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Different Temperatures and Extraction Solvents significantly affected the Phenolic, Flavonoid content and Antioxidant activity from *Diospyros buxifolia* Leaf extracts

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ABSTRACT
Diospyros buxifolia, a member of Ebenaceae family is a tropical evergreen shrub or small tree that possesses various biotic activities such as antibacterial, anti-inflammatory and cytotoxicity activities. The present study was conducted to analyse the antioxidative properties, total flavonoid content and total phenolic content of leaf extracts of Diospyros buxifoliaespecially during variations in temperatures. Tests such as ferric reducing antioxidant power (FRAP) and 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) were used to determine the antioxidant properties of the plant extract. Distilled water and methanolic extracts with disparate temperatures at 40°C and 60°C were analysed for potential antioxidant properties, total flavonoid content and total phenolic content. The results showed that methanolic extracts of Diospyros buxifolia achieved higher total phenolic content at 40°C (35.43 ± 0.19 mg GAE/g extract), total flavonoid content at 60°C (257.78 ± 12.62 mg CE/g extract), FRAP value at 60°C (0.369 ± 0.036 mM/L at the concentration of 1.0 mM) and DPPH radical scavenging activity at 60°C (22.31 ± 1.180 % at the concentration of 100 µg/mL) compared to aqueous extracts.IC_{50} values of methanolic extracts were found to be 276.30 µg/mL at 60°C for DPPH. The results showed moderate antioxidant activity in all sample extract and compared with ascorbic acid which showed IC_{50} values 56.56 µg/mL for DPPH. The results of in vitro models showed that methanolic extract attain higher antioxidant activity as more phenolic and flavonoid constituents present in methanolic extract when equate to aqueous extract.
***Keywords**: Diospyros buxifolia; antioxidant activity; total flavonoid content; total phenolic content*

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INTRODUCTION

Medicinal plants, rich with their secondary metabolites, offer a reservoir of preventive and therapeutic options[1]. Through the efforts of ongoing scientific researches, increasing number of phytochemicals have been tested and developed into effective modern drugs. Antioxidant substances have high reduction potential and it is difficult to synthesize. Natural antioxidants which include carotenoids, vitamins, phenols, and flavonoids exist naturally in plants with compounds that possess antioxidant activity. The use of natural antioxidants has been highly promoted when compare to the synthetic drugs due to the public concerns on the safety[2].Wide range of different biology aspects of *Ebenaceae* is presented in a collection of related data. Many species are valued for their wood, particularly ebony, for fruit, and as ornamental plants. *Diospyros buxifolia* from the family of *Ebenaceae* is a kind of small evergreen tree which could be seen all over the tropical region. *Diospyros* species possesses various biological activities such as cytotoxicity activity, antibacterial and anti-inflammatory activities [3]. The genus is large in amount and more species have been estimated based on the date of the source. More than 1000 entries as well as synonyms and items of low confidence have been listed in the Royal Botanic Garden, Kew List. However, high confidence is marked and assigned on up to 700 species. Maridass (2008) had reported the pharmacological and phytochemical studies of 13 *Diospyros* species. The result of methanolic fruit extract yield with total of 29 *Diospyros* species. Presence of bioactive constituents of terpenoids (100%), essential oils (100%), tannin (55.17%), flavonoids (68.97%) and alkaloids (82%) were detected in those 29 *Diospyros* species. All the metabolites of *Diospyros* have been shown in response for therapeutic activity of plants [4].

Extraction method with sorts of extracting solvents to isolate antioxidant compounds from plants is the widely used technique. Nevertheless, the antioxidant activities and the yields of extraction of plants depends on the solvent nature, which involve the existence of different chemical features and polarities of polyphenolic compounds that may be solubilized or insolubilized in certain solvent. Polar solvents are commonly used to recover the polyphenols from a plant matrix. Aqueous mixtures containing acetone, methanol, ethyl acetate and ethanol are few better solvents employed for plant extraction[5].Ethanol and methanol play a vital role and have been generally supplicate to extract the potential antioxidant compounds directly from the plant-based foods. Even though there are many past researches carried out to determine the antioxidant capacity of various types of herbs, there are no systemic researches carried out on determination of antioxidant activity of *D. buxifolia*. Therefore, current study was executedto analyse the total flavonoid and total phenolic content and also the potential of total antioxidant activity of *D. buxifolia*.

MATERIALS AND METHODS

Plant Materials

Fresh leaves of *Diospyros buxifolia*were collected from Agro Park of Universiti Malaysia Kelantan and used as the main material in this experiment.

Chemicals

The chemicals that were used in this experiment are distilled water, methanol, Folin and Ciocalteu's phenol reagent, sodium carbonate, gallic acid, aluminium chloride, sodium nitrite, sodium hydroxide, catechin, ascorbic acid, 2, 2-diphenyl-1-picrylhydrazyl powder, glacial acetic acid, sodium acetate trihydrate, TPTZ (2,4,6-tri[2-pyridyl]-s-triazine), iron (III) chloride hexahydrate, hydrochloric acid and iron (II) sulphateheptahydrate ($FeSO4•7H2O$). All chemicals used were of analytical grade.

Sample Preparation

Fresh leaves were harvested, washed and rinsed. The leaves were over dried at 40°C for 24 hours. The dried leaf samples were blended into powder form by using electrical blender (Elba). 50 g of dried leaf powder were weighed and mixed with 200 mL distilled water and heated at 2 different temperatures, 40ºC and 60ºC for 30 minutes in a water bath machine (Memmert). Whatman No.1 filter papers were used to separate the extracted solvent and the impurities in the solvent. The solvent was evaporated by a vacuum rotary evaporator (Buchi) under pressure of 40°C. Lastly, the extracts were transferred to sterilised glass petri dishes and weighed. The plant extracts was then kept in chiller under 4°C for further use. The same procedure was followed for methanol.

Total Phenols Determination

Total phenolic content (TPC) of the extracts were evaluated by using modified Folin – Ciocalteu assay[6].10mL of distilled water was mingled with 0.01g of extract. Then 1mL of 10% v/v Folin and Ciocalteu's phenol reagent is added to the separated 1mL (1000µg/mL) leaf extract. After 3 minutes, 1mL of 10% w/v sodium carbonate solution was added and followed by the addition of distilled water to 10 mL. The mixture was placed in the dark at room temperature for 90 minutes; the absorbance was then read at 750 nm. Gallic acid was used to make calibration curve. The total phenolic content was carried out in triplicate. TPC was expressed as mg of gallic acid equivalents (GAE) per g of extract.

Total Flavonoids Determination

Aluminium chloride colorimetric method was used for flavonoids determination [7].5ml of distilled water was added to each of the 1mL plant extracts (1000µg/mL) followed by the addition of 0.3mL of sodium nitrite (5% w/v). After 5 minutes, 0.6 mL of aluminum chloride (10% w/v) was added, followed by the addition of 2mL of sodium hydroxide (1 M) 5 minutes later. The volume was then adjusted to 10 mL by adding 2.1mL distilled water. The mixture was shaken vigorously to ensure adequate mixing. The absorbance of the reaction mixture was measured at 510 nm with spectrophotometer. Total flavonoid was determined from extrapolation of calibration curve which was made by preparing catechin standard solution. The estimation of the flavonoid compounds was carried out in triplicate. The results were expressed as mg of catechin equivalents (CE) per g of extract.

Free Radical Scavenging Activity Determination

The antioxidant activity of the plant extracts and the standard was tested by using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay with slight modification[8]. The stock solution of DPPH was prepared with 0.009 g of 2, 2-diphenyl-1-picrylhydrazyl powder and mixed with 100 mL methanol. Serial dilution technique was used to make the sample in 5 concentrations which 20 µg/mL, 40 µg/mL, 60 µg/mL, 80 µg/mL and 100 µg/mL. 1 mL of extract was mixed with 2.7 mL methanol. Control for the baseline correction was prepared without using any extract or methanol. The mixture was kept in the dark for 90 minutes after vigorously shake. The antiradical activity was measured by noting a decrease in absorbance at 517 nm of a solution of coloured DPPH in methanol bring about by the samples. The presence of

ascorbic acid was used as positive control for all the triplicate determinations. Radical scavenging activity (RSA) equation was used to compute the DPPH radical scavenging ability.

$$\% \text{ RSA} = \frac{\text{Absorbance of control} - \text{Absorbance of Sample}}{\text{Absorbance of control}} \times 100$$

Ferric Reducing Antioxidant Power (FRAP) Determination

FRAP method was used to determine the antioxidant activity in easy way according to Benzie and Strain [9]. Acetate buffer was firstly prepared (300 mM, pH 3.6) by mixing 16 mL of glacial acetic acid with 3.1 g of sodium acetate trihydrate and made up to 1 L by using distilled water. After that, TPTZ (2,4,6-tri[2-pyridyl]-s-triazine) solution (10 mM) was prepared by adding 0.031 g of TPTZ to 10 mL of hydrochloric acid (40 mM) and placed it into a water bath at 50°C for 10 minutes. Iron (III) chloride solution (20 mM) was prepared by adding 0.054 g iron (III) chloride hexahydrate into 10mL distilled water. 200 mL acetate buffer was mixed with 20 mL TPTZ solution together with 20 mL iron (III) chloride solution (ratio of 10:1:1) to make FRAP reagent and kept in a water bath at 37˚C for 10 minutes. Serial dilution technique was used to make the sample in 6 concentrations which 0.1 mM, 0.2 mM, 0.4mM, 0.6 mM, 0.8 mM and 1.0 mM. 200 µL of extract solution was mixed with a volume of 1.5 mL freshly prepared FRAP reagent. The mixture was shaken and incubated at a temperature of 37oC for 4 minutes. Then the absorbance values were recorded at 593nm. The results are expressed as mM of Fe(II) per litre of extract and all samples were carried out in triplicate. Iron (II) sulphate was act as reference antioxidants in this assay.

Statistical Analysis

The data from the experiment were expressed as mean ± standard deviation of triplicate measurement. One-way analysis of variance (ANOVA) at $P < 0.05$ with statistical software SPSS Ver. 16.0 was used to analyze the data.

RESULTS

Total Phenols Determination

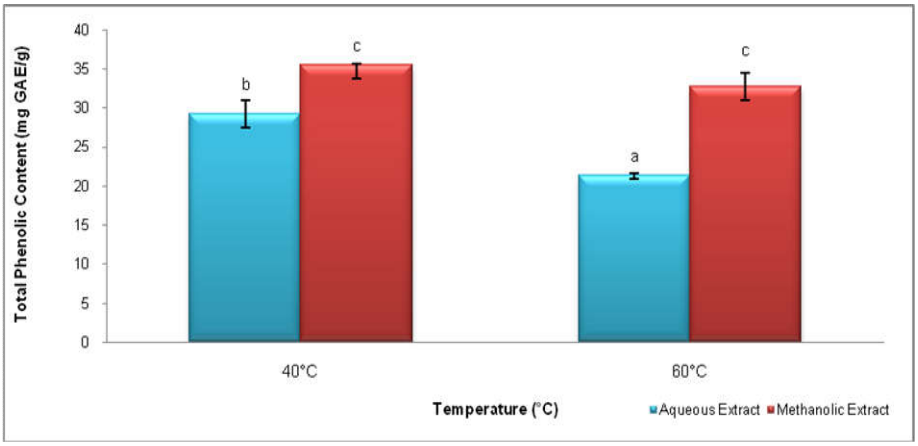

Figure 1: Total phenolic content of aqueous and methanolic extracts at 40°C and 60°C respectively. Data expressed as mg GAE/g of extract. Means with different superscript letters are significantly different at P<0.05 (Tukey Test).

Total Flavonoids Determination

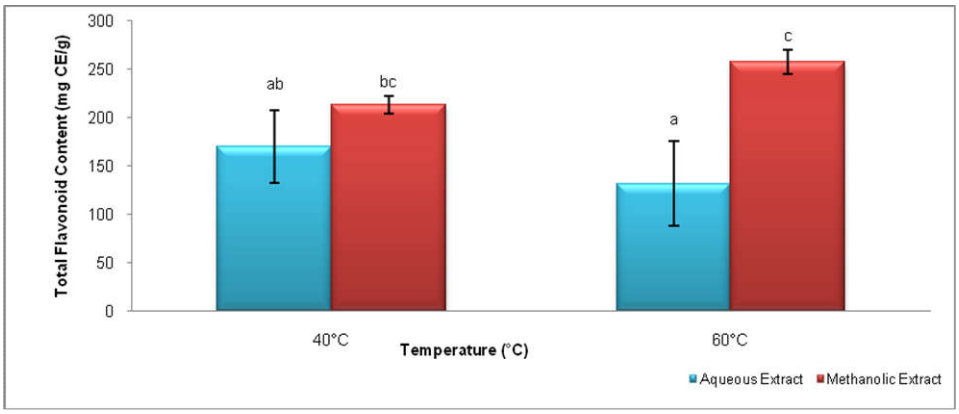

Figure 2: Total flavonoid content of aqueous extracts and methanolic extracts at 40°C and 60°C respectively. Data expressed as mg CE/g of extract. Means with different superscript letters are significantly different at P<0.05 (Tukey Test).

Free Radical Scavenging Activity Determination

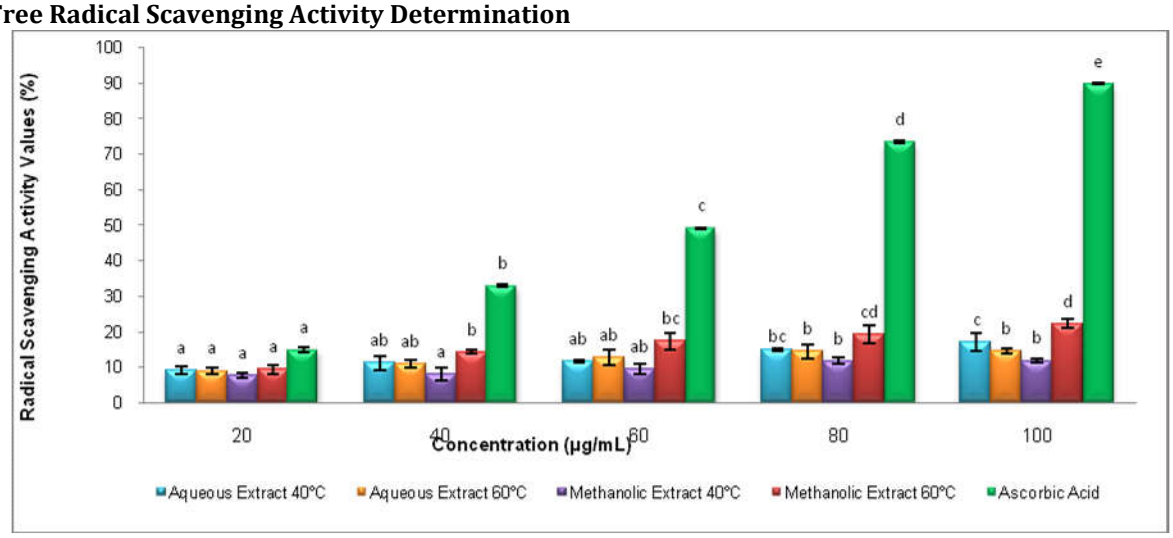

Figure 3: Radical scavenging activity of aqueous extracts and methanolic extracts at concentration 20 to 100 µg/mL. Data calculated by using the RSA equation. Means with different superscript letters are significantly different at P<0.05 (Tukey Test).

Ferric Reducing Antioxidant Power (FRAP) Determination

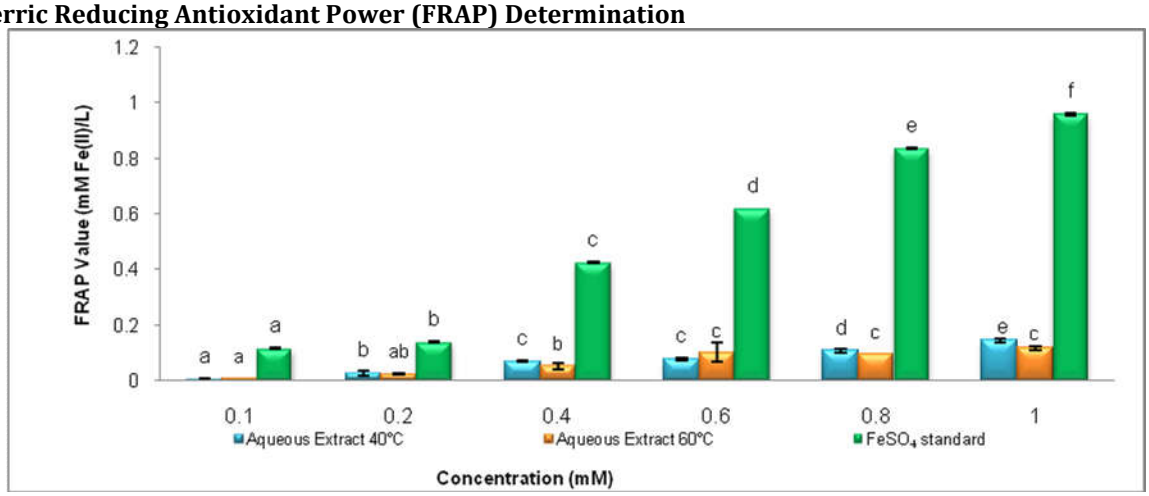

Figure 4: FRAP value of iron (II) sulphate standard and aqueous extracts at concentrations 0.1 to 1.0 mM. Data expressed as mMFe(II)/L of extract. Means with different superscript letters are significantly different at P<0.05 (Tukey Test).

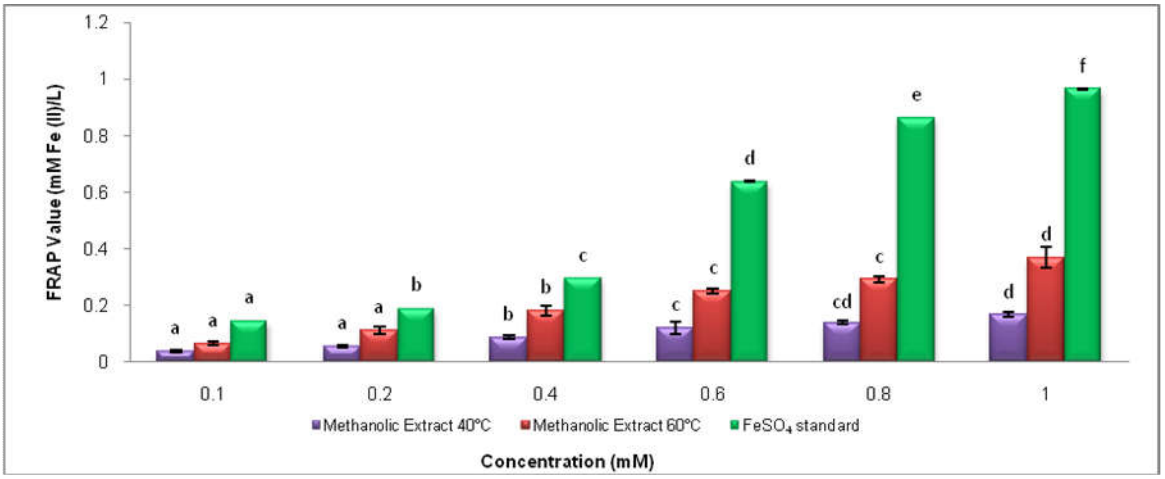

Figure 5: FRAP value of iron (II) sulphate standard and methanolic extracts at concentrations 0.1 to 1.0 mM. Data expressed as mMFe(II)/L of extract. Means with different superscript letters are significantly different at P<0.05 (Tukey Test).

DISCUSSION

Total Phenols Determination

Methanolic extracts at 60°C (32.76 ± 1.72 mg GAE/g) obtained the highest total phenolic content due to massive amount of antioxidant compounds such as tannin[10], whereas lowest total phenolic content found in aqueous extracts at the same temperature. Total phenoliccontent of *Diospyrosebenum*Roxb. leaf extracts reported by Baravalia*et al*. (2009)[11] was highest in the methanolic extract. Another recent study also reported that total phenolic content of calyx and seed of persimmon (*Diospyroskaki* cv. Fuyu) was recorded as the methanolic extract higher than aqueous extract[12, 13].

Total phenolic content in methanolic extract of *Nepetanepetella* leaves (58.11 ± 1.24 mg GAE/g DW) was observed higher than aqueous extract (41.65 ± 2.18 mg GAE/g DW) [14]. The solubility of phenolic compounds is relatively lower in water than in polar organic solvents [15]. Moreover, the basic theory in extraction process is that solvents only dissolve the same polarity of phytochemicals that appear in the plant extracts. Methanolic extracts provided the highest value for total phenolic content based on the result, thus, it is suggested that most of the phenolic compounds in *Diospyros buxifolia*had a strong polar characteristic.

Different variables may influence the phenolic extraction efficiency as studies revealed that temperature could be one of the aspects which affected the recovery of phenols from plant material beside of that extraction solvent[16]. In the current study, 40°C was found to improve the recovery of phenolic compounds in leaf extracts more effectively when compared with temperature 60°C. Despite, temperature is a major factor to influence the analytical quantification by causing polymerization or/and thermal degradation between the polyphenolic compounds present in the extraction yield. The results obtained in this section deduced that the phenolic compounds presented in *D. buxifolia* were heat sensitive. As the extraction temperature increased more than 40°C, these heat sensitive phenolic compounds would be destroyed. In another word, temperature had significant difference ($P<0.05$) influence on phenol extraction in the present study.

Total Flavonoids Determination

Flavonoids are large compounds occurring ubiquitously in plants. It was believed that majority of the flavonoids were strong antioxidants which able to scavenge reactive oxygen species effectively due to the phenolic hydroxyl groups. The content of flavonoids in extracts of *D. buxifolia* obtained from solvents values was experimentally determined in the current investigation. Negative result was obtained when screened on total flavonoid content in *D. buxifolia[3].* Total flavonoid content for aqueous extract at 40°C and 60°C was 170.00 ± 37.80 mg CE/g and 131.91 ± 43.64 mg CE/g respectively whereas total flavonoid content in methanolic extract at 40°C and 60°C was 213.33 ± 8.82 mg CE/g and 257.78 ± 12.62 mg CE/g. Methanolic extract at 60°C was higher than other leaf extracts yet aqueous extract 60°C was significantly ($p<0.05$) lower than other leaf extracts. These results are supported by Sathishkumar*et al*. (2008) who demonstrated that greater speed of the molecule movements in higher temperature can affects the flavonoids to diffuse more quickly from cell to extracting agent[17]. Hence, methanolic extract at 60°C contained the greatest total flavonoid content.

The result showed in Figure 2 revealed that flavonoids content were significantly higher ($P<0.05$) in methanolic extracts than aqueous extracts. However, methanolic extract at 40°C and 60°C had almost similar total flavonoid content which achieved a data of 213.33 ± 8.82 mg CE/g and 257.78 ± 12.62 mg CE/g respectively. The highest total flavonoid content was obtained in the methanolic extracts followed by aqueous extracts at both temperature (40°C and 60°C). A similar trend was observed in the determination of total phenolic content of *D. buxifolia* as flavonoids were the dominating phenolic group in plant [18]. Although there was no literature data related to leaf extract of *D. buxifolia*, reported the total flavonoid content of *Diospyrosebenum*Roxb. leaf extract was highest in the methanolic extract when compared with aqueous extract, which was also from *Diospyros* species[11].

Free Radical Scavenging Activity Determination

DPPH was one of the widely used method due to the easiness, time saving and high accuracy technique to evaluate the total antioxidant activity of the plant extracts. Furthermore, the results are more consistent when put into comparison with other free radical scavenging method like ABTS [19].The activity seems to be similar for both methanolic extracts and aqueous extracts. Quantitative assessment produced the results of radical scavenging activity (RSA) ranging from 11.91% to 22.31% for both aqueous and methanolic extracts at the concentration of 100 μg/mL.Methanolic extracts at 60°C exhibited highest percentage of radical scavenging activity (22.31%) among all others leaf extract. This result was represented that methanolic extract of *D.buxifolia* contained more radical scavengers and possessed a strong DPPH radical scavenging action at 60°C. It was comparable with those described in the literature for *Annona squamosa* L. leaves which the radical scavengers were more soluble in methanolic extracts (1409.15 ± 58.83 μmol of ascorbic acid/g of extract) than aqueous extracts (639.65 ± 22.17 μmol of

ascorbic acid/g of extract). Total antioxidant activity of *Flacourtiaindica* leaves was 180 µg/mL and 260 µg/mL ascorbic acid for aqueous and methanolic extracts respectively [20]. It was shown that methanolic extract exhibited maximum radical scavenging activity [21]. Furthermore, many researchers had reported that alcoholic extracts contain higher DPPH inhibition percentages than aqueous extracts [22].

Herein, temperature had a significant effect on total antioxidant activity of *D. buxifolia*. Temperature increased in methanolic extract at 60°C leads to increase in antioxidant potential in leaf extract, in contrast, the antioxidant activity in aqueous extract was spotted to be effective at lower temperature which is 40°C. Nonetheless, the highest DPPH scavenging capacity was observed in methanolic extract at 60°C among all others extracts. This suggest that compounds with the highest DPPH radical scavenging ability in methanolic extract at 60°C probably more soluble in methanol at 60°C than aqueous extract at 40°C and 60°C. The findings were similar with the study of Ruenroengklin*et al*. (2008) use of 45°C to 60 °C exhibited a relatively high antioxidant activity in the extracted litchi anthocyanins[23].

The antioxidant activity may be attributed to secondary metabolites present in different parts of *D. buxifolia*. There was a research investigated on other parts of *Lanneabarteri* such as stem bark (83.64 ± 4.51)% and root (91.43 ± 1.85)% gave positive reaction in DPPH test. Studies of Chew *et al*. (2012) observed the methanolic root extract of *Leucasaspera* (32.36 ± 1.19)% exhibited higher free radical scavenging activity than leaf extract (17.04 ± 0.82)%[24]. Even though different species of plants were compared, however the distribution of secondary metabolites in various parts of plant playa major role for antioxidant activity should be taken into consideration.

Ferric Reducing Antioxidant Power (FRAP) Determination

The antioxidant activity in aqueous and methanolic extracts at 40°C and 60°C was determined by FRAP assay because result was high reproducibility over a wide concentration range [25]. On the other hand, FRAP assay appears to be an attractive and potentially useful test due to the procedure is speedy and straight-forward. FRAP of extracts and reference antioxidants (iron (II) sulphate) are presented in Figure 4 and 5. Standard solution showed higher reducing power in methanolic and followed by aqueous extract. At the concentration of 1.0 mM, the reduction of Fe^{3+} to Fe^{2+} in aqueous extract at 40°C and 60°C was 0.146 ± 0.008 mMFe(II)/L and 0.119 ± 0.007 mM Fe(II)/L whereas for the methanolic extracts at 40°C and 60°C was 0.166 ± 0.009 mM Fe(II)/L and 0.369 ± 0.036 mM Fe(II)/L respectively. All of the leaf extracts at the concentration of 1.0 mM had no significant difference ($P<0.05$) except for methanolic extract at 60°C. Hence the optimum extraction solvent and temperature to obtain highest antioxidant activity by using FRAP assay was methanol and 60°C respectively.

Sharma *et al*. (2013) demonstrated that the FRAP value of methanol extract (498 µM Fe(II)/g) of *Parkinsoniaaculeata* L. Leaves was found to be higher than aqueous extracts (461 µM Fe(II)/g)[26]. Similar to the result, methanolic extract of *N. nepetelle* has stronger reducing power when put into comparison to aqueous extracts. Between, BHA and TROLOX standards were used as standards instead of iron (II) sulphate.FRAP value of methanolic extracts of *Prunusmahaleb* L. seed (51.9%) were higher than hexane extract (32.8%) at 100 µg/mL while the reference antioxidants were vitamin C (91.4%), vitamin E (90.8%) and BHT (91.6%). An increase in the FRAP values with increasing extraction temperature in methanolic extracts were observed. According to Spigno*et al*. (2007), increasing in temperature may favours extraction thus enhancing both diffusion coefficient and solubility of solute[27]. However, it was believed that antioxidant compounds can be denatured beyond a certain temperature and caused a decrease in the antioxidant capacities of crude extract. Besides that, since there was no significant difference ($p<0.05$) observed in both extraction temperature of aqueous extracts, thus in the economic point of view, 40°C was selected as the best extraction temperature for aqueous extract as this temperature exhibits the highest antioxidant activity.

The pattern of antioxidant activity results between DPPH and FRAP method almost the same as the activity was higher at 60°C in methanolic extracts. In FRAP assay, the fundamental capability of leaves extract to transfer electrons was tested while reduction by hydrogen atoms were also involved in the DPPH assay. Therefore, it can be concluded that all of the leaf extracts exhibit antioxidant activity. Nonetheless, the outcomes demonstrated that methanolic extract at 60°C was active in both electron donation and scavenging for free radical. This study was in line with the results of Armin *et al*. (2012) as methanolic extract of *Prunusmahaleb* L. seed showed higher reducing power compared to hexane extract. Different mechanisms may contribute to oxidative processes in the complex system. Consequently, it is crucial to identify the extracts by using various types of antioxidant assays.

CONCLUSION

Different temperatures and extraction solvents significantly affected the extraction yield of phenols, flavonoids and antioxidant activity from *D. buxifolia* leaf extracts. Application of polar solvent which is methanol was the best solvent for the full exploitation of the phenolics, flavonoid and antioxidant

potentials of *D. buxifolia* leaf extracts. Extraction temperature of 60°C exhibited the most efficient extraction of flavonoids and antioxidant activity of *D. buxifolia* while the temperature of 40°C was the optimised temperature for extraction yield of phenolics. Furthermore, the temperatures at 60°C and the application of methanol displayed a generally high antioxidant activity. Even though the research showed that the antioxidant activity was low in *D. buxifolia* leaf extracts, the further research is warranted to isolate the bioactive compounds which are responsible for antioxidant activity.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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