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Review

Mode of action and determination of antioxidant activity in the dietary sources: An overview

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ABSTRACT

Determination of antioxidant/capacity in the dietary, food, drugs, and biological samples is an interesting approach for testing the safety of these compounds and for drug development. Investigating the google searching engines for the words (measurement + antioxidant + capacity) yielded more than 20 million results, which makes it very difficult to follow. Therefore, collecting the common methods to measure the antioxidant activity/capacity in the food products and biological samples will reduce the burden for both the students and researchers. Nowadays, it is widely accepted that a plant-based diet with a high intake of dietary sources such as vegetables, fruits, and other nutrient-rich plant foods may decrease the effect of oxidative stress-related diseases. In this review article, we have provided the most recent advances in the most common *in vitro* methods used for evaluating the antioxidant potential of numerous food products, plant extracts, and biological fluids. We have also provided detailed procedures on how to perform them and analyze the results. This review article shall be a comprehensive reference for all techniques used in this area.

Contents

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1. Introduction

Oxidative stress is defined as an increase in cellular free radicals generation to the cell antioxidant levels which create an imbalance toward shifting the cellular environment toward an oxidant (Pizzino et al., 2017). Currently, oxidative stress is the major leading cause of tissue damage and is a central mechanism associated with numerous chronic disorders including cancer, liver disorders, neurological disorders, diabetes mellitus (DM), and cardiovascular disorders (Litescu et al., 2010). The free radicals in the forms of reactive oxygen species (ROS) are continuously produced in the human cells. However, these cells are also well-equipped with an efficient antioxidant system that is composed of both enzymes and other non-enzymatic molecules that can neutralize the adverse effect of these free radicals (Alam et al., 2013). At the biological levels, the term antioxidant is referred to as any compound which is capable to block or slow down the reaction of a substance with ROS which in turn inhibits the oxidation process (Litescu et al., 2010). Therefore, a principal issue to identify an antioxidant depends on the assessment of the concentration ratio between the free radical and the antioxidant (Litescu et al., 2010). Under some disease or stress conditions, the production of antioxidants in the cell is reduced leading to increases in the ROS/antioxidant ratio which is identified as oxidative stress (Pizzino et al., 2017).

The plants, vegetables, fruits, nuts, and spices have been reported to be a major source of dietary active antioxidant that protects the body against several oxidative stress-induced (Carlsen et al., 2010). For this reason, much attention during the last decades in the industry of food technology, production, and processing have focused on the isolation and development of natural antioxidants from plant resources which are suitable and effective for human use with the least side effects (Nantz et al., 2006). Therefore, efforts have been made to ensure the lifespan, safe use, commercial value, and effectiveness of these natural antioxidants. However, the biological food complexity, as well as the obstacles in the analytical system make it very difficult to easily detect, isolate, quantify, and test these natural antioxidants (Apak et al., 2013). Besides, the evaluation of the total antioxidant power and health benefits of an individual antioxidant is not an easy process especially when a collaboration between diverse antioxidants is required to achieve the desired effect (Apak et al., 2013).

Currently, laboratory assessment aims to measure the expected total *in vivo* and *in vitro* antioxidant potential and correlate it with the expected effects in the food system (Wolfe and Liu, 2007). The currently established assays and tests to measure the abilities, activities, and the effect of a certain antioxidant or antioxidant compound depends on multiple factors the food environment (i.e. pH), the physical structure of the antioxidant, stability, and its ability to interact with other molecules (i.e. ROS) and food constituents (Wolfe and Liu, 2007). Also, the effective cellular power of an antioxidant depends on the plant species, the availability of the antioxidant in that species, the degree of ripeness, culture conditions, and cultivation times (Fogliano et al., 1999-; -Singh and Singh, 2008). The target of our food system is to determine the relative antioxidant efficiency. However, the evaluation of the total antioxidant capacity of a certain antioxidant should apply to water-soluble (hydrophilic) and lipid-soluble (lipophilic) antioxidants (Karadag et al., 2009). Depending on the mechanisms, the two major antioxidant capacity methods can be classified as 1) A hydrogen atom transfer (HAT) method which donates a hydrogen ion from a stable molecule thus allowing the antioxidant to scavenge the ROS, and 2) A single electron transfer (SET) which depends on the potential of the antioxidant to reduce certain molecules and compounds by transferring an electron (Karadag et al., 2009). Up-to-date, several *in vitro* analytical methods have been established to evaluate the potential, capacity, and activity of natural antioxidants including spectrometric, electrochemical, and chromatography techniques (Onyeoziri et al., 2016). However, this review aims to enrich the field with the recent update on the role and action of natural antioxidants and the current methods used to determine their activities.

2. Methods

Internet browsing from different databases in the English language was used to recognize and to download review and research articles linked to antioxidant determination study using appropriate key terms (antioxidant activity, *in vitro*, extract, antioxidant determination, *in vivo*) March 2020. Three hundred and twenty papers seemed and those were exposed to the initial screening. The collection of the papers was according to antioxidant assays of extracts and methods of antioxidant. Many abstracts and papers were reviewed for *in vitro* methods of antioxidant, while a few papers for *in vitro* methods. Any other topics related to this overview were also reviewed from the prepared scientific papers.

2.1. Natural antioxidants of dietary sources

During the last decades, considerable attention has been focused on vegetables, fruits, herbs, and spices as major resources of natural effective antioxidants triggered by the scientific concern raised with the continuous usage of the commercially available synthetic antioxidants (Azizkhani and Zandi, 2010). In general, the term natural antioxidants is referred to as any substance with

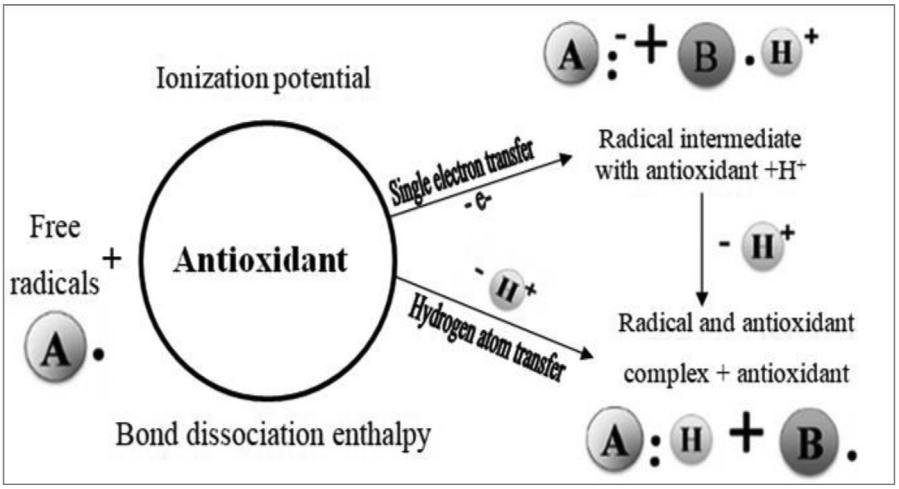

Fig. 1. Mode of action of antioxidant reacting with free radicals.

an antioxidant activity that is isolated purely from plants, microorganisms, or animal tissues or to those which compose as a result of food/meat processing (Pokorny, 1999). The chemical structures of natural antioxidants are very similar to those, which are synthesized commercially. The most common natural antioxidants with phenolic properties include flavonoids, dihydrochromanols (DHC) pyrocatechol, and pyrogallol derivatives (Karpińska et al., 2001). Some substances are also considered synergists, which enhance and boost the antioxidant potentials. These include ascorbic acid, citric acids, Millard products, amino acids, and polysubstituted organic acids (Pokorný, 1991). Of note, it is advisable to use the polar antioxidants in fatty foods due to their low solubility which produces antioxidant-rich stable fatty food (Hudson, 2012).

2.2. Mechanism of oxidation

The main reverse impact of food oxidation is a change in organoleptic quality, especially the development of rancidity, toxic compounds, off-flavors, color, and food quality loss and vitamin destruction (German, 1999). As known, the oxidation of the lipid-containing food is induced by a chain of reactions mediated by free radicals that produce alkyl, alkoxyl, peroxyl radicals, molecular singlet oxygen, and superoxide anion radical (Lobo et al., 2010). In brief, the mechanism of food oxidation involves three phases, namely initiation, propagation, and termination (Polumbryk et al., 2013). During the initiation phase, the free radicals (peroxyl radicals) in the cell are formed due to the removal of a hydrogen atom (Cadenas and Davies, 2000; Lobo et al., 2010) These peroxyl radicals stimulate the production of lipid hydroperoxides which have a damaging effect on the cells by attacking and oxidizing the membrane lipids (Lobo et al., 2010). Besides, the hydroperoxides have other adverse effects on the cell function and metabolism by stimulating the breakdown of numerous compounds such as hydrocarbons, ketones, aldehydes, etc. (Cadenas and Davies, 2000). During the propagation phase, a reaction that involves catalysis (a transition metal ion) breaks down the hydroperoxides to produce the lipid peroxyl and lipid alkoxyl radicals (Frankel and Meyer, 2000). Nonetheless, the termination step produces non-radical products and involves a delicate combination of these radical molecules (Lobo et al., 2010). The emerging role of the antioxidants in preventing these reactions involve delaying/inhibiting the reactions of the initiation and propagation phases by interacting with the radical itself or the radical mediators (Madhavi et al., 1995-; Bagchi and Puri, 1998; -Aruoma, 2003-; -Lobo et al., 2010-; Hunyadi, 2019). Besides, dietary antioxidants can slow down the oxidative reaction and protect against radical damaging effects directly by scavenging ROS through quenching the singlet oxygen and substrate removal (Madhavi et al., 1995; Hunyadi, 2019).

2.3. Antioxidant assays

The assay of the antioxidants is generally classified into 2 assays based on their action mode mechanisms, namely SET, and HAT assays. In most methods that utilize the SET, the antioxidants activity or capacity is determined spectrophotometrically by their ability to reduce a fluorescent or colored probe (oxidizing agent/oxidant/substrate) (i.e. instead of common peroxyl radicals) (Fig. 1). During this process, the changes in the absorbance are drawn against the concentration of the antioxidant to yield an expected linear curve. The degree in the change in absorbance is correlated with the concentration and activity of the antioxidant (Moharram and Youssef, 2014). However, the SET method is pH and solvent dependent (Karadag et al., 2009). In other words, the pH is the most contributing factor that determines the reducing capacity of the antioxidant (Karadag et al., 2009). Generally, the antioxidant reactivity during the SET assay depends mainly on two major antioxidant functional group preterits namely, the ionization and deprotonation potentials. Under acidic pH, the protonation on the antioxidant is increased leading to a decrease in the ionization potential and so suppressing the overall antioxidant reducing abilities (Prior et al., 2005). On the contrary, proton dissociation is increased under the basic condition, which leads to increasing the antioxidant reducing capabilities (Prior et al., 2005). Besides, the SET reaction is relatively slower than the HAT due to the time needed for the reaction to have competed and solvent stabilization (Karadag et al., 2009). However, The HAT-based method evaluates the antioxidant potential to scavenge ROS by its ability to donate hydrogen atom(s) (i.e. break the radical chain) as shown in the reaction below (Karadag et al., 2009; MikaMi et al., 2009).

ROO• + AH/ArOH → ROOH + A•/ArO•(HAT)

In general, the HAT assay protocol depends mainly on the bond dissociation enthalpy (energy) of the hydrogen-donating group of the antioxidant (Karadag et al., 2009). Accordingly, the weaker hydrogen ion release forms the donating group of the antioxidant will produce a faster more stable HAT reaction (Fig. 1). Like SET, HAT is also pH and solvent dependent. However, unlike SET, HAT assay is faster and is obtained from kinetic curves (Karadag et al., 2009). Such kinetic HAT assay is composed form antioxidant, an

Table 1
The most common *in vitro* antioxidant activity methods.

Method	Mode of action (mechanism)	Method and end-product determination
Scavenging of superoxide radical formation by alkaline	Hydrogen atom transfer	Spectrometry/colorimetry
N,N-dimethyl-p-phenylene diamine dihydrochloride	Electron transfer	Spectrometry/colorimetry
Ferric reducing power	Electron transfer	Spectrometry/colorimetry
DPPH radical scavenging activity	Electron transfer	Spectrometry/colorimetry
Total phenols by Folin-Ciocalteu	Electron transfer	Spectrometry/colorimetry
Copper (II) reduction capacity	Electron transfer	Spectrometry/colorimetry
Scavenging of hydrogen peroxide	Hydrogen atom transfer	Spectrometry/colorimetry
Trolox equivalent antioxidant capacity	Electron transfer	Trolox standard curve (Trolox equivalents in mM)
ABTS scavenging activity	Hydrogen atom transfer	Spectrometry/colorimetry
β-carotene scavenging activity	Electron transfer	Spectrometry/colorimetry
Scavenging of hydroxyl	Hydrogen atom transfer	Spectrometry/ loss of fluorescence of fluorescein
Oxygen radical absorbance capacity	Hydrogen atom transfer	Spectrometry/ loss of fluorescence of fluorescein
Scavenging of nitric oxide	Hydrogen atom transfer	Spectrometry/ colourimetry (reaction is taken place with Griess reagent)
Cupric ion reducing antioxidant capacity	Electron transfer	Spectrometry/colorimetry
Scavenging of xanthine oxidase	Electron transfer	Spectrometry/colorimetry
Scavenging of thiobarbituric acid	Hydrogen atom transfer	Malondialdehyde
Scavenging of ferric thiocyanate	Electron transfer	Spectrometry/colorimetry
Scavenging of Electron transfer	UV	phosphomolybdenum spectrophotometer equivalents of α-tocopherol
Total radical-trapping antioxidant	Hydrogen atom transfer	Spectrometry/chemiluminescence quenching
Peroxynitrite radical scavenging activity	Electron transfer	Fluorescence/spectrophotometer
Lipid peroxidation inhibition capacity	Hydrogen atom transfer	Malondialdehyde
Inhibited oxygen uptake	Hydrogen atom transfer	Polarographic probe, fluorescence or pressure gauge
Gas chromatography	Repartition between a liquid stationary phase and a gas mobile phase	Flame ionisation or thermal conductivity detection
High-performance liquid chromatography	Repartition between a solid stationary phase and a liquid mobile phase with different polarities	UV–VIS detection, fluorescence, mass spectrometry or electrochemical detection.

Table 2
The other methods of antioxidant activity methods are used in dietary sources.

Method name	Reference
Enhanced chemiluminescence	(Gheldof and Engeseth, 2002)
Inhibition of Briggs – Rauscher oscillation reaction	(Gülçin, 2010)
Cellular antioxidant activity	(Arnao et al., 2001)
Dye-substrate oxidation method	(Frankel and Meyer, 2000)
Fluorometric analysis	(Haiwei, 2010)
TLC bioautography	(Huang et al., 2005)
Total oxidant scavenging capacity	(Huda-Faujan et al., 2009)
Chemiluminescence	(Hudson, 2012)
Electrochemiluminescence	(Hunyadi, 2019)

oxidizable fluorescent probe (dichlorofluorescein), and a synthetic radical generator such as AAPH, AMVN, and ABTS) (Adom and Liu, 2005; Wolfe and Liu, 2007).

2.4. Antioxidant activity/capacity determination

An effective way to compare the antioxidant levels and the effectiveness of antioxidants in the treatment of oxidative stress-related chronic disorders is to evaluate their levels in the food and biological samples including the serum and the tissue itself (Apak et al., 2007). The total peroxyl radical-trapping potential measures the capacity of antioxidants in human serum and (Valkonen and Kuusi, 1997; Xu et al., 2014). However, the antioxidant activity describes the kinetics (speed) of any reaction that is formed between the antioxidant and the target radical which reflects the ability of an antioxidant to block the propagation stage in the oxidative chain (Couttolenc et al., 2020). Primary antioxidants generally scavenge free radicals directly whereas the secondary antioxidants prevent the generation of ROS through other reactions (i.e. Fenton's reaction) (Couttolenc et al., 2020). On the other hand, the term total antioxidant capacity refers to the overall antioxidant status in the biological samples and measures the total amount (concentration) of scavenged free radicals (Apak et al., 2013; Rubio et al., 2016). *In vitro*, the most common techniques to assay the antioxidants include an antibody, fluorescence, Folin–Ciocalteu spectrophotometric, chromatography, and light emission-based techniques (Moharram and Youssef, 2014). Among the *in vivo* methods, the dietary source's sample that is to be checked is often managed to the testing animals at a specific dose diet as recommended by the respective method with a certain period, the animal is often immolated, then, the tissues or blood is used for the analysis (Baud et al., 1991).

For any method to measure the antioxidant potential, the key features include an oxidant generator, oxidation initiator (i.e. temperature and metal catalysts), a substrate, and an appropriate measure of the endpoint. However, different methods may result in variable results for the same antioxidant which could be attributed to the variation in several factors for including the source of the oxidant, temperature, light (photosensitization), the partial pressure of oxygen, and experimental conditions that affect the reactant contact such as tubes shaking (Karadag et al., 2009).

In general, the methods used to evaluate the antioxidant capacity/activity are based on 1) scavenging the peroxyl radicals such as oxygen radical absorbance capacity assay (ORAC), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), metal (i.e. ferric) reducing/antioxidant power (FRAP), total reactive antioxidant potential (TRAP), and cupric reducing/antioxidant power (CUPRAC) (Ou et al., 2001; Ou et al., 2002; Rivero-Pérez et al., 2007; Karadag et al., 2009), and 2) evaluating some products that formed after the oxidation such as malondialdehyde (MDA), thiobarbituric acid reactive substances (TBARS), and oxidized lipids such as oxidized low-density lipopro-

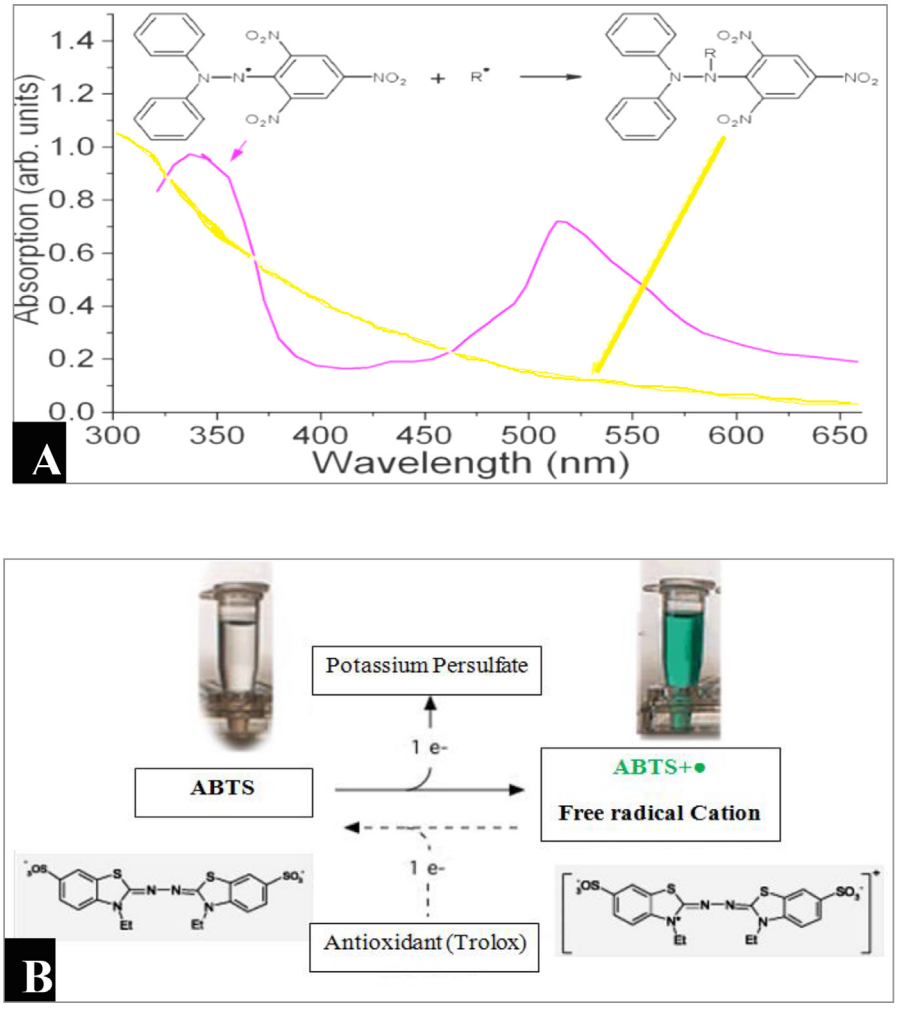

Fig. 2. A: Reaction mechanism of 2,2-diphenyl-1-picrylhydrazyl (DPPH) with antioxidant; **B:** ABTS chemical reaction during the antioxidant activity test.

tein cholesterol (ox-LDL-c) (Gheldof and Engeseth, 2002, Perez-Jimenez and Saura-Calixto, 2008; Karadag et al., 2009). In this review article, the most important *in vitro* methods have been updated and reported (Tables 1 & 2).

DPPH method

The DPPH methods depend on measuring the scavenging potential of certain antioxidants by using the synthetic-free radical generator, DPPH (Prior et al., 2005). Upon reacting with the H^+-donating antioxidant, the reduction in DPHH to hydrazine (Fig. 2**A**) leads to a reduction in the absorbance of the reaction as measured at 515–517 nm (Kedare and Singh, 2011). The reaction also results in an obvious discoloration from purple to yellow. The degree in the absorbance decrease, as well as, in discoloration correlates with the H^+-donating abilities, concentration, and activity of the antioxidant in the sample (Reddy et al., 2012). At the practical levels, the DPPH assay is conducted by mixing certain volumes of the samples (or control) with a certain volume (3.5 ml) DPPH solution followed by incubation for 30 min at room temperature and reading the tubes at 517 nm. The antioxidant activity (%) is calculated as = $[(A_{control} - A_{sample(standard)})/A_{control} \times 100]$ (Azhari et al., 2014).

Trolox equivalent antioxidant capacity (TEAC) or 2,2′-azinobis (3-ethylbenzothiazolline-6-sulfonic acid (ABTS) assay

In the presence of hydrogen peroxide (H_2O_2), ABTS, a peroxidase substrate, is oxidized by the antioxidant yielding a radical cation ($ABTS^+$). This $ABTS^+$ is a colored product that can be measured by a spectrophotometer at 600–750 nm (Huang et al., 2005). Using this test, the antioxidant capacity presented in a sample is determined by the decrease in the absorbance which indicates a decrease in the production of $ABTS^+$ which can be expressed relative to Trolox or as a percentage of scavenging. Therefore, The ABTS assay is a test that directly measures the quantity of H_2O_2 in the sample or to reflect the activity of H_2O_2-producing enzymes (Roginsky and Lissi, 2005). At the practical levels, an ABTS working reagent is prepared by mixing equal quantities of freshly prepared 7 mM ABTS solution with 2.45 mM potassium persulphate solution. The ABTS solution is then mixed with 60 ml methanol to prepare the working solution. Different concentrations of the sample or standard (control sample) (i.e. ascorbic acid or PHT) is mixed 1:1 (v/v) with the ABTS working solution, incubated at room temperature for 6 min, and reading the absorbance at 734 nm. The radical scavenging potential (activity) of ABTS in the sample (percent of inhibition) is evaluated from the standard curve (Arnao et al., 2001).

2.5. β-Carotene method

The β-carotene method is based on the bleach of carotenoids after autoxidation induced by peroxyl radicals (i.e. triggered by

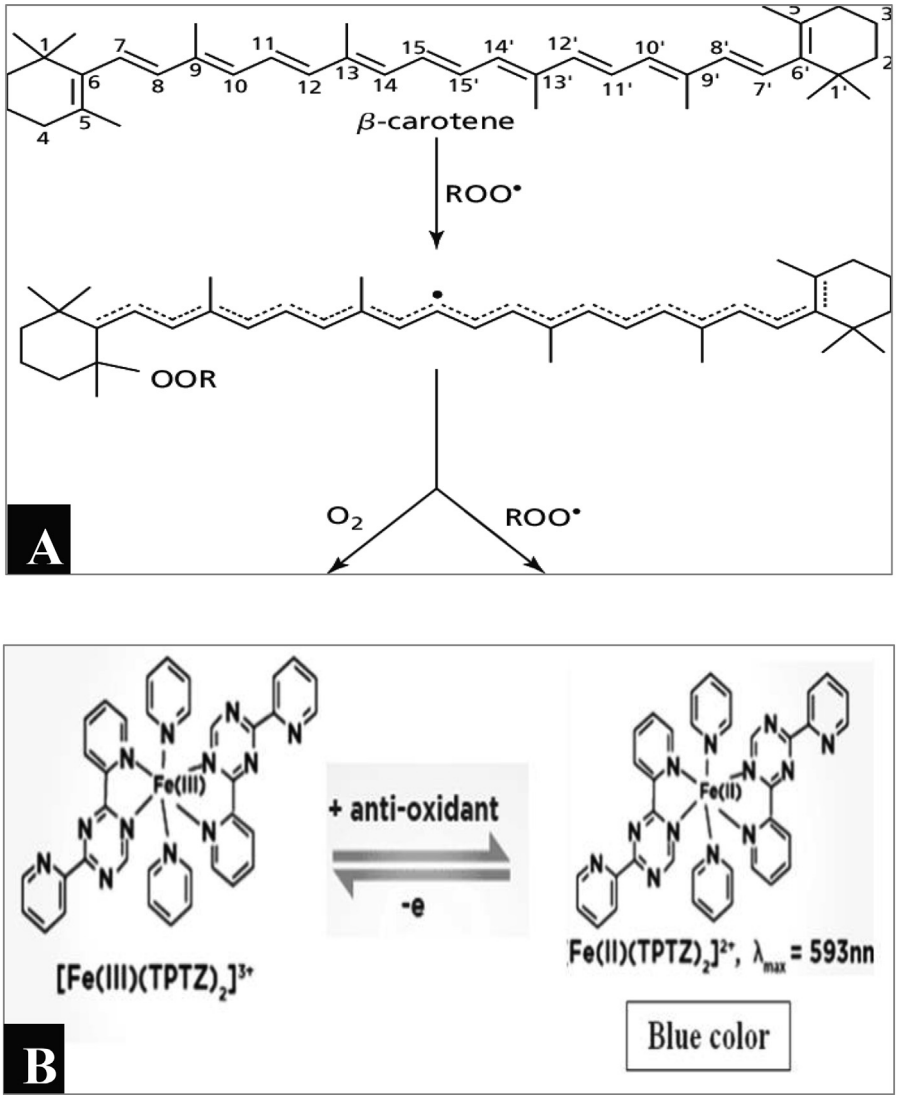

Fig. 3. A: Reaction of β-carotene with peroxyl radicals, **B:** Ferric reducing antioxidant power assay reaction.

the oxidation of linoleic acid (LA)). This yields a colored product that can be read at 470 nm. The degree of the bleach of the carotenoids is inhibited by the antioxidants in the sample **(**Fig. 3**A)** (Laguerre et al., 2007; Haiwei, 2010) This method is best to be used with the emulsion compounds (Faso, 2011). To perform the test, 1 ml of 3.34 mg/ml β-carotene solution prepared in chloroform is mixed with 50 mg linoleic acid and 500 mg Tween 20. Then, the solvent is discarded by evaporation (40 °C/5 min) to collect the residue which is then slowly mixed with 100 ml of distilled water to form an emulsion. In a separate tube, 0.2 ml of different concentrations of the samples (mg/ml) or the control is mixed with 5 ml of the prepared emulsion solution, incubated for 15 min at 40 °C, and the absorbance is immediately measured at 470 nm (Faso, 2011). The antioxidant potential of the sample is calculated as = $[(A_{control} - A_{sample\ (standard)})/A_{control} \times 100]$.

2.6. Reducing the power method

The reducing potential of a sample is measured by its ability to convert Fe^{3+} to Fe^{2+}. The increase in the absorbance is correlated with reducing potential (power) of the sample (Anandjiwala et al., 2008; Gülçin, 2010) . To perform the assay, different concentrations of the samples (1 ml) or the standard (Gallic acid and tannic acid) are mixed in saturates tubes with 2.5 ml of phosphate buffer saline (PBS/pH7.4) and 2.5 ml of 1% potassium ferricyanide (K_3Fe (CN)) (1%) and incubated for 20–25 min at 50 °C. Then, 2.5 ml of 10% trichloroacetic acid (10%) is added. After that, the whole mixture is centrifuged at 1200 × g for 10 min to collect the upper layer. In a separate tube, 2.5 ml of this isolated layer is mixed with an equal volume of distilled water and 0.5 ml of 0.1% $FeCl_3$ solution. The absorbance of the samples is then read at 700 nm and the reducing power of the sample is driven from the standard curve (Anandjiwala et al., 2008).

2.7. Metal chelating activity method

Some antioxidants have portent metal-chelating potentials. The principle of this assay relies on the ability of the sample to compete for ferrozine, which is a chemical that can bind to Fe^{2+} and produces a red color. The decrease in the red color at 562 nm indicates a Fe^{2+} chelating potential of the sample (Gao et al., 2007). To conduct the analysis, 0.1 ml of different concentrations of the sample extract are mixed with 0.5 ml of 0.2 mM $FeCl_2$ solution followed by the addition of 0.2 ml of 5 mM ferrozine. The whole mixture is incubated for 10 min at room temperature and the absorbance is

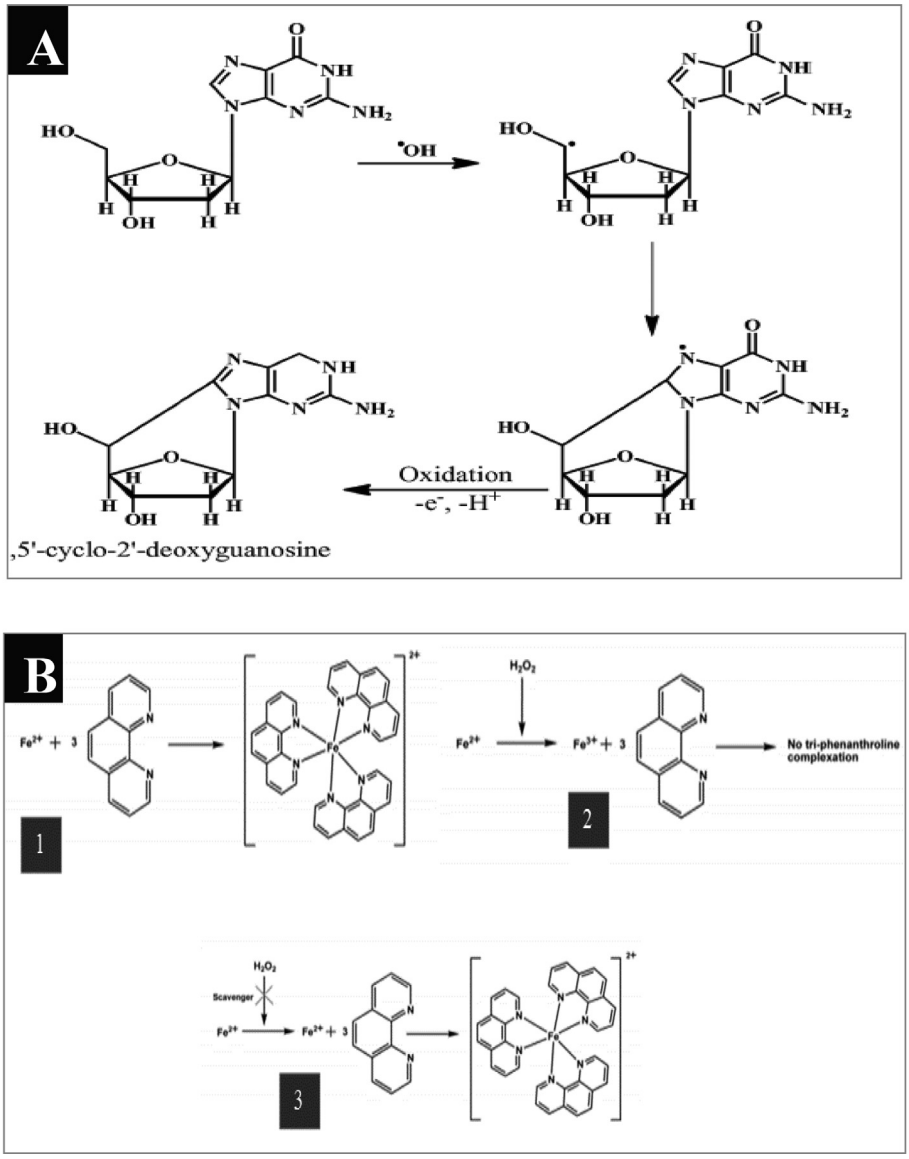

Fig. 4. A: Reaction of hydroxyl radical with the sugar moiety of DNA, **B:** Reaction pathway leading to the detection of hydrogen peroxide scavenging ability by antioxidants.

read at 562 nm. The metal chelating activity of the extract (%) is as = [($A_{control}$ –$A_{sample\ (standard)}$)/$A_{control}$ × 100].

2.8. Hydroxyl radical scavenging assay

The hydroxyl (HO•) radicals are the most damaging free radicals in the cells that are produced through a Fenton reaction mediated by the interaction of H_2O_2 with Fe(II) (Zhu et al., 2000; Dunford, 2002) . The presence of Fe (II)/H_2O_2 conjugate is not ideal for the scavenging assay ass some antioxidants can chelate Fe (II) **(**Fig. 4**A)**. Therefore, it will be very difficult to know if the antioxidant activity of the sample is mediated by scavenging HO• or chelating Fe (II) (Bossmann et al., 1998). Ascorbic acid (AA) may act as a HO• generation catalytic by reducing Fe(III) to Fe(II) (Huang et al., 2005). To measure the HO• scavenging activity of a sample, 1 ml of different extract concentrations (standards) are mixed with 1 ml Fe-EDTA solution (13% $FeH_8N_2O_8S_2$ & 0.26% EDTA), 0.5 ml of 0.22% AA, 0.5 ml of 0.018% EDTA, and 0.1 ml of 0.1% DMSO diluted in PBS pH = 7.4). The whole mixture is heated at 90 °C for 10–15 min and the reaction is stopped by the addition of 1 ml of 17.5% ice-cold trichloroacetic acid (TCA). Then, 3 ml of the NASH reagent (75 g of NH4CH3CO2, 3 ml of glacial acetic acid, 2 ml of acetylacetone, and distilled water (to 1 L)) is added to the mixture and the whole setting is incubated at room temperature until the yellow color develops. The absorbance is read at 412 nm. The HO• scavenging ability of the extract is calculated as the percentage of inhibition using Gallic acid as a standard.

2.9. Hydrogen peroxide (H_2O_2) scavenging assay

The daily human exposure rate to H_2O_2 is approximately 0.28 mg/kg/day mainly from the leaf crop intake (Duttagupta et al., 2015). Besides, other routes of H_2O_2 exposure include inhalation and skin (Duttagupta et al., 2015). H_2O_2 is the major source of OH• radicals through Fenton's reaction **(**Fig. 4**B)** (Alam et al., 2013). To determine the sample H_2O_2 scavenging capacity 100 ml of 0.05% (w/v) of the sample is mixed with an equal volume of 0.002% H_2O_2 followed by the addition of 100 mM NaCl and 0.8 ml of 0.1 M phosphate buffer (Na_2HPO_4: KH_2PO_2) and incubation at 37 °C for 10 min. To this mixture, phenol red dye (0.2 mg/ml) and horseradish peroxidase (HRP) (0.1 mg/ml, prepared in 0.1 M phosphate buffer) is added and incubated for another 15 min at the same

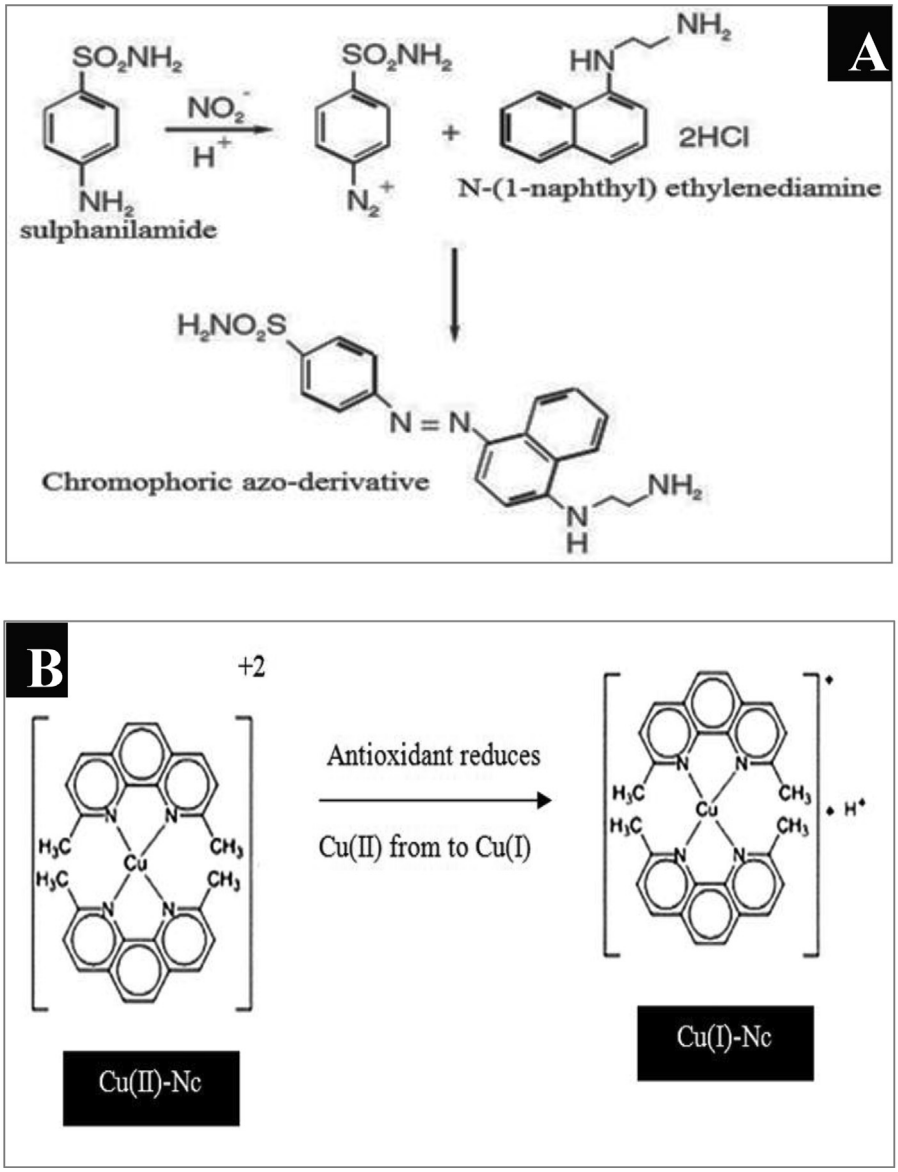

Fig. 5. A: The primary reaction of nitric oxide scavenging activity, **B:** CUPRAC ion reducing antioxidant capacity.

temperature. After that, the reaction is terminated by the addition of 1 ml of 1 M NaOH, and absorbance is read at 610 nm. The H_2O_2 scavenging ability of the sample (%) is as = $[(A_{control} - A_{sample\ (standard)})/A_{control} \times 100]$.

2.10. Nitric oxide scavenging activity

Nitric oxide (NO) can react with other free radicals to produce highly toxic peroxynitrite (NOO–) radicals (Radi, 2018). The capacity of a sample to scavenge NO can be measured using the Griess-llosvay reaction (Benevides Bahiense et al., 2017). The test relies on the decomposing of sodium nitroprusside to NO (at physiological pH) which can react with O_2 to produce a stable compound, which can be further determined by the Griess reagent **(**Fig. 5**A)**. In the presence of NO scavenging molecules, the production of nitrite ions is reduced and so the absorbance (Kumaran and Karunakaran, 2007). For this assay, different concentration of the sample (μg/ml) (standard, ascorbic acid (AA)) is mixed with 10 mM sodium nitroprusside solution (prepared in PBS/pH = 7.4) and the whole mixture is incubated for 2 h at 30 °C. Then, 0.5 ml of Griess reagent containing 2% H_3PO_4, 1% sulfanilamide, and 0.1% N-(1-Naphthyl) ethylenediamine is added to all reaction tubes, and the produced color is read at 550 nm. The NO scavenging ability is calculated as a percentage of inhibition from the standard curve (Nabavi et al., 2010).

2.11. Cupric ion reducing antioxidant capacity (CUPRAC)

CUPRAC measures the antioxidant potential of a sample by measuring the rate of conversion of Cu^{+2} to Cu^{+1} in the presence of neocuproine (Nc) or athocuproine (BC) (chelating agents). In the test, the chelators complex with the Cu^{+1} containing compound to yield a stable color that can be read at 450 nm **(**Fig. 5**B)**. It is one of the best tests to measure the antioxidant potential of the thiol-group and plasma antioxidants like AA, β-carotene, bilirubin, tocopherols, and albumin. For the analysis, the following solution should be prepared: 1×10^2 M $CuCl_2$ (in water) 1 M ammonium acetates (NH_4Ac) buffer (pH = 7), 7.5×10^3 M Nc (in absolute ethanol), and Trolox (standard) 1×10^3 M (in absolute ethanol). For the assay, add 1 ml of each of the prepared stocks of $CuCl_2$, NH_4Ac buf-

fer, and Nc to a test tube followed by the addition of (x) ml standard or antioxidant sample (x ml). The final volume of each sample was made to 4.1 ml by the addition of water. The whole mixture is incubated for 30 min and then the absorbance is read at 450 nm and the antioxidant reducing activity is calculated from the standard curve.

2.12. Xanthine oxidase (XO) inhibition potential

The superoxide radicals (O•) savaging abilities of antioxidants can be determined enzymatically and non-enzymatically (Karadag et al., 2009). Under the physiological stable condition, the normally expressed XO in the tissue is a dehydrogenase that moves electrons to NAD (Rivero-Pérez et al., 2007). Under stress, XO becomes a potent oxidase that generates large quantities of H2O2 and O• radicals (Rivero-Pérez et al., 2007). The activity of XO is measured spectrophotometrically at 295 nm by measuring the formation of uric acid from its substrate, xanthine (Table 1) (Noro et al., 1983). To perform the analysis, the following components: 1.0 ml of the sample (prepared in appropriate solvent), 1.9 ml of PBS (pH = 7.4), 0.1 ml (0.045 U/ml) XO solution, and 1.0 ml of 100 μM xanthine solution are added to each other and inculcated for 15 min at 25 °C. The reaction is terminated by the addition of 1 ml of 1 M HCl and was read against the blank solution (no enzyme) at 295 nm. The inhibitory activity of the antioxidant on XO (%) is calculated as = $[(A_{control} - A_{sample\ (standard)})/A_{control} \times 100]$.

2.13. N,N-Dimethyl-p-phenylenediamine dihydrochloride (DMPD)

In the availability of Fe^{3+} and under acidic conditions, DMPD produces a highly reactive radical, $DMPD^{\bullet+}$ radical that has a stable purple color which can be detected at 514 nm. This radical can scavenge the antioxidant in the sample (AOH) which transfers an electron to the $DMPD^{\bullet+}$ radical, thus decoloring the solution Table 1 (Verde et al., 2002; Talaz et al., 2009). However, since the plasma contains high levels of Fe^{3+}, this method is not recommended to detect the antioxidant capacity in this blood fraction (Talaz et al., 2009). Therefore, the decrease in the light intensity (absorbance) is correlated negatively with the concentration of the antioxidant in the sample. In a reaction tube, 100 ml of 0.1 M $C_2H_3NaO_2$ (sodium acetate) buffer (pH 2.25) and 0.4 ml of 0.05 M $FeCl_3$ solution, and 1 ml of 200 mM DMPD solution are mixed and kept in dark for 10 min at 4 °C. Then, 50 μl of the diluted samples or standard (Trolox) is added to 950 μl of the above mixture containing $DMPD^{\bullet+}$ radical and the absorbance is read immediately at 514 nm. The antioxidant capacity of the samples is driven by the Trolox standard curve.

2.14. Thiobarbituric acid (TBARS) method

This method is very commonly used with dietary sources, plasma, cell cultures, and tissues. TBRAS are by-products that are commonly formed during the process of lipid peroxidation (Ghani et al., 2017). The test is based on the formation of HO• by a Fenton reaction between H2O2 and Fe–EDTA complex which degrades benzoate resulting in the release of TBARS. The decrease in the formation of TBARS reflects the antioxidant activity of the sample (Koracevic et al., 2001). For the measurement of the antioxidant activity using this method, 10 μl of the sample (or uric acid (UA), 1 mmol/l prepared in 5 mmol/l NaOH), 490μ ml of the Na2HPO4 (100 mmol/l, pH 7.4), 500 μl of Na-benzoate (1o mmol/l), 200 μl of Fe-EDTA complex (Fe(NH4)2SO4 (2 mmol/l) mixed with an equal volume of EDTA solution (2 mmol/l in Na2HPO4 buffer/pH = 7.4), 200 μl of H2O2 (10 mmol/l) are mixed °C and incubated for 60 min at 37°C. Then, 1 ml of acetic acid and 1 ml of TBA (0.8% v/v prepared in 50 mmol/l NaOH) are added to the blank (no sample or uric acid), standard, and sample tubes and incubated for 10 min in boiling water path. The tubes are then cooled in ice and the absorbance is read at 532 nm. The activity of the antioxidant in the sample (nmol/l) is calculated as = $[(A_{control} - A_{sample\ (standard)})/A_{control} \times 100]$ (Koracevic et al., 2001).

2.15. Ferric thiocyanate method

This method studies the ability of antioxidants to prevent the peroxidation of linoleic acid (LA) in the presence of ammonium thiocyanate and TBA. This method detects and measures the peroxides, which form during the early phase of lipid peroxidation in which the peroxides are allowed to react with FeCl3 where the Fe3 + ions combine ammonium thiocyanate to yield ferric thiocyanate which has a stable red color that can be measured at 500 nm (Huda-Faujan et al., 2009; Jiang et al., 2017) . This method is very common for food samples. For the analysis, the sample and LA are dissolved in ethanol at a final concentration of 0.02% and 2.51% (v/v), respectively, 4 mg of the diluted sample is mixed with 4.1 ml of the LA in 8 ml of 0.02 M phosphate buffer (pH 7.0). The volume of the reaction is made 20 ml by the addition of 3.9 ml distilled water. The whole mixture is heated for 10 min at 40 °C (in dark). Then, 100 μl of this mixture is mixed with 100 μl 0.02 M ferrous chloride (prepared in 3.5% hydrochloric acid), 100 μl ammonium thiocyanate, and 9.7 ml of 75% ethanol and the absorbance is read at 500 nm. Gallic or ascorbic acids can be used as standards. The percent of inhibition (antioxidant activity) (%) is calculated as = $[(A_{control} - A_{sample\ (standard)})/A_{control} \times 100]$.

2.16. Phosphomolybdenum method

The tests depend on the potential of an antioxidant to convert (reduce) the phosphate-Molybdenum (VI) to Phosphate-Molybdenum (V) which forms a stable green color that is read at 695 nm (Alam et al., 2013). It is a valid method to be used with crude extract for the detection of carotenoids, ascorbic acid, and α-tocopherol (Alam et al., 2013). For the analysis, the sample (1 ml/100 μg) is mixed with 1 ml of the working reagent composed of 4 mM ammonium molybdate, 28 mM sodium phosphate, 0.6 M sulfuric acid. The whole mixture is incubated for 1.5 h at 95 °C in a water bath, cooled at room temperature, and the absorbance is read at 695 nm against the blank (no ample). Ascorbic acid can be used as a standard and the concentration of the antioxidant in the sample is calculated from the standard curve (mol/g) as equivalent to ascorbic acid.

2.17. Oxygen radical absorbance capacity (ORAC)

This assay measures the decrease in the fluorescence of the fluorescein probe which detects peroxy radicals generated by 2,2-azobis2-amidopropane dihydrochloride (AAPH) due to free radicals scavenging by the antioxidant (Table 1) (Prior et al., 2003; Alam et al., 2013) in the presence of free radical scavengers (Prior et al., 2003). This test is usually performed in commercially available 96-well polypropylene fluorescence plates and the decreases are fluorescence is read at excitation/emission 485/520 nm every 1 min for 35 min. Different concentrations of Trolox are used as standards and the antioxidant activity (concentration) is calculated from the standard curve or the area under the curve.

2.18. Superoxide radical ($O2^{-}$) scavenging activity

This test depends on the ability of an antioxidant to scavenge the $O2^{-}$ radical in a test tube based on the oxidation of NADH

and the concomitant assay of the deduction of nitroblue tetrazolium (NBT) (Robak and Gryglewski, 1988; Chun et al., 2003). Briefly, the reaction mixture contains 1 ml of the extract, 0.5 ml of 0.936 ml NADH, 0.5 ml nitroblue tetrazolium (NBT), and 1 ml Tris-HCl buffer (pH = 8). The reaction is started by the addition of 0.5 ml of 0.12 mM phenazine methosulfate. The whole mixture is incubated for 5 min at room temperature and the absorbance is read at 560 nm against the blank (no sample). Ascorbic acid can be used as a control. Superoxide radical scavenging activity (%) is calculated using the following equation = $[(A_{control} - A_{sample\ (standard)}) / A_{control} \times 100]$.

2.19. Total radical-trapping antioxidant parameter (TRAP)

TRAP is the best test to be used for plasma samples. The TRAB method relies on preserving the decomposition of fluorescence of the R-phycoerythrin (R-PE) by ABAP (2,20-azo–bis(2-amidino-pro pane) hydrochloride) (a radical generator). The antioxidant activity of the extract is proportional to the decoloration of the product (Jacobs et al., 1999). TRAP may be either directly measured by a fluorescence-based method (TRAPm) or calculated (TRAPc) by a mathematical formula, considering the serum levels of four natural antioxidants (Ceriello et al., 1997). To measure antioxidant activity by this method, the 120 μl of the sample (standard) is mixed with the reagent mixture containing 75 μl of ABAP, 30 μl of diluted R-PE, 375 μl of distilled water, and 2.4 ml of phosphate buffer (pH = 7.4). The reaction is monitored over 45 min at 38 °C using a luminescence spectrometer. TRAP values are calculated from the standard curve.

2.20. Peroxynitrite radical scavenging activity

Peroxynitrite ($ONOO^{\bullet}$) free radicals are the most active and damaging free radicals in the cells, which are usually produced from the interaction of superoxide radicals with the NO. $ONOO^{\bullet}$ is the leading cause of many chronic disorders in humans (Alam et al., 2013). The method used to evaluate the $ONOO^{\bullet}$ scavenging activity of a sample is based on the oxidation of dihydroxyrhodamine 123 (DHR 123). The decrease in the fluorescent signal (of inhibition) is an indicator of the $ONOO^{\bullet}$ scavenging activity of the sample (Kooy et al., 1994).

2.21. Other assays of antioxidant activity of dietary sources

In addition to the above-mentioned assays, the literature also mentioned many other assays to measure the antioxidant capacity/activity in food, plant extract, and biological fluids (Table 2).

2.22. In vivo methods

However, in this review, details about *in vitro* methods and the techniques which are used for antioxidant activity of the dietary sources have been reported as the main topic, but here summarised information about the *in vivo* models (Table 3). For the majority of the *in vivo* methods, the antioxidant potential is measured by the administration of the dietary sources, food supplement, drug, and plant extract to the experimental animals intraperitoneally, subcutaneously, and orally (Alam et al., 2013). The antioxidant potential of the active ingredients of the crude extract is determined in the tissues by measuring different parameters including total antioxidant capacity, ferric reducing ability of plasma, serum and tissue levels of ROS, MDA, TBARs, ox-LDL, and glutathione (GSH) (total, reduced, oxidized), as well as the serum and tissue expression/levels/and activities of antioxidant enzymes such as glutathione-s-transferase, catalase, superoxide dismutase (SOD), thioredoxin1/2, etc. (Badarinath et al., 2010).

Table 3
The most *in vivo* antioxidant activity methods is used in the dietary sources.

Method	Mode of action	End-product determination
Glutathione-s-transferase Reduced glutathione estimation Ferric reducing ability of plasma Superoxide dismutase γ-Glutamyl transpeptidase Catalase assay LDL assay Lipid peroxidation assay Glutathione peroxidase estimation Glutathione reductase	Testing animals	Blood or tissues test

3. Conclusion

Mode of action, mechanism, and the *in vitro* determination of antioxidant activity/capacity from dietary sources, food, and biological samples were the focus in this review article, which were adopted from previous studies. The application of any of the mentioned methods is a useful method to measure the antioxidant activity of the samples some methods are restricted to some samples and others can be applied to any samples. However, the variation in these methods varied according to the measurement of the product. This research seeks to provide an update of a brief overview of the mode of action and determination of antioxidant activity in the dietary sources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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