W_{18}O_{62}^{-7} \rightarrow H_4P_2W_{18}O_{62}^{-8}$)
(Molybdate series $H_2P_2Mo_{18}O_{62}^{-6} \rightarrow H_6P_2Mo_{18}O_{62}^{-6}$)

FRAP Assay (Ferric reducing antioxidant potential): Total antioxidant activity is measured by ferric reducing antioxidant power (FRAP) assay given Benzie and Strain 40. FRAP assay uses antioxidants as reductant in a redox-linked colorimetric method, employing an easily reduced oxidant system present in stoichiometric excess. At low pH (3.6), reduction of ferric tripyridyl triazine (Fe III TPTZ) complex to ferrous form (which has an intense blue colour) can be monitored by measuring the change in absorption at 593nm as shown in **Figure 4**.

The reaction is non specific, in that any half reaction that has lower redox potential, under reaction conditions, than that of ferric ferrous half reaction, will drive the ferrous (Fe3+ to Fe2+) ion formation (redox potential 0.77V). The change in absorbance is therefore, directly related to the combined or "total" reducing power of the electron donating antioxidants present in the reaction mixture. Standard ferrous sulphate solution is used as reference solution.


$Fe(TPTZ)_2^{3+} + ArOH \rightarrow Fe(TPTZ)_2^{2+} + ArO^{\cdot} + H^+$
TPTZ= 2, 4, 6-tripyridyl-s-triazine ligand (λ_{max}=593 nm)

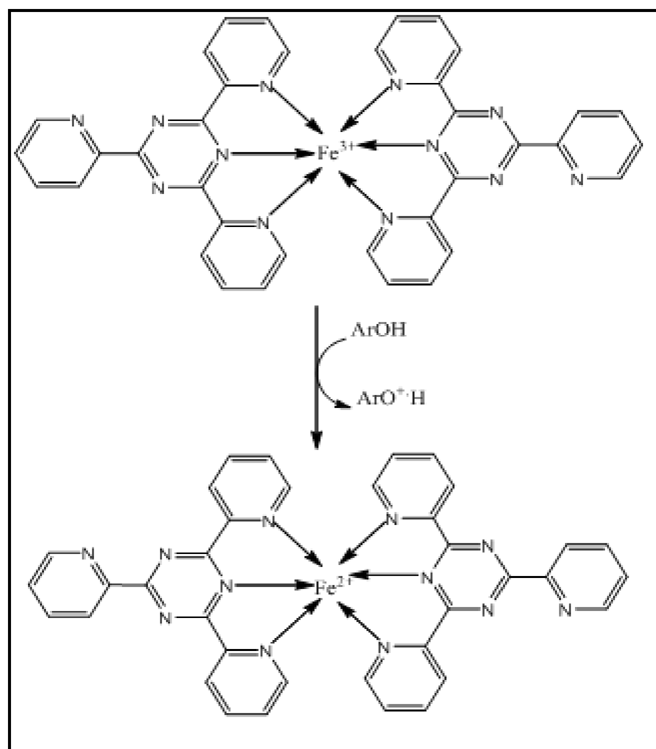


FIG.4: REDUCTION OF Fe^{3+}-TPTZ to Fe^{2+}-TPTZ

The FRAP assay involves the FRAP reagent prepared by mixing TPTZ (2.5 ml, 10 mM in 40 mM HCl), 25 ml acetate buffer and 2.5 ml $FeCl_3.H_2O$ (20 mM). The final solution contains 1.67 mM Fe^{3+} and 0.83mM TPTZ. To measure FRAP value, 300μL of freshly prepared FRAP reagent is warmed to 37°C and a reagent blank reading is taken at 593 nm; then 10 μL of sample and 30 μL of water are added. Absorbance readings are taken after 0.5 s and every 15 s until 4 min.

The change in absorbance ($\Delta A=A_{4\ min}-A_{0min}$) is calculated and related to ΔA of Fe^{2+} standard solution. ΔA is linearly proportional to the concentration of antioxidant. One FRAP unit is arbitrarily defined as the reduction of 1 mol of Fe^{3+} to Fe^{2+}. Pulido et al [41] measured the FRAP values of several polyphenols in water and methanol. The absorption at λ_{593} does not stop at 4 min. and slowly increased even after several hours for polyphenols like caffeic acid, tannic acid, ferulic acid, ascorbic acid and quercetin. Reducing power appeared to be related to the extent of conjugation in phenols as well as the number of hydroxyl constituents [42].

DPPH Assay: $DPPH^{\cdot}$ (2, 2-diphenyl-1-picrylhydrazyl) (**Figure 5a** shows the structure of

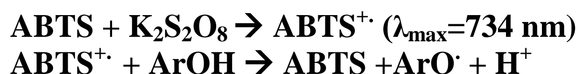
the violet chromophore) is a stable radical owing to stabilization by delocalization on to aromatic rings. $DPPH^{\cdot}$ can trap other radicals easily but does not dimerize. Because a strong absorption band is centered at about 515 nm, the solution of DPPH radical form in deep violet in colour and it becomes colorless to pale yellow when reduced upon reaction with hydrogen donor. The decrease in absorbance depends linearly on antioxidant concentration. Trolox is used as standard antioxidant [43, 44].

$DPPH^{\cdot}$ (λ_{max}=520 nm) + ArOH → DPPH +$ArO^{\cdot}$ + H^{+}

FIG. 5: STRUCTURES OF VARIOUS CHROMOPHORES USED IN DPPH, ABTS AND FRAP ASSAYS

ABTS Method: This assay requires 2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) which on treatment with sodium / potassium persulphate [45] or MnO_2 [46] give a bluish-green radical cation ($ABTS^{+\cdot}$). The radical cation was obtained by reacting 7mM ABTS stock with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark for 12-16 h before use. The radical cation is reduced in presence of hydrogen donating antioxidants (both lypophilic and hydrophilic compounds and food extracts including flavonoids, hydroxycinnamates and carotenoids). The radical cation shows absorption maxima at wavelengths 415 nm, 645 nm, 734 nm and 815 nm. Trolox can be used as standard antioxidant. The influences of both the concentration of antioxidant and duration of the reaction on the inhibition of the radical cation absorption can be taken into account when determining the antioxidant activity. This method is better than original Trolox equivalent antioxidant capacity assay, TEAC-I (which utilized metmyoglobin-H_2O_2 to generate $HO^{\cdot}$, which then reacted with ABTS to generate the radical cation) assay in number of ways [47,48]. Firstly, direct

generation of ABTS radical cation with no involvement of an intermediary radical is done hence the stock solution of $ABTS^{+\cdot}$ is stable for many months in refrigerator. Secondly, the radical cation is pre-formed prior to addition of antioxidant test systems. Thirdly, it is applicable to both lypophilic and hydrophilic systems [45].

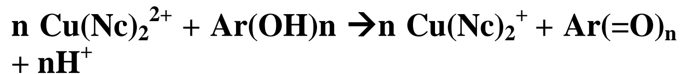


$$\mathbf{ABTS + K_2S_2O_8 \rightarrow ABTS^{+\cdot} (\lambda_{max}=734\ nm)}$$
$$\mathbf{ABTS^{+\cdot} + ArOH \rightarrow ABTS + ArO^{\cdot} + H^+}$$

The $ABTS^{+\cdot}$ radical cation is blue in color and absorbs light at 734 nm. **Figure b** indicates the electron transfer reaction in $ABTS^{+\cdot}$. When food substances are being tested using this assay, kinetic profiles should first be examined before end points are determined [49].

The ABTS radical cation is reactive towards most antioxidants including phenolics, thiols and Vitamin C [49]. During this reaction, the blue ABTS radical cation is converted back to its colorless neutral form. The reaction may be monitored spectrophotometrically. The reaction may complete in four minutes to several hours. This assay is often referred to as the Trolox equivalent antioxidant capacity (TEAC-II) assay. The reactivity of the various antioxidants tested is compared to that of Trolox, which is a water-soluble analog of vitamin E [50]. A drawback of ABTS based assay is the promiscuity of reactions of $ABTS^{\cdot}$ which is a non physiological free radical.[51]

CUPRAC Assay: Copper ion reducing antioxidant capacity assay utilizes the copper (II) neocuproine reagent as the chromogenic oxidizing agent. This method is more advantageous over other ET based assays as the working pH range for this assay is the physiological pH (7) in contrast to alkaline pH used in Folin method or acidic pH used in FRAP method. It is applicable to both hydrophilic and lipophilic antioxidants (unlike folin and DPPH assays), has a selective action on antioxidant compounds without affecting sugars and citric acid commonly present in foodstuffs and has a capacity to assay –SH bearing antioxidants (unlike FRAP) [24]. The CUPRAC assay method describes the development of a simple and widely applicable antioxidant capacity assay for flavonoids, phenolic acids, hydroxycinnamic acids, thiols, synthetic antioxidants and vitamin C and E [52].



$$\mathbf{n\ Cu(Nc)_2^{2+} + Ar(OH)n \rightarrow n\ Cu(Nc)_2^{+} + Ar(=O)_n + nH^+}$$

Figure 5c shows the reduction of Cu(II) neocuproine reagent. The polyphenol is oxidized to the corresponding quinone, the reduction product is bis(neocuproine) copper(I) chelate shows absorption maximum at 450 nm. Neocuproine is (2,9-dimethyl-1,10-phenanthroline). The colour change observed is from light blue (due to Cu(II)-Nc cation) to orange-yellow (due to reduced Cu(I)-Nc cation). Liberated protons are buffered in ammonium acetate medium.

CUPRAC reactions complete in 30 min for most the samples. Slow reacting antioxidants may need elevated temperature incubation to complete their reaction with CUPRAC reagent. Although the concentration of Cu^{2+} ions is in stoichiometric excess of that of Nc in the CUPRAC reagent for driving the above redox reaction to the right, the actual oxidant is $Cu(Nc)_2^{2+}$ species and not the sole Cu^{2+}, because the standard redox potential of the Cu (II/I)-Nc is 0.6 V which is much higher than that of Cu^{2+}/Cu^{+} couple (0.17 V). Therefore, the polyphenols are more easily oxidized with Cu(II)-Nc than with Cu^{2+} [53].

The CUPRAC reagent does not involve any radical reagent hence is insensitive to number of physical parameters like, temperature, sunlight, pH, humidity etc. The reagent can be adsorbed on a cation-exchanger membrane to build a low cost, linear response antioxidant sensor [54]. CUPRAC methodology can be best described as a self-sufficient and integrated train of measurements providing a useful "antioxidant and antiradical assay package" [55].

CERAC Assay: Cerric (IV) ion reducing antioxidant capacity assay was developed by D. Ozyurt et al [56, 57]. CERAC assay selectively oxidized antioxidant compounds such as trolox, quercetin, gallic acid, ascorbic acid, catechin, naringin, caffeic acid, ferulic acid, glutathione and cysteine but not citric acid and reducing sugars.

This method is based on the electron transfer (ET) reaction between Ce (IV) ion and antioxidants in optimized acidic sulphate medium (i.e., 0.3 M H_2SO_4 and 0.7 M Na_2SO_4 and subsequent

determination of the produced Ce (III) ions by a fluorometric method. In this method, increasing amounts of antioxidant were added to a fixed amount of Ce (IV). For this purpose, 1 ml of 1.0×10^{-3} M Ce (IV) solution, 7 ml of 1 M Na_2SO_4 solution, x ml (increasing variable) antioxidant solution were placed in a 20-mL test tube, sufficient sulphuric acid was added to yield a final acid concentration of 0.3 M H_2SO_4, and diluted to 10 ml with distilled water.

After standing for 30 min at room temperature, the fluorescent product, Ce (III), exhibited strong fluorescence at 360 nm with an excitation wavelength of 256 nm, the fluorescence intensity being correlated to antioxidant power of the original sample. The titration curve was constructed as signal intensity against antioxidant concentration. The method is reproducible and shows a good correlation with those of reference methods like ABTS and CUPRAC assay.

Lipid peroxidation inhibition assay (LPO): Lipid peroxidation, a well-established mechanism of cellular injury in plants and animals, is used as an indicator of oxidative stress in cells and tissues. Lipid peroxides are unstable and decompose to form a complex series of compounds including reactive carbonyl compounds. Polyunsaturated fatty acid peroxides generate malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) upon decomposition and the measurement of MDA and HAE has been used as an indicator of lipid peroxidation.

The assay is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA and 4-hydroxyalkenals at 45°C. One molecule of either MDA or 4-hydroxyalkenal reacts with 2 molecules of N-methyl-2-phenylindole to yield a stable chromophore (carbocyanine dye) with maximal absorbance at 586 nm as indicated in **Figure 6a** [58, 59].

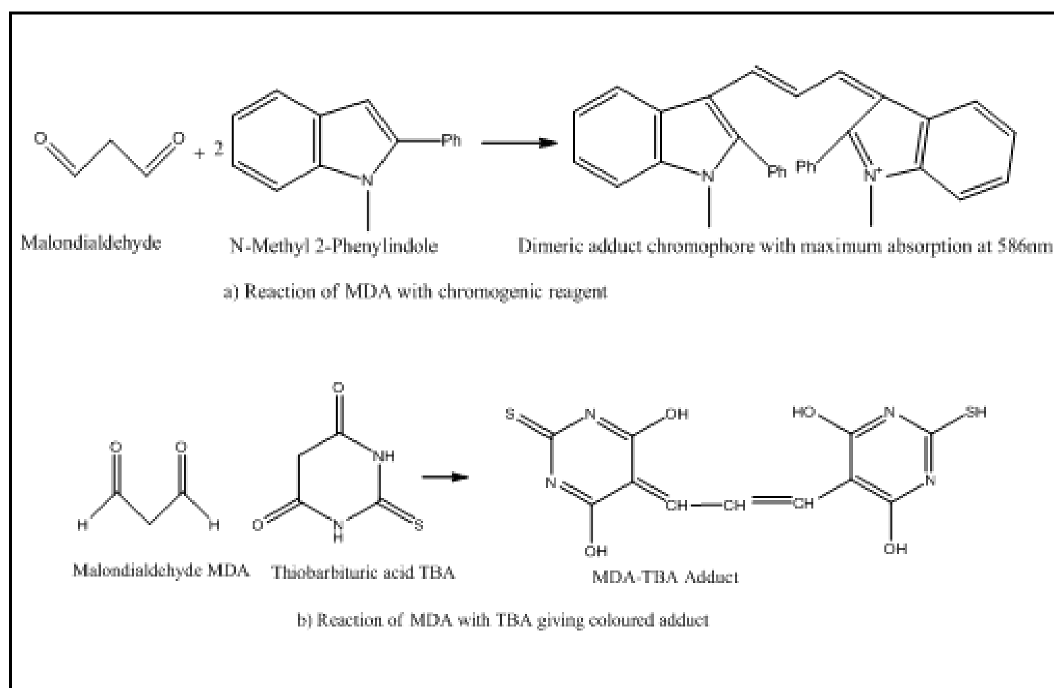


FIG. 6: REACTIONS INVOLVED IN TBARS ASSAY AND LIPID PEROXIDATION ASSAY

Thiobarbituric acid reactive substances (TBARS) assay: The measurement of thiobarbituric acid reactive substances is a well established method for screening and monitoring lipid peroxidation [60]. The assay measures the inhibition of production of thiobarbituric acid reactive substances (TBARS) from sodium benzoate under the influence of the free oxygen radicals derived from Fenton's reaction. A solution of 1mmol/L uric acid was used as standard. A standardized solution of Fe-EDTA complex reacts with hydrogen peroxide by a Fenton type reaction, leading to formation of hydroxyl radicals.

The reactive oxygen species degrade benzoate, resulting in the release of TBARS. Antioxidants from the added sample (human and animal tissues, fluids, drugs and food) cause suppression of the

production of TBARS. At low pH and elavated temperature (90-100˚C), MDA readily participates in nucleophilic addition reaction with 2-thiobarbituric acid (TBA), generating a red, fluorescent 1:2 MDA: TBA adduct (**Fig.6b**) [58]. This reaction can be measured colorimetrically at 530-540 nm or fluorometrically at an excitation wavelength of 530 nm and emission wavelength of 550 nm [61, 62].

Hydroxyl radical averting capacity (HORAC): The hydroxyl radical is generated by a Co^{2+} mediated Fenton-like reaction, and the hydroxyl radical formation under the experimental condition is indirectly confirmed by the hydroxylation of p-hydroxybenzoic acid. The fluorescence decay curve is monitored in the absence and presence of antioxidant which is the index of the hydroxyl radical prevention capacity. Gallic acid is chosen as a reference standard and activity is measured in terms of Gallic acid equivalents (GAE). The hydroxyl radical prevention capacity is mainly due to the metal-chelating capability of the compounds [63].

Ferrous ions chelating assay: One of the mechanism of antioxidative action is chelation of transition metals preventing catalysis of hydroperoxide decomposition and Fenton type reactions [64, 65]. In the presence of chelating agents, the complex formation is disrupted leading to colour reduction. Measurement of colour reduction allows the estimation of the chelating activity of the coexisting chelator. The transition metal ion, Fe^{2+} possess the ability to move single electron by virtue of which it can allow the formation and propagation of many radical reactions, even starting with relatively non reactive radicals.

A volume of 1.6 ml of 70% ethanol and 50μL of $FeCl_2$ (2mM) were treated with solution containing antioxidant compounds or plant extracts. The mixture was mixed thoroughly and incubated for 5 min. Then 100μL of ferrozine (5mM in 70% EtOH) was added, mixed and left to incubate in the dark at room temperature for 5 min.

Ferrozine react with diavalent iron to form a stable red or purple complex species that is very soluble in water. The absorbance of Fe^{2+}- Ferrozine complex was measured at 562 nm. Both EDTA and BHA were used as positive controls and reaction mixture without extract was used as negative control. The metal ion chelating activity of each extract for Fe^{2+} was calculated as:

Chelating effect (%) = [(A_0-Ae) / A_0] x 100%

Where A_0 is absorbance reading of the negative control (blank, without extract/ standard) and A_e is absorbance reading in the presence of sample. EDTA is usually added to check the stability of metal complex [66].

Nitric oxide free radical scavenging activity: Sodium nitroprusside in aqueous solution at physiological pH generates nitric oxide, which interacts with oxygen to produce nitric ions that can be estimated by use of Griess reagent [67, 68, 69]. Scavenger of nitric oxide competes with oxygen leading to reduced production of nitric oxide. Sodium nitroprusside (5 mM) in phosphate-buffered saline (PBS) was mixed with plant extracts and incubated at 25° C for 150 min. The above solution was reacted with Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% napthylethylenediamine dihydrochloride NED).

The absorbance of chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphylethylenediamine was read at 546 nm. Colour of the solution changed from colourless to light pink to deep purple. The percentage scavenging of nitric oxide of plant extract and standard solution of potassium nitrite is calculated using the following formula:

NO Scavenged (%) = (A_c-A_e)/A_c x 100

Where A_c = Absorbance of control reaction and A_e = Absorbance in presence of sample.

Potassium ferricyanide reducing power assay (PFRAP): This method is based on the reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}), which is accomplished in the presence of antioxidants. Substances having a reduction potential react with potassium ferricyanide forming potassium ferrocyanide which further reacts with $FeCl_3$ to form an intense prussian blue complex having maximum absorbance at 700 nm. The amount of

complex formed is directly proportional to the reducing power of test sample [70].

PCL Assay: In the PCL assay (Photochemiluminescence) the photochemical generation of free radicals is combined with the sensitive detection by using chemiluminescence [71, 72]. The reaction is induced by optical excitation of a photosensitiser S which results in the generation of the superoxide radical $O_2^{\cdot -}$.

$$S + h\upsilon + O_2 \rightarrow [S^*O_2] \rightarrow S^{\cdot +} + O_2^{\cdot -}$$

The free radicals are visualized with a chemiluminescent detection reagent. Luminol (5-amino-2, 3-dihydro-1,4-phthalazinedione) acts as photosensitiser and oxygen radical detection reagent. Luminol upon excitation gives L^* an intermediate and triplet oxygen $^3O_2$. Once the $O_2^{\cdot -}$ and luminol radicals are generated, they proceed through a series of reactions resulting in the production of blue luminescence. In presence of any exogenous antioxidant species the $O_2^{\cdot -}$ radical out compete the luminal radical via a HAT reaction leading to halt in luminescence until the concentration of antioxidant is exhausted. The resultant lag/log relationships of antioxidant compounds are compared with effectiveness of standards.

For the analysis 1.5 ml of buffer solution of pH 10.5, 1ml of distilled water, 25µl of photo sensitiser and 10 µl of standard solution were mixed and measured, the antioxidant potential was assayed by means of the lag phase at different concentrations.

N, N-dimethyl-p-phenylenediamine (DMPD) assay: An improved decolourization method for measuring the antioxidant activity of samples using DMPD has been developed by Asghar et al in 2007 for measurement of antioxidant activity in food samples.

The purple coloured long lived DMPD radical cation ($DMPD^{\cdot +}$) is generated through a reaction between DMPD and potassium persulphate and is subsequently reduced in presence of H donating antioxidants [73]. The determination is done at pH 5.25 using 0.1 M acetate buffer. The DMPD radical cation is stable upto 12 h. One µL of $DMPD^{\cdot +}$ solution and 50 µL antioxidant solution were mixed continuously for 10 min. at 25°C, after which the absorbance of solution was taken at 517.4 nm.

This method has advantage over method used earlier in which Fe (II) ions were involved in generation of radical cation which through Fenton's Reaction could cause negative deviation in the antioxidant activity of food extracts. This assay can equally be applied to both lipophilic and hydrophilic antioxidants. This method is rapid and inexpensive and reproducible. It has a promising aspect of use in screening large number of fruit samples [74].

Cellular Antioxidant Activity (CAA): The cellular antioxidant activity model better represents the complexity of biological systems and is an important tool for screening foods, phytochemicals and dietary supplements for potential biological activity. Many of the chemical assays are performed at non physiological pH and temperature and may therefore be unreliable indicators of true biological antioxidant levels.

The technique accounts for some aspects of uptake, metabolism and distribution of antioxidant compounds within cells, so it provides a clearer picture of how the antioxidants act within a living cell (in vivo) and by extension, a living cell culture rather than in a test tube (in vitro). Although CAA helps in determining actual efficacy of antioxidants within the body of animal as it takes into account aspects of cellular uptake, distribution and metabolism of antioxidant compounds but the method used is very expensive and is not suitable for initial antioxidant screening of foods and dietary supplements.

Since liver is the major place for xenobiotic metabolism therefore liver cells can be used as model cells for determination of oxidative stress in cultured cells for evaluation of chemoprotective effect of dietary compounds. Human HepG2, a differentiated cell line of hepatic origin is used as reliable model for such assays [75]. The CAA method is a cell based assay that loads a cell with the diacetate precursor of an indicator compound, 2', 7'-dichlorofluorescin (DCFH), i.e., DCFH-DA which is oxidized to DCF when reactive oxygen species such as peroxyl radicals are present. The concentration of DCF, a fluorescent compound, can

be measured using a fluorescent plate reader. The assay involves the use of peroxyl radicals generated from azobis (amidinopropane) dihydrochloride (ABAP).

When a sample of phytochemical origin such as fruit or vegetable extract or dietary supplements containing antioxidants is added to the assay, the antioxidants react with the peroxyl radicals, preventing the peroxyl radicals from oxidizing the DCFH and thereby preventing the formation of DCF. Consequently the fluorescence decreases due to the scavenging effects of the antioxidants.The representative examples of antioxidants include vitamins, carotenoids, phenolics and flavonoids[76].

In -vitro Cellular Antioxidant Activity can also be assessed using a Light-Scattering Properties (turbidity) of Human Erythrocytes. It relies on differences in scattering properties between lysed and intact human erythrocytes. AAPH, a peroxyl radical generator is used to enhance lipid peroxidation. The consequent hemolysis triggered a loss of the light-scattering ability in the lysed erythrocytes. When an antioxidant is added, the area under the absorbance decay curve (AUC) was linearly proportional to the concentration of antioxidant compound.

This erythrocyte cellular antioxidant activity (ERYCA) method is found to be relatively fast, sensitive, accurate, and repeatable, even when using erythrocytes from different donors and for different storage times [77]. The ERYCA assay has the advantage of assessing different mechanisms of antioxidant protection, including direct scavenging of free radicals in the surrounding medium and cell mediated antioxidant protection (Cell-MAP), in one step.

Cell-MAP addresses the following: the physiochemical properties of antioxidants such as their lipo-solubility, the ability of both lipid and water soluble compounds to diffuse effectively into lipoproteins and cell membranes and eventually enhance from there, the erythrocytes defenses through mediation of both, plasma membrane redox system (PMRS), and the anti oxidative defense enzyme system [78].

CONCLUSIONS: This review compiles the methods for determination of antioxidant potentials of food and dietary supplements. 99% of the diseases caused to humans are stress related and only 1% is genetic disorders. Obesity is also one of the key reasons of developing diseases like coronary disease, blood pressure, cancer, diabetes etc. Many of these diseases can be reversed through nutritional excellence. Low nutrient eating (and toxic eating) leads to increased cellular toxicity with undesirable levels of free radicals and advanced glycation end products (AGE's).

"The amount of antioxidants in your body is directly proportional to how long you will live." says Dr. Richard Cutler, former Director of the National Institute of Aging, Washington. Choosing right food in correct proportion can decrease the risk of life threatening disorders and increase life longevity. The antioxidant potential measurement assays can help in choosing naturally occurring antioxidant rich food containing ascorbic acid, vitamin E, carotenoids and natural polyphenols for preservation of food and decreasing the ROS in body.

This review is a showcase of types of radical species encountered / generated in our body in various metabolic processes and in vitro methods used to quantify them. The outcome of the antioxidant action for different assays (regardless of mechanism) is similar in the sense of quenching/reducing reactive radical species but the reaction conditions, kinetics and potential side reactions may vary.

HAT and ET mechanisms occur parallel in most systems although one may dominate depending upon the antioxidant structure and properties. No single assay can be used as universally accepted method for determination of total antioxidant potential. It may be concluded that raw and mildly processed fruits, vegetables, nuts, dry fruits, cereals, spices and condiments have significantly higher level of bioactive compounds richer in antioxidants [52, 79]. Fruits like blueberry, cranberry, blackberry, prunes, raspberry, strawberry, red apples, cherry, plums, peaches, grapefruits, lemons, oranges, kiwi, pomegranate are richer in antioxidant content [80, 81, 82]. Among vegetables garlic, red, yellow and white onions, red and green

peppers, white and green cabbage, leek, spinach, beetroot, broccoli have higher health benefits due to their phenolic compounds [83, 84, 85]. Tomatoes contain lycopene, a stable, active antioxidant. Many vegetables contain quercetin and related polyphenolic compounds. Tea is a source of epigallocatechin gallate, in green tea, and the aflavin and the associated thearubigins, in black tea. Red wine contains resveratrol [86].

The higher antioxidant activities among cereals were found in buckwheat, oats, amaranth, quinoa and rice [87-91]. Oilseeds like flaxseeds, sesame seeds, sunflower seeds, rapeseeds have high lignin content contributing towards their antioxidant potential [92]. Chemiluminescence and fluorescence assays are also being used for determining quality parameters of edible oils, such as oxidative stability, antioxidant activity, and lipid hydroperoxides content, as well as classification or adulteration of vegetable oils.[93] Among spices cloves, cinnamon, Black pepper, turmeric, fennel and cumin seeds, bay leaves, nutmeg were found to have higher antioxidant capacity in their essential oils owing to the presence of various active molecules [94].

Assessment of the antioxidant activities done by Paganga et al of a serving of 100g fresh weight fruit, vegetable and comparison with 150 ml beverage in micromol Trolox equivalents showed that the antioxidant activities of 1 glass (150 ml) red wine equivalent to 12 glasses white wine equivalent to 2 cups of tea equivalent to 4 apples equivalent to 5 portions of onion equivalent to 5.5 portions egg plant equivalent to 3.5 glasses of blackcurrant juice equivalent to 3.5 (500 ml) glasses of beer equivalent to 7 glasses of orange juice equivalent to 20 glasses of apple juice [95].

Antioxidants derived from naturally occurring plant and plant derived products have gained importance in recent years as the synthetic antioxidants like BHA, BHT and TBHQ have restricted use in foods (suspected carcinogens) and the quest for finding better natural antioxidants and a universal method for their determination is still on.

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