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Determination of Antioxidant Activity in Different Kinds of Plants *In Vivo* and *In Vitro* by Using Diverse Technical Methods

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Abstract

The phenolic compounds content and antioxidant activity in plant are very necessary for human health in light of their high active compounds which are very important to protect human body from hard diseases. Therefore, due to evaluated antioxidant activity of various samples (plants), we used 18 methods which were mentioned in 100 articles in different explanations. All of these diverse methods were discussed to demonstrate and show which method is more frequent either *in vivo* or *in vitro*. Moreover, there were different kinds of solvents which were used to extract phenolic compounds from plants. So, the results show that a,a-diphenylpicrylhydrazyl (DPPH) was more frequent method to determine of antioxidant activity *in vitro*, while Lipid Peroxidation (LPO) was more common to evaluate antioxidant activity *in vivo*. Ultimately, methanol was found to be most common solvent which was used to extract phenolic compounds from plants.

Keywords: Antioxidant; DPPH; Reducing power; Oxidation

Introduction

The food safety maintains the nutritional value, and reduces damage during handling and storage, and it pays attention of producer and consumer alike. Therefore, the development of modern technologies in food processing should meet the requirements of rising food for human's consumption and it has an incessant desire to make consumer who want to stay away from industrial additives which might cause a lot of diseases for the human. Lately, antioxidants were increased to use in food preservation in light of fat oxidation process which is the main cause of damage to fats, oils and fatty food. Then, it leads to the loss of nutritional value and appearance of unwanted flavors [1].

Lipid oxidation process occurs when fats and oils react with oxygen, so short chain fatty acids, alcohols, aldehydes, and ketones are considered the final products for lipid oxidation, and they are responsible on rancid flavors [2]. It has become easy today to recognize that free radicals generated by lipid oxidation and then cause of heart disease [3]. Protection against free radicals can be improved by ample intake of dietary antioxidants, and some most substantial testimony indicates that foods containing antioxidants which may have a major importance in disease prevention. The goal in this article was that there are some attempts which have been taken to include *in vivo* and *in vitro* methods because of analyzing the frequency of the use of different methods.

Extraction of Natural Antioxidant from Plants

Fruit group

There are a lot of fruits which have many bioactive compounds, and these fruits were used to treat human body from diverse diseases. For example, the researchers used pomegranate in their research, in spite of its high phenolic compounds such as tannin and pelleterne. Moreover, they brought to light that its seed has the ability to prevent many diseases such as diarrhea [4]. So, we can see that several studies have reported that pomegranate had the highest antioxidant activity when was compared to other fruit [5]. The second fruit of interest is lemon. It was used as antioxidant, for it has phenyl proponoid which might protect human from cough. Also, it contains a complex mixture of phenolic compounds such as flavonoids, coumarins, B-sitosterols and organic acids [6].

Similar to pomegranate, lemon and other fruits, blueberries are rich in types of anthocyanins, flavonoids and tannins. Furthermore, the blueberry skin, which is used as natural antioxidant because it is a particularly rich source of pterostilbene [7]. Moreover, there were many researches which have studied different kinds of fruits, and they are consumed in large amounts by many people such as orange, apple, and grapefruits. These fruits are good sources of vitamin C and polyphenolic compounds including hydroxycinnamic acids and flavanoids [8].

vegetable groups

Onion (*Allium cepa L.*) a versatile vegetable of Allium family is very essential because it has a good relish, in addition, it is an important source of many useful elements. Many of the studies highlighted diverse kinds of flavonoids in different types of onions [9].

Basil is one of locally vegetable that was known as "reyhan", and it is preferred as a healthy vegetable for people who are living around, Yesiloglu [10] have reported that the average of phenolic compounds content in this kind of vegetable were (116, 100, 66 GAE/g) successively by using ethanol, acetone, and water.

Celery (*Apiumgraveolens L.*) leaves are rich in phenolic compounds content, and it has 113.0 mg gallic per 100 g of fresh celery, while in the whole celery was 436.1 mg/100 mg gallic per 100 g of dry celery [11]. Moreover, the previous studies mentioned that a cutting process will change the total phenolic compounds and the flavonoids which can identify by HPLC [11].

Curry leaf (*Murrayakoenigii L.)* is one of the native plants that were grown in Asia, and it has a good taste when it was added in different kinds of food. Curry leaf extract contains monoterpene which has been found

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a good source for natural antioxidant in light of its high antioxidant activity [12,13]. *Mentha spicata* is considered very important to treat a lot of diseases in India, and many studies were confirmed that it had a high antioxidant activity. Moreover, antioxidant activity for this plant may reach to the synthetic antioxidant such as BHT.

Phenolic Compounds

Classifications of phenolic compounds

There are about eight thousands kinds of phenolic compounds, which are mostly containing an aromatic ring in their chemical composition. Scientists in the modern classification tried to split phenols to the simple phenols and polyphenols, and they have suggested that the phenolic acids as a part of simple phenols in light of their phenol subunit. Flavonoids are considered as a part of the polyphenols, which contain two or three of the phenol subunits. Therefore, the researchers have concluded that polyphenols have more phenol subunit in their chemical structure [14].

Phenols and phenolic acid: Phenolic acids contain carboxylic acid in the chemical composition, and each of the hydroxycinnamic and hydroxybenzoic is a main pillar of phenolic acids according to figure 1. Moreover, scientists have noted that p-coumaric, caffeic, ferulic, and sinapic acids are the main component part of the hydroxycinnamic (Figure 1).

Flavonoids: The molecular weight for flavonoids is low (Figure2) [15]. Flavane is the main part of flavonoids which contains two benzene rings (A and B) within its chemical composition. As these two rings connected to each other through pyrane ring (C), so all of flavones, isoflavones, flavonoids, flavonols, flavanones, anthocyanins, and pro anthocyanidins are part of flavonoids in according to a new classification (Figure2).

Anthocyanins: The anthocyanidin is one of the simple structures of the anthocyanin, and anthocyanidins consists of an aromatic ring which linked and connected to a heterocyclic ring. Moreover, the heterocyclic ring is connected to the third aromatic ring through a carbon bond [17]. Scientists have noted that anthocyanins are often found in glycoside form with sugar. Moreover, many kinds of anthocyanins were found in nature makes these kinds of phenolic compounds very complex. Scientists have noted that anthocyanins in different kinds of fruit presented and showed a great interesting in enriching the modern classification of these types of antioxidants (Figure 3).

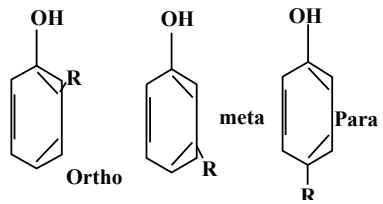

Figure 1: Phenolic acid.

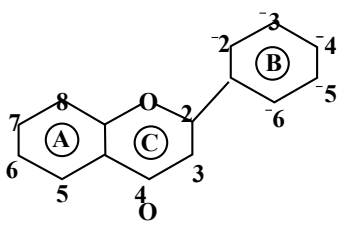

Figure 2: Flavonoids.

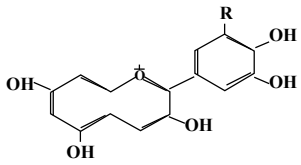

Figure 3: Anthocyanins.

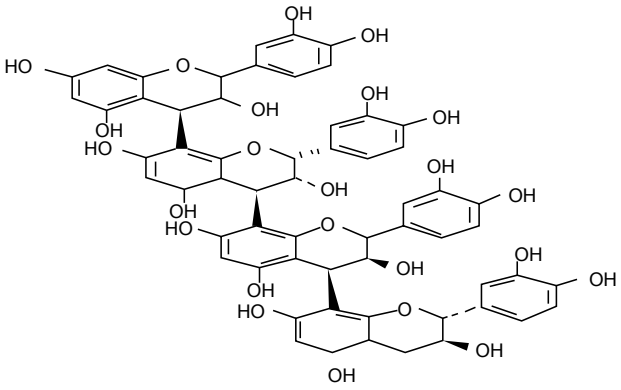

Figure 4: Tannin.

Tannins: Tannins are natural products which were presented in several plant families, and they have large amounts of phenolic rings in their structure. Also, tannins are classified into two groups: hydrolysable and condensed. Condensed tannins contain flavonoids units with several degrees of condensation, but the hydrolysable tannins are considered a mixture of simple phenols with ester linkages in their structure. So, there are many factors such as alkaline, mineral acids and enzymes which have the ability to hydrolysis tannins [18] (Figure 4).

Extraction of Phenolic Compounds

Solvents

The scientists have studied and analyzed the impact of different types of solvents such as methanol, hexane and ethyl for the purpose of extraction of antioxidants from plants through the use of different parts of the plant such as leaves and seeds. Scientists also said that the purpose of used solvents have different polarity to extract phenolic compounds from plants with a high degree of accuracy [19]. Moreover, scientists have noted an important point when they wanted to discover an essential solvent, so they mentioned that the high polar solvents have a high effectiveness of antioxidants such as methanol. Also, Anokwuru et al. [20] have explained that acetone and N,N dimethylformamide (DMF) possess highly effective extraction of antioxidants, in addition, researchers confirmed that hexane and methanol more widely used in light of the high extractive efficiency Koffi et al. [21] have found that methanol was more effective to obtain a big amount of total phenolic contents from walnut fruits extracts when they were compared with ethanol [20]. Finally, Koffi et al. [21] were found that ethanolic extract of Ivorian plants extracted higher phenolics when were compared with acetone, water, and methanol.

Microwave-assisted extraction

Microwave-assisted extraction (MAE) has used as an alternative to conventional techniques for possessing the ability to reduce both time and extraction solvent volume when was used to extract antioxidants [22]. The main objective of using MAE is, it is a new technology which has the ability to heat the solvent and encouraging them to extract antioxidants from plants without a shortfall in the amount of these solvents [23]. So, scientists have worked to promote usage of this kind of method instead of traditional methods. Li et al. [24] reported that conventional methods by using diverse types of solvents were presented less antioxidant activity and phenolic content than MAE. Therefore, they were confirmed that MAE was more effective to increase antioxidant activity when they tried to measure antioxidant activity by using FRAP, ORAC, and TPC.

Ultrasonic

Tabaraki and Nateghi [25] have noted that the evolution in the world requires the use of green technology and friendly in order to keep the environment from the risk of toxic substances. Therefore, extraction of phenolic compounds by using ultrasound has grown significantly during few years, for its big role to reduce the used amount of solvent with low energy. Corrales et al. [26] have suggested that ultrasonic has the ability to break down plant tissue and work on the production and release of active compounds in solvents with a high efficiency. Moreover, Scientists have confirmed to increase antioxidant activity from 187.13 μmol TE g^{-1}DM to 308μmol TE g^{-1}DM by using ultrasonic as a sophisticated and effective method to extract antioxidants from different sources.

Antioxidant Capacity Assays

In vitro

Antioxidant activity might not be concluded based on a technical test for a single antioxidant, and there are several practice *in vitro* test procedures which are carried out for evaluating antioxidant activities with the samples of benefit. Another aspect is that antioxidant test models differ in different respects. Therefore, it is difficult to compare exactly one method to other one.

Thiobarbituric acid (TBA) method: The possibility of using plant extracts to decrease oxidation was described by Ottolenghi [27] is as follows: Using approximately 0.02% of the plant extract with the addition of 2 ml of thiobarbituric acid concentration of 20%. Then, added 2 ml of the previous mixture to 1 ml of sample. The mixture was placed in a boiling water bath for 10 minutes. The mixture was moved later to the centrifuge equipment on 3000 rpm for 20 minutes. The absorbance activity of a filtrate part was measured by using spectrophotometer at 552 nm.

DPPH: The researchers pointed out that the molecule 1, 1-diphenyl-2-picrylhydrazyl (a,a-diphenylpicrylhydrazyl DPPH) is one of the stable free radicals that contain a decentralized electron. The decentralized of electron has the ability to increase violet color as evidence of the antioxidant activity. When DPPH solution is mixed with any type of antioxidants that have the ability to grant a hydrogen atom, this mixture will have the ability to reduce violet color as a sign of resistance to oxidative stress efficiently. The possibility of using plant extracts to prevent the formation of hydrogen peroxide can be measured according to the method of Ruch et al. [28]. This method DPPH was used (0.05 M pH 7.4) of phosphate buffer inspite of preparing (0.04 M) solution of hydrogen peroxide. Then, spectrophotometer was used at 230 nm to estimate the amount of hydrogen peroxide in a good light. Approximately (0.02-0.06 mg/mL) of plant extracts was melted and added to the hydrogen peroxide to measure the absorbance at 230 nm. At the same time, it was measured the absorbance of control that contains phosphate buffer without hydrogen peroxide. The percentage of inhibition was calculated according to the following equation:

$$\%\text{inhibition}=[(A_i - A_t)/A_i]\times 100$$

Where A_i is the absorbance of control and A_t is the absorbance of test.

Chelating of minerals: The metal ions has changed a significant impact in the generation of oxygen free radicals in the diets and within the body of the organism, so the iron has two oxidation forms which called ferrous ion Fe^{+2} and ferric ion Fe^{+3}. The latter is an inefficient iron, and anyway it can be reduced to ferrous Fe^{+2}, depending on ambient conditions and pH [29]. In this method chelating of minerals are recognized on the ability of ferrozine to form a complex compound with a red color by using Fe^{+2} as a chelate material [30]. Approximately 0.5 ml of ferrous chloride (0.2 mM) was added to the 0.1 mL of plant extracts). Using 0.2 mL of ferrozine (0.005 M) was more effective to start reaction between the chemical materials, and then it was kept for 10 min at room temperature. Finally, the absorbance is measured by using spectrophotometer at 562 nm. This method was used a control sample such as EDTA and citric acid, for showing the differences between plants extracts and the control sample.

Reducing power: This method is identified on the high capacity of the antioxidant compounds through the increase in the absorbance of the reaction mixture. Therefore the increase in absorbance will rise directly to an increase in antioxidant effectiveness. So, antioxidant compounds will produce a color complex with potassium ferricyanide, trichloro acetic acid and ferric chloride, which are measured at 700 nm. The increase in absorbance of the reaction mixture gives a clear picture about the possibility of using these kinds of plants extracts as antioxidants [31]. 1 ml of sample is dissolved in distilled water, and then addition 2.5 mL of $K_3Fe(CN)_6$ (1% w/v) with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) according to method was described by Oyaizu [32]. The mixture is incubated for 20 minutes at a temperature of 50°C with the addition of 22.5 mL of Trichloro acetic acid (10% w/v) after the completion of the incubation period. An upper layer (2.5 mL) was obtained through a centrifuge 3000 rpm for 10 minutes, and then it was mixed with (2.5 mL, 0.5 mL) of distilled water and $FeCl_3$ (0.1%, w/v) respectively.

Oxygen radical absorbance capacity (ORAC) Method: The researchers noted that AAPH (2,2-azobis 2-amidopropane dihydrochloride) has the ability to generate free radicals. Also, Benzie and Strain [33] have stressed that the substance used as a standard sample to show the antioxidants activity as trolox equivalent. Prior et al. [34] were used in their methods about 0.2 ml of a final volume, and they were prepared samples at pH 7.0 [35]. Then, they were added 0.0625, 0.0125, 0.025, and 0.05 mM/L of trolox for lipophilic as well as they were prepared 0.0125, 0.025, 0.05 and 0.1 mM/L of trolox for hydrophilic as the standard. Ultimately, using 75 mM/L of phosphate buffer as a blank to compare the results. The plate is placed immediately in a multi-label counter preheated to 37°C after the addition of AAPH. Approximately for 10 s, the plate was shaken and read the fluorescence for 35 min in an orbital mannered. The scientists have suggested using Wallac Workout 1.5 software in light of calculation the area under curve for each sample. The final results are made to determine the difference between blank, sample, and standard trolox. Then, it is expressed as trolox equivalents per gram sample.

Xanthine oxidase method: Allopurinol was prepared by adding 0.1 mg in 1 ml of methanol. Approximately 0.2 ml of xanthine oxidase was mixed with each of 1.3 ml at pH 7.5, 0.5 mg for phosphate buffer and extracts successively. The mixture was added to substrate for xanthine enzyme 1.5 ml of 0.15 M, and then this mixture was incubated for 10 min at 24°C. The absorbance was measured by using spectrophotometer at 293 nm. A blank sample was prepared by adding 1.5 ml of xanthine substrate to the 0.5 ml, 1.3 ml, and 0.2 ml for methanol, phosphate buffer, and xanthine oxidase successively. The percentage of inhibition was calculated according to the following equation:

$$\text{Percentage of inhibition } \% = \left[1 - \left(\text{As} / \text{Ac}\right)\right] \times 100$$

Where As and Ac are the absorbance values of the test sample and control, respectively.

Ferric reducing/antioxidant power (FRAP): The FRAP method was used to determine the antioxidant activity in easy way according to Benzie [33]. The FRAP reagent was firstly prepared by mixing of 300 mM acetate buffer and pH 3.6 with 10 mM iron reagent (TPTZ) solution which was dissolved in 40 mM and 20 mM for HCl and $FeCl_3.6H_2O$ successively. Then, about 1.9 ml of the FRAP solution was kept at 37°C and reacted with 0.1 mg of plant extracts in light of preparing a good condition for reaction. The mixture was incubated for 4 min, and then the absorbance was measured at 593 nm by using spectrophotometer. In light of calculating the results, the standard curves was prepared by using an appropriate concentration of $FeSO_4$.

Beta-carotene bleaching activity: The scientists were determined antioxidant activity of plants extracts by using β-carotene bleaching method according to Wettasinghe and Shahidi [36]. Beta-carotene solution was prepared by dissolving 5 mg of beta-carotene in 50 ml of chloroform, and then 3 ml of β-carotene was mixed with 40 mg and 400 mg for linoleic acid and Tween 20 successively. To make form of beta carotene with linoleic acid, it was added approximately 100 ml of distilled water to the mixtures which was dried under nitrogen. 1.5 ml of beta carotene-linoleic acid was added to 0.02 mg of plant extracts in light of determination the β-carotene bleaching activity. The incubated mixture was estimated at 470 nm by using spectrophotometer. Standard curve of Alpha-tocopherol was used to determine the percentage of antioxidant activity by using the following equation:

$$\text{AA } \% = 100 \left(\text{DR}_c - \text{DR}_s\right) / \text{DR}_c$$

Whereas AA=antioxidant activity; DR_c=degradation rate of the control, DR_s=degradation rate of the sample

In vivo

Glutathione peroxidase (GSHPx) estimation: 2 ml of cytosolic GPx was prepared at pH 7 by using phosphate buffer (75 mM/L). Then, the whole mixtures were prepared by adding 0.05 ml , 0.05 ml , 0.1 ml and 0.1 ml for glutathione reductase solution (60 mM/L), NaN_3 (0.12 M/L), Na_2EDTA (0.15) mM/L, and NADPH (3.0 mM/L) respectively. It was added 0.1 mL of 7.5 mM/L H_2O_2 to encourage and start reaction between the chemical materials. To know if there were any changes in the absorbance at 340 nm, it was concentrated on the conversion of NADPH to NADP. The calculation of enzyme activity was determined as mg of proteins and according to Wood [37].

Ferric reducing ability of plasma: The method described by Benzie and Strain [38]. The scientists were used blood of rats to determine the ferric reducing ability of plasma after putting blood of rats on an anticoagulation glass tubes for 0, 7, and 14 days. FRAP was prepared at 37°C by mixing 1 ml, and 10 ml of 2,4,6 tripyridyl-s-triazine (10 mM) in 40 mM HCl, $FeCl_2$ $6H_2O$ (20mM), and acetate buffer (0.3 M) respectively. Then, adding and mixing of 3 ml FRAP with 0.375 and 0.025 ml distilled water and plants extract successively to determine the absorbance at 593 nm because of developed color in organic layer. The temperature is kept at 37°C. The readings are selected for the calculation of FRAP values at 180 s.

Glutathione-S-transferase (GSt): Glutathione-S-transferase is one of the important materials that have the ability to remove toxic compounds including active pharmaceutical compounds. Catalyze enzyme has the ability to interact with SH group which is presented in the chemical composition of glutathione. Thus this action acts to control electron sites and make them more soluble in water. The method might be used as mentioned by Jocelyn [39]. The substrate was prepared by mixing 1 ml, and 1 mM/L of potassium phosphate (0.1 N), GSt (1 mM/L), and l-chloro-2,4-dinitrobenzene (1mM/L) respectively. Then, this mixture was added to 6 mg/ml of cytosol. The reaction was started when the substrate added after incubation for 5 min at 37°C, and the increase of absorbance was estimated at 340 nm by using spectrophotometer.

Superoxide dismutase (SOD) method: This method might be used for determination of antioxidant activity of a sample, and it was described by McCord and Fridovich [40]. The main purpose of this method that was estimated 5% of a red blood cell after adding 75 mM, 30 mM, and 2 mM from Tris-HCL (pH 8.2), EDTA, and pyrogallol respectively. Then, the absorbance was measured at 420 nm. The percentage of inhibition was calculated depending on that the ability of enzyme to inhibit of oxidation. So, any changes might be happened on the absorbance, it will give a clear picture on the ability of enzyme activity to prevent oxidation.

Glutamyltranspeptidase activity (GGT) assay: The scientific basis for this method is that preparing the substrate of enzyme by using nitroanilide, glycylglycine, and $MgCl_2$. Then, 0.05 M of tris at pH 8.2 is added to the substrate solution [41]. The absorbance is measured at 405 nm after keeping the stock solution for 1 min at 37°C.

Catalase (CAT): Catalase activity might be estimated in red blood cells by using Aebi's method [42]. The first step was to prepare the stock solution by using 2 ml and 1 ml from phosphate buffer at pH 7 and H_2O_2 (30 mM) respectively. Then, 50 µl of the lysate was added to the stock solution. The ability of catalase to work a reducing factor was measured by determining the changes of absorbance at 240 nm. Finally, the percentage of catalase activity was calculated through the basic equation. The scientists were mentioned to an important point when they wanted to determine enzyme activity. They said that any damage will happen to 1 mmol of H_2O_2 will equal to 1 unit of enzyme activity.

LDL assay: The isolated LDL is used to show if there are any effects on 150 mmol/L and 1 mmol/L for NaCl and Na_2EDTA respectively, when were kept to interact with each other at 4°C. Using of filtration (0.00045 mM) is necessary to sterilized LDL. The stock solution of LDL is prepared by using 0.1 mg of protein/mL. So, to determine the effects of LDL on oxidation, samples are added to LDL solution at 37°C for 1 min. $CuSO_4$ is added about 0. 005 mmol/ L, and they are incubated at 37°C for 2 hours. The method is advised to add 0.01 mg of butylated hydroxytoluene (BHT) because it is necessary to inhibit any damage that might be happened by Cu^{+2}. This element has the ability to encourage oxidation in a good light. After the end of the incubation period, the LDL oxidation is estimated by calculating amount of hydrogen peroxide which formed during the storage period. It will give a good way to estimate the amount of malondialdehyde which is

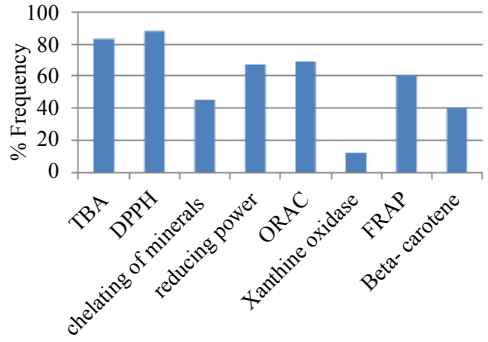

Figure 5: *In vitro* methods.

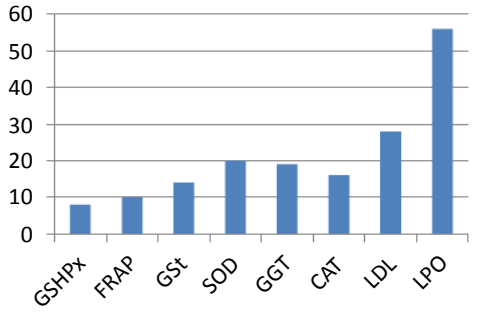

Figure 6: *In vivo* methods.

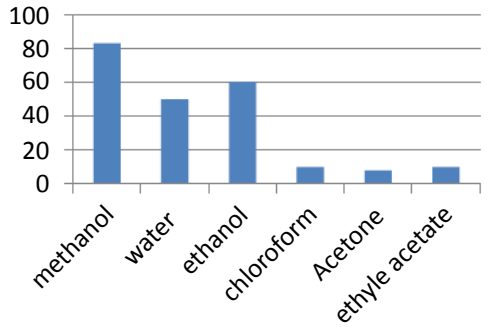

Figure 7: Diverse solvents.

the final compound of oxidation as evidence the end of the oxidation process [43].

Lipid peroxidation (LPO) assay: The first step used was, a Teflon glass homogenizer to crush tissues by using buffer which concentration 0. 1 M with alkaline pH about 7.4 according to the method described by Ohkawa et al. [44]. Then, determination of LOP in this homogenate is measured the amounts of malondialdehyde (MDA) produced primarily. The second step is that preparing 3.4 ml from 0.2 ml, 0.2 ml, 1.5 ml, and 1.5 ml of Tissue homogenate, 8.1% sodium dodecyl sulfate, 20% acetic acid, and 8% TBA respectively. To make the volume of the mixture reach to 4 ml, there is the amount of distilled water was added in the prepared mixture. Then, this method is used glass balls to make the temperature rise quickly to 95°C by using water bath for 60 min. The volume of mixture is reached to 5 ml after cooling all of tubes a 37°C. The final stage is that prepare (15:1) from each of butanol and pyridine. Then, adding 5 ml of butanol: pyridine which was prepared to the mixture with 3000 rpm of centrifugation for 10 min. After centrifugation finished, the absorbance was measured especially for upper layer at 532. To compare the results, a blank sample was used to show the differences between each other.

Results and Discussion

The results for *in vitro* and *in vivo* methods are shown in figure 5 and 6 respectively. Figure 5 is given clear picture that two *in vitro* methods were most frequently used and these were in order of decreasing frequency: DPPH>TBA. Therefore, DPPH method is the most simple and easy in light of its low costly for the antioxidant activity evaluation of a sample. But, figure 6 appears that the frequency of use is higher for LPO assay and it was followed by LDL (Figures 5 and 6).

Figure 7 is shown that three solvents are prominently being used for the extraction purpose in relation to the stated experiment. These solvents are methanol, ethanol, and water. So, methanol, ethanol, and water have good polarity, and they are used in a good light to extract polar compounds such as phenolic compounds and flavonoids which are believed to be effective antioxidants (Figure 7).

Conclusion and Future Directions

This review article is demonstrated *in vitro* and *in vivo* methods of antioxidant evaluation. It was prepared based on plenty literature review. This article is presented 8 *in vitro* and 8 *in vivo* methods which are being used for antioxidant evaluation purpose. DPPH method was the most frequently method for *in vitro* antioxidant activity evaluation while LPO was found as the mostly used *in vivo* antioxidant method. Methanol extract was found with the highest frequency for antioxidant study. This article will be a comprehensive ready reference for anyone who is interested on antioxidant study.

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