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Total phenolic content and primary antioxidant activity of methanolic and ethanolic extracts of aromatic plants' leaves

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Abstract: The aim of this study is to determine the total phenolic content and primary antioxidant activity of methanolic and ethanolic extracts of four aromatic plants' leaves namely knotweed (*Polygonum minus*), curry (*Murraya koenigii),* kaffir lime (*Citrus hysrix*) and fragrant screwpine (*Pandanus odurus*). Total phenolic content (TPC) assay using Folin-Ciocalteu method was used to assess the presence and level of phenolic compounds in each sample. The present study showed that both methanolic and ethanolic extracts of *P. minus* had the highest TPC and followed by *M. koenigii*, *C. hystrix* and *P. odorus*. Primary antioxidant activity in terms of free radical scavenging activities of both methanolic and ethanolic extracts was then measured by 2, 2, diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity assay. The lowest EC_{50} values based on the DPPH· radical scavenging activity were shown by *P. minus* extracts as compared to the other samples. For both ethanolic and methanolic extracts, the correlations between TPC and EC_{50} based on the DPPH· radical scavenging activity assay were negative and weak. Relatively, the present results suggest that of the four aromatic plants, *P. minus* and *M. koenigii* have shown potential as sources of natural antioxidants.

Keywords: aromatic plants' leaves, TPC, DPPH· radical scavenging activity, natural antioxidants, *Polygonum minus*, *Murraya koenigii*

Introduction

Antioxidant is a substance that has the ability to delay the oxidation of a substrate by inhibiting the initiation or propagation of oxidising chain reactions caused by free radicals. It plays important roles to prevent fats and oils from becoming rancid and protects human body from detrimental effects of free radicals. Synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tert-butylhydroquinone (TBHQ) and propyl gallate (PG) have been widely used around the world for decades. However, they are being scrutinised for possible toxic and carcinogenic effects. As a result, an intense new area of research has been developed concerning the search for identification and characterisation of naturally occurring antioxidants. Natural antioxidants are more ideal as food additives, not only for their free radical scavenging properties, but also on the belief that natural products are healthier and safer than synthetic ones; thus they are more readily acceptable to the modern consumers.

Numerous aromatic, spicy and medicinal plants have been examined for their antioxidative potential (Chan *et al*., 2007; Faridah *et al*., 2006; Hinneburg *et al*., 2006; Vogel *et al*., 2005; Wong *et al*., 2006). Herbs and spices that are usually used to flavour dishes are among the tremendous sources of phenolic compounds, which have been reported to show good antioxidant activity (Zheng & Wang, 2001). In Malaysia, knotweed (*Polygonum minus*), curry leaves (*Murraya koenigii*), kaffir lime (*Citrus hysrix*) and fragrant screwpine (*Pandanus odorus*) leaves are commonly used in Malaysians' food preparation. *P. minus* is famous to flavour dishes such as "*laksa*" and "*asam pedas*". Traditionally, *P. minus* is used to remove dandruff and it is boiled in water

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as a drink to treat digestion problem and consumed after childbirth. As for aromatic leaves of *M. koenigii*, the leaves are extensively used for flavouring dishes such as curries. Parts of this plant have also been used as raw materials for traditional medicine formulation in India. Its leaves and roots can be used to cure piles and allay heat of the body, inflammation and itching. Furthermore, essential oil of *C. hystrix* has been used for aromatherapy, neutraceutical and personal care products. The crude extract of *C. hystrix* leaves, peels and stems have shown good antioxidant activity in palm olein system and in linoleic acid model system (Irwandi *et al*., 2004). Regarding *P. odorus*, it has many uses in traditional Malay food preparation such as colouring, flavouring and appetite enhancer. Compounds in its root have been studied for hypoglycemic effect in rats (Peungvicha *et al*., 1998).

Previously, Huda-Faujan *et al*. (2007) investigated the antioxidant activities of water extracts of knotweed (i.e. *P. minus*) and curry leaves (i.e. *M. koenigii*) based on reducing antioxidant power, ferric thiocyanate (FTC) and thiobarbituric acid (TBA) assays. They found that water extracts of the aromatic leaves especially *P. minus* may be a potential source of natural antioxidants with similar characteristics to the synthetic antioxidant, BHT. In present study, methanolic and ethanolic extracts of knotweed (*P. minus*) and curry (*M. koenigii)* leaves along with kaffir lime (*C. hysrix*) and fragrant screwpine (*P. odorus*) leaves were tested for their total phenolic content (TPC) and primary antioxidant activity in terms of 1, 1- diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activities.

Materials and Methods

Sample collection

Four aromatic plants' leaves namely knotweed (*Polygonum minus*), curry (*Murraya koenigii),* kaffir lime (*Citrus hysrix*) and fragrant screwpine (*Pandanus odorus*) were purchased from local markets and supermarkets in Kuantan, Pahang Darul Makmur, Malaysia. The samples were randomly selected off the shelves based on their freshness.

Sample preparation and extraction

The leaves were cleaned and dried in warm room at 40°C (not exceeding 50°C) following the suggestion by Khamsah *et al.* (2006). However, our previous antioxidant studies suggest no huge detrimental effects on the total phenolic compounds when drying the samples at 60°C and 70°C (Norshazila *et al*., 2010; Nurliyana *et al*., 2010). Then, the dried leaves were ground separately into fine powder using a dry grinder. 25 g dried powder of each plant's leave was weighed and transferred into a beaker. 400 mL of solvent (i.e. absolute methanol or 70% ethanol) was added into the beaker and the mixture was shaken using mechanical shaker for 24 h at room temperature. Each extract was filtered using Whatman No.1 filter paper. The filtrate was collected and the residue was re-extracted twice. The two extracts were then pooled. The solvents (i.e. absolute methanol and 70% ethanol) in the extract were removed under reduced pressure at 40°C using rotary evaporator. For 70% ethanolic extract, the residual water was lyophilised using freeze dryer. The extracts were filled in the bottles and stored at 4°C until further uses.

TPC assay

TPC of the extracts were measured using Folin-Ciocalteu method as described by Amin *et al*. (2004). All samples and readings were prepared and measured in triplicate. Gallic acid was used as standard. 0.5 mg/mL stock standard solution of gallic acid was prepared by dissolving 250 mg of dry gallic acid in 1 mL of extracting solvent and then diluted to 500 mL of distilled water. The stock solution was stored at 4°C. Working standards of between 0.01 and 0.05 mg/mL were prepared by diluting the stock solution with distilled water. The extract was prepared at concentration of 1 mg/mL. 100 µL of extract was transferred into a test tube and 0.75 mL of Folin-Ciocalteu reagent (previously diluted 10-fold with deionised water) was added and mixed. The mixture was allowed to stand at room temperature for 5 min. Then, 0.75 mL of 6% (w/v) sodium carbonate was added to the mixture and mixed gently. After standing at room temperature for 90 min, the absorbance was read at 725 nm using Perkin Elmer Lambda 25 UV/Vis spectrophotometer. The standard calibration curve of gallic acid (0.01–0.05 mg/mL) was plotted.

DPPH· radical scavenging activity assay

The DPPH· radical scavenging activity assay used by Chan *et al*. (2007) was adopted with slight modification. Different dilutions of the extract (0.001, 0.025, 0.050, 0.075 and 0.100 mg/mL) were prepared. DPPH· solution was also prepared by dissolving 6.0 mg of DPPH· in 100 mL methanol. Then, 1 mL of extract from each dilution was added into the test tube containing 2 mL of DPPH· solution. Control was prepared by adding 1 mL of methanol to 2 mL of DPPH· solution. BHA, ascorbic acid and trolox were used as standards. The mixture was shaken vigorously and was left to stand in the dark for 30 min. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm. The scavenging activity of each extract on DPPH radical was calculated using the following equation:

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

All samples and readings were prepared and measured in triplicate. DPPH· radical scavenging activities of the extracts were expressed as EC_{50} values. $EC_{50,}$ effective concentration of the extract required for 50% scavenging of DPPH· radicals was calculated from the plotted graph of scavenging activity against sample concentration using Goal Seek (Microsoft Office Excel).

Statistical analysis

Data were analysed using Statistical Package for Social Science (SPSS) for Windows version 11.0.2 (Chicago: SPSS Inc.). One-way ANOVA was used to analyse the mean differences among all plants' leave extracts while independent *t*-test was used to analyse differences between methanolic and ethanolic extracts.

Results

TPC assay

A linear calibration curve of gallic acid with r^2 value of 0.997 was obtained (not shown). Figure 1 shows mean TPC of the plants' leave extracts measured using the GAE equation of y = 7.895x + 0.008 (R^2 = 0.997), whereby y = absorbance at 765nm and x = concentration of total phenolic compounds in mg per mL of the extract. Among the methanolic extracts, *P. minus* had the highest TPC (31.38 ±0.13 mg/g), followed by *M. koenigii* (20.46 ±0.20 mg/g), *C. hystrix* (7.12 ±0.09 mg/g) and *P. odorus* (4.90 ±0.15 mg/g). For ethanolic extracts, *P. minus* (21.06 ±0.44 mg/g) still had exceptionally high TPC, followed by *M. koenigii* (12.31 ±0.18 mg/g), *C. hystrix* (6.65 ±0.05 mg/g) and *P. odorus* (5.63 ±0.11 mg/g). ANOVA analysis showed significant differences ($p < 0.05$) between TPCs of the samples.

DPPH· radical scavenging activity assay

Figure 2 and Figure 3 show that in both extracting solvents, *P. minus* and *M. koenigii* had considerably high DPPH· radical scavenging activities as compared to the standards (i.e. BHA, ascorbic acid and trolox). In contrast, *C. hystrix* and *P. odorus* indicated low DPPH· radical scavenging activities. As for the EC_{50} (Table 1), the lowest concentration was shown by *P. minus*, followed by *M. koenigii*, *P. odorus* and *C. hystrix* with significant differences between the methanolic and ethanolic extracts.

Correlation between TPC and primary antioxidant activity

Correlations between TPC and EC_{50} based on the DPPH· radical scavenging activity assay of methanolic and ethanolic plants' leave extracts were negative and weak (methanolic extracts – r= -0.587, R^2= 0.345; ethanolic extracts - r= -0.772, R^2= 0.597).

Discussion

Antioxidant level – TPC assay

Folin-Ciocalteu reagent, a mixture of phosphotungstic ($H_3PW_{12}O_{40}$) and phosphomolybdic ($H_3PMo_{12}O_{40}$) acids, is reduced to blue oxides of tungstene (W_8O_{23}) and molybdene (Mo_8O_{23}) during phenol oxidation. This reaction occurs under alkaline condition provided by sodium carbonate. The intensity of blue colour reflects the quantity of phenolic compounds, which can be measured using spectrophotometer (Conforti *et al*., 2006). In present study, both methanolic and ethanolic extracts of *P. minus* had the highest TPC, followed by *M. koenigii*, *C. hystrix* and *P. odorus*. ANOVA analysis showed significant differences ($p < 0.05$) between TPCs of the samples. The results suggest that the TPC varied significantly from one plant to another. Likewise, Huda-Faujan *et al*. (2007) reported that *P. minus* had the highest TPC (i.e. 44.35 mg tannic acid equivalent (TAE)) and followed by *M. koenigii* (i.e. 24.62 mg TAE), *Cosmos caudatus* (ulam raja), *Oenanthe javanica* (selom) and *Centella asiatica* (pegaga); however, the levels of TPC for *P. minus* and *M. koenigii* are different from present study. As mentioned by Huda-Faujan *et al*. (2007), the different levels of TPC may be attributed to the different plants, procedures and standards used to express the TPCs; the colour measurement of Folin-Ciocalteu reagent which was non-specific on phenol, and perhaps there were other components that can react with Folin-Ciocalteu reagent such as ascorbic acid.

Apart from that, the results also suggest that extraction by methanol could give higher phenolic content as compared to ethanol, even though it was not shown for *P. odorus*. The findings were likely in agreement with Pérez *et al*. (2007) who found that methanol was the most efficient solvent as compared to ethanol and water for extracting phenolic compounds from control rosemary leaves and from those decontaminated by gamma irradiation. In addition, Yang *et al*. (2007) reported that methanol extract of lotus rhizome had the highest yield and total phenolic recovery. Methanol are said to be the most suitable solvent in the extraction of phenolic compounds due to its ability to inhibit the reaction of polyphenol oxidase that causes the oxidation of phenolics and its ease of evaporation compared to water (Yao *et al*., 2004). However, Moure *et al*. (2000) suggested that both methanol and ethanol offered the best results to extract phenolic compounds from *Gevuina avellana* hulls as compared to acetone. They

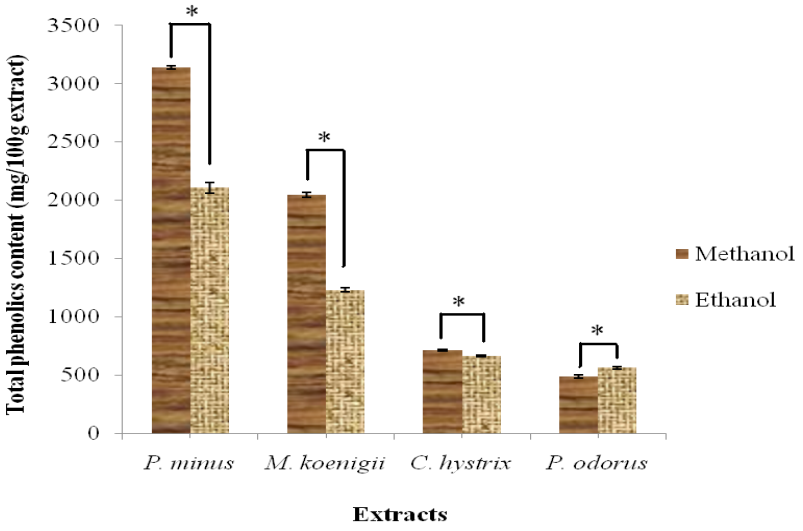

Figure 1. Mean total phenolic content of the aromatic plants' leave extracts. Results were expressed as gallic acid equivalent (GAE). The values were expressed as mean ± standard deviation (n=3). Asterisk (*) indicates a significant difference at the level $p < 0.05$ between methanolic and ethanolic extracts.

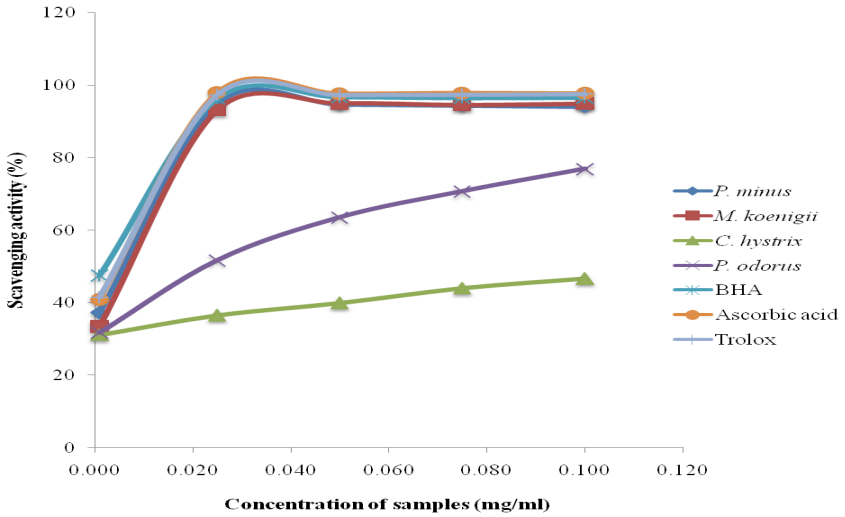

Figure 2. Scavenging activities of the methanolic extracts of aromatic plants' leaves and standards on DPPH· radicals. The values were expressed as mean ± standard deviation (n=3). BHA, ascorbic acid and trolox were used as the standards.

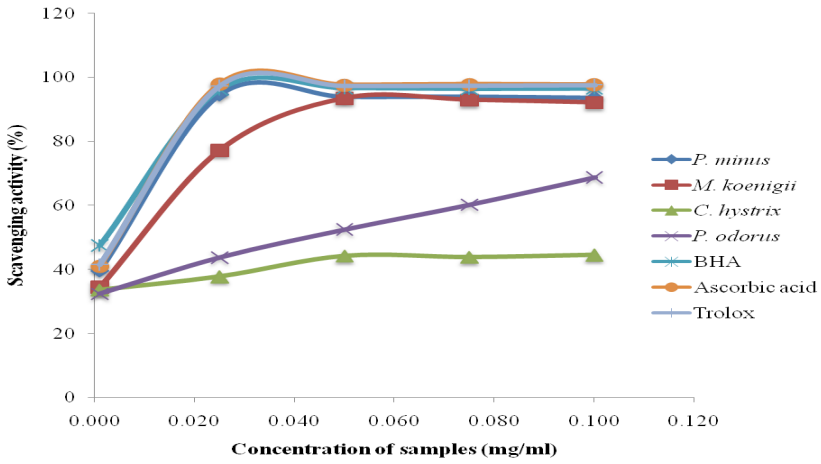

Figure 3. Scavenging activities of the ethanolic extracts of aromatic plants' leaves and standards on DPPH· radicals. The values were expressed as mean ± standard deviation (n=3). BHA, ascorbic acid and trolox were used as the standards

Table 1. DPPH· radical scavenging activity (EC_{50}) of the plants' leave extracts and standards

Samples	Extract	EC_{50} (mg/mL)
P. minus	Methanol	0.005 ± 0.001
	Ethanol	0.004 ± 0.001
M. koenigii	Methanol	0.006 ± 0.001
	Ethanol	0.008 ± 0.001
C. hystrix	Methanol	0.126 ± 0.001
	Ethanol	0.080 ± 0.001
P. odorus	Methanol	0.022 ± 0.001
	Ethanol	0.042 ± 0.001
BHA (standard)		0.002 ± 0.001
Ascorbic acid (standard)		0.003 ± 0.001
Trolox (standard)		0.003 ± 0.001

found that as polarity of the solvent increased, higher extraction yields of total soluble solids and total extractable polyphenolics were obtained. Moreover, Sun and Ho (2005) discovered that extracting solvent significantly affected the yield of phenolic content of buckwheat extract. Therefore, this work shows that different extracting solvents influenced different yields of TPC in present study.

Primary antioxidant activity - DPPH· radical scavenging activity assay

DPPH· radical scavenging activity assay assessed the ability of the extract to donate hydrogen or to scavenge free radicals. DPPH· radical is a stable free radical and when it reacts with an antioxidant compound which can donate hydrogen, it is reduced to diphenylpicrylhydrazine. The changes in colour (i.e. from deep-violet to light-yellow) can be measured spectrophotometrically.

Same pattern of DPPH· radical scavenging activity was observed for *P. minus*, *M. koenigii*, BHA, ascorbic acid and trolox, where their scavenging activities increased sharply to more than 90% before becoming plateau (Figures 2 and 3). The DPPH· radical scavenging activities of *C. hystrix* and *P. odorus*, on the other hand, increased gradually as the concentration increased. Decrease in absorbance of DPPH· solution (i.e. from purple to yellow) depends on intrinsic antioxidant activity of antioxidant as well as on speed of reaction between DPPH· and antioxidant. EC_{50} value, defined as the concentration of antioxidant required for 50% scavenging of DPPH· radicals, is a parameter widely used to measure antioxidant activity; smaller EC_{50} value corresponds to a higher antioxidant activity of the plant extract (Maisuthisakul *et al*., 2007). In present study, for both methanolic and ethanolic extracts, *P. minus* had the lowest EC_{50} value, which indicated its powerful free radical scavenger ability and followed by *M. koenigii*, *P. odorus* and *C. hystrix*. However, there were significant differences in EC_{50} between methanolic and ethanolic extracts. The variations again might be due to intrinsic characteristics of plants compounds and application of different extracting solvents. Azizah *et al*. (2007) found that there were significant differences between EC_{50} values of ethanolic and water extracts of cocoa beans. Apparently, the extracting solvents were likely to influence measurement of antioxidant activities of the extracts.

Correlation between TPC and primary antioxidant activity

There were weak negative correlations between TPC and EC_{50} for methanolic extracts (r= -0.587, R^2= 0.345) and for the ethanolic extracts (r= -0.772, R^2= 0.597). According to Prior *et al*. (2005), the Folin-Ciocalteau assay gives a crude estimate of the TPC present in an extract, whereas the free radical scavenging assay is not only specific to polyphenols. Besides, various phenolic compounds respond differently in DPPH assay, depending on the number of phenolic groups they have (Singleton and Rossi, 1965). Tawaha *et al*. (2007) further suggested that negative correlation between TPC and antioxidant activity may be due to the TPC that does not incorporate necessarily all the antioxidants that may be present in an extract. These may explain the negative correlation between the TPC and the DPPH· radical scavenging activity observed in present study.

Conclusions

In general, it is found that the extracting solvents significantly affected the TPC and antioxidant activities of the four aromatic plants' leaves namely knotweed (*Polygonum minus*), curry (*Murraya koenigii),* kaffir lime (*Citrus hysrix*) and fragrant screwpine (*Pandanus odorus*). Relatively, *P. minus* and *M. koenigii* had high TPC and primary antioxidant activities. However, the exact phenolic compounds or other compounds responsible for the antioxidant properties of the extracts are still unknown. Since previous and present studies suggest that *P. minus* and *M. koenigii* have shown potential as sources of natural antioxidants, further studies need to be directed to isolate and characterise antioxidant active compounds from the extracts which could be responsible for the high antioxidant activities.

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