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RESEARCH ARTICLE

Phytochemical analysis and antioxidant activity of different plant parts of *Pellacalyx axillaris*

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Abstract

Pellacalyx axillaris or locally known as 'membuloh' is a mangrove species belonging to the Rhizophoraceae family. Till date, there has been only one phytochemical study found on this particular plant species and that without any documentation on its biological activities. Therefore, the present work aimed to reveal the phytoconstituents and the antioxidant activity of different crude extracts from different plant parts of *P. axillaris*. Experimentally, three organic solvents of different polarities i.e. *n*-hexane, ethyl acetate and methanol were used to prepare the crude extracts from the dried leaves, twigs and barks of *P. axillaris*. The preliminary phytochemical screening of this species indicated the presence of terpenoids, phenolic compounds, tannins, flavonoids, alkaloids, anthraquinone glycosides and carbohydrates. The *in vitro* antioxidant activity of the species evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging assay, and ferric reducing antioxidant power (FRAP) suggested that the methanolic bark extract contained potential source of natural antioxidants. Further research into isolation of antioxidant compounds from this species is highly recommended.

Keywords: Pellacalyx axilaris, leaves, twigs, barks, Malaysia

INTRODUCTION

Red mangrove, or else known as Rhizophoraceae is comprised of about 16 genera with more than 100 species and covered most of the tropical and subtropical areas around the world including the seasonal regions. Mangrove forests in Malaysia contain 23 species belonging to seven genera; *Rhizophora*, *Ceriops*, *Bruguiera*, *Pellacalyx*, *Kandelia*, *Carallia*, and *Gynotroches* (Mastaller *et al*., 2004; Abed *et al*., 2013). Most of the species from this family play important roles in many aspects of human life. For instance, the woods of *R. mucronata* are used as construction timber, charcoal and firewood in Kenya (Ahdouh-Guebas *et al*., 2000). Branches of *R. apiculata* are used in brush-pile fishing in Sri Lanka (Jayasuriya, 1991), while some of the species possess great remedial properties, making them good candidates for alternative medicine.

The ethnobotanic importance of this family is aroused when most of the Rhizophoraceae species are traditionally used by indigenous populations to treat a variety of ailments and conditions. For examples, the leaves of *B. gymnorhiza* are used to control blood pressure in India (Mastaller, 1997), while the Chinese uses the roots and barks extracts to heal burns (Bandaranayke, 2002). Interestingly, modern day studies have shown that the extracts of Rhizophoraceae species contain numerous secondary metabolites e.g. terpenoids, steroids, phenylpropanoids, lignans, glycosides, alkaloids, coumarins, flavonoids, phenolic acid, and others, which display important biological properties including anti-tumor (Yang *et al*., 2015), anti-inflammatory (Barik *et al*., 2016) and anti-diabetic activities (Adhikari *et al*., 2016).

Till date, there have not been many phytochemistry reports found on the genus *Pellacalyx* except for two phytochemical analyses on of the barks and stems of *P. saccadianus*, revealing the presence of alkaloids (Carrick *et al*., 1968; Abed *et al*., 2015). Only in 2016, phytochemicals isolation had been done on this particular species, resulted in the isolation of two new metabolites, along with six known compounds (Abed *et al*., 2015). Similarly, *P. axillaris* is also one of the species that received little attention among researchers since there was only one paper reported on the isolation of a new tropane alkaloid from the leaves of the species (Arbain *et al*., 1991). Following this view, the present work aimed to investigate the presence of different classes of phytochemicals as well as to evaluate the antioxidant property of different crude extracts from the leaves, twigs and barks of *P. axillaris*.

EXPERIMENTAL

Plant materials

P. axillaris samples i.e leaves, twigs and barks were collected in July 2014 from Kuala Berang, Terengganu. The species was identified by Dr. Shamsul Khamis from Universiti Putra Malaysia and a voucher specimen of this species (SK1941/11) was deposited at the Herbarium of Universiti Putra Malaysia.

Chemicals and reagents

Analytical grade *n*-hexane, ethyl acetate, methanol and concentrated hydrochloric acid were purchased from Sigma Aldrich

Company (U.S.A). 2-2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), potassium persulfate, iron(III) chloride hexahydrate, iron(III) sulphate pentahydrate, sodium acetate, 2,4,6-tris(2-pyridiyl)-s-triazine (TPTZ), butylated hydroxytoluene (BHT) and trolox were obtained from Sigma-Aldrich Chemie (Steinheim, Germany).

Extraction of *P. axillaris*

Powdered samples of *P. axillaris* i.e. leaves (200 g), twigs (750 g) and barks (1 kg) were extracted in a Soxhlet extractor with three solvents of different polarities; *n*-hexane, ethyl acetate and methanol. The extraction cycle was continued until the solution became colourless. The solvents were evaporated to dryness using a rotary evaporator to afford nine different crude extracts.

Phytochemical Analysis of *P. axillaris*

A stock solution with a concentration of 1% w/v of each extract was prepared using the respective solvent. These extracts were analysed qualitatively to detect the presence of phenolic compounds, flavonoids, tannins, alkaloids, carbohydrates, terpenoids, proteins, saponins, cardiac glycosides and anthraquinone glycosides by following standard methods (Roopalatha & Nair, 2013).

Antioxidant activity

The determination of antioxidant capacities in all crude extracts was done using 2, 2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant potential (FRAP) and 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assays. The absorbance was recorded on an UV/Vis absorbance microplate reader (EPOCH).

DPPH free radical scavenging assay

The free radical scavenging activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) was carried out on all the crude extracts with BHT and trolox as the standards (Fu *et al*., 2014). Stock solutions of extracts (1.0 mg/mL) were diluted with methanol to get ~~to~~ the final concentration range of 100-1000 mg/mL. The purple colour solution of DPPH was prepared by dissolving DPPH (4 mg) in methanol (100 mL). The prepared sample solution (100 µL) was allowed to react with the methanolic DPPH solution (100 µL) at room temperature for 30 min. A UV-Vis absorbance microplate reader was used to measure the absorbance of the reaction mixtures at a wavelength of 517 nm. The absorbance of the control (DPPH and methanol) was measured immediately at 0 min. The percentage of antioxidant activity was calculated using Eq. 1.

$$Scavenging\ (\%) = [(Abs(r) - Abs(s))/Abs(r)] \times 100 \quad (1)$$

Abs(r) = Absorbance of radicals (DPPH or ABTS)
Abs(s) = Absorbance of extract or standards (BHT and trolox)

By plotting a graph of I% versus concentration of sample or standard, the concentration of sample at 50% inhibition (IC_{50}) was calculated based on the formula obtained from the plot. All data was run in triplicates and expressed in average value.

ABTS free radical scavenging assay

The ABTS assay was carried out based on the method described by Zou *et al*. (2011), with slight modification. ABTS and potassium persulfate were dissolved in distilled water to obtain respective concentrations of 7 mM and 4.9 mM. Equal amount of these solutions was mixed and incubated in the dark for 12-16 hours prior to usage. The ABTS radical solution was diluted with distilled water until its absorbance reached 0.7 at 754 nm. The prepared sample (10 µL) was then allowed to react with the ABTS solution (190 µL) in the 96 well plates. The absorbance was recorded at 734 nm after 30 minutes of incubation period. The percentage of antioxidant activity was calculated using Eq. 1.

By plotting a graph of I% versus concentration of sample or standard, the concentration of sample at 50% inhibition (IC_{50}) was calculated based on the formula obtained from the plot. All data was run in triplicates and expressed in average value.

Ferric reducing antioxidant power (FRAP) assay

Determination of antioxidant activities of the crude extracts was conducted according to Channarong *et al.* (2012) with minor modification. The working FRAP reagent was prepared by mixing acetate buffer (300 mM), TPTZ (10 mM) in HCl and $FeCl_3.6H_2O$ (20 mM) in the ratio of 10:1:1. In a 96-well plate, 5 µL of sample was allowed to react with methanol (15 µL) and FRAP reagent (150 µL) and was kept in a water bath at 37°C. The absorbance was recorded at 593 nm after 10 minutes of incubation time. Various concentrations of $FeSO_4.7H_2O$ solutions (0.1 mM – 1 mM) were used to construct a calibration curve and the FRAP values were expressed as mM $FeSO_4$ equivalents.

RESULTS AND DISCUSSION

Preliminary phytochemical analysis of *P. axillaris*

Based on the analysis, terpenoids, proteins, carbohydrates, glycosides, phenolic compounds, flavonoids, tannins, alkaloids and saponins showed different results in all different solvent extracts. Most notably, methanolic extracts of the leaves, twigs and barks showed rich variety of secondary metabolites i.e. phenolic compounds, tannins, anthraquinone glycosides and carbohydrates. Whereas in all *n*-hexane extracts of *P. axillari*s, none of the phytochemicals was tested positive, except for terpenoids. Maximum amount of cardiac glycosides was reported in the methanolic bark extract while flavonoids were determined to be in minimum presence with low degree of intensities (+) in all methanolic extracts. The Dragendorff's and Mayer's tests for alkaloids indicated the presence of high amount of alkaloids in the methanolic bark extract while they were weakly detected in both methanolic leaf and twig extracts. This finding somehow matches the two earliest phytochemical surveys documented by Chan *et al.* in 1969 and 1977. Both proteins and saponins however, were completely absent in all plant extracts.

DPPH radical scavenging activity

A stable, purple coloured free radical known as 2, 2-diphenyl-1-picrylhydrazyl (DPPH) will changes to yellow upon electron transfer (ET) mechanism to an antioxidant. DPPH can react with many samples within a short period of time and is able to detect activities even at very low concentrations (Suman *et al*., 2015). In regard to that, nine different crude extracts from P. axillaris were screened for their abilities to quench free radicals using DPPH. Table 1 shows the result of this investigation.

The highest scavenging activities were observed in PABM, PATM and PATE with respective IC_{50} values of 2.24, 5.21 and 6.28 µg/mL, followed by methanolic leaf extract, PALH with an IC_{50} value of 37.91 µg/mL. These results are consistent with the earlier preliminary phytochemical analysis as the ferric chloride test indicated the presence of high amount of phenolic compounds in the two aforementioned methanolic extracts. This class of compound is known to be able to decolourize DPPH solution with its ability to donate proton, thus serving as primary antioxidants (Alabri *et al*., 2014). However, despite of having quite interesting antioxidant properties, its IC_{50} value was still higher than the two standards; BHT (1.35 µg/mL) and trolox (1.43 µg/mL). On the other hand, the weakest antioxidant capacity was demonstrated by two *n*-hexane extracts PALH and PATH with corresponding IC_{50} values of 844.1 and 752.9 µg/mL. Low polarity solvent like *n*-hexane is not able to significantly extract polar compounds. This is due to the presence of hydroxyl and sometimes carbonyl groups in most polar molecules which make them to be preferably extracted in much more polar solvent like methanol or ethanol (Zazouli *et al*., 2016).

Table 1 DPPH radical scavenging activity of different solvent extracts from *P. axillaris.*

Samples	IC_{50}
Leaves	
PALH	844.1
PALE	206.4
PALM	37.91
Twigs	
PATH	752.9
PATE	6.28
PATM	5.21
Barks	
PABH	224.0
PABE	139.1
PABM	2.24
Controls	
BHT	1.35
Trolox	1.43

*All values are expressed as a mean of triplicate experiment; $p < 0.05$.

ABTS radical scavenging activity

The 2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ABTS assay is a commonly used method to evaluate the antioxidant capacity of either naturally occurring or chemically-synthesized compounds used in various fields e.g. food processing industries, topical medication and pharmacology. Typically, the ABTS radicals are generated through a reaction between the ABTS salt with a strong oxidizing agent like potassium persulfate or potassium permanganate. This blue-green ABTS radical coloured solution will turn to yellow or colourless when being neutralized with a proton from a free radical scavenging antioxidant, resulting in the formation of reduced $ABTS^{\bullet+}$. This will eventually lead to the suppression of its characteristic of long wave absorption spectrum at 734 nm. The inhibition concentration (IC) to acquire 50% of the maximum inhibition capacity of ABTS by the crude extracts was presented in Table 2.

Table 2 ABTS radical scavenging activity of different solvent extracts from *P. axillaris..*

Samples	IC_{50}
Leaves	
PALH	>1000
PALE	812.2
PALM	564.8
Twigs	
PATH	>1000
PATE	364.3
PATM	266.4
Barks	
PABH	>1000
PABE	992.2
PABM	211.7
Controls	
BHT	213.0
Trolox	353.4

*All values are expressed as a mean of triplicate experiment; $p < 0.05$.

The methanolic bark extract (PABM) appeared to contain the significant amount antioxidants by immediately quenching the ABTS radicals and demonstrating the lowest IC_{50} value, above all tested extracts. The scavenging activity of PABM was in fact lower than the two positive controls; BHT (IC_{50} = 213.0 µg/mL) and trolox (IC_{50} = 353.7 µg/mL). Moreover, the ethyl acetate (PATE) and methanol (PATM) extracts also showed significant antioxidant activities with IC_{50} values of 364.3 and 266.4 µg/mL, respectively. In contrast, the *n*-hexane extracts (PALH, PATH and PABH) exhibited weak ABTS scavenging activities with IC_{50} values of more than 1000 µg/mL.

Ferric reducing antioxidant power (FRAP)

FRAP is a method that measures the total antioxidant capacity based on the reaction principle. Other scavenging assays measure the capacity of antioxidants in scavenging specific radicals, inhibiting lipid peroxidation or chelating metal ions. In FRAP assay, quantification of antioxidants' reducing power can be done by accessing the reduction of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) occurrence. The Fe^{2+} can be monitored by the formation of Pearl's Prussian blue which is measured at characteristic wavelength of 593 nm. BHT and trolox were used as positive controls (Table 3). The FRAP values of each extract were calculated from the calibration curve (R^2=0.9898) and were expressed as mM $FeSO_4.7H_2O$ equivalent.

Table 3 Ferric reducing antioxidant power (FRAP) of crude extracts from *P. axillaris.*

Samples	FRAP (mM equivalent to $FeSO_4.7H_2O$)					
	0.1	**0.2**	**0.4**	**0.6**	**0.8**	**1.0**
Leaves						
PALH	-	-	-	-	-	-
PALE	-	-	-	-	-	-
PALM	-	-	-	0.36 ± 0.01	0.55 ± 0.05	0.61 ± 0.61
Twigs						
PATH	-	-	-	-	-	-
PATE	-	-	-	0.05 ± 0.00	0.20 ± 0.01	0.66 ± 0.02
PATM	-	-	-	0.36 ± 0.05	0.55 ± 0.02	2.69 ± 0.10
Barks						
PABH	-	-	-	-	-	-
PABE	-	-	-	-	-	-
PABM	-	0.23 ± 0.00	0.29 ± 0.01	0.68 ± 0.04	1.22 ± 0.00	2.97 ± 0.01
Controls						
BHT	-	0.03 ± 0.03	0.14 ± 0.04	0.54 ± 0.03	2.38 ± 0.04	3.01 ± 0.05
Trolox	-	0.06 ± 0.00	0.11 ± 0.02	0.27 ± 0.00	0.46 ± 0.05	2.85 ± 0.06

*All values are expressed as a mean of triplicate experiment; $p < 0.05$.

In this study, the methanolic twig and bark extracts (PATM and PABM) exhibited the highest FRAP with equivalent values at 1.0 mM concentration; 2.69 ± 0.10 and 2.97 ± 0.01, respectively. In fact, their FRAP equivalent values showed no statistical difference ($p > 0.05$) with both positive controls; BHT (FRAP equivalent value = 3.01 ± 0.05 mM) and trolox (FRAP equivalent value = 3.01 ± 0.05 mM). The result somehow, is in accordance with the previous scavenging activities (DPPH and ABTS) in which good antioxidant activities are noticeable in both PATM and PABM. However, the inactivity observed in other crude extracts may be due to the presence of non-polar compounds which are unable to act as reducing agents (Benzie & Strain, 1996).

CONCLUSION

Qualitative screening of *P. axillaris* of Rhizophoraceae revealed the identification of different classes of natural products, including phenolic compounds, tannins, flavonoids, alkaloids, terpenoids, anthraquinone glycosides and carbohydrates. The antioxidant activity of all crude extracts screened by three assays i.e. DPPH radical scavenging activity, ABTS radicals scavenging activities and ferric reducing antioxidant power (FRAP) revealed that the highest antioxidant activity was observed in the methanolic bark extract of *P. axillaris*. Positive correlations were also noticed between the preliminary phytochemical surveys with the obtained antioxidant values. Further research on the crude methanolic extracts of all parts is necessary to explore the bioactive components that responsible for their high antioxidant capacities.

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