The Principle of Some \textit{In vitro} Antioxidant Activity Methods: Review

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Abstract. The antioxidant is defined as any substance that could delay or prevent oxidative damage by free radicals. The antioxidant inhibits the oxidative damage by free radicals through several mechanisms. The sources of the antioxidant are abundantly available in nature and can be found in the daily diet such as fruit, vegetables, seeds, nuts, leaves, roots, and barks. Several major compounds identified as antioxidants such as polyphenols, vitamins, and carotenoids. This article provides a general summary of the most common \textit{in vitro} methods for determining antioxidant activity. It emphasizing the working principle, methodology, advantages, and disadvantages of different methods. The determination of antioxidant activity could not only follow one single approach due to the diversity of antioxidant mode of actions. Therefore, the choices of the appropriate method of analysis need to be considered to achieve the purpose of the research.

1. Introduction
The report of World Health Organization (WHO) mentioned the burden of non-communicable diseases (NCDs) (e.g., cancer, cardiovascular diseases, chronic respiratory diseases, neurodegenerative diseases and diabetes) in many countries, as their numbers are gradually increasing [1]. It accounts for almost two-thirds of annual mortality, especially in low- and middle-income countries [2]. The NCDs have been linked with unhealthy lifestyles such as imbalanced diet (high calories and fat intake) and moderate physical activity. Besides, the occurrence of NCDs has been associated with oxidative stress, where the production of free radicals [reactive oxygen species (ROS) and reactive nitrogen species (RNS)] and the biological antioxidant defense system is not proportional [3]. ROS include superoxide anion radical (O$_2^-$), singlet oxygen (O$_2^+$), hydroxyl radical (·OH) and perhydroxyl radical (HO$_2^-$) [4]. RNS include radical nitrogen oxide (NO), nitrogen dioxide (NO$_2$), and non-radicals S-nitrosothiols, peroxynitrite (ONOO$^-$), nitroxy anion (NO$^-$), nitrate (NO$_3^-$), nitrosonium cation (NO$^+$), dinitrogen trioxide (N$_2$O$_3$), dinitrogen tetroxide (N$_2$O$_4$), nitril chloride (NO$_2$Cl), and nitrous acid (HNO$_2$) [5]. The free radicals are unstable, reactive, and tend to capture other stable molecules. Consequently, if the number of free radicals is in high concentration, the oxidative tissue damage can occur in the body [6].

The antioxidant, mentioned by Halliwell and Gutteridge, is the chemical compounds that appears in small number compared to the oxidant receptors, have function to inhibit, postpone, prevent or counteract the damaging effect of oxidation to substance [7]. The antioxidant correspond to the free radicals and countering the damaging effect through several pathways such as removing radicals that ignite the peroxidation, prevention of reactive species initiation or peroxides decomposition by
binding the metal ions, inhibition of peroxides formation through quenching anion superoxide (\(\cdot \text{O}_2^-\)), cut off the auto oxidation chain reaction, and/or reduction of oxygen (\(\text{O}_2\)) concentrations [8]. In the last few years, there are numerous attempts to reduce the NCDs occurrence through the intake of antioxidant-containing diet. The natural sources of antioxidant are commonly found in plants and be part of a daily diet, e.g. fruits, vegetables, seeds, nuts, leaves, roots, and barks. Vitamins and secondary metabolites from plants, namely polyphenols, are the primary class of chemical compounds with free radicals quenching properties [9]. There are substantial shreds of evidence of diet-containing antioxidant may be of significant importance in NCDs prevention [10].

Numerous papers are reporting the investigation of antioxidant efficacy of the various samples of interest using the \textit{in vitro} methods. Through several modes of actions, the effectiveness of compounds with antioxidant activity supposed to be measured by multiple \textit{in vitro} assay [10]. Generally, this approach of antioxidant tests using free radical traps is relatively straightforward, simple, rapid, and relatively inexpensive to perform [10]. Many researchers have developed the assays based on their inhibition mode of actions, such as DPPH scavenging activity, hydroxyl radical averting capacity (HORAC), cupric ion reducing antioxidant capacity (CUPRAC), trolox equivalence antioxidant capacity (TEAC); total radical trapping antioxidant parameter (TRAP); reaction of substances with thiobarbituric acid (TBARS), antioxidant reduction power of ferric ion (FRAP), and absorbance capacity of oxygen radicals (ORAC). The principle of the most common \textit{in vitro} antioxidant methods that are used for the measurement of antioxidant activity is summarized in this paper.

2. \textit{In vitro} antioxidant activity
The complexity of \textit{in vitro} antioxidant methods through several mechanisms of actions makes the comparison of each antioxidant method impossible. However, the \textit{in vitro} antioxidant approach can provide a measurement of the effectiveness of compounds. Generally, the assessment of \textit{in vitro} antioxidant activity can be divided into two main mechanisms. The first category is assessment in relation to free radicals transfer (hydrogen atom transfer, single electron transfer, or the combination of both). The second category is related to the evaluation of the damaging effect on biological markers and substrates, which is based on lipid peroxidation [7].

3. Discussion

3.1. DPPH (2,2-diphenyl-1-picrylhydrazyl) Scavenging Activity
DPPH is the most popular free radical for the study of \textit{in vitro} antioxidants according to data obtained from the PubMed database, where there are more than 13000 studies employ DPPH till July 2019. The \textit{in vitro} method using DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) is an antioxidant assay based on scavenging of the DPPH by antioxidant [11]. In its radical form, DPPH shows an active absorption band at \(\lambda_{\text{max}}\) 515-517 nm. Upon reaction of the antioxidant with DPPH, the DPPH accept the hydrogen donor, and the solution loses its color from purple to pale yellow. Solvent properties and pH mainly influence the scavenging activity. The protic solvents with strong hydrogen bonds such as the mixture of water, methanol, and ethanol are typical solvents for analyzing hydrophilic and hydrophobic antioxidants [12]. The assay is a rapid, simple, reproducible, and comparable to other antioxidant methods such as ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid). It can also be used to quantify antioxidants in complex biological systems, in either solid or liquid samples, such as fruit juices, vegetables, milk, and edible seeds oil [12,13]. Nevertheless, it should be noted that the quantification of the antioxidant must consider the effect of solvents used in order to avoid false-positive results [10].

3.2. FRAP (Ferric Reducing Ability Plasma)
Based on PubMed, the FRAP method has been described and used to measure the antioxidant efficacy in 3300 papers. In this method, the evaluation of antioxidant activity is characterized by the
calculation of the low pH mixture of iron (III) and 2,3,5-triphenyl-1,3,4-triazolo-2-azoniacyclopenta-1,4-dienechloride (TPTZ) reduction to its ferrous form (II), change the solution into violet-blue color. The changes was evaluated using spectrophotometer at the wavelength of 593 nm [14]. It has been applied to detect the antioxidant activity of honey [15]. FRAP method has some limitations. It is unable to accurately detect the antioxidant activity of slow-reacting compounds such as polyphenols, unable to measure samples below physiological pH values (pH 3.6), and could give false-positive results if the samples have lower redox potential value than the redox pair Fe$^{3+}$/Fe$^{2+}$[15].

3.3. **TEAC (Trolox Equivalent Antioxidant Capacity)**

The data searching from PubMed shows that the TEAC assay has been utilized for measuring the antioxidant capacity, approximately 1100 papers. The principle of this method is based on the scavenging of stable ABTS' (2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) radical by antioxidants in comparison with Trolox. The reduction causes the color loss of ABTS. The color loss is measured at $\lambda_{\text{max}}$ 750 nm. Generally, the polar analog of vitamin E, namely Trolox, is used to be the standard of the antioxidant calculation, and the result is interpreted as Trolox equivalents. The advantages of this method are simple, reproducible, a wide range of samples (e.g. sorghums and sorghum products), a wide range of pH, and a wide range of samples polarity (hydrophobic and hydrophilic compounds) [16]. However, it should be noted that the different reaction rates between the antioxidants and ABTS radical, cause the different reaction times for the complete measurement of antioxidant capacity [17].

3.4. **ORAC (Oxygen Radical Absorbance Capacity)**

ORAC is one of the popular methods for determining the antioxidant activity according to the data from PubMed, where there are 1600 papers conducting this method. The measurement of antioxidant activity is based on the scavenging activity of oxyradical-induced oxidation of 2,2'-azo-bis-(2-methylpropionamidine) dihydrochloride (AAPH) by samples with antioxidants. In this method, phycoerythrin or fluorescein is used as a target molecule. Over time, the fluorescent signal of these compounds will be lost when the antioxidant compounds appear. When the solution tested containing antioxidant properties, the fluorescent reduction is inhibited and measured at the excitation wavelength of 485 nm and emission wavelength of 520 nm [10,16]. The test uses Trolox as a reference standard, and the values usually expressed as Trolox Equivalent (TE). The antioxidant capacity results have a correlation with the total fluorescence area under reaction curves where the values of antioxidant are measured by subtracting the area with the blank, which contain no antioxidant [18]. ORAC method is suitable to be conducted in a microtiter plate with constant temperature for automation of experiments in screening of biological samples matrices, relevant to in vivo conditions, considers both inhibition time and degree of inhibition of free radical action, and compliant for various sample matrices, including food (e.g., sorghum, orange juice, blueberries, nuts), pharmaceutical, and blood plasma [16,19]. However, some difficulties occur when applying this method such as the control of ORAC reaction temperature, reagent concentration, and oxygen [20].

3.5. **HORAC (Hydroxyl Radical Averting Capacity)**

The working principle of this assay is the measurement of the antioxidant activity by the calculation of the inhibitory activity of antioxidants against the oxidation of the fluorescent probe (fluorescein) by hydroxyl radicals. This radical is produced through the radical initiation process by the catalyzer hydrogen peroxide ($H_2O_2$) and Fenton reagent. Over time, the radicals bind the fluorescein, until the appearance of antioxidant, gradually preventing the oxidation of the probe [21]. The standard of an antioxidant such as gallic acid is used to make the calibration curve. The value of antioxidants is then compared and correlated to the total area fluorescence decay curve (AUC) of the samples. The strong point of this assay is that the value of antioxidant capacity is directly calculated against the hydroxyl radicals produced from the breaking of the hydrophilic chain [22]. This method has been utilized for
determining the antioxidant activity of vegetables (tomato, parsley, celery, chili pepper, radish, capsicum, eggplant, broccoli) [31].

3.6. CUPRAC (Cupric Ion Reducing Antioxidant Capacity)
CUPRAC is one of electron-transfer based assay for measuring the antioxidant capacity. The method is based on the utilization of the pigment-oxidizing agent (chromogenic), namely bis-copper (II) neocuproine. When the antioxidants mix with the reagent, the reduction of chromogenic probe occurs and changes the color of the solution. The degree of color change is correlated with the concentration of antioxidants in the sample. The change is measured at a wavelength of 450 nm [23]. The CUPRAC method is simple and adjustable. It is beneficial for high-throughput screening of the antioxidants in matrices of food (egg white, whey protein, gelatin) and blood serum. It was also applicable to measure the hydrophilic and lipophilic antioxidant, due to the high solubility of the reagent in a polar and non-polar solvent [24]. In addition, CUPRAC assay works at physiological pH (pH 7) compared to another the electron-transfer assay such as ABTS and FRAP [18].

3.7. Total Radical Trapping Antioxidant Parameter (TRAP)
The TRAP method was firstly described by Wayneret al. to measure the total antioxidant capacity of blood plasma or serum [25]. This method is based on the generation of peroxy-radicals from 2,2′-azobis(2-aminopropionitrile) hydrochloride (AAPH). The surface of the oxygen electrode can be used to monitor the lag time of oxidation. The induction period of AAPH was measured to determine the ability of antioxidants to inhibit the oxidation. Then, the total antioxidant capacity of samples was measured by a comparison of the time interval of the reaction induction and the interval time generated by the reference compound, Trolox. It has been applied to measure the antioxidant activity of vegetables, berries, apple, banana, pepper, and green bean [32]. This method, however, has a significant drawback, such as lack of oxygen electrode stability. Therefore, some modification has been done by the addition of chemiluminescence such as R-phycocerythrin. This pigment is able to give a brief point where the reaction reaches the completion. The addition of the antioxidants could quench the chemiluminescence in the system [26].

3.8. Folin-Ciocalteau
This method measures the total antioxidant capacity in the samples based on the electron-transfer, which reduces the Folin-Ciocalteau (FC) reagent (phosphowolframate–phosphomolybdate complex) from antioxidant sources to form blue chromophore. This blue color exhibit a broad light absorption with a maximum wavelength at 765 nm. The higher of phenols concentration in the system, the greater intensity of light absorption. The common reference standard used in this method is gallic acid. Total antioxidant capacity could be calculated, and then expressed as Gallic acid equivalents (GAE). Initially, the application of this method is for the determination of protein content. Since then, the FC method is widely extended to determine the total phenolic content in food matrices, for both water-soluble and lipophilic phenolic compounds (e.g. fresh fruit, vegetables, fruit juices) [27,33]. It should be noted that the method is not specific for phenols because any reducing agent will get a good result of antioxidant capacity [28].

3.9. TBARS (Thiobarbituric Acid Reactive Substances)
TBARS is widely known as an assay for measuring the inhibition of lipid peroxidation product by antioxidantin vitro. This assay measures the susceptibility of the samples to the peroxy radical-induced by Cu²⁺/H₂O₂. When peroxidation occurs, the lipid peroxides and peroxy radicals are formed subsequently and decompose the lipid to create aldehydes and derivatives, such as 4-hydroxy-2-nonenal, malonic aldehyde (MDA), and hexanaldehyde [29]. This metabolite will react with TBA (thiobarbituric acid) and form red or pink chromophore. The changes of color are then measured at the wavelength of 530-540 nm. TBARS assay is comparable with other assays because it needs simple instrumentation, good reproducibility, adaptable for measuring lipophilic and hydrophilic antioxidant,
and suitable for running ‘high-throughput’ analysis [30]. It has been used for determining the antioxidant activity in fried fast food, essential oils, and medicinal plants [34,35,36]. However, some drawbacks of this method have been described such as absorbance interference by samples containing aldehydes and sugars, unsuitable for measuring lipid peroxidation in vivo, takes longer time, and more complex preparation [29,30].

4. Conclusion
This review is focused on the most common method of in vitro antioxidant activity. Each method has its advantages and disadvantages, depends on the characteristic of sample materials and mode of action of antioxidants. There is no single approach could describe all antioxidant activity. The evaluation of antioxidant activity should be carried out using several methods to measure the diverse inhibition mechanism of free radicals. Researchers have to critically consider the method of the antioxidant analysis before carried out that for the research purpose.

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