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In-vitro Antioxidant Potential in Leaf, Stem and Bark of *Azadirachta indica*

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ABSTRACT

Azadirachta indica (Meliaceae; Neem tree) is a resourceful medicinal plant. Almost all parts of the plant are used in traditional and folklore medicine. This study was conducted to measure antioxidant activity, total phenolic content (TPC) and total flavonoid content (TFC) in a crude extract of different parts of the neem tree. 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging and Ferric-Reducing Antioxidant Power Assay (FRAP) were used to evaluate antioxidant activity, while total phenolic and total flavonoid compounds were measured using the Folin-Ciocalteu method and aluminium chloride ($AlCl_3$) assay, respectively. Bark and leaf extract showed higher free radical scavenging activity, with IC_{50} value of 23.27 and 55.07 (µg/ml) respectively, while seed extract showed the lowest activity, with IC_{50} of 672.36 (µg/ml; ($P<0.05$). The bark extract revealed significantly higher antioxidant activity and phenolic content than the leaf and seed extract ($P<0.05$). Flavonoid content in leaf extract was found to be significantly higher ($P<0.05$) than in seed and bark extract; however, bark extract showed significantly lower flavonoid content than seed extract. These results suggest that the potency of *Azadirachta indica* makes it a good source of natural antioxidant compounds.

Keywords: Antioxidants, flavonoids, Folin-Ciocalteu, IC_{50}, neem, phenolics

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INTRODUCTION

Azadirachta indica is an evergreen tree that grows up to 15 m in height. It has round, large crown branches, its bark is moderately thick, dark grey outside but reddish inside and it has a colourless, sticky foetid sap. Its light green pinnate leaves are crowded at the end of the branches and are

about 20-40 cm long. *Azadirachta indica*, popularly known as neem, originated from India and belongs to the Meliaceae family of plants (Pandey et al., 2014). The United States National Academy of Sciences published a report entitled, 'Neem – A Tree for Solving Global Problems'. Life on earth ultimately depends on plants. Every plant can be utilised for different purposes by man and animals; in fact, plants have long been used in traditional medicine as well as modern medicine (Nahak & Sahu, 2010). The neem plant has been used in Unani, Ayurveda and Homeopathy as a medicine to treat fever and infestation by intestinal worms as well as for tooth cleaning and appetite boosting (Biswas et al., 2002; Ghimeray et al., 2009). According to Nahak and Sahu (2010) more than 100 bioactive compounds, the most important being azdirachtin, are present in different parts of the neem plant. Azadirachtin, a potential antecedent, controls growth as well as reproductive regulating activities (Ghimeray et al., 2009; Pandey et al., 2014). The neem plant contains a high amount of phenolic and polyphenolic compounds, depending on the geographical location and environmental factors (Kaushik et al., 2007).

The need for natural antioxidants has increased because of demand from science and industry. The pharmaceutical, cosmetic and food industries rely on natural antioxidants because of the strong biological activity of medicinal plants as well as to avoid the side effects that come with the use of synthetic antioxidants (Nahak & Sahu, 2010). Plants that contain vitamins, flavonoids and polyphenols show high antioxidant activity (Gupta & Sharma, 2006). Natural antioxidants not only improve the quality of food but also that of human health as they prevent the chain reaction triggered by free radicals (Nahak & Sahu, 2010). While much research into the neem plant has been done, none has focussed on neem plants grown in the environmental conditions of Malaysia. Therefore, the aim of this research was to study the antioxidant potential of neem plants native to peninsular Malaysia.

MATERIALS AND METHOD

Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH), Dimethylsulphoxide (DMSO), quercetin, Folin-Ciocalteau, Na_2CO_3 and 2,4,6-Tripyridyl-s-Triazine (TPTZ), Gallic acid, methanol, $AlCl_3$ and K-acetate were the chemicals used in this experiment.

Materials from the Plant

Fresh leaves, fruit and bark of neem plants were collected from Universiti Sultan Zainal Abidin (UniSZA), Gong Badak campus, Terengganu, Malaysia. The Faculty of Bioresources and Food Industry, UniSZA, Tembila, Terengganu, Malaysia authenticated the plant, which was kept at the university herbarium.

Extract Preparation

The plant samples were washed properly and separated into leaf, fruit and bark

and then dried. The samples were oven-dried at 43°C, then extracted with 95% ethanol. The crude extract was filtered using Whatman number 1 filter paper and further concentrated on a rotary evaporator (EYELA N-1110, Tokyo) at 45-50°C, dried and kept at 4°C for further assay. The sample and solvent mass ratio was 1:2 during extraction; this was dissolved in DMSO, methanol and/or diluted double-distilled water for the final concentration according to requirement (Abdulkadir et al., 2016a).

Total Phenolic Content Assay (TPC)

Total phenolic content of the extracts was determined according to Ainsworth (2007), with some few modifications (Abdulkadir et al., 2015a). Folin-Ciocalteu (F-C) reagent was used in the experiment, where 250 µL of extract diluted in DMSO was put in a test tube and subsequently mixed with 1.25 ml of F-C reagent diluted in distilled water with a ratio of 1:9 followed by incubation for 10 minutes. A volume of 1 ml of 7.5% Na_2CO_3 solution was added and the extract was subsequently incubated for 30 minutes in the dark prior to data taking using a spectrophotometer at 650 nm. Gallic acid solution was used as a standard. Three replicates were maintained for this experiment.

Total Flavonoid Content Assay (TFC)

TFC was used to determine total flavonoid content using a previous method (Kalita et al., 2013) with some modification (Abdulkadir et al., 2015b). Methanol solutions of quercetin of volume 0.32 mg/mL were used and further dilutions of different concentrations were read by spectrophotometer at 415 nm. A calibration curve was prepared and used. A volume of 0.5 mL of extract from each part was mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water. Aluminium chloride reacts with flavonoids such as flavones and flavonols and forms stable acid complexes through the C-4 keto group and or between the C-3 and C-5 OH group. Three replicates were maintained.

The 1,1-diphenyl-2-picryl-hydrazyl (DPPH) Assay

Antioxidant activity in the extract of different parts of *Azadirachta indica* in DPPH assay was tested according to Clarke et al. (2013), with some slight modification (Abdulkadir et al., 2015c) i.e. 500 µL of extract diluted in methanol was mixed with 1 ml of DPPH in methanol (0.3 mM). The tubes containing the extract were kept in the dark for 30 minutes, after which the absorbance of the solution was measured at 517 nm using a spectrophotometer (SHIMAZU). Blanks, containing methanol only, were run concurrently with quercetin solution dissolved in methanol serving as a standard. IC_{50} concentration was established as the concentration able to reduce DPPH absorbance by 50%. The different samples of extract were first tested at the single concentration of 0.3 mM followed by

subsequent serial dilution that resulted in a range of concentration values. The DPPH scavenging effect (% inhibition) = $[A_0 - A_1)/A_0] \times 100$, where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of the plant extract.

Ferric-Reducing Antioxidant Power Assay (FRAP)

The FRAP assay was carried out according to Benzie and Strain (1996) with slight modification (Abdulkadir et al., 2016b). The FRAP reagents were prepared using 10 mmol TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mmol HCl, 20 mmol iron (III) chloride aqueous solution and acetate buffer (pH 3.6) with the ratio 1:1:10 (v/v), respectively. The FRAP reagents were freshly prepared before starting the experiment and warmed at 37°C in a water bath for 30 minutes before use. Fifty microliters of sample were added into 1.5 mL of the FRAP reagent. The absorbance of the reaction mixture was measured using spectrophotometer at 593 nm after 30 minutes of incubation. A volume of 2000 µM of iron (II) sulfate solution was used as a standard and further diluted to 1000, 500, 250, 125, 62.5 and 31.25 µM. The results were expressed as µmol Fe(II)/g dry weight of the plant material. Three replications were maintained and the mean values were calculated.

Statistical Analysis

Each assay was subjected to one-way analysis of variance using the Statistical Package for the Social Sciences (SPSS, version 17.). A significance level of 0.05 % was used to test differences among the samples used.

RESULTS

Total Phenolic Content

The standard curve of Gallic acid and quercetin solution was used to calculate total phenolic and total flavonoid content in the fruit, stem and leaf of the neem plant (Table 1). The data collected on GAE (Gallic acid equivalents) and QAE (Quercetin equivalents) of the extract from the different parts of the plant were interpreted. In the equation formulated, Y represented average absorbance of the sample and X, the amount of Gallic acid or quercetin acid in µg/ml**.**

Table 1
Linear equations and their R^2 values obtained from the standard calibration curve

Assay	Calibration curve	R^2
TFC	Y=0.0025X+0.0708	0.9986
TPC	Y=0.0088X+0.0932	0.9991
FRAP	Y=0.0003X+0.7706	0.9995

Table 2
Estimation of total phenolic content (TPC; mg GAE/g) in different plant parts of Azadirachta indica

Sample	TPC (mg GAE/g)
Leaf	136.45±0.99[b]
Seed	30.43±1.16[c]
Bark	285.77±4.49[a]

[a, b & c]Indicate a significant difference at ($P < 0.05$)

Table 3
Estimation of the total flavonoid content (TFC; mg GAE/g) in different parts of Azadirachta indica

Sample	TFC (mg QAE/g)
Leaf	77.01±3.11[a]
Seed	8.08±0.69[b]
Bark	2.21±0.23[c]

[a, b & c]Indicates a significant Different at ($P < 0.05$)

Phenolic content in the samples were significantly different. Bark extract contained the highest level of phenolic content, 285.77±4.49 (mg GAE/g; P<0.05), which was significantly higher than 136.45±0.99 (mg GAE/g) in the leaves of the neem plant (Table 2). Furthermore, the leaves showed significantly higher (P<0.05) phenolic content than the seed, which showed a content of 30.43±1.16 (mg GAE/g; Table 2).

Total Flavonoid Content

The total flavonoid content in different samples was calculated via linear equation obtained from the standard calibration curve of the quercetin acid (Table 1). The flavonoid content of the leaf extracts was 77.01±3.11 (mg QAE/g; P<0.05), which was significantly higher than the value of 8.08±0.69 in the seed extract (Table 3). A similar result was observed when we compared the flavonoid content between the seeds and the bark. The seeds showed a significantly higher flavonoid content than the bark, which showed a flavonoid content of 2.21±0.23 (Table 3).

DPPH Antioxidant Activity

DPPH radical scavenging was used to determine antioxidant activity in the extract; this method reduces stable radical compounds, turning the substance from purple to yellow. The inhibition percentage in the leaf extract was 90.32% in bark, which was similar to the control (quercetin) but significantly higher (P<0.05) than the value of 88.43% recorded for the leaf extract (Table 4). A similar result was observed when we compared the inhibition percentage of the seeds and the leaves. The leaves showed a significantly higher percentage of inhibition (P<0.05) than the seeds at 69.83% (Table 4). Antioxidant potential of the sample was determined by the amount of antioxidant needed to scavenge 50% of DPPH free radicals (IC_{50}) at 517 nm. The IC_{50} result showed significant difference (P<0.05). Quercetin (control) was found to have lowered IC_{50} values of 15.64 µg/ml compared to the extract used, which significantly differed between the seeds and leaves, showing slight difference of value from that of the bark extract, which had an IC_{50} of 23.27 µg/ml.

Table 4
DPPH percentage inhibition and IC_{50} in the different parts of Azadirachta indica

Sample	DPPH % inhibition (µg/ml)	IC_{50} (µg/ml)
Leaf	88.43[b]	55.07[b]
Seed	69.83[c]	672.36[a]
Bark	90.32_{a}	23.27[c]
Quercetin	90.28[a]	15.64[d]

[a, b & c] Indicates a significant difference at ($P<0.05$)

Ferric-Reducing Antioxidant Power Assay (FRAP)

The result revealed changes in absorbance of the control ferrous (II) sulphate along the concentration gradient; therefore, it could be concluded that absorbance depends on the increase of concentration. The ability of the samples used to reduce Fe^{3+} to Fe^{2+} was expressed as equivalence of ferrous sulphate (mM), and the results were calculated using the equation obtained from the standard curve of Fe (II) sulphate (Table 1). The FRAP value of 1668.44±11.10 (mM Fe (II)/g; $P<0.05$) in the extract of bark was significantly higher than that of 1131.78±34.65 (mM Fe (II)/g) and 267.89±50.04 (mM Fe (II)/g) in the leaves and seeds, respectively (Table 5). In addition, the FRAP value of the leaf extract was significantly higher ($P<0.05$) than that of the leaf extract (Table 5).

Table 5
Ferric-reducing antioxidant power of different parts of Azadirachta indica

Sample	FRAP value (mM Fe (II)/g)
Leaf	1131.78±34.65[b]
Seed	267.89±50.04[c]
Bark	1668.44±11.10[a]

[a, b & c] Indicates a significant difference at ($P<0.05$)

DISCUSSION

Total Flavonoid Content

Naturally occurring flavonoid compounds have a polyphenolic structure, which is mostly soluble in water and mainly occurs in a plant as a sugar derivative known as glycoside. Pigments in flowers, fruit and seeds are responsible for flavonoids (Maria de Lourdes, 2013). In this study, flavonoid content in the leaf extract was higher than in other parts of the neem plant; it was highest in the seed extract, followed by the bark (Table 3), which is consistent with the results of Ghimeray et al. (2009) and Kiranmai et al. (2012) but slightly different from those of Naseer et al. (2014), concluded that the highest flavonoid content was found in the leaf extract followed by the bark and then the seed extract. Nevertheless, Abdulkadir et al. (2015a) recently reported high flavonoid content in *Moringa oleifera* leaves compared with that of bark and seed extract of the plant. This may be related to the higher cellular activities in the leaf. These activities are related to providing protection for the leaves from environmental stress. Total flavonoid content observed in this study was found to be high in the leaf extract but

low in the seed extract (Kiranmai et al., 2012). These differences may be attributed to solvent polarity, geographical location and concentration used in the experiment.

DPPH Assay

Free radical scavenging assay is the most rapid, reliable and sensitive method for measuring antioxidant activity in plant samples, measuring the absorbance of DPPH stable radicals spectrophotometrically (Waghulde et al., 2011). This study showed a decrease in percentage (%) inhibition in the following order: seed > leaf > bark > control (Table 4). This figures recorded in this study were higher than those recorded in previous studies such as those by Naseer et al. (2014), Nahak and Sahu (2010) and Abdulkadir et al. (2016a) but consistent with those recorded by Waghulde et al. (2011). However, the result stated by Ramamurthy et al. (2012) using a different solvent of *Solanum torvum* fruit at 500 µg/ml showed higher scavenging activity than was found in this study. Quercetin had a lower IC_{50} value 15.64 (µg/ml) than did the bark and leaf extract. Nevertheless, the seeds showed a significantly higher amount of IC_{50} than the control, probably due to the higher antioxidant potential but low antioxidant activity (Table 4). This result is consistent with the findings of Kiranmai et al. (2012) of low IC_{50} in leaf extract compared to seed extract of the same plant. However Pandey et al. (2014) reported a higher IC_{50} for the leaves of *Azadirachta indica* than was found in this current research. This difference may be attributed to the differences in solvent polarity, concentration and environmental factors.

FRAP Assay

The ferric-reducing power (FRAP) assay encompasses transfer of an electron, through which Fe^{3+} / ferricyanide complex is reduced to the ferrous (Fe^{2+}) form. The reducing compounds are an indicator of the substance's electron-donation potential and antioxidant activity (Cheynier, 2012). The FRAP value of bark extract was found to be higher than that of the leaf to seed extract (Table 5), indicating higher electron mobilisation in the bark due to direct contact with environmental conditions. This result is consistent with the result obtained by Kiranmai et al. (2012) using ethanolic extract of *A. indica* and by Abdulkadir et al. (2015b) using methanolic extract of *Moringa oleifera.* This difference may be related to the differences in solvent polarity, concentration and environmental factors.

CONCLUSION

Many studies have focussed on natural antioxidants from fruit, vegetables and medicinal plants. The results obtained may be related to high prevalence of oxidative diseases as well as the lethal effect of some synthetic antioxidants. Medicinal plants play a vital role in the treatment of many oxidative-related problems. The results obtained in this study from the DPPH, FRAP assay and total phenolic

and flavonoid content tests showed the potentiality of *Azadirachta indica* as a source of natural antioxidants. Bark and leaf extract of the plant revealed that these parts of the plant held the most potent antioxidant activity. Other species of neem under different environmental conditions can be examined in future studies for a more complete understanding of the antioxidant potential of this plant.

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