Effect of Total Phenolic Content on Metal Chelation and Free Radical Scavenging Activity of *Rhizophora apiculata*

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Abstract

The present study is aimed to study and determine the total phenolic content, the metal chelation and the antioxidant activity of the extracts of *Rhizophora apiculata* bark and leaf from the organic solvents which were hexane, ethyl acetate and methanol. The total phenolic contents of six extracts were analyzed by the Folin-ciocateu colorimetric method. Their antioxidant properties were determined by free radical scavenging and metal chelation. The *R.apiculata* leaf and bark extracted in ethyl acetate and methanol had more total phenolic content than those extracted in hexane, especially the bark extracts, for which total phenolic content was calculated at 1.620±0.082 mg GAE/ g CE for ethyl acetate bark extract and 1.595±0.033 mg GAE/ g CE for methanolic bark extract. The results of the free radical scavenging activity study showed that the antioxidant activity of *R.apiculata* was more pronounced in the bark than in the leaf. This was clearly demonstrated by the lower IC_{50} values, especially in the ethyl acetate and methanolic extracts (IC_{50} = 6.31±0.23 and 6.80±0.19 g/mL, respectively). These antioxidant activities were stronger than those of the BHT standard, which produced IC_{50} values of 10.13±0.45 g/mL. Furthermore, these data for antioxidant activity were supported by the results of the metal chelation assay, in which ethyl acetate and methanolic extracts of *R.apiculata* bark showed the highest capacity of metal chelation by ferrous ion at 10.497±0.051 and 10.234±0.101 mg Fe^{2+}/ g CE, respectively. It can be concluded that the *R.apiculata* bark extracts had interesting bioactive substances, and were effective in scavenging free radicals and chelating metal ions. Its extract has the potential to be a powerful antioxidant agent. Further research is required on the recognition and purification of the interesting bioactive and phenolic compounds from ethyl acetate and methanol extracts of this species.

Keywords: Phenolic content, Free radical scavenging, Metal chelating, *Rhizophora apiculata*, Extracts

1. INTRODUCTION

Rhizophora species are mangrove plants and have shown a variety of medical and pharmaceutical uses. Extracts of different parts of the plant have applications against various animal, plant, and human pathogens. They have been used as an in vitro HIV-1 inhibitor in human cells [1] and as antiviral [2], antibacterial [3], and antioxidant agents [4-7]. These applications are made possible by major secondary metabolite or bioactive compounds like polyphenol, which occurs as tannin in this species [8-12]. Tannins are natural

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polyphenols, occurring in leaf and bark, which combine with proteins to form stable complexes [13-14]. Their antioxidant properties were characterized by free radical scavenging activity [6-7].

R. apiculata, locally known as bakauminyak, is one of the major species of the RHIZOPHORACEAE family. This plant is a rich source of antioxidants due to its high total phenolic content [4-5]. Moreover, it exhibited higher free radical scavenging activity and ferric reducing power [4]. Previous reports have revealed that extracts from *R. apiculata* showed strong antioxidant activity, due to its polyphenolic content [9-12], especially in ethanolic bark extracts [2,6-7]. In a preliminary researches rendered various important bioactive compounds which showed potential as antioxidant agents [5,15]. This was confirmed by a recent report on the petroleum ether, methanol and chloroform extractions of another *Rhizophora* species, *Gynotroches axillaris*. It was found that the methanolic solvent of its leaf exhibited significantly higher antioxidant activity than the petroleum ether and chloroform extracts [15]. However, there has been little study of the antioxidant activity of *R. apiculata* leaf and bark extracted in different solvents. So, it is important to screen different solvent extracts of this plant to expand our knowledge of its antioxidant activity and possibly discover a new source of economically valuable materials and metabolites with therapeutic properties.

Therefore, this study aims to: 1) sequentially extract bioactive compounds from *R. apiculata* leaf and bark using the three organic solvents consisting of hexane, ethyl acetate and methanol; and 2) determine their total phenolic content, antioxidant activity, and metal chelating action. To the best of our knowledge, this research studies the effect of phenolic content on metal chelation and antioxidant capacity. The association between total phenolic content, antioxidant activity and metal chelating action will be discussed.

2. MATERIALS AND METHODS

2.1 Materials

Fresh leaf and bark of *R. apiculata* were collected from Rajamangala beach, Rajamangala University of Technology Srivijaya, Trang province in Thailand. The voucher specimen (BKF no.194836) is deposited at The Forest Herbarium, Thailand. The plant materials were washed under tap water and dried for a week. The dried materials were then ground to a fine powder in an electric blender and stored until maceration prior to analysis. Folin-ciocalteu reagent, gallic acid, butylated hydroxytoluene (BHT), 1,1-diphenyl -2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich Chemical Co. Other chemicals used were of 99% or greater purity.

2.2 Preparation of *R. apiculata* leaf and bark

One thousand grams of leaf and bark were extracted by maceration for a week in sequentially polar organic solvents. These solvents were, in order of increasing polarity, hexane, ethyl acetate and methanol. The resulting extracts were filtered with Whatman No. 1 filter paper and the filtrates concentrated by rotary evaporator in a vacuum at 50^{o}C to give dry residues which were kept in a refrigerator until use. The percentage yields of the hexane, ethyl acetate and methanol crude leaf extracts were 0.35, 2.8 and 23.04%, respectively, and 0.85, 0.96 and 54.41% for the bark extracts. The extracts were then analyzed for the determination of total phenolic content, antioxidant activity and metal chelating action.

2.3 Determination of total phenolic content

The contents of total phenolic compounds of the leaf and bark crude extracts were determined using the Folin-ciocalteau method modified by the method

of Miliauskas et al [16] using gallic acid as standard. Each sample was prepared by dissolving 10 mg of each dried extract in 10 mL methanol to obtain 6 sample solutions at a concentration of 1 mg/mL.Then, 0.2 mL of each sample was pipetted into a vial containing 2.5 mL of H_2O. Folin-ciocalteu reagent (0.2 mL) was then added, mixed well and left for one minute. Finally, 2 mL of 7 % sodium carbonate solution was added and the mixture was incubated for 60 min in the dark, at room temperature. The absorbance was recorded at 765 nm using a UV-vis spectrophotometer. The total phenolic content for each sample was calibrated alongside the standard curve of gallic acid at concentration 20-100 mg/mL and expressed in terms of gallic acid equivalents (GAE) per gram of dried crude extract.

2.4 Free radical scavenging activity onto 1, 1-diphenyl-2-picrylhydrazyl (DPPH)

The free radical scavenging activity was determined using the DPPH method as described by Seethalaxmi et al [17]. The procedure is as follows: 2 mL of each test sample and standard were mixed with 2 mL of 0.15 N methanolic DPPH solution. The mixture was vortexed and allowed to stand at room temperature in the dark for 30 min. The wavelength of maximum absorbance of DPPH was measured at 517 nm using a spectrophotometer. Methanol was used as a blank. BHT (Butylated hydroxyltoluene) was used as a positive control. The percentage of free radical scavenging activity of each concentration was calculated based on the formula $(1-(A_{sample}-A_{sample\ blank})/A_{control})) \times 100$ where A_{sample} is the absorbance of the test sample with DPPH solution, $A_{sample\ blank}$ is the absorbance of the test sample only, and $A_{control}$ is the absorbance of DPPH solution. Higher free radical scavenging activity indicates higher antioxidant activity by lowering absorbance of the reaction mixture. The decrease in absorbance is due to the reduction of the DPPH radical, which was measured using a UV-visible absorption spectrometer at 517nm (U-1800, SHIMAZU Co., Japan). The antioxidant activity of the test sample was expressed as the effective concentration of the extract (mg/mL) required for scavenging the free radicals. All measurements were performed in triplicates and expressed as the average values. IC_{50} was calculated by plotting inhibition percentage against sample concentration. BHT was used as standards.

2.5 Metal chelating activity onto 1,10-phenanthroline (Phen) assay

The antioxidant capacity of the *R.apiculata* leaf and bark extracts measured using the Phen method was studied according to the method of Szydłowska-Czerniak et al [13] with minor modifications. In a 10-mL volumetric flask, 0.6 mL of each sample solution (1 mg/mL) was added to 1 mL 0.2% $FeCl_3$ solution and 0.5 mL 0.5% 1,10-phenanthroline solution. The reaction mixture was made up to volume with methanol. The solution obtained was mixed and left at room temperature in the dark. After 30 min, the absorbance of the orange-red solution was measured at 510 nm against a reagent blank (1 mL of $FeCl_3$ (0.2%) and 0.5 mL of Phen (0.5%) made up to 10 mL with methanol). The activity was recorded by comparison with the calibration curve of working solutions of $FeSO_4.H_2O$ between 0.62-10.00 mg/mL. The least-squares method was applied to calculate the lines $y = 0.019x$ ($R^2 = 0.990$) Phen method.

2.6 Statistical analysis

The results were expressed as means ± standard deviation. The data were analyzed by one way ANOVA and different group means were compared by Duncan's multiple ranges (DMR) test. $P < 0.05$ was considered significant in all cases. The pearson correlation test was used to

determine the correlation between variables in total phenolic content results, metal chelation and scavenging activity for *R. apiculata* leaf and bark extracts in different solvents. $P < 0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

Phenolic contents of *R. apiculata* leaf and bark were extracted in different solvents. Several extracts of R.apiculata leaf and bark from organic solvents are reported to provide diverse medicinal properties [10-11]. In this work, we prepared extracts from R.apiculata leaf and bark using three solvents: hexane, ethyl acetate and methanol to obtainsix sample extracts. The total phenolic content of the six extracts was determined by the Folin-ciocalteu method and the data were showed in Figure 1.

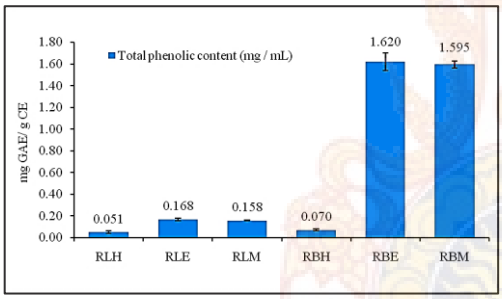


Figure 1. Total phenolic contents and metal chelating activity of leaf and bark *R. apiculata* extract. RLH, RLE, RLM are leaf of *R. apiculata* extracted by hexane, ethyl acetate and methanol, respectively. RBH, RBE, RBM are bark of *R. apiculata* extracted by hexane, ethyl acetate and methanol, respectively.

The amounts of phenolic content can be clearly compared in Figure 1. The total phenols of the extracts were expressed in μg per gram of dried sample based on a standard curve generated with gallic acid, according to the equation $y = 0.003x + 0.001$ ($R^2 = 0.999$). A distinct difference in total phenolic contents was observed between the leaf and bark extracted with hexane and the other extracts. The amount of total phenolic content was markedly higher in the ethyl acetate and methanolic extracts of both leaf (0.158 to 0.168 mg GAE/g crude extract, CE) and bark (1.595 to 1.620 mg GAE/g CE). This difference in total phenolic content was especially pronounced in the bark extracts. Moreover, the results obtained indicated that extracts of the bark had higher total phenolic content (0.070 to 1.620 mg GAE/g CE) than extracts of the leaf (0.051 to 1.158 mg of GAE/g CE) from the same solvent. These results showed that ethyl acetate and methanol efficiently extracted the significantly bioactive metabolite substances and released most of the secondary metabolites from the bark. This may be due to the fact that phenolic compounds are extracted in higher amounts by using polar solvents like ethanol and methanol [15, 18-19]. Differences in the polarity of the extracting solvents could result in a wide variation in the polyphenolic contents of the extract [19-21]. A study by Hong et al reported that phenolic compounds, extracted as gallic acid from hydrolysable tannin in the bark of *R.apiculata*, showed excellent biological activity [9] and also constituents of the leaf of *R. apiculata* are useful in several fields [22]. Their activity may be supported by the composition of the leaf and bark extracts as reported in the literature [11].

3.1 Metal chelation assay

The purpose of this test was to determine the capacity of the bioactive compounds in the samples to bind to the ferrous ion, catalyzing oxidation. The evaluation of metal chelation is especially important for the analysis of antioxidant activity because iron in its ferrous ion form, or $Fe^{2+,}$ is an essential catalyst in the catalyzed oxidation reaction, which generates several antioxidants, presented in the following equation:

Fe(III)–L + antioxidant → Fe(II)–L + oxidized antioxidant

where L is the ferrous-selective chromagenic ligand (1,10-phenanthroline). The obtained results are listed in Figure 2.

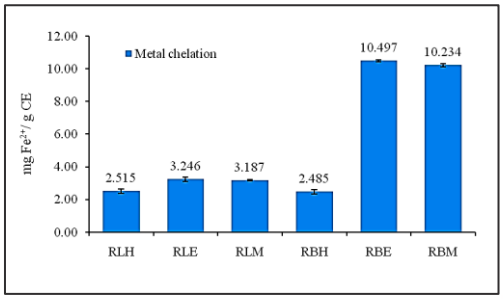
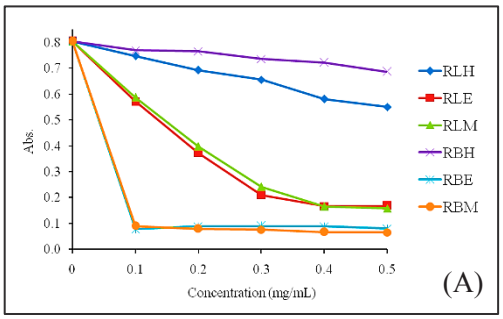


Figure 2. Metal chelating activity of leaf and bark *R. apiculata* extracts. RLH, RLE, RLM are leaf of *R. apiculata* extracted by hexane, ethyl acetate and methanol, respectively. RBH, RBE, RBM are bark of *R. apiculata* extracted by hexane, ethyl acetate and methanol, respectively

The antioxidant capacity of bark extracts significantly differed from that of leaf extracts. Moreover, the obtained results showed that ethyl acetate and methanol were more efficient solvents than hexane for the extraction of antioxidants. Therefore, it is possible that phenolic antioxidants are products of secondary metabolism in plants and they show not only free radical scavenging activity but also a metal chelation potential [23-24]. Our results showed that the bark extracts from ethyl acetate (10.497±0.051mg Fe^{2+}/ g CE) and methanol (10.234±0.101mg Fe^{2+}/ g CE) were more effective at chelating iron than the other extracts. This corresponds to the results for free radical scavenging activity and total phenolic content, all of which may be a consequence of the bioactive substances in this part of the plant.

3.2 Antioxidant activity of *R. apiculata* leaf and bark extracted in different solvents DPPH assay

In general, phenolic compounds are commonly found in plants and have long been considered as potential antioxidants and free radical scavengers [25-26] due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. Naturally-occurring antioxidants may have the potential to prevent diseases and the oxidation of food and here we considered the antioxidant potential of *R.apiculata* extract [4-8]. The free radical scavenging capacity of the leaf and bark extracts was determined using the DPPH method. The reduction capacity of DPPH was determined by the decreased in its absorbance at 517 nm, which is reduced by antioxidants [4]. Figure 3(A) showed a significant decrease in the concentration of DPPH due to the scavenging ability of the extracts. As shown in Figure 3(B), the extracts showed the increasing in free radical scavenging activity when the concentrations were increased. The DPPH scavenging activity observed in ethyl acetate and methanol extracts of both leaf and bark were higher than in the hexane extracts from both parts. The results for the bark extracts showed the highest DPPH activity in ethyl acetate (96.919±0.157%) and methanol (95.271±0.108%) and were comparable to the standards, BHT (93.910±0.117%). These DPPH scavenging activity results correlated with the results from total phenolic content (r^2 = 0.999).



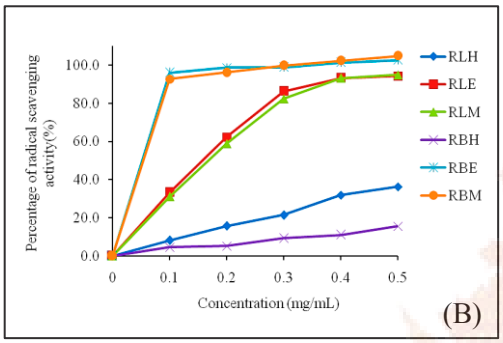


Figure 3. DPPH Free radical scavenging activity of leaf and bark *R. apiculata* extract(A); Percentage of free radical scavenging activity of leaf and bark *R. apiculata* extract (B). Values are the average of triplicate experiments and represented as mean±standard deviation.

The quality of the antioxidants in the extracts was determined by the IC_{50} values shown in Table 1. The IC_{50} value was studied to compare the effective concentration of each sample at which DPPH radicals were scavenged by 50% from the linear regression analysis. A lower IC_{50} value indicates a greater antioxidant activity of the extract. In this study, the free radical scavenging antioxidant capacities (IC_{50}) of ethyl acetate and methanolic extracts from both parts of *R.apiculata* were greater than those of the two hexane extracts. In particular, the ethyl acetate and methanolic bark extracts presented great capacity to scavenge free radicals. In contrast, the free radical scavenging activities of the controls, BHT 10.13±0.45 µg /mL, were higher than those of all the leaf extracts and also the bark extract from hexane. Interestingly, both the ethyl acetate (6.31±0.23 µg/mL) and methanolic (6.80±0.19 µg /mL) extracts from the bark had lower IC_{50} values than the BHT synthetic standards. The results indicate that the ethyl acetate and methanolic bark extracts were able to act as free radical inhibitors and primary antioxidants reacting with free radicals. We assume that the effectively prevented radical scavenging occurred from the phenolic compounds.

Table 1. IC_{50} of R.apiculata leaf and bark extracts by DPPH method

Parts	Solvents	DPPH radical scavenging activity IC_{50} (µg/mL)
Leaf	Hexane	> 500
	Ethyl acetate	166.47 ± 7.70
	Methanol	147.33 ± 5.47
Bark	Hexane	> 500
	Ethyl acetate	6.31 ± 0.23
	Methanol	6.80 ± 0.19
BHT	-	10.13 ± 0.45

4. CONCLUSIONS

Leaves and bark of *R. apiculata* were extracted sequentially with hexane, ethyl acetate and methanol. The ethyl acetate and methanol were found to be good solvents for the extraction of phenolic compounds than hexane. Especially, the ethyl acetate and methanolic of bark extracts appeared a good source of polyphenols and possessed good antioxidant activity on DPPH radicals. Thus, *R. apiculata* bark may be source of natural antioxidants.

5. ACKNOWLEDGMENTS

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