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Natural Antioxidant from *Hibiscus Sabdariffa* Extract: Assessments on Extraction Yield, Antioxidant Capacity and Total Polyphenol Content of Different Polarities of Solvent Extracts

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Abstract
Hibiscus sabdariffa L. (H. sabdariffa, roselle; Malvaceae) has traditionally been used as food, in herbal drinks, hot and cold beverages, as a flavoring agent in the food industry and as herbal medicine. The effect of solvents (hexane, ethyl acetate and ethanol) on extraction yield, antioxidant capacity and polyphenol content were studied. The antioxidant capacities of the H. sabdariffa extracts were evaluated using a ferric reducing antioxidant power (FRAP) assay and the free-radical scavenging capacity was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays. The polyphenols from H. sabdariffa extracts were determined by total phenolic content (TPC) and total flavonoid content (TFC). The ethanol extract showed the highest extraction yield (38.39 ± 0.29%) and total antioxidant activity: DPPH (73.96 ± 0.7%) and FRAP (72.93 ± 2.4 mmol Fe^{2+} g^{-1}). The same extract also exhibited the highest phenolic content (312.25 ± 0.17 mg gallic acid equivalent g^{-1}). Meanwhile, for flavonoid content, the ethanol and ethyl acetate extracts shared the same amount, at 6.29 ± 0.35 mg quercetin equivalent g^{-1}. High antioxidant capacity was significantly correlated with high phenolic content. This study suggests that ethanolic extracts of H. sabdariffa can be used as good sources of natural antioxidants for health benefits.

Keywords
Solvent extraction; Antioxidant; Phenolic compound; Flavonoid

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1 Introduction

Various plants, particularly medicinal plants, have been extensively studied for their antioxidant activity in recent years. Antioxidants from aromatic, spicy, medicinal, and other plants have been studied to develop natural antioxidant formulations for food, cosmetics and other applications[1]. There are three major classes of plant chemicals, namely, terpenoids, phenolic metabolites and

alkaloids[2]. Among these three groups, phenolic compounds are the most important for dietary applications and the most extensively studied[3]. Phenolic compounds include phenolic acids (hydroxybenzoic and hydroxycinnamic acids), polyphenols (hydrolyzable and condensed tannins), and flavonoids. These compounds protect plants, fruits, and vegetables from oxidative damage and have been used as antioxidants by humans. Finding new and safe antioxidants from natural sources is of great interest for applications in natural antioxidants, functional foods, and nutraceuticals.

There are many techniques to recover antioxidants from plants, such as soxhlet extraction, maceration, supercritical fluid extraction, subcritical water extraction, and ultrasound-assisted extraction. However, extraction yield and antioxidant capacity depend not only on the extraction methods but also on the solvents used for extraction. Solvent extraction is the most frequently used technique for the isolation of plant antioxidant compounds. In addition to that, the plant material extract yields and the resulting antioxidant activities are strongly dependent on the nature of the extracting solvent due to the presence of different antioxidant compounds of varied chemical characteristics and polarities that may or may not be soluble in a particular solvent[4]. Polar solvents are frequently used for recovering polyphenols from plant matrixes. The most suitable solvents are aqueous mixtures containing ethanol, methanol, acetone, and ethyl acetate. Ethanol has been known as a suitable solvent for polyphenol extraction, depending on the percentage and quantity. Methanol is generally found to be more efficient in extraction of lower molecular weight polyphenol, whereas aqueous acetone is commonly used to extract higher molecular weight flavanols[5]. There are reports concerning optimization of conditions for extraction of phenolic compounds, and the antioxidant activities of some plant food. However, as some researchers have indicated, the optimal procedure is usually different for different plant matrices[6,7].

Hibiscus sabdariffa is from the Malvaceae family, described as an annual, bushy plant with a height of up to 2.5 m, characterized by smooth, cylindrical red stems, reddish veins and long, green leaves[8]. The flowers are borne singly, having different colours: yellow or buff with a rose or maroon eye and turning pink during maturation. The calyx is red, consisting of five valves containing 3-4 kidney-shaped light-brown seeds. *H. sabdariffa* flowers are a rich source of the anthocyanins which is responsible for the red colour[8]. It has been reported that the extract of *H. sabdariffa* has bioactive properties that play crucial role in preventing hypertension, reducing hepatic diseases and diabetes. The main constituents of *H. sabdariffa* relevant in its pharmacological context are organic acids, anthocyanins, polysaccharides and flavonoids[9,10]. In the present study, the antioxidant capacities of the different solvent extracts of *H. sabdariffa* were studied by 1,1-diphenyl-2-picrylhydrazyl hydrate (DPPH), together with the ferric reducing antioxidant power (FRAP). The contents of total phenolics content (TPC) and total flavonoid content (TFC) were also determined.

2 Materials and Methods

2.1 Materials

The *H. sabdariffa* plant used in this study was purchased from the Federal Agricultural Marketing Authorities (FAMA) at Rengit, Johor, Malaysia. The plant was authenticated by an expert and deposited at the Herbarium Unit of the Institute of Bioscience, Universiti Putra Malaysia with Voucher No. SK 3269/18. DPPH and Folin-Ciocalteu's (FC) phenol reagent was obtained from Merck (Darmstadt, Germany). Sodium carbonate, gallic acid, 1,1-diphenyl-2- picrylhydrazyl (DPPH) and 2,4,6-tris(1-pyridyl)-5-triazine (TPTZ) were purchased from Sigma (Steinheim, Germany), and ferrous sulfate was obtained from R&M Chemicals (Essex, UK).

2.2 Preparation of Plant Extracts and Determination of Yield

The fresh *H. sabdariffa* (500 g) was washed and air-dried for seven days. The air-dried sample was ground into powder using an electric grinder. The *H. sabdariffa* powder (10 g) was mixed with the solvents (100 mL) and the mixture was soaked for 12 hours. After 12 hours, the mixture was filtered with Whatman filter paper No. 4 and the residues were further extracted with a fresh batch of solvent (100 mL). This procedure was repeated three times and the extracts were combined and concentrated in a rotary evaporator (IKA RV-10 Digital V, German). The concentrated extract was weighed to determine the extraction yield. All concentrated extracts were kept at 4 °C prior to analyses.

2.2 Determination of Polyphenol Content

2.2.1 Total Phenolic Content (TPC)

Total phenolic content was determined with FC reagent, similar to the method employed by Kähkönen et al.[11]. Each extract sample (0.2 mL,1 mg mL^{-1}) was added with 1 mL of a 10-fold dilution of FC reagent and 0.8 mL of 7.5% (w/v) bicarbonate solution. Then, the reaction mixture was allowed to stand at room temperature for 30 min. The absorbance was measured at 765 nm, using a UV-vis spectrophotometer (Tecan Infinite 200 Pro, Switzerland). The TPC was expressed as gallic acid equivalents (GAE) in milligram per gram (mg g^{-1}) of dry material. This experiment was performed in triplicate. The data obtained were reported as mean and standard deviation.

2.2.2 Total Flavonoid Content (TFC)

The total flavonoid content of each extract was investigated using the aluminum chloride colorimetric method as described by Chang et al.[12], with slight modifications. In brief, the extract was diluted with methanol to 100 mg mL^{-1}. The calibration curve was established using various concentrations of quercetin (as standard reference), which prepared by a serial dilution from an initial concentration of 100 mg mL^{-1} in methanol. The diluted extract or quercetin (2.0 mL) was mixed with 0.1 mL of 10% (w/v) aluminum chloride solution and 0.1 mL of 0.1 mM potassium acetate solution. The mixture was kept at room temperature for 30 min. Then the maximum absorbance of the mixture was measured at 415 nm using a UV-vis spectrophotometer (Tecan Infinite 200 Pro, Switzerland). TFC was expressed as milligram quercetin equivalent per gram defatted *L. aromatica* (mg QCE g^{-1} DFLA). This experiment was performed in triplicate. The data obtained were reported as mean and standard deviation.

2.3 Determination of Antioxidant Activity

2.3.1 DPPH Radical Scavenging Activity

The antioxidant activity of the extract was measured by the DPPH method adapted from Shimada et al.[13]. One milliliter of freshly prepared 1 mM DPPH in methanol was added to test tubes containing 5 mL of the sample extracts. A control was prepared by adding 1 mL of DPPH solution to 5 mL of 80% methanol. After being stored in the dark for 30 min, the absorbance was read at 517 nm using a UV-vis spectrophotometer (Tecan Infinite 200 Pro, Switzerland). Ascorbic acid was used as a positive control and measurements were made in triplicate. The percentage of free-radical scavenging activity was calculated, using the following Equation 1:

$$\text{Free-radical scavenging activity (\%)} = \frac{Abs_{517(blank)} - Abs_{(sample)}}{Abs_{517(blank)}} \times 100 \qquad (1)$$

2.3.2 Ferric Reducing Antioxidant Power (FRAP)

FRAP assay was employed using the method employed by Wojdylo et al.[14], with minor modification. Acetate buffer (300 mM), dilute HCl (40 mM) and 2,4,6-tripyridyl-s-triazine (TPTZ) solution (10 mM) were prepared. Ferric chloride solution (20 mM) and FRAP reagent consisting of acetate buffer, TPTZ and ferric chloride in the ratio 10:1:1 (v/v) were freshly prepared simultaneously. The

tested sample (15 μL) was added to 270 μL of TPTZ solution, and the measurement at 593 nm was carried out immediately (t = 0 min) after addition of 15 μL of ferric chloride solution[15] using a UV-vis spectrophotometer (Tecan Infinite 200 Pro, Switzerland). The measurement was also carried out after the sample was incubated for 4 min in a dark room. Ascorbic acid was used as positive control in this assay.

3 Results and Discussion

3.1 Effects of Solvent on Extraction Yield and Polyphenol Content

3.1.1 Extraction Yield

Extraction is the essential step for recovering and isolating phytochemicals from plant materials. The extraction efficiency is affected by the chemical nature of the phytochemicals, the extraction method used, sample particle size, the solvent used, as well as the presence of interfering substances[16]. Meanwhile, the extraction yield depends on the solvent of varying polarity, pH, temperature, extraction time, and sample composition under the same extraction time and temperature conditions; solvent and composition of sample are known to be the most important parameters[17]. Hence, the selection of extraction solvents is critical for complex plant samples. An extraction solvent system is generally selected according to the purpose of extraction, polarity of the components of interest, polarity of undesirable components, overall cost, safety and environmental concerns[18]. Thus, in this study, the extraction yields were obtained using three different solvents, and the selection of solvent used was based on increasing the polarity solvent: hexane < ethyl acetate < ethanol. The percentage yield was obtained from Equation 2:

$$\text{Extraction yield (\%)} = \frac{W_2 - W_1}{W_0} \times 100\% \qquad (2)$$

where W_2 is the weight of the extract and the container, W_1 is the weight of the container alone, and W_0 is the weight of the initial dried sample.

The percentage yields of *H. sabdariffa* are shown in Table 1. Ethanol showed significantly ($p < 0.05$) the highest extraction yields of 38.39%, compared to ethyl acetate with 31.95%. Meanwhile, hexane showed the lowest extraction yield with only 10.69%. This result shows that the percentage yield increases with increasing polarity of the solvent used in extraction. These facts are in accordance with the polarity of the solvent used for the extraction and solubility of phenolic compounds in it[19]. It is interesting to note that hexane, ethyl acetate and ethanol polarities are 0.009, 0.228 and 0.654 respectively.

3.1.2 Total Phenolic Content (TPC)

Phenolics or polyphenols are plant secondary metabolites and are very important by virtue of their antioxidant activity by chelating redox-active metal ions, inactivating lipid free-radical chains and preventing hydroperoxide conversions into reactive oxyradicals[20]. The results of TPC in the *H. sabdariffa* extracts are summarized in Table 1. TPC values were obtained from the calibration curve, using Equation 3:

$$y = 0.004x + 0.0499 \qquad (3)$$

with $R^2 = 0.9876$, where x is the absorbance and y is the concentration of gallic acid solution (mg mL^{-1}) expressed as mg GAE g^{-1}.

The results of TPC in the *H. sabdariffa* extracts is summarized in Table 1. The ethanol extracts contained the highest TPC (312.25 mg GAE g^{-1}), followed by ethyl acetate (127 mg GAE g^{-1}) and hexane extracts (37 mg GAE mg^{-1}). It is evident that the recovery of phenolic compounds depends on the solvent used, its polarity and the solubility of phenolic compounds in the extraction solvent[21] and solvent polarity plays a key role in increasing phenolic solubility[22]. This may be caused by the possible formation of complexes of some phenolic compounds in the extract, which are soluble in ethanol and ethyl acetate. Hence, polar phenolic compounds in *H. sabdariffa* extracts increase with increasing polarity, according to the like dissolves like

principle. These phenolic compounds may possess more phenol groups or have higher molecular weights than the phenolics in the hexane extract[17]. Based on TPC results, the best extracting solvent was ethanol; which are in agreement for several medicinal plants such as *Withania somnifera*[23], horseradish root[24] and others selected medicinal plants[25].

3.1.3 Total Flavonoid Content (TFC)

Flavonoids are secondary metabolites with high therapeutic potential, including cardioprotective[26], anti-inflammatory, antimicrobial[27] and antitumor[28] activities. In this study, quercetin was used as a standard reference to detect flavonoid content in *H. sabdariffa* extracts. The regression equation for TFC determination used was given in Equation (4):

$$y = -0.4592x + 3.0966 \quad (4)$$

with R^2 = 0.9930, where x is the absorbance and y is the concentration of quercetin solution (mg mL^{-1}) expressed as mg QCE g^{-1}. Results in Table 1 indicated that both ethanol and ethyl acetate showed the same TFC value (6.29 mg QCE g^{-1}), while the lowest TFC was found for the hexane extracts (4.50 mg QCE g^{-1}). These results suggested the possible influence of extracting solvent on TFC for the *H. sabdariffa* extracts, especially polar solvents, which have higher solubility for flavonoids, including flavones, flavanols and condensed tannins; thus, it gives higher TFC value. These observations could be owing to flavonoids having the capability as radical scavengers due to the molecular structure of flavonoids, which contain hydroxyl groups that could donate an electron (H^+) to radicals such as hydroxyl (HO•), superoxide ($O_2^{•-}$) and peroxyl (ROO•), thus neutralizing them[28]. These results agree with those of Do et al.[17] who suggested that the highest TFC belongs to the ethanol extract of *Limnophila aromatica*. Besides, Abozed et al.[29] also reported similar observation with a different plant - Beni-suef-3 wheat. Therefore, it could be suggested that both ethanol and ethyl acetate are the best options to extract flavonoids in higher amounts.

Table 1. Extraction yield and total polyphenols content of different solvent extracts of *H. sabdariffa*.

Solvent	Extraction yield (%)	TPC (mg GAE g^{-1})	TFC (mg QCE g^{-1})
Hexane	10.69 ± 0.46	37.00 ± 0.04	4.50 ± 0.33
Ethyl acetate	31.95 ± 0.21	127.00 ± 0.01	6.29 ± 0.66
Ethanol	38.39 ± 0.29	312.25 ± 0.17	6.29 ± 0.35

Note: Values (mean ± standard deviation (SD)) are the average of three samples of each solvent of *H. sabdariffa* extracts, analyzed individually in triplicate (n = 1×3×3), ($p < 0.05$).

3.2 Solvent Effects on the Antioxidant Activities

3.2.1 2,2-diphenyl-1-picryl hydrazyl (DPPH)

DPPH radical has been used as a stable free radical to determine the antioxidant activity of natural compounds[30]. The antioxidant activity of plant extracts containing polyphenol components is due to their capabilities to be donors of hydrogen atoms or electrons and to capture free radicals[31]. Thus, 2,2-diphenyl-1-picryl hydrazyl (DPPH) (which is purple) will reduce to α,α-diphenyl-β-picrylhydrazine (yellow)[32]. According to Suhaj[33], scavenging of the stable radical (DPPH) is considered a valid and easy assay to evaluate the scavenging activity of antioxidants.

Results of the free-radical scavenging activity of *H. sabdariffa* extracts are presented in Figure 1. Results showed that ethanol extract contained the highest DPPH radical scavenging activity (73.96 ± 0.7%), followed by ethyl acetate extract (40.08 ± 0.6%) and hexane extract (22.46 ± 2.4%). The findings justify that secondary metabolite, including phenolics and flavonoids, are rich in ethanol and ethyl acetate extracts which have antioxidant activity due to their redox properties and chemical structures[34]. It is also well known

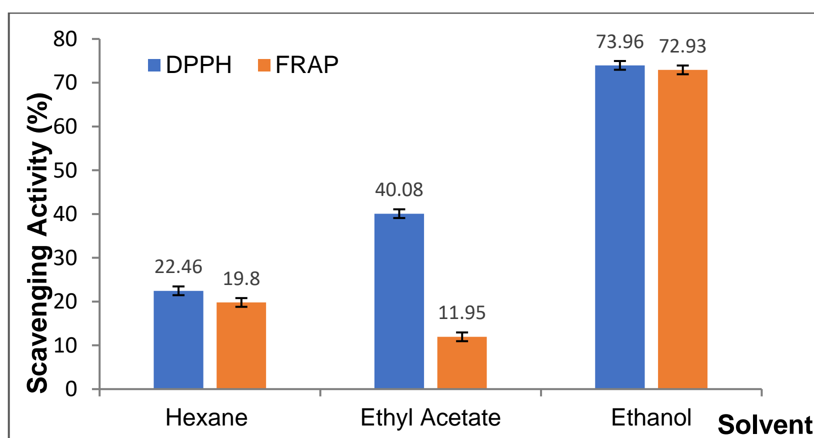
that plant phenolic compounds can play an important role in shaping the plant's biological properties, including antioxidant properties[35]. Meanwhile, flavonoids are highly effective scavengers of most oxidizing molecules, including singlet oxygen and other free radicals implicated in several diseases[36]. This is also supported by the studies of Graversen et al.[37] and Roberts and Gordon[38], who found that plant polyphenols have a synergistic effect with other antioxidants present in plant material. In addition, other studies have also been carried out to analyze the synergistic effect of antioxidants[39,40,41]. However, antioxidant activity in this study did not show a synergistic effect in plant extract mixtures.

3.2.2 Ferric Reducing Antioxidant Power (FRAP)

FRAP assay is commonly used to study the antioxidant capacity of plant materials. The antioxidant capacity of *H sabdariffa* extracts is determined by the ability of the antioxidants to reduce ferric iron to ferrous in the FRAP reagent, which consists of 2,4,6-tris(1-pyridyl)-5-triazine (TPTZ) prepared in sodium acetate buffer, pH 3.6. The reduction of ferric iron in the FRAP reagent will form a blue product (ferrous–TPTZ complex) whereby the absorbance can be read at 593 nm. The absorbance data was calculated against a series of dilutions of ferrous sulfate, $FeSO_4.7H_2O$ as standard calibration curve, given in Equation (5):

$$y = -0.0004x + 0.0401 \quad (5)$$

with $R^2 = 0.9397$ (0.278 g of ferrous sulfate in 1 L of distilled water) and expressed as equivalent to mmol Fe^{2+} g^{-1}. The results which show the effects of different extracting solvents on the reducing potential of *H. sabdariffa* at a concentration of 1 mg mL^{-1} are shown in Figure 1. The reducing power of the *H. sabdariffa* extracts increased in a concentration-dependent manner (Figure 1). The values of absorbance for the tested extract solutions at a concentration of 1 mg mL^{-1} determined in this assay, ranged from 11.95 to 72.93 Fe^{2+} g^{-1} and followed the order of effectiveness: ethanol (72.93 ± 2.4 mmol Fe^{2+} g^{-1}) > hexane (19.8 ± 0.9 mmol Fe^{2+} g^{-1}) > ethyl acetate (11.95 ± 0.2 mmol Fe^{2+} g^{-1}). The ethanol extract exhibited greater TPC, resulting in good reducing power in the present analysis. These results correspond well with those reported by Wong et al.[42], that ethanolic extract exhibited high antioxidant capacity, especially in plant extraction. This is possibly due to the most ferric ion being reduced to ferrous ions by an antioxidant. This was evidenced by the formation of a more intense blue colour that implies the electron donation of the antioxidant compound. Besides, the reducing potential of antioxidant components is very much associated with their TPC. The plant extracts with higher levels of total phenolics also exhibit greater reducing power[43,44,45].



Note: Data are expressed as mean ± standard deviation. ($n = 3$)

Figure 1. Antioxidant capacities of different solvent extracts of *H. sabdariffa*.

4 Conclusion

The results of the present study showed that *H. sabdariffa* extracts exhibited potent antioxidant activity. Among the three extracts, the ethanolic extract exhibited higher potency of free-radical scavenging and ferric reducing activity, which is highly related to the presence of phenolic compounds and flavonoids. Thus, this study suggests that ethanolic extracts of *H. sabdariffa* can be used as good sources of natural antioxidants for health benefits. Further isolation of bioactive compounds is required to identify the unknown compounds to establish their pharmacological properties.

Conflict of Interest

The authors declare no conflict of interest that is related to the work.

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Author Contribution

Conceptualization: Mohamad, N.R.
Data curation: Mohamad, N.R.
Methodology: Mohamad, N.R.
Formal analysis: Mohamad, N.R. & Darham, W.
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Validation: Abdul Gani, S.S. & Abdul Wahab, A.
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