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Considerations of Choosing Antioxidant Assays: A Review

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Abstract:*Antioxidant activity assay is conducted to determine the ability of drug or plant extract to scavenge free radicals. Several methods are often used based on the mechanisms, which include the Oxygen Radical Absorbance Capacity method, Total Radical-trapping Antioxidant Parameter method, Ferric Reducing Antioxidant Power method, DPPH (α, α-diphenyl-β-picrylhydrazyl) method, and Folin-Ciocalteu method. These methods certainly have limitations and predominances. However, most of the limitations are caused by the environments support. Moreover, the interference of various substances could also affect the measurements. For the predominances, every method have their characteristics.*

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I. Introduction

Free radical compounds are the byproducts of the body's regular metabolism such as cell metabolism, arachidonic acid metabolism, phagocytosis, and fertilization. Free radicals can cause oxidation that triggers membrane and DNA damage, modification of protein in the body, and cell death because of lipid peroxidation and the fragment of the DNA. In addition to being obtained from the byproducts of normal metabolism, free radical compounds can also be obtained from external factors such as living air, food consumed and other exposures in the surrounding environment[1].

In aerobic metabolism free radicals can be made when the body do some regular processes such as apoptosis, gene expression and also proliferation. Because when these processes excess, free radicals can be made from the antoxidant system by oxidizing cell proteins, DNA, enzymes and membrane to defense the radicals[2].

When the level of free radicals continues to increase in the body due to external influences, the defense system in the body, e.g., endogenous antioxidants, will no longer work effectively as a protector of the free radical attacks, and subsequently will lead to oxidative stress. Thus, antioxidant supplements are needed to prevent this oxidative stress[3]. Antioxidants, often termed as reductors, can react with free radicals and neutralize free radicals[4].

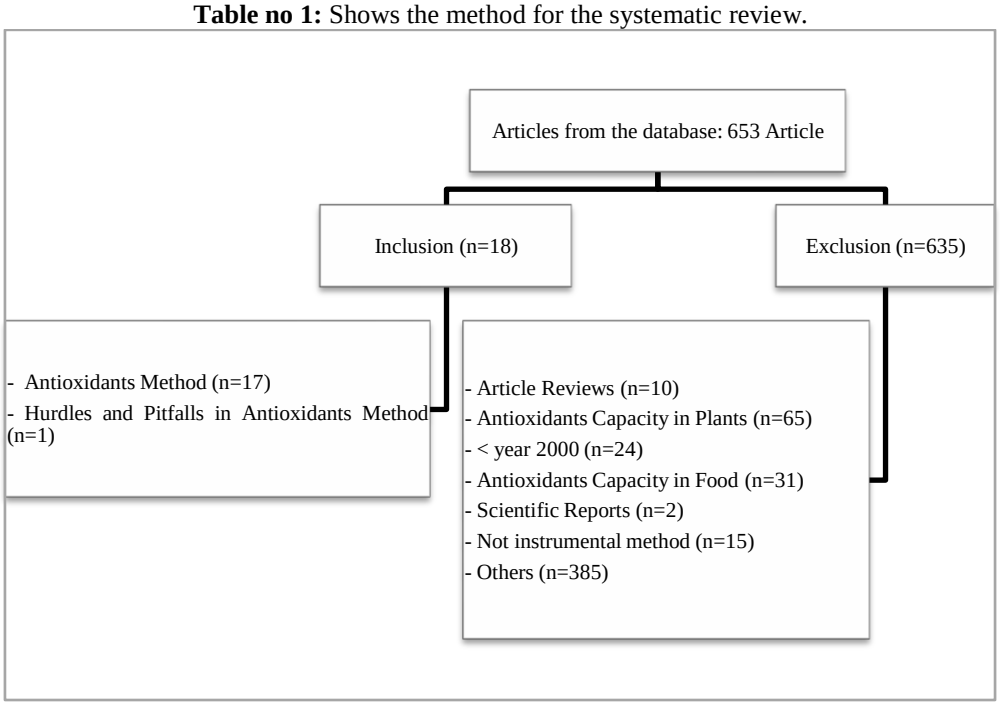
Antioxidants can be obtained either synthetically or naturally. Natural antioxidants are derived from plants. The antioxidant activity can be assayed by using several methods, e.g., the ORAC method, TRAP method, FRAP method, DPPH method, and Folin-Ciocalteu method. These methods are classified based on their mechanisms, e.g. (1) Hydrogen atom transfer or HAT mechanism (ORAC method and TRAP method); and (2) Single-electron transfer (SET) mechanism (FRAP method, DPPH method, and Folin-Ciocalteu method)[5].

This article reviewed the methods of antioxidant activity assays and the considerations in selecting the most appropriate method for plant extracts.

II. Methods

This review included studies published in PubMed database obtained using the keywords “Comparison antioxidant assays[All Fields]” that showed 55 articles [8 included, 48 excluded (35 antioxidants capacity in plants, 13 others)], keywords “Determination[All Fields]”, “Antioxidant[All Fields]”, “Capacity[All Fields]” that showed 598 articles [10 included, 588 excluded (10 reviews, 30 antioxidant capacity in plants, 24 <2000, 31 antioxidants capacity in food, 2 scientific reports, 15 not an instrumental method, 375 others)].

Table no 1: Shows the method for the systematic review.



III. Result

The antioxidant can be defined as a compound that giving their electron to the free radicals or to the oxidant. By giving its electron, it can work to inhibit the oxidant in the substances. Antioxidants stabilize free radicals by complementing the lack of electrons possessed by free radicals, and also the chain of radical formation got inhibitted inhibiting[6]. The excellence works of antioxidants is generally caused by several factors, including temperature, the structure, concentration from its antioxidant, type of substrate that got oxdized and also physical state[7].

Antioxidants have structure that determines its intrinsic reactivity to free radicals and other ROS/NOS and delivering antioxicand capacity. The efficient values from antioxidant also depends on the location in the system also the concentration[8, 9].

Table no 2: General sources of Antioxidants[5].

Antioxidants			
Non Enzymatic			Enzymatic
Large Molecules	Small Molecules	Hormones	
e.g. Ferritin, albumin, ceruloplasmin.	e.g. Uric acid, ascorbic acid, gluthatione, tocopherol, polyphenols, carotenoids.	e.g. Melatonin, sterogen, angiotensin.	e.g. glutathione peroxidase, superoxide dismutase, and catalase.

There is a lot of functions that antioxidant have, one of them is protect oils and lipids in food due the oxidative degradation, because when antioxidant is added to food it will control the rancidity development, maintain the quality of nutrients, prevent the toxic oxidant to formed, and also the shelf-life of the product will extend. But synthetic antioxidants should be limited to be used because of some safety considerations.

Hydrogen Atom Transfer (HAT) Mechanism

The hydrogen atom from the antioxidant will be donated to quench the free radicals, and can be explained by the reaction:

$$X^* + AH \rightarrow XH + A^*$$

In this kind of mechanism, the method is not dependent in solvent nor pH. The reaction will also be completed in seconds to minutes. However, in this mechanism, if there are any reducing agents founded, including metals, the reaction will get a complication[5].

Table no 3: Limitations of Hydrogen Atom Transfer Mechanisms

No.	Method	Limitations
1	Oxygen radical absorbance capacity (ORAC)	Temperature sensitive[10]. Time-consuming, need a specific instrument, limited to measure lipophilic chain[5].
2	Total radical-trapping antioxidant parameter (TRAP)	Complex, time-consuming, need a high degree of expertise, too many end points in the measurements[5].

Table no 4: Predominances of Hydrogen Atom Transfer Mechanisms

No.	Method	Predominances
1	Oxygen radical absorbance capacity (ORAC)	Can determine antioxidants with lipid in food[11]
2	Total radical-trapping antioxidant parameter (TRAP)	Can measure nonenzymatic antioxidants[5]

Single Electron Transfer (SET) Mechanisms

In this mechanism, the antioxidants will be oxidized by the oxidants, and when it happens, antioxidants will transfer its electron to the oxidants. By that reaction, we can see that there will be a change on the absorbances of the oxidant or the antioxidant and can be measure with the UV-Vis spectrometry[11] (Ou, et al., 2002). The mechanism can be explained by the reaction:

$$M(n) + AH \rightarrow M(n-1) + AH+$$
$$ROO* + AH \rightarrow ROOH + A*$$
$$ROO* + FL\text{-}H \rightarrow ROOH + FL*$$

The antioxidant activity test is carried out to determine and measure three values, which are:

1. Antioxidant Capacity - that is measuring the total level of donated electrons, usually in the form of electrons from the -OH group.
2. Antioxidant Activity - which measures the concentration of antioxidants needed to give a certain rate or level of reaction.
3. Potential Antioxidants - namely, measuring expectations that antioxidants can extinguish free radicals under certain conditions[10] (Shaich and Xie, 2015).

SET mechanism is usually based on ionization potential and on deprotonation mechanism of the reactive functional group, this transfer method also considering pH condition, because when pH values is increasing the ionization potential is decreasing. SET mechanism is usually slow in transfering electron because it based on product precentage decrease.

Knowing the limitations and also the predominances of some methods for doing the antioxidant assay is useful to choose which method that is suitable to our conditions to measure the antioxidant capacity of any sources that we use.

The considerations why we should know the limitations and predominances are its analytical range, reproducibility, and recognition of interfering substances[5] (Prior, et al., 2005).

Table no 5: Limitations of Single Electron Transfer (SET) Mechanisms

No.	Method	Limitations
1	Ferric reducing antioxidant power (FRAP)	The sample form must be in solution[12] Only estimates the Fe (III) reducing activity, can not determine compunds that act by doing radical sequencing (thiols and protein)[11]
2	DPPH free radical scavenging	Very sensitive to O2, light, pH, and solvents[10]
3	Folin-Ciocalteu assay	Interfering substances can affects the result, need to do the correction steps[5]

Table no 6: Predominances of Single Electron Transfer (SET) Mechanisms

No.	Method	Predominances
1	Ferric reducing antioxidant power (FRAP)	Simple, speedy, inexpensive, robust, and does not need any specialized instruments[11]
2	DPPH free radical scavenging	Easily monitored by the spectrometer[5]
3	Folin-Ciocalteu assay	Simple, sensitive, precise[11]

IV. Discussion

ORAC method can measure antioxidant capacity by H atom transfer, on this H atom transfer ORAC method focused on how peroxyl radicals induced oxidation. Some of fluerescent poretin like R-phycoerythrin and dichlorofluorescein are used in ORAC method, this subtances are used as substrates and helps the reaction[10]. This substrates will react with some peroxyl radicals to form a nonfluorescent substance. ORAC method needs >30min for the reaction between the fluorescent substrate and the peroxyl radicals and the antioxidant activity will be determined with AUC from the fluorescence, and can be determined by LC-MS instrument and be

calculated with its AUC. Even though this method have a limitation for the lipophillic compound, it can be modified by using a solution that contain 50% water/50% acetone (v/v) that containing 7% methylated alpha-cyclodextrin (RMCD), this solution can solubilize the lipophillic compound[13]. The temperature limitation that ORAC method have can be modified by controlling the temperature on the plate by doing an incubation on 37o C, because sometimes temperature changes may affect the determination of the antioxidant capacity[14].

For the TRAP method, the antioxidant capacity usually determined by monitoring how many peroxyl radicals got interfere by the antioxidant compound, there are some substrate that usually used in the process e.g. 2,2′ azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) or R-phycoerythrin, and when the substrate meets the peroxyl radicals it can be determined also throught fluorescence or spectrophotometry[15]. This method used in measuring antioxidant capacity in serum or plasma because it can measure a nonenzymatic oxidation in the body. Altough sometimes the results of this method is containing a lot of end points sometimes it can be a predominance because it can make a lot of comparison data.

FRAP method is a method that can determined antioxidant activity on the tissue and also cells but this method can not detect some compunds that doing radical squencing, even though this method can not detect that kind of compounds this method is good at low pH reaction which is pH 3.6. This low pH reaction is conducted to maintain iron solubility and can increases the redox potential. This method is known as a method that totally doing an electron transfer, so the combination between this method and the other method can be useful for determined antioxidant capacity with different kind of antioxidants[5]. There are so many various result of the time that this method conducted, it can be 4 minutes and also 30 minutes, this various time scale depending on how the compounds bind and break down the iron. But most of the time this method needs 4 until 6 minutes to complete the reaction and being detected[16]. The results also showed the changes in the color. The reaction that occurs in this method is the reduction of ferric into a colored product. When antioxidant is oxidized, subsequently the Fe (III) complex is reduced to Fe (II) under acidic condition, and solid blue color will form with maximum absorption at 593nm[12].

DPPH method is one of the method that use a organic nitrogen radicals that have a purple colour, by this method the antioxidant capacity can be measured by how the radical compound reducing the DPPH. This reducing ability can be detect by its absorbance. If free radicals with electron in the DPPH are paired, the result of the color's solution changes from dark purple to bright yellow and absorbance at 515-517 nm itcan be measured[17, 18]. This method is easy to perform and only needs UV-Spectrophotometry as its instrument but can be complicated when the results have overlap spectra at 515nm. An important thing to know that DPPH assay also have a radical reaction (HAT) but still have the SET reaction as its dominant reaction, because DPPH is an oxidant and also a radical probe[5].

Folin-Ciocalteu assay is usually used to measure the total phenols in the sample with a reduction/oxidation mechanisms. Altough this assay is simple, precise sensitive, and can be useful for characterizing the botanical samples but the reaction is really slow if the pH is low. The analytical wavelength of measurement is 765 nm[5]. This F-C assay is an assay that needs to consider the volume of F-C reagent and alkali that is used, the reference standards, the time and temperature that is used, and the optical density at 765nm also must be considered. The reference standard that have been used in this assay is gallic acid but but can be replaced by tanin, cathecin and also caffeic acid. If all of these things are considered correctly the good analysis could be established.

V. Conclusion

This article discusses the limitations and the predominances of the ORAC method, TRAP method, FRAP method, DPPH method, and Folin-Ciocalteu method. Most of the limitations are caused by the environments support, and also the substances that interfere with the measurements. For the predominances, every method have their own characteristics. This kind of limitations and also the predominances can be considered for choosing which method suitable for the situation that happened.

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