

COMPARATIVE ANALYSIS OF PHENOLIC CONTENT AND ANTIOXIDATIVE ACTIVITIES OF EIGHT MALAYSIAN TRADITIONAL VEGETABLES

(Analisis Perbandingan Kandungan Fenolik dan Aktiviti Antipengoksida Lapan Sayuran Tradisional di Malaysia)

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Abstract

Vegetables have been believed to exhibit antioxidant activities due to its phenolic content. Thus, this study was carried out to determine the total phenolic content of water and ethanolic extracts of Malaysian traditional vegetables and assess their antioxidant activities. Eight samples of Malaysian traditional vegetables were dried and extracted its phenolic compounds using water and ethanolic solvent. Total phenolic content of the extracts were compared and evaluated using Folin-Ciocalteu and Prussian Blue reagent. The antioxidant activity were assessed using ferric thiocyanate assay and DPPH free radical scavenging assays. Results found that total phenolic content of water extracts ranged from 7.08 to 14.76 mg GAE (Folin-Ciocalteu assay) and 3.50 to 7.82 mg GAE (Prussian Blue assay). However, the content of phenolic of ethanolic extracts ranged from 5.21 to 15.86 mg GAE (Folin-Ciocalteu assay), and 1.84 to 11.54 mg GAE (Prussian Blue assay). The highest antioxidant activity was observed in water extracts of *Etilingera elatior* (75.6%) and ethanolic extracts of *Sauropus androgynus* (78.1%). Results also found that the best half maximal inhibitory concentration or IC₅₀ were demonstrated by water and ethanolic extracts of *Sauropus androgynus* which demonstrated 0.077 mg/mL and 0.078 mg/mL, respectively. Hence, this study obtained that most of the Malaysian traditional vegetables have a potential source of natural antioxidant.

Keywords: Folin-Ciocalteu method, Prussian Blue assay, antioxidant activities, Malaysian vegetables

Abstrak

Sayuran dipercayai boleh mempamerkan aktiviti antipengoksida disebabkan oleh kandungan fenoliknya. Oleh itu, kajian ini telah dijalankan untuk menentukan jumlah kandungan fenolik dalam sayuran tradisional Malaysia serta menilai aktiviti antipengoksidanya. Lapan jenis sayuran tradisional Malaysia telah dikeringkan dan diekstrak sebatian fenoliknya dengan menggunakan air dan pelarut etanol. Kandungan fenolik daripada ekstrak sampel telah dibandingkan dan dinilai menggunakan kaedah Folin-Ciocalteu dan reagen Prussian Biru. Aktiviti antipengoksida telah dinilai menggunakan asai ferik tiosianat dan asai pemerangkapan radikal bebas DPPH. Keputusan kajian telah menunjukkan bahawa jumlah kandungan fenolik ekstrak air adalah dari 7.08 ke 14.76 mg GAE (asai Folin-Ciocalteu) dan, dari 3.50 ke 7.82 mg GAE (asai Prussian Biru). Walau bagaimanapun, kandungan fenolik ekstrak etanol bernilai dari 5.21 ke 15.86 mg GAE (asai Folin-Ciocalteu) dan 1.84 ke 11.54 mg GAE (asai Prussian Biru). Aktiviti antipengoksida tertinggi telah diperhatikan pada ekstrak air *Etilingera elatior* (75.6%) dan ekstrak etanol *Sauropus androgynus* (78.1%). Keputusan kajian juga telah mendapati bahawa separuh perencatan maksimum atau IC₅₀ yang terbaik telah dipamerkan oleh ekstrak air dan etanol *Sauropus androgynus* dengan nilai 0.077 mg/mL dan 0.078 mg/mL, masing-masing. Oleh itu, kajian ini menunjukkan bahawa kebanyakan sayuran tradisional Malaysia berpotensi sebagai sumber bahan antipengoksida semula jadi.

Kata kunci: Kaedah Folin-Ciocalteu; Asai Prussian Biru; aktiviti antipengoksida; sayuran Malaysia

Introduction

The importance of health implication by consumption of vegetables has been acknowledged by many scientists. Epidemiological studies reported that consumption of vegetables have been linked in prevention of some chronic and degenerative diseases such as cancer, diabetes, osteoporosis, and cardiovascular disease [1-3]. The discoveries of the importance of dietary micronutrients in vegetables have made them being demanded in many sectors include food, pharmaceutical, and agricultural industries. This eventually increased the awareness of public on the importance and beneficial effects of consumption of vegetables in their diet.

Malaysia is well known to have rich and diverse kinds of traditional vegetables or locally known as *ulam*. These vegetables have been consumed by people in Malaysia for many years ago especially for old people in countryside. In fact, Mansor [4] reported that there were more than 120 species of traditional vegetables in Malaysia. It is commonly eaten as fresh or raw as salad, or cooked through boiling or blanching and very popular especially among the Malay communities [5]. The fruit kernels, rhizomes, young leaves, and shoots are normally selected for consumption. These vegetables usually can be found mainly in the countryside area, which planted by the villagers or grow by itself in the forest. However, in the last few years, some of these vegetables has been grown commercially for market place [3].

Vegetables are very high in fiber but low in calories. It provides with the main source of carbohydrates, proteins, minerals, and vitamins, which are crucial for growth and health as well as mind. There are also contain phytochemicals such as phenolics, carotenoids, lignans, and lycopenes. These phytochemicals have been reported to have potential health benefits such as anti-tumour [3], anti-allergenic, anti-inflammatory, anti-microbial, and anti-thrombotic [6-7]. The health effect of consumptions of vegetables might be due to the antioxidant activities of the plants especially from phenolic compounds [8-9]. Phenolic compounds were believed to have redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers as well as have a potential as metal chelation action [10].

The explorations of natural antioxidants from vegetables have received much attention to apply as suitable antioxidants to replace synthetic antioxidant in food products. These naturally-occurring antioxidants can also be formulated into capsules or tablets as nutraceutical products can assist to prevent oxidative damage from occurring in human body [11]. Thus, the extensive study of traditional vegetables may provide advantages for more awareness of potential phenolic antioxidant and might have beneficial effect to both food and health [12]. In fact, for the last few decades, there have been convergences of interest among researchers in this field since clear scientific information is very necessary. Studies reported that antioxidant activities in the Malaysian traditional vegetables include *Averrhoa bilimbi* [13-15], *Cosmos caudatus* [3,5,14], *Centella asiatica* [3,5,16], *Morinda citrifolia* [17], *Polygonum minus* [3,5,18], *Oenanthe javanica* [3,5], and *Melicope Lunu ankeda* [18] were associated with their phenolic contents.

Phenolic contents and its antioxidant activities in plants have been evaluated by numerous methods. These methods include ferric reducing antioxidant power (FRAP) assay, β -carotene bleaching assay, thiobarbituric acid (TBA) assay, oxygen radical absorbance capacity (ORAC), 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity, and cupric reducing antioxidant capacity (CUPRAC) reagents. All these methods have their own advantages and disadvantages [19], and one procedure cannot identify all possible mechanisms of antioxidant activity. Therefore, most researchers combine several methods to evaluate the relationship between the phenolic content and their antioxidant activities of the plants.

Hence, the objectives of this study were to assess and compare total phenolic content of eight Malaysian traditional vegetables using Folin-Ciocalteu and Prussian Blue assays and its antioxidant activities using *in vitro* methods of FRAP and DPPH assays. The correlation between total phenolic content and its antioxidant activities were also investigated in this study.

Materials and Methods

Chemicals

Ethanol, Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), ferric chloride (FeCl_3), potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$), linoleic acid, disodium hydrogen phosphate, hydrochloric acid (HCl), gallic acid, and trichloroacetic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ammonium thiocyanate (NH_4SCN) and ferrous chloride (FeCl_2) were purchased from Merck (Merck KGaA, Darmstadt, Germany).

Sample Collection

Eight Malaysian traditional vegetables were selected in this study were *Anacardium occidentale* shoot, *Carica papaya* shoot, *Curcuma longa* leaves, *Etilingera elatior*, *Manihot esculenta* shoot, *Pithecellobium jiringa*, *Psophocarpus tetragonolobus*, and *Sauropus androgynus*. These samples were locally known as *pucuk gajus*, *pucuk betik*, *daun kunyit*, *bunga kantan*, *pucuk ubi*, *jering*, *kacang kelisa*, and *cekur manis*, respectively. All samples were purchased from wet market around Bandar Baru Nilai, Negeri Sembilan, Malaysia. Prior to extraction, samples were prepared based on Mohd Zin et al. [17]. The samples were washed under running tap water before being chopped into smaller pieces. Then, the samples were dried at 45°C for 24 to 48 hrs using dehydrator (Excalibur Food Dehydrator 3000) and were grinded using domestic blender (Panasonic MX-337) into powder.

Water Extraction

Samples were extracted using water according to Wong et al. [11] with slight modification. Firstly, 5.0 g samples were soaked with distilled water in ratio water: sample (10:1) and the mixtures were left at room temperature (25°C) for an hour in the dark condition with occasional agitation. Then, the aqueous extracts were filtered through Whatman No. 1 filter paper to obtain crude extract. The residual water was removed by evaporating the extract using rotary evaporator (BÜCHI Rotavapor R-215) at 80°C and 50 rpm rotation. The crude extract were collected, weighed, and diluted to 5 mg/mL and were then stored at 4°C until further analysis.

Ethanolic Extraction

The dried and fine homogenized sample were weighed and transferred into a beaker. Prior to the extraction, 70% ethanol was added into the samples with the ratio of 1:7 (w/v) and was stored in the dark condition for three days. The extracts were then filtrated through Whatman No. 1 filter paper before evaporating the residual solvent using rotary evaporator at 75°C and 50 rpm rotation. The crude extract obtained were collected, weighed, and diluted to 5.0 mg/mL and were then stored at 4°C until further analysis.

Determination of Total Phenolic Content: Folin-Ciocalteu Assay

Total phenolic content of extracts were determined using Folin-Ciocalteu assay method according to Singleton and Rossi [20] and Hoff and Singleton [21] using external calibration of gallic acid solution (10, 20, 30, 50, and 100 ppm). Briefly, 2.5 mL of 10-fold diluted Folin-Ciocalteu reagent and 2.0 mL of 7.5% solution of Na_2CO_3 were added into 1.0 mL of 0.5 mg/mL sample extract. Finally, the absorbance of reaction mixture was measured at 765 nm using spectrophotometer (Spectronic GENESYS 20) after 15 mins heating at temperature of 45°C. Results were expressed as mg gallic acid equivalent (GAE) using an equation obtained from gallic acid calibration curve. All analysis were carried out in triplicate.

Determination of Total Phenolic Content: Prussian Blue assay

Prussian Blue assay was done according to the method of Gupta and Verma [22] with a slight modification. Briefly, 1.0 mL sample was diluted with 50 mL distilled water. Next, 3 mL of 0.5 M ferric chloride (FeCl_3) in 0.1 N HCl and 3.0 mL potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$) were added into the diluted samples. The color immediately developed after 10 to 15 mins. Then, the absorbance was measured at 725 nm using spectrophotometer (Spectronic GENESYS 20). Gallic acid was also used as the standard reference phenolic compound. The total phenolic content were measured by GAE determined from standard gallic acid calibration (10, 20, 30, 50, and 100 ppm) curve. Results were expressed as mg GAE and were carried out in triplicate.

Determination of Antioxidant Activity: Ferric Thiocyanate Assay

The antioxidant capacity of all extracts was carried out according to the ferric thiocyanate assay [23]. Four milligram of samples were dissolved in 4 mL of 99.5% (w/v) ethanol and were mixed with linoleic acid (2.51% v/v) in 99.5% (w/v). Next, ethanol (4.1 mL), 0.05 M phosphate buffer pH 7.0 (8 mL) and distilled water (3.9 mL) were added into the mixture and was kept in screw-cap container in a dark storage at 40°C. Then, 0.1 mL of this solution was added with 9.7 mL of 75% (v/v) ethanol and 0.1 mL of 30% (w/v) NH₄SCN. Precisely, 3 mins after the addition of 0.1 mL of 20 mM ferrous chloride (FeCl₂) in 3.5% (v/v) HCl to the reaction mixture, the absorbance of the producing red color was measured at 500 nm using spectrophotometer (Spectronic GENESYS 20) for seven days. The percentage of inhibition of linoleic acid was calculated according to the following formula (equation 1):

$$\text{Linoleic acid peroxidation inhibition (\%)} = \left[100 - \left(\frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \right) \times 100 \right] \quad (1)$$

Determination of Antioxidant Activity: DPPH Radical Scavenging Activity Assay

The DPPH radical scavenging activity assay was determined according to the method of Azlim Almey et al. [24]. Different concentrations of diluted sample extracts (1.0, 2.0, 3.0, 5.0, and 10.0 mg/mL) were prepared. First, 6.0 mg of DPPH was dissolved in 100 mL methanol to make a DPPH solution. Then, 2.0 mL of DPPH solution were transferred into a test tube and 1.0 mL of each diluted sample extracts were mixed with the solution. After that, 1.0 mL of methanol was added into 2.0 mL DPPH solution as a control. The standard reference used was gallic acid. The solution mixture was shaken vigorously and placed in a dark condition for 30 mins. Then, the absorbance of the mixture was determined at 517 nm using spectrophotometer (Spectronic GENESYS 20). The scavenging activity of the extracts was calculated using the following (equation 2):

$$\text{Scavenging activity (\%)} = \left[1 - \left(\frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \right) \times 100 \right] \quad (2)$$

DPPH radical scavenging activities of the samples were expressed as half maximal inhibitory concentration (IC₅₀) values, which indicate the concentration of extracts that required to scavenge 50 % of DPPH free radicals. The IC₅₀ values were estimated by plotting the graph of scavenging activity against concentration (10, 20, 30, 50, and 100 ppm) of gallic acid.

Statistical Analysis

All experimental results were analyzed using Minitab software (Minitab Version 15.1.10). Every measurement of each assay and sample was done in triplicate. The experimental data were calculated using complete randomized design (CRD) and analysis of variance (ANOVA). The Duncan's multiple range test (DMRT) and one-way ANOVA were used to determine the significant differences among means from triplicate analysis at p<0.05. Pearson's correlation test was also determined to assess correlation between means.

Results and Discussion

Extraction yield and Total Phenolic Content

The yields of water and ethanolic extracts of vegetables are shown in Figure 1. From the Figure, the highest yield of extracts were demonstrated in water and ethanolic extracts of *Anacardium occidentale* shoot with percentage of yield of 3.10% and 1.98%, respectively. The lowest extraction yields were obtained in water extracts of *Manihot esculenta* shoot (0.22%) and ethanolic extracts of *Etilingera elatior* (0.05%). Results found that the yields of water extracts were varied in the range between 0.22 and 3.10% while the ethanolic extracts yields were in the range between 0.05 and 1.98%.

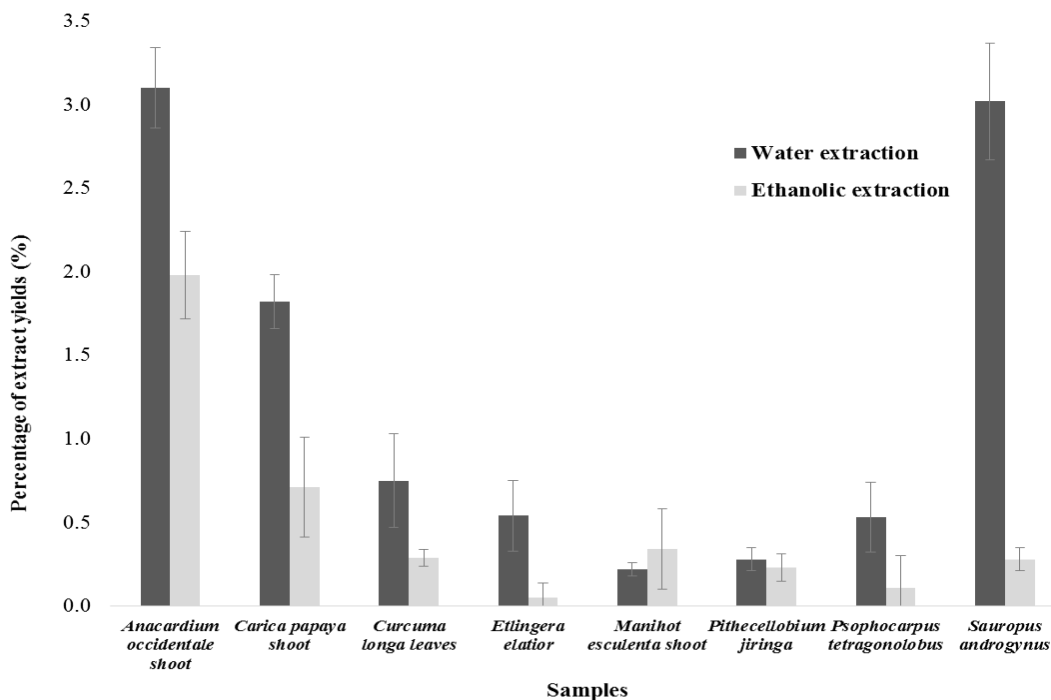


Figure 1. Yields of water and ethanolic extracts of vegetables

The amount of extraction yields are depends on the solvent and method of extraction. Water and ethanol are the most commonly solvents used in plant extraction because of their low toxicity and safer to be used [13, 18, 25]. The different polarity of different solvent will obtain different amount of extraction yield. Furthermore, water was always used for blanching and boiling the traditional vegetables before people consuming vegetables [11, 18]. This result also revealed that water extraction of vegetables was higher than ethanolic extraction except for *Manihot esculenta* shoot (Figure 1). Hence, polar solvent such as water, ethanol, and methanol were proven to be more efficient to produce high yield in leaves as well as to extract phenolic compounds [13, 18, 26-27].

Table 1 shows the total phenolic content of water and ethanolic extracts determined using Folin-Ciocalteu and Prussian-Blue assays. From the table, results showed that the total phenolic content of water extracts of vegetable ranged from 7.08 to 14.76 mg GAE when were determined using Folin-Ciocalteu assay. However, the total phenolic content of water extracts of vegetables varied in the range between 3.50 and 7.82 mg GAE when were measured using Prussian-Blue assay. In the ethanolic extracts, total phenolic content of vegetables were varied in the range between 5.21 and 15.86 mg GAE, and between 1.84 and 11.54 mg GAE when determined using Folin-Ciocalteu and Prussian-Blue assays, respectively. Analysis of total phenolic content using Folin-Ciocalteu assay clearly found that water and ethanolic extracts of *Anacardium occidentale* shoot had the highest ($p < 0.05$) total phenolic content. However, water and ethanolic extracts of *Manihot esculenta* shoot obtained the highest ($p < 0.05$) total phenolic content when were measured using Prussian-Blue assay. In general, results found that most of the phenolic content of extracts were higher when determined using Folin-Ciocalteu assay compared to Prussian-Blue assay, except for ethanolic extracts of *Manihot esculenta* shoot and *Psophocarpus tetragonolobus*.

Previously, González et al. [28] reported that the percentage of phenolic content of propolis from different area in Argentina ranged between 3.25 and 33.49 GAE (analyzed using Folin-Ciocalteu), and 2.36 and 22.86 GAE (analyzed using Prussian Blue assay). Total phenolic content in red wine was also higher when were determined using Folin-Ciocalteu assay (5.14 to 13.3 mg/L GAE) compared with Prussian-Blue assay (1.8 to 4.8 mg/L GAE).

However, the content of phenolic determined using Folin-Ciocalteu assay and Prussian Blue assay was approximately similar in white wines [29].

Table 1. Total phenolic content of water and ethanolic extracts measured by Folin-Ciocalteu and Prussian-Blue assays

Samples	Total phenolic content (mg GAE / g extracts)			
	Water extracts		Ethanolic extracts	
	Folin-Ciocalteu assay	Prussian-Blue assay	Folin-Ciocalteu assay	Prussian-Blue assay
<i>Anacardium occidentale</i> shoot	14.76 ^a	3.57 ^e	15.86 ^a	7.72 ^b
<i>Carica papaya</i> shoot	10.63 ^c	3.69 ^e	7.17 ^c	5.10 ^c
<i>Curcuma longa</i> leaves	7.08 ^e	4.09 ^d	8.86 ^{bc}	7.73 ^b
<i>Etlingera elatior</i>	10.76 ^c	3.50 ^e	9.72 ^b	7.49 ^b
<i>Manihot esculenta</i> shoot	12.37 ^b	7.82 ^a	8.90 ^{bc}	11.54 ^a
<i>Pithecellobium jiringa</i>	7.91 ^e	5.48 ^c	5.38 ^d	1.84 ^e
<i>Psophocarpus tetragonolobus</i>	8.86 ^d	4.22 ^d	5.21 ^d	6.70 ^c
<i>Sauropus androgynus</i>	12.38 ^b	6.71 ^b	6.97 ^{cd}	3.62 ^d

Data were expressed in triplicate analysis. Values with the same lowercase within each column were not significantly different (p>0.05).

The different in phenolic contents values from both methods is accounted by reagent sensitivity, different redox potential of the system used, and reducing capacity [28]. In fact, method of Folin-Ciocalteu reagent is not strictly specific for phenolic compounds measurements because there are other components that can react with this reagent such as ascorbic acid [13, 24], and anthocyanin [29]. In red wine, the contents of anthocyanin is higher than in white wine, thus, the different values of phenolic content determined in both assays could be explained by reaction of anthocyanin with Folin-Ciocalteu reagent [29]. This fact may also explain the finding in this study. Furthermore, Folin-Ciocalteu reagent can also act as medium for ionizing phenols, making them as reducing agents. The reaction is enhanced in alkaline condition since the additional of sodium carbonate create the alkaline environment. On contrary, Prussian-Blue reagent work in acidic condition where phenolic compounds were unionized resulting in their low reducing power as compared to Folin-Ciocalteu reagent [28].

Folin-Ciocalteu and Prussian-Blue assays are the two most common methods in determining total phenolic content in plants. Both are based on color changes a result of sample oxidized by specific reagent. Folin-Ciocalteu reagent consist of phosphotungstic ($H_3PW_{12}O_{40}$) and phosphomolybdic ($H_3PMo_{12}O_{40}$) acids that will oxidize phenolic compound to blue oxides of tungstene (W_8O_{23}) and molybdene (Mo_8O_{23}) [24]. In Prussian Blue assay, the method is based on the formation of ferricyanide-ferrous $[(Fe(CN)_6]^{3-}Fe^{2+}$ ion complex as a result of reduction of red ferric (Fe^{3+}) to blue ferrous (Fe^{2+}) ion by phenolic compounds. HCl is added in the reagent mixture in the assay preparation to increase the stability of ferric chloride ($FeCl_3$), thus, speed up the reaction to complete [31]. The formation of oxidized blue color in both reactions indicates the quantity of phenolic compounds that can be measured using spectrophotometer at specific wavelength [29, 30]. Various types of phenolic compounds also generate different responses of blue color intensity to the different assay [20, 32-33].

In fact, both assays have their own advantages for phenolic contents measurement in extracts. Folin-Ciocalteu assay method is more stable and reproducible method compared to Prussian-Blue assay. Prussian-Blue assay method is unstable due to the formation of undesirable precipitation of extract with the reagent and it will increase with incubation time. However, Prussian-Blue assay is more rapid and sensitive as this assay allows the detection of

phenolic contents at sample extracts concentration lower than 1.10^{-6} mg/mL. Thus, a highly skilled operator is required to conduct Prussian-Blue assay preparation. Furthermore, the handling of the reactive mixture of $K_3Fe(CN)_6$ and $FeCl_3$ is a critical in conducting Prussian-Blue assay and laborious [28].

In this study, it was also obtained that most of water extracts of vegetables contained higher phenolic compounds compared with ethanolic extracts when determined using Folin-Ciocalteu assay. Othman et al. [18] reported that total phenolic content of *Melicope Lunu ankeda* (locally known as *tenggek burung*) and *Polygonum minus* (locally known as kesum) were higher in water extracts than in ethanolic extract. However, phenolic contents of *Murraya koenigii* or curry leaves and *Eugenia polyantha* or *Salam* leaves were higher in ethanolic extracts. Previously, phenolic compounds in henna leaves [34] and from sorghum leaf [35] were more efficient to extract with water as compared with methanol solvent. Water was also the best solvent for extracting tea catechins, than 80% methanol or 70% ethanol [36].

The choice of extraction solvents such as water, acetone, ethyl acetate, alcohols (methanol, ethanol, and propanol) and their mixtures [37] will influence the yields of phenolic contents. In fact, most of aqueous solvent extracts have been shown to give higher total phenolic content as compared to absolute solvent. Turkmen et al. [38] reported that the use of aqueous acetone to extract phenolic compounds from black tea obtained the highest values of total phenolic content compared to other absolute acetone. Alcoholic solvents are not highly selective for phenolic compounds. However, they are more preferred for extracting phenolic compounds from natural sources as they provides comparatively high yield with water. The efficiency and extractability of alcohol as solvent can be enhanced by increasing their polarity and the addition of water with absolute concentration of alcohol solution [39]. Total phenolic content of vegetative parts of *Pluchea indica* is higher when extract using 50% ethanol solvent followed by aqueous ethanol and absolute ethanol [40]. Furthermore, the phenolic contents of *Lathyrus maritimus* L. seed extracted using acetone-water system obtained higher amount than using absolute ethanol-water or methanol-water systems [41]. Perhaps, the addition of water in absolute solvent help to increase the extractability of sample as the polarity of solvent increased [42].

The efficiency of phenolic contents of sample extraction can be enhanced by increasing the amount of water to the solvent. This could be explained in term of hydroxyl bond occur between water and phenolic compounds. It is known that oxygen molecule from solvent will form hydrogen bond with hydroxyl groups of phenolic compounds. Therefore, as more water molecules present in extraction system, more oxygen atom in water molecules are available for the hydrogen bond formation with phenolic compounds. This will allow more phenolic compounds is extracted and resulting in higher extraction yield and total phenolic compounds. Water extracts lead in more hydrogen bond formation with all hydroxyl groups even with the phenolic compounds that bound to the other compounds such as sugar [42]. However, absolute solvent may only restricted to bond with compound with hydroxyl groups of phenolic compounds. Hence, total phenolic content extracted were significantly influenced by different types of solvent and the properties of the phenolic components of the plants types [42-43].

Antioxidant Activities of Vegetables Using Ferric Reducing Antioxidant Power Assay

Results of FRAP analysis of water and ethanolic extracts of vegetables after incubation with linoleic acid for seven days are shown in Figure 2 and Figure 3, respectively. The Figures show that the absorbance reading of the mixture of linoleic acid and vegetables extracts increased during seven days incubation at 40 °C. All samples of linoleic acid in assays oxidized when incubating with vegetables but with different reaction rate. At initial incubation, the lowest absorbance reading measurements were shown in water extract of *Etilingera elatior* (0.0019) and ethanolic extract of *Carica papaya* shoot (0.0060). After seven day incubation, the absorbance values of negative control (without any extracts) was 0.4010 and was significantly ($p < 0.05$) increased with all water and ethanolic extracts of vegetables as predicted. The highest percentage of inhibition of linoleic acid peroxidation was observed in water extract of *Etilingera elatior* (75.6%) and ethanolic extract of *Sauropus androgynus* (78.1%).

Different extracts may have different types and amounts of phenolic compound that can slow down peroxidation rate of unsaturated bond in linoleic acid. The highest value of absorbance reading in negative sample assay was due to the highest rate of linoleic acid peroxidation since it was not inhibited by any compounds in any extracts. The percentage of inhibition linoleic acid was measured at seven days incubation compared with the initial incubation.

The percentage of inhibition of linoleic acid peroxidation of water extracts in the descending order were *Etlngera elatior* (75.6%), *Psophocarpus tetragonolobus* (70.8%), *Sauropus androgynus* (69.3%), *Manihot esculenta* shoot (69.3%), *Carica papaya* (68.3%), *Curcuma longa* leaves (64.8%), *Pithecellobium jiringa* (62.3%), and *Anacardium occidentale* shoot (59.6%). The percentage of inhibition of linoleic acid of ethanolic extracts ranged between 63.6 and 78.1%.

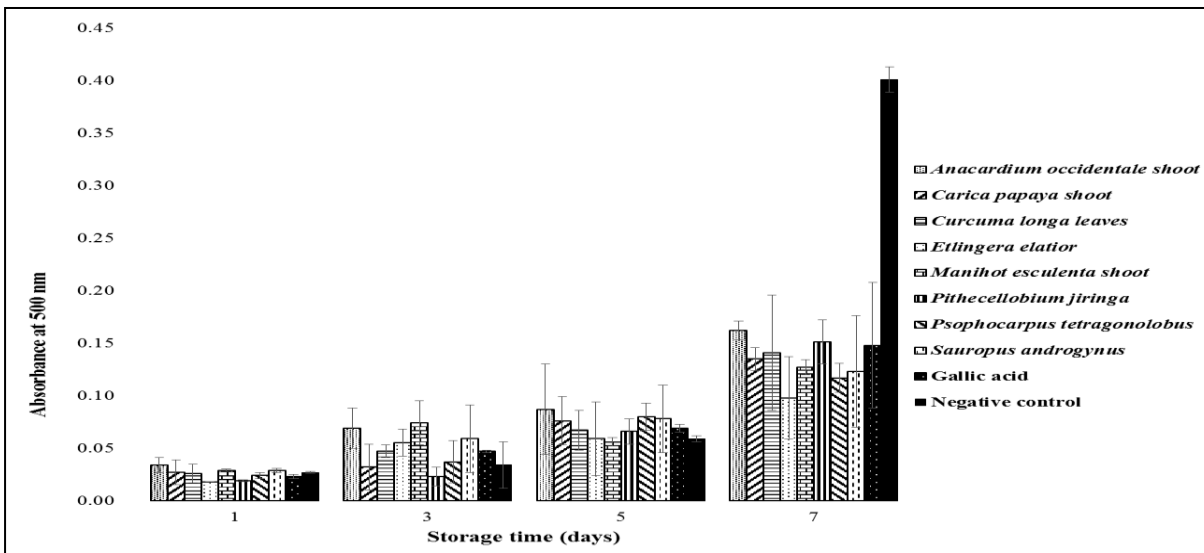


Figure 2. FRAP analysis of water extracts of vegetables. Data were expressed as mean and were analyzed in triplicate

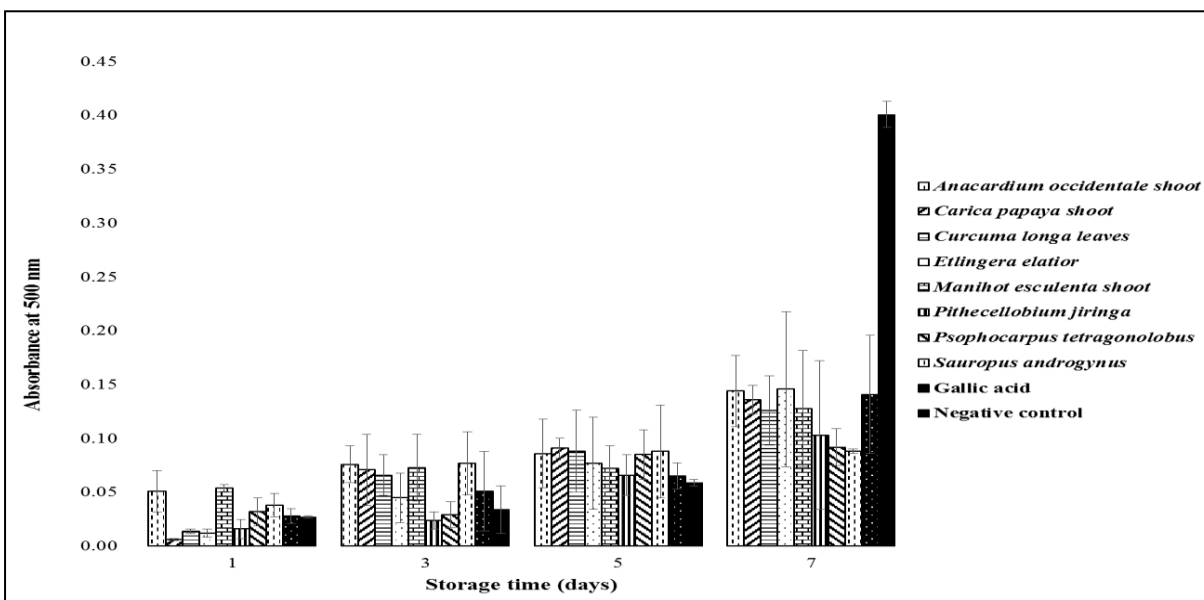


Figure 3. FTC analysis of ethanolic extracts of vegetables. Data were expressed as mean and were analyzed in triplicate

Previously, Huda-Faujan et al. [44] reported that percentage of inhibition of linoleic acid peroxidation of water extracts of five Malaysian vegetables ranged between 52.1 and 67.7%. The vegetables tested was *Murraya koenigi*, *Polygonum minus*, *Centella asiatica*, and *Oevanthe javanica*. However, percentage of linoleic acid inhibition of methanolic extracts of the vegetables ranged between 63.6 and 70.6% [5]. Furthermore, the highest percentage (78.4%) of inhibition of linoleic acid peroxidation in *Terminalia arjuna* was performed in the stem bark of 80% methanolic extract. However, the leaves part of the samples extracted with 80% ethanol demonstrated the lowest percentage (61.9%) of inhibition of linoleic acid peroxidation [45].

In the present study, it was found that the percentage of linoleic acid peroxidation inhibition by gallic acid was 63.9%. The percentage of the inhibition was similar ($p>0.05$) that demonstrated by water extract of *Carica papaya* shoot and *Curcuma longa* leaves. According to Catha et al. [45] percentage of inhibition of linoleic acid peroxidation by Butylated Hydroxyl Toluene (BHT) which is a synthetic antioxidant (91.3%) exhibit significantly higher ($p<0.05$) activity that all extract of *Terminalia arjuna*. In several experiments of antioxidant activity, gallic acid exhibited more effective to inhibit oxidation than BHT. However, in general, natural antioxidant was obtained less effective to protect linoleic acid against peroxidation. An effective natural antioxidant may suggested to be used in combination of two or more mixtures to protect linoleic acid.

Antioxidant Activity of Vegetables Extracts using DPPH Assay

The radical scavenging activity of water and ethanolic extracts of vegetables using DPPH assay are shown in Figure 4 and Figure 5, respectively. Results demonstrated that the DPPH free radical scavenging activity of extracts increased with increasing in sample concentration. At 1.0 mg/mL, water extract of *Curcuma longa* leaves obtained the highest scavenging activity ($p<0.05$), while at the same concentration *Manihot esculenta* shoot demonstrated the lowest scavenging activity of DPPH free radical [Figure 3(a)]. However, the scavenging activity of *Manihot esculenta* was not significantly different with *Pithecellobium jiringa* at 1.0 mg/mL. From Figure 3(b), the highest scavenging activity of ethanolic extract was obtained in *Psophocarpus tetragonolobus* at concentration 1.0 mg/mL.

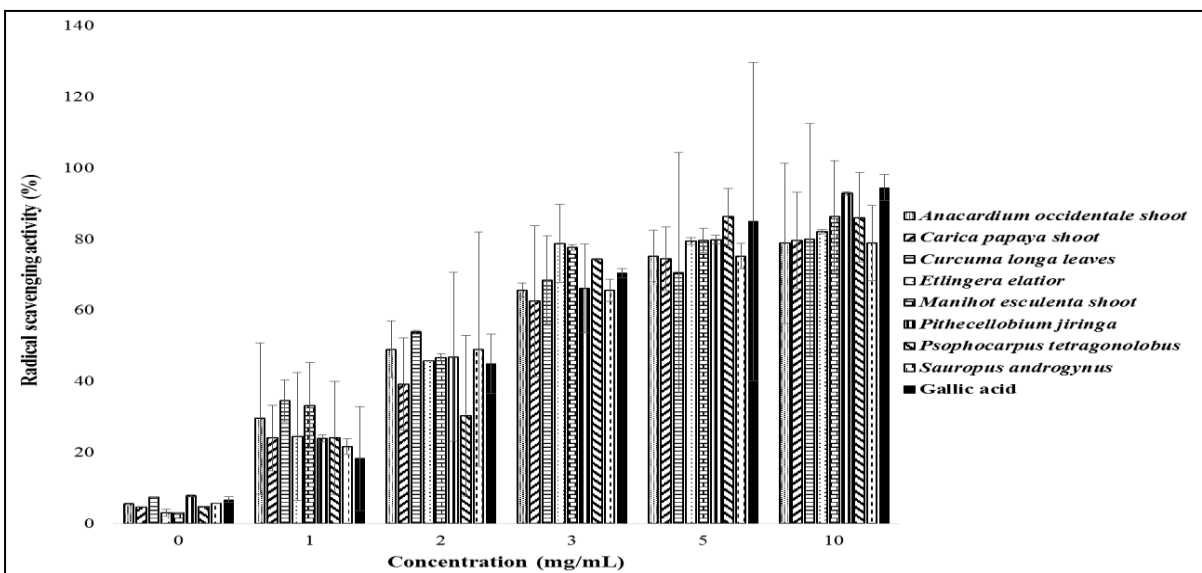


Figure 4. The radical scavenging activities of water extracts of vegetables using DPPH assay.

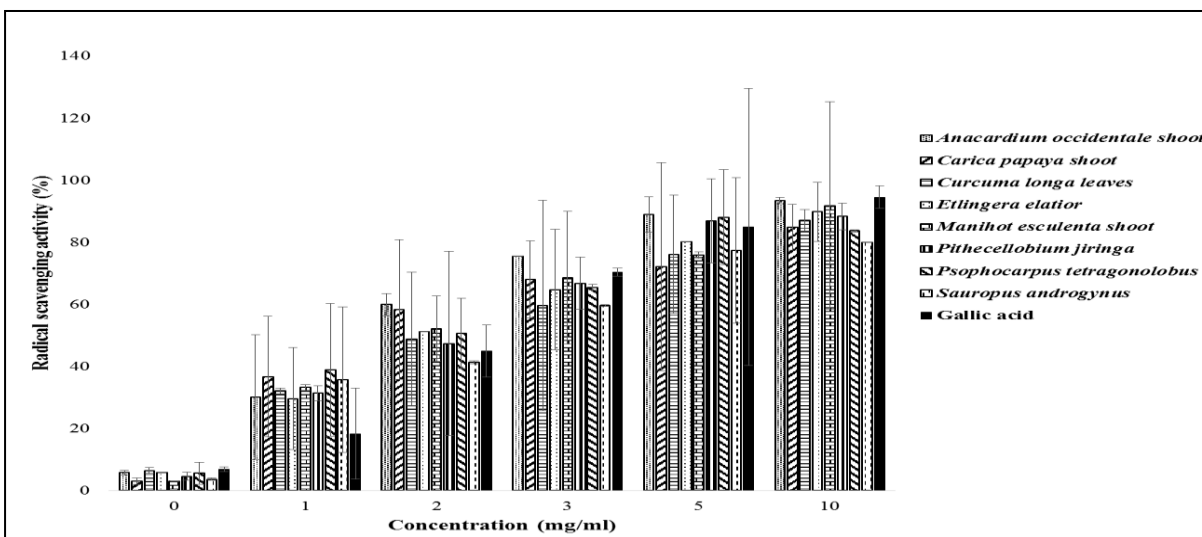


Figure 5. The radical scavenging activities of ethanolic extracts of vegetables using DPPH assay.

One of the most widely used methods for determining antioxidant activity of sample extracts is DPPH radical scavenging activity assay [18, 33, 45]. The assay was developed based on the ability of antioxidant properties in the extracts to scavenge the stable radical DPPH [48]. In the present study, it was demonstrated that the antioxidant activity of extracts was depending on the extract concentration. In general, antioxidant activity of extracts increased with concentration of extracts in assay. Other studies also reported the similar trend of the DPPH radical scavenging activity of various extracts [13, 18, 33, 45]. However, at concentration of 6 mg/mL, the scavenging activity of sample extracts start to become plateau and this could due to the decreasing amount of DPPH radical in the assay. The scavenging activity of extracts might be due to the presence of phenolic hydroxyl groups in phenolic compounds. Results also obtained that most of ethanolic extracts demonstrated higher antioxidant activity compared to water extracts. In fact, Prior et al. [46] reported that this could be due to the better solubility of DPPH radicals in organic acid especially in ethanol compared to water solvent. Furthermore, Spigno et al. [39] reported that higher antioxidant activity of extracts should be found in alcoholic extracts compared to water extracts because alcoholic solvents maximize DPPH radicals interaction with antioxidant present in extracts.

Table 2 shows data of IC_{50} of water and ethanolic extracts of vegetables and expressed as mg/mL. The extract concentration that provide 50% inhibition or IC_{50} was calculated from the graph plotted inhibition percentages against tested samples extracts. Result obtain that the best IC_{50} activity of extracts were demonstrated by water and ethanolic extract of *Sauropus androgynus* (0.077 mg/mL and 0.078 mg/mL, respectively). However, the IC_{50} value of water extract of *Pithecellobium jiringa* (0.091 mg/mL) and ethanolic extract of *Anacardium occidentale* shoot (0.092 mg/mL) were not significantly ($p > 0.05$) different with gallic acid. In fact, the lower the IC_{50} value, the higher the antioxidant activity is examined as the IC_{50} values defined the concentration of extract that causes 50% loss of the DPPH activity [47]. Previously, Olajuyigbe and Afolayan [48] obtained that the IC_{50} of water and ethanolic extracts of *Ziziphus mucronata* subsp. *mucronata* were 0.065 mg/mL and 0.042 mg/mL, respectively. The IC_{50} of ethanolic extracts of various parts of *Cinnamomum cassia* ranged between 0.072 and 0.208 mg/mL.

Correlation of Phenolic Contents and Antioxidant Activities of Vegetables Extracts

Table 3 shows result of correlation analysis between total phenolic content and antioxidant activity of vegetables. The results obtain that total phenolic content were low and negatively correlated with antioxidant activities in linoleic acid. Nevertheless, all positive correlation between total phenolic content and scavenging activity of DPPH free radical were observed in all samples of extracts. Ethanolic extracts were strongly correlated with DPPH free radical assay and was consistent with other studies [13, 18, 29, 49]. This study also demonstrated that phenolic contents of ethanolic extracts obtained higher correlation with DPPH free radical assay compared with water

extracts. DPPH is known to react specifically with low molecular weight phenolic compounds [50] as well as DPPH interaction and solubility is maximized in alcoholic solvent with the existence of extract [38, 46]. However, several studies also found that total phenolic content did not correlate with antioxidant assay [51-52]. The reasons for the reports might be due to the high amount of reducing agent in the extracts [25] that can interfere the results. In fact, extraction procedure can greatly influence the antioxidant activity as different solvent affected the antioxidant activity significantly [18]. Furthermore, different methods used in analysis of antioxidant activity may also give different value of antioxidant activity for same extract. Various types of components in sample extracts will also react differently with different reagents and different mechanisms. Thus, there was no specific method that can produce accurate result for certain analysis and various methods with different principles must be applied to obtain more accurate and precise results.

Table 2. Inhibitory concentration IC₅₀ of water and ethanolic extracts (mg/mL).

Samples	Inhibitory concentration (IC ₅₀)	
	Water extraction	Ethanolic extraction
<i>Anacardium occidentale</i> shoot	0.088 ^a	0.092 ^a
<i>Carica papaya</i> shoot	0.078 ^d	0.083 ^c
<i>Curcuma longa</i> leaves	0.078 ^d	0.085 ^c
<i>Etilingera elatior</i>	0.080 ^c	0.088 ^{ab}
<i>Manihot esculenta</i> shoot	0.085 ^b	0.090 ^a
<i>Pithecellobium jiringa</i>	0.091 ^a	0.087 ^b
<i>Psophocarpus tetragonolobus</i>	0.084 ^b	0.082 ^c
<i>Sauropus androgynus</i>	0.077 ^d	0.078 ^d
Gallic acid	0.093 ^a	0.093 ^a

Data were expressed as mean and were analyzed in triplicate. Value with the same lowercase within each column are not significantly different (p>0.05).

Table 3. The result of correlation analysis of water and ethanolic extracts of vegetables.

Total phenolic content	Pearson correlation coefficient (r)	
	Inhibition of linoleic acid oxidations	Scavenging activity of DPPH free radical
1. Water extraction		
Folin-Ciocalteu assay	-0.083	0.049
Prussian-Blue assay	0.072	0.128
2. Ethanolic extraction		
Folin-Ciocalteu assay	-0.718	0.702
Prussian-Blue assay	-0.569	0.589

Conclusion

From this study, it can be concluded that all water and ethanolic extracts of Malaysian traditional vegetables contain various amount of total phenolic compounds when were determined using both Folin-Ciocalteu and Prussian Blue assays. In general, most of the ethanolic extracts of the vegetables obtained slightly higher antioxidant activity in

both FRAP and DPPH assays. However, total phenolic content and antioxidant activity was only have positive correlation when were determined using DPPH assay. Thus, this study obtained that the traditional Malaysian vegetables can be a potential source of natural antioxidant. Nevertheless, further work is needed to identify and isolate the individual phenolic compounds to determine the *in vitro* and *in vivo* of antioxidant mechanism in this vegetable.

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