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PHYTOCHEMICAL ANALYSIS AND DPPH RADICAL SCAVENGING ACTIVITY OF *Plectranthus amboinicus* (Lour.) Spreng LEAF EXTRACTS

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Abstract

Plectranthus amboinicus (Lour.) Spreng (Lamiaceae) is traditionally used in folk medicine to treat countless illnesses. This study aimed to determine the phytochemicals analysis including, phytochemical screening, total phenolic and flavonoid contents as well as DPPH radical scavenging activity of *P. amboinicus* leaf extracts. The extraction of phytochemicals was performed using sequential maceration method using *n*-hexane, ethyl acetate and methanol. Phytochemical screening was conducted using standard chemical tests, while total phenolic and flavonoid contents were performed using Folin-Ciocalteu and aluminum chloride colorimetric methods, respectively. All extracts were subjected to 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical assay. Preliminary phytochemical analysis revealed that the leaves of *P. amboinicus* consisted of flavonoids, phenols, terpenoids, glycosides and tannins, but showed negative results for alkaloids and saponins tests. The greatest phenolic content was observed in the ethyl acetate extract (73.31 ± 0.97 mg GAE/g), while the lowest value was reported in the *n*-hexane extract (24.63 ± 0.84 mg GAE/g). The ethyl acetate extract also composed of the highest flavonoid content (95.72 ± 0.80 mg QE/g), while the methanol extract had the lowest flavonoid content (9.84 ± 0.69 mg QE/g). Among the extracts, the methanol extract demonstrated better DPPH radical scavenging activity with an IC_{50} of 878.37 µg/mL.

INTRODUCTION

Antioxidants are substances which are used to slow down or eliminate oxidative damage to target molecules. These substances are significant to trap free radicals due to their redox hydrogen donor and single oxygen sequencer properties [1]. The ability of antioxidants to delay the oxidation of biomolecules makes this substance become more important and benefits for food preservation, dietary supplements and health promotion [2, 3]. Available synthetic antioxidants such as BHT (butylated hydroxyl toluene) and BHA (butylated hydroxyanisole) are applied in foods such as oil, bread, cookies, biscuits and dairy products to help prevention of lipid oxidation, withstand various treatments and conditions as well as to prolong the shelf life [4, 5]. Nowadays, besides the synthetic antioxidants, natural antioxidants mainly polyphenols, carotenoids and vitamins obtained from foods, medicinal plants and agricultural by-products are also utilized in the food industry as well as for health maintenance [6-8]. Currently, the phenolic- and flavonoid-rich natural diets with antioxidant activity have raised interest in nutrition and food science owing to their ability to reduce free radical formation and to scavenge free radicals [9, 10].

Plectranthus amboinicus (Lour.) Spreng (Lamiaceae) is a medicinal herb commonly known in Malaysia as “daun bangun-bangun”, “pokok bangun-bangun”, “sedingin” or “hati-hati hijau”, and synonymous with *P. aromaticus* Roxb., *Coleus aromaticus* Benth. and *C. amboinicus* Lour [11, 12]. The plant mainly grows in the subtropics and tropics regions and can be found in a well-drained and semi-shaded habitat [11, 13]. *P. amboinicus* is commonly used as

culinary herbs, ornamental plants and vegetables, and traditionally applied in folk medicine. The leaves of this plant are being utilized to treat multiple ailments such as digestion, skin conditions, respiratory conditions, infections and fever [14]. *P. amboinicus* had displayed remarkable bioactivities such as antimicrobial, antiviral, antiepileptic, antitumorigenic, anti-inflammatory, wound healing, insect bites lactogenic, antioxidant and analgesic activities. These bioactivities are attributed to the presence of a variety of phytochemicals in essential oils and extracts. Phenolics, flavonoids, tannins, terpenoids, glycosides, saponins, carbohydrates, steroids, proteins, amino acids, quinones and alkaloids were classes of compounds which can be found in its extracts [11, 15].

Although several phytochemistry and bioactivities research on *P. amboinicus* had been conducted [11, 16, 17], however, there is still a gap which needs to be explored in term of plant sample that are originated from other regions. To date, there is no information regarding the phytochemistry and antioxidant effect of *P. amboinicus* growing in Penang, Malaysia. Thus, the present study is carried out to detect the presence of phytochemicals, and determine total phenolic and flavonoid contents as well as antioxidant effect by DPPH radical scavenging assay of *P. amboinicus* leaf extract from different solvents.

MATERIALS AND METHODS

Plant Materials

The leaf of *P. amboinicus* (voucher number 1D019/2021) was purchased from Herbagus Trading. The leaf was collected from Bertam Perdana, Kepala Batas, Penang, Malaysia in September 2021. The specimen was deposited at UKMB Herbarium, Universiti Kebangsaan Malaysia (UKM) and was identified by Dr. Shamsul bin Khamis, a botanist from UKM.

Preparation of Crude Extracts

The leaf of *P. amboinicus* was dried in an oven at 55ºC for 10 hours, followed by grinding into a powdered form with size 2-3 mm [18]. The crude extracts were prepared by the sequential maceration of powdered samples (234 g) using *n*-hexane, ethyl acetate and methanol (2.0 L each) for three days at room temperature [19]. The extraction was carried out using an orbital shaker at 130 rpm [20]. The extract was filtered with Whatman filter paper and the filtrate was concentrated under reduced pressure to afford *n*-hexane (4.39 g; 1.88%), ethyl acetate (4.10 g; 1.75%) and methanol (16.62 g; 7.10%) crude extracts [21].

Preliminary Phytochemical Analysis

The *n*-hexane, ethyl acetate and methanol crude extracts of leaf of *P. amboinicus* were subjected to preliminary assessed for the presence of several phytochemicals including flavonoids, phenols, terpenoids, glycosides, alkaloids, saponins and tannins.

Test for Flavonoids (Ammonia Test)

The crude extracts were separately dissolved in ethyl acetate, followed by the addition of dilute ammonia solution (1% v/v, 1 mL) until the formation of layer. The presence of flavonoids in the extracts were confirmed by the appearance of yellow colour at the ammonia layer [21].

Test for Phenols (Ferric Chloride Test)

A small amount of each crude extract was dissolved in ethanol in a separate test tube. Two mililiter of the ethanolic extract was mixed with distilled water (1 mL) and was filtered. Then, the filtrate was treated with 2 drops of ferric chloride solution (5% w/v). Formation of blue, green, purple or black colour indicated the presence of phenols [22].

Test for Alkaloids (Dragendroff's Test)

Approximately 1 g of crude extract was dissolved in hydrochloric acid solution (5% v/v, 5 mL). A few drops of Dragendroff's reagent were added to the acid solution, followed by heating for a few minutes. Formation of red-orange precipitate in the mixture indicates the presence of alkaloids [23].

Test for Saponins (Foam Test)

Crude extract (3 mL) was mixed with distilled water (2 mL) and shaken vigorously for 10 min. The appearance of persistence foam within 10 min is an indicator of the occurrence of saponins [22].

Test for Terpenoids (Salkowski's Test)

The crude extract (500 mg) was dissolved with chloroform (2 mL). Then, concentrated sulphuric acid (3 mL) was added along the test tube wall. The formation of reddish-brown colouration at the interface infers a positive result for terpenoids [21].

Test for Glycosides (Fehling's Test)

Test A: Crude extract (2.0 g) was mixed with distilled water (20 mL), heated on a water bath for 5 min and filtered. The filtrate (5 mL) was treated with Fehling's A and B solutions (0.2 mL) until the reaction mixture becomes alkaline (tested using litmus paper). The positive result was indicated by the appearance of brick-red precipitate during heating.

Test B: Crude extract (2.0 g) was dissolved in sulphuric acid solution (1 M, 15 mL), heated on a water bath for 5 min and

filtered. Fehling's A and B (0.2 mL) was added to the filtrate (5 mL) followed by addition of a few drops of sodium hydroxide (5% w/v) until the solution becomes alkaline (tested with litmus paper). The formation of high brick-red precipitate in test B than that of the test A confirms the existence of glycosides [24, 25].

Test for Tannins (Braymer's Test)

The ethanolic extract (2 mL) was mixed with distilled water (2 mL) followed by filtration. A few drops of ferric chloride solution (5% w/v) were added to the filtrate. The formation of green precipitate indicates the existence of tannins [22, 26, 27].

Determination of Total Phenolic Content

Preparation of Calibration Curve using Standard Gallic Acid

The total phenolic content (TPC) of *P. amboinicus* leaf extracts were evaluated using the Folin-Ciocalteu colorimetric method as described by the previous studies with slight modifications [28]. The stock solution of gallic acid was prepared by dissolving gallic acid (25 mg) with methanol (25 mL) to acquire a stock concentration of 1000 µg/mL. The serial gallic acid solutions were made at various concentrations (0, 50, 100, 150, 200, 250, and 300 µg/mL). For each concentration, gallic acid (0.2 mL) was mixed with Folin–Ciocalteu reagent (0.2 mL) followed by the addition of distilled water (1.8 mL). The mixture was then incubated in the dark. After 5 min of incubation, sodium carbonate solution (Na_2CO_3) (7% (w/v), 2 mL) and distilled water (0.8 mL) were added. The mixtures were allowed to stand for 30 min and the absorbance resulting blue-coloured mixture was measured on a UV–Visible spectrophotometer at 765 nm. The solution for each concentration were prepared in triplicate and the average value of absorbance was used to construct the calibration curve.

Preparation of Samples for Total Phenolic Content

The extract solution was prepared in methanol at concentration 1000 µg/mL. The extracts were subjected to the similar procedure as described for standard gallic acid. For each analysis, the extracts were prepared in triplicate. The results were presented as mg Gallic Acid Equivalent per gram of dry weight extract (mg GAE/ g) based on a standard curve of gallic acid and the data were expressed as mean values with the standard deviation. Total phenolic content was calculated as follows:

$$C = cV/m$$

Where,
C = total phenolic content in mg GAE/g dry weight extract
c = concentration of gallic acid derived from the standard curve in mg/mL
V = volume of extract in mL
m = mass of extract in g

Determination of Total Flavonoid Content

Preparation of Calibration Curve using Standard Quercetin

The total flavonoid content (TFC) in all extracts was assessed using the aluminum chloride colorimetric method with minor adjustment [29, 30]. The stock solution of quercetin (1000 µg/mL) were prepared by dissolving quercetin (25 mg) in methanol (25 mL). Serial dilutions of quercetin solution (0, 50, 100, 150, 200, 250, 300 µg/mL) were prepared in methanol. For each concentration, standard quercetin solution (0.5 mL) was mixed separately with methanol (1.5 mL) and aluminium chloride (10% w/v, 0.1 mL). The solution was then mixed with potassium acetate (1 M, 0.1 mL) and distilled water (2.8 mL) and was incubated for 30 min at room temperature in the dark. The absorbance of the reaction mixture was measured at 415 nm on a UV–Visible spectrophotometer against methanol as a blank. Blank sample was prepared by using a similar procedure except the aluminium chloride was replaced with distilled water. The presence of flavonoids in the standard solution was shown by the formation of an orange yellowish colour. Triplicate determinations were performed, and the average value of absorbance was used to construct the calibration curve.

Preparation of Samples for Total Flavonoid Content

The extract solution with concentration of 1000 µg/mL was prepared by dissolving extract (1 mg) in methanol (1 mL). The procedure as described for standard quercetin was followed, and their absorbance was recorded using a spectrophotometer at 415 nm. The flavonoid content represented as mg of quercetin equivalents per g of dry weight extract (mg QE/g). All determinations were performed in triplicate. TFC values were expressed as means ± standard deviation and were determined using the following formula:

$$C = cV/m$$

Where,
C = total flavonoid content in mg QE/g dry weight extract
c = quercetin concentration based on calibration curve in mg/mL
V = volume of extract in mL
m = mass of extract in grams

DPPH Radical Scavenging Assay

The antioxidant activity of the crude extracts were determined using DPPH radical scavenging assay [31]. Each sample (1 mg) was dissolved in methanol (1 mL) to achieve the stock solution with concentration of 1000 µg/mL. Then, the stock solution was further diluted using methanol to obtain a final concentration of 500, 250, 125, 62.5, 31.3, 15.63 and 7.81 µg/mL. Each of the diluted sample solutions (200 µL) included the stock solution were individually mixed with the methanolic DPPH solution (50 µM, 3.8 mL) and incubated at room temperature in the dark for 30 min. The absorbance was recorded at 510 nm against methanol as a blank. DPPH blank was prepared by mixing DPPH solution (3.8 mL) and methanol (0.2 mL), while a blank sample is a mixture of sample solution (0.2 mL) and methanol (3.8 mL). Ascorbic acid was used as a positive control. The scavenging effect of the DPPH radical was calculated as follows:

$$\text{Scavenging effect (\%)} = \left[\frac{(A_{\text{DPPH blank}}-[A_{\text{sample}}-A_{\text{blank sample}}])}{A_{\text{DPPH blank}}}\right] \text{x } 100$$

where $A_{DPPH\ blank}$: Absorbance of DPPH solution in methanol, A_{sample}: Absorbance of DPPH solution with sample and $A_{blank\ sample}$: Absorbance of sample in methanol. The IC_{50} value of sample (concentration of the sample in which the percentage of inhibition is equal to 50) were determined using GraphPad Prism 6 software. All determinations were conducted in triplicate and the results were expressed as means ± standard deviation.

RESULTS AND DISCUSSION

Preliminary Phytochemical Analysis

Phytochemical screening of the *n*-hexane, ethyl acetate and methanol leaf extracts of *P. amboinicus* (Table 1) disclosed the existence of a variety of phytochemicals, including terpenoids, flavonoids, phenolic compounds, tannins and glycosides. Terpenoids was the only phytochemical which were detected in all extracts of *P. amboinicus*. On the contrary, saponins and alkaloids were completely absent in all tested crude extracts. Generally, terpenoids had been reported to display antioxidant, anticancer, anti-inflammatory, antibacterial, antiviral and antimalarial properties [32, 33]. Other compounds, i.e., flavonoids, phenolic compounds and tannins were existed in the ethyl acetate and methanol extracts. Flavonoids are typically known to display health promoting properties such as antioxidant and anti-allergic activities as well as effective against nerve diseases [11, 34]. Phenolic compounds are considered as an important phytochemical which showed antioxidant properties and useful for the treatment of skin aging, wounds and burns [34, 35]. Tannins are a type of polyphenol which have been reported to possess antimicrobial, antitumor and antiviral activities [32, 33]. Moreover, glycosides commonly act as antioxidant, antimicrobial and antidiarrheal agents [36-38], were discovered in methanol extract only.

As can be seen from Table 1, the obtained preliminary phytochemical analyses revealed that all phytochemicals were detected in methanol and ethyl acetate extracts, excepts for glycosides that were found absent in the ethyl acetate extract. The *n*-hexane extract contained the lowest number of phytochemicals present, in which only terpenoids were detected in the extract. This implies that methanol is effective at extracting bioactive compounds owing to their its polarity properties [34]. In line with the results of this study, phenols, glycosides, flavonoids, terpenoids and tannins were also detected in the leaf extracts of *P. amboinicus* collected from India, Egypt and Pahang, Malaysia [39-47]. However, the absence of alkaloids and saponins in this research were contrary as compared to the previous reports [15, 39-47]. The dissimilarities of the finding may be caused by the effect by different mineral composition, soil type, temperature, light and water content that give great influence on phytochemical contents of the plant [48, 49].

Table 1. Preliminary phytochemical analysis of the leaf extracts of *P. amboinicus*

Phytochemical	Test	Crude extract		
		***n*-Hexane**	**Ethyl acetate**	**Methanol**
Flavonoids	Ammonia test	-	+	+
Phenols	Ferric chloride test	-	+	+
Terpenoids	Salkowski's test	+	+	+
Glycosides	Fehling's test	-	-	+
Alkaloids	Dragendroff's test	-	-	-
Saponins	Foam test	-	-	-
Tannins	Braymer's test	-	+	+

Total Phenolic and Flavonoid Contents

The total phenolic content (TPC) of the *n*-hexane, ethyl acetate and methanol extracts obtained from the leaf of *P. amboinicus* was determined using the calibration curve of standard gallic acid ($y = 0.0043x + 0.0501$; $R^2 = 0.9953$). The TPC of all extracts at concentration of 1000 µg/mL were tabulated in Table 2. The highest phenolic content was observed in ethyl acetate extract (73.31 ± 0.97 mg GAE/g), followed by methanol (54.09 ± 0.71 mg GAE/g), while the lowest TPC was recorded in *n*-hexane extract (24.63 ± 0.84 mg GAE/g extract). High phenolic content was also reported for the ethyl acetate extract of leaf of *P. amboinicus* collected from Mysore, India [50]. According to Mariod *et al.* [51], ethyl acetate extract was found to be more effective in extracting low molecular weight compounds and high molecular weight polyphenols and this might correspond to the high TPC in ethyl acetate extract.

Table 2. Total phenolic and flavonoid contents of *P. amboinicus* leaf extracts

Crude extract	Total phenolic content (mg GAE/g)	Total flavonoid content (mg QE/g)
n-Hexane	24.63 ± 0.84	56.65 ± 0.85
Ethyl acetate	73.31 ± 0.97	95.72 ± 0.80
Methanol	54.09 ± 0.71	9.84 ± 0.69

The value was presented as mean ± standard deviation of three replicate experiments; GAE = Gallic acid equivalent; QE = Quercetin equivalent.

The total flavonoid content of all extracts of leaf of *P. amboinicus* were calculated using a linear quercetin standard curve ($y = 0.0047x + 0.0011$; $R^2 = 0.9971$). The obtained results showed that the ethyl acetate extract exhibited the highest flavonoids content (95.72 ± 0.80 mg QE/g), followed the *n*-hexane (56.65 ± 0.85 mg QE/g) and methanol (9.84 ± 0.69 mg QE/g) extracts (Table 2). High flavonoid content also reported for *Salvia pomifera* (Lamiaceae) leaf after extracted using ethyl acetate [52]. Ethyl acetate is the solvent typically used for less polar flavonoids extraction [53]. Therefore, most of the flavonoids in the leaves of *P. amboinicus* were less polar or semi-polar in nature [54].

In a survey of earlier literature reports, it was found that the amounts of phenolic and flavonoid compounds in this present study were slightly varied than those of leaves extracts from other regions, including India, Vietnam, Poland and Egypt as well as Selangor, Malaysia [17, 47, 50, 55-59]. The difference values of these compounds could be affected by several factors such as geographical variation, genetic diversity, environmental conditions and post-harvest techniques such as drying, storage and extraction methods and solvents [9, 60].

DPPH Radical Scavenging Activity

The antioxidant activity of the leaf extracts of *P. amboinicus* was determined by its capacity to scavenge DPPH free radicals. The scavenging effect was represented as half maximum inhibitory concentration (IC_{50}) and percentage inhibition (%) at a concentration of 1000 µg/mL, as indicated in Table 3. Among all tested extracts, the methanol extract (I% = 57.10%, IC_{50} = 878.37 µg/mL) possessed better DPPH radical scavenging activity as compared to the ethyl acetate extract (I% = 51.72%, IC_{50} = 984.60 µg/mL). However, both extracts exhibited lower scavenging effect in comparison to ascorbic acid (IC_{50} = 29.87 µg/mL). Besides, the *n*-hexane extract was found inactive against DPPH radicals, which only gave a percentage of inhibition of 9.00%. The present result was in agreement with the previous study by Swamy *et al*. who also reported that the methanol leaf extract of *P. amboinicus* from Selangor, Malaysia showed scavenging properties against DPPH free radical [17]. The weak activity demonstrated by the methanol and ethyl acetate extracts may be due to the weak capacity of compounds in the extract to donate hydrogen to the DPPH free radicals [61].

Table 3. DPPH free radical scavenging activity of the leaf extracts of *P. amboinicus*[a]

Antioxidant activity	Sample			
	***n*-Hexane**	**Ethyl acetate**	**Methanol**	**Ascorbic acid[b]**
Inhibition (%)	9.00 ± 0.56	51.72 ± 0.40	57.10 ± 0.33	94.96 ± 0.08
IC_{50} (µg/mL)	ND	984.60 ± 8.74	878.37 ± 3.30	29.87 ± 0.81

[a]Values are mean ± SD of three replicates; [b]positive control; ND = Not determined; Inhibition (%) at 1000 µg/mL

CONCLUSIONS

The results of phytochemical analysis of *P. amboinicus* leaf extract from different solvents suggested that the ethyl acetate extract could be a good source of bioactive phytochemicals due to the presence of a variety of phytochemicals. High TPC and TFC in this extract may contributed to its DPPH radical scavenging activity. Further study on the isolation of therapeutically active compounds with antioxidant activity from the potent extracts can be done in the future in order to develop pharmaceuticals. The toxicity assay of the extract is also warranted to determine the safety of the plant.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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