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Comparative analyses of saponin, phenolic, and flavonoid contents in various parts of *Rhizophora mucronata* and *Rhizophora apiculata* and their growth inhibition of aquatic pathogenic bacteria

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ABSTRACT

Mangrove plants are the source of various secondary bioactive metabolites and have been used in traditional medicine for many diseases. This study aimed to investigate bioactive phytochemical components of two mangrove plants, *Rhizophora mucronate* and *Rhizophora apiculata* in the Rhizophoraceae family. Eight extracts of four parts (pod, leaf, twig, and bark) of each plant were prepared by maceration with a methanolic solvent. Phytochemical analysis was conducted through the aid of a standard test and was confirmed by quantitative analysis based on the determination of the total saponin, phenolic, and flavonoid contents. Free radical scavenging activity was studied *in vitro* by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) methods. Antibacterial activity was performed by the hole-plate diffusion method and determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration. The pod of *R. mucronata* predominantly showed the highest total saponin content [8.05 ± 0.50 mg escin equivalent (EE)/g CE], and the bark presented a great amount of phenolic and total flavonoid contents (2.12 ± 0.11 mg gallic acid equivalent/g CE and 6.73 ± 0.25 mg RU/g CE). *R. mucronata* showed greater free radical scavenging activity than *Rhizophora apiculata* at a concentration of 100 _g/ml supported by the DPPH and ABTS assays. Antibacterial screening showed that the maximum zone of inhibition was noted for *R. mucronata* extracts against *Streptococcus agalactiae*, *Aeromonas hydrophila*, *Vibrio harveyi*, and *Vibrio parahemolyticus* (8.50–13.56 nm). The *R. mucronata* pod extract had the lowest MIC to only *A. hydrophila.* Meanwhile, the *R. mucronata* bark extract had the lowest MIC to *S. agalactiae* and *A. hydrophila*. The higher antibacterial activity of *R. mucronata* extracts was consistent with the greater saponin, phenolic, and flavonoid contents and antioxidant activity of the extracts of these species with a significant value of $p < 0.05$. The present study highlighted that *R. mucronata* could be used as a potential source of bioactive compounds against aquatic pathogenic bacteria.

INTRODUCTION

Mangrove plants grow under stressful habitat conditions where marine and freshwater systems meet. They are able to adapt morphologically and survive steep temperature gradients and extreme conditions of salinity at the interface of sea and land. To survive in these complex conditions, these plants are endowed with unique and diverse classes of phytochemicals. The use of these plants in the treatment of various ailments may be attributed to the presence of bioactive phytochemicals and secondary metabolites such as phenolics, flavonoids, tannins, alkaloids, and saponins (Das *et al*., 2020; Gurudeeban *et al*., 2013; Sobolewska *et al*., 2020). Several mangrove plants are used in traditional medicine or insecticides and pharmaceuticals (Gajula *et al*., 2020; Premanathan *et al*., 1999; Ravikumar *et al*., 2011). Mangrove plants have been reported by a number of authors as potential sources of natural antioxidant and antimicrobial agents that could be used in medicines for the treatment of bacterial infections and

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cancer (Arulkumar *et al*., 2020; Eswaraiah *et al*., 2020a, 2020b). Therefore, the identification of the phytochemical constituents of bioactive components of these plants is necessary to predict the biological activities that may be exhibited by them (Vasanthi *et al*., 2014).

Several phytochemical surveys of mangrove plants have been published. For example, the major phytochemical substances of interest in these reports are polyphenols and tannins (Das *et al*., 2020). Other naturally occurring significant level substances, like alkaloid and saponin groups, have also been reported (Aberoumand, 2012; Samatha *et al*., 2012). In medicine, the antioxidant, anticancer, and anti-inflammatory activities of saponins are used to treat hypercholesterolemia (Haslam *et al*., 1989) as well as cardiovascular diseases (Samatha *et al*., 2012), and saponins have also been used to facilitate antibody access to intracellular proteins (Gowri and Vasantha, 2010). Although many studies on bioactivity have been performed in the last few decades (Ahmad *et al*., 2013; Arulkumar *et al*., 2020; Eswaraiah *et al*., 2020b; Gajula *et al*., 2020; Hong *et al*., 2011; Loo *et al*., 2007; Premanathan *et al*., 1999; Rahim *et al*., 2008; Saad *et al*., 2011; Sulaiman *et al*., 2011), relatively fewer reports have been published on the antibacterial potential of mangrove plant in inhibiting the growth of aquatic pathogenic bacteria.

Rhizophora mucronata and *Rhizophora apiculata* are mangrove trees belonging to the family Rhizophoraceae. They generally grow in tropical and subtropical areas. When applied against various animal, plant, and human pathogens, the extracts of different parts of both plants exhibited antiviral (Vijayavel *et al*., 2006), antibacterial (Ahmad *et al*., 2013), and antioxidant (Loo *et al*., 2007; 2008; Rahim *et al*., 2008) activities. These activities were derived from phytochemical or bioactive substances, including polyphenols (Hong *et al*., 2011; Sulaiman *et al*., 2011). Present in several parts of the plants, these substances combine with proteins to form stable complexes and antioxidants characterized by free radical scavenging activity (Szydłowska-Czerniak *et al*., 2008). Besides polyphenols, other phytochemicals presented various properties already mentioned above. However, the antibacterial activities of these two plants with various parts against aquatic pathogens have not been investigated.

Due to the lack of research in this area, aquatic pathogens are susceptible to diseases caused by many different bacteria such as *Streptococcus agalactiae*, *Aeromonas hydrophila*, *Vibrio harveyi,* and *Vibrio parahemolyticus*. As a result of this point, synthetic chemicals or synthesized antibiotics are generally used to combat pathogenic bacteria. Thus, finding bioactive substances in plants could provide essential data for the development of novel bioactive agents in order to use them as an alternative replacement for synthetic chemicals against bacterial pathogens in aquaculture and future application in the pharmaceutical industry. This work aims to study phytochemical analyses and antioxidant activities in the pod, leaf, twig, and bark of *R. mucronata* and *R. apiculata* and to evaluate the correlation between the plants and plant parts and three bioactive components (saponin, phenolic, and flavonoid) against aquatic pathogens.

MATERIALS AND METHODS

Plant materials

The fresh pods, leaves, twigs, and bark of *R. mucronata* and *R. apiculata* were collected in November 2015 from Trang Province, Thailand (7˚52′56.2″N 99˚32′75.8″E for *R. mucronata* and 7˚52′29.5″N 99˚31′07.7″E for *R. apiculata*), as shown in Figure 1. The specimen voucher (BKF 194808 for *R. mucronata* and BKF.194836 for *R. apiculata*) was deposited at Bangkok Forest Herbarium, Department of National Parks, Wildlife and Plant Conservation, Thailand.

Extraction procedure

Each part collected from *R. mucronata* and *R. apiculata* was prepared by drying for 10 d and then reduced mechanically. The dried sample of 1,000 g pod, leaf, twig, and bark was soaked in a sealed container for 5 days with methanol in the ratio of 1:4

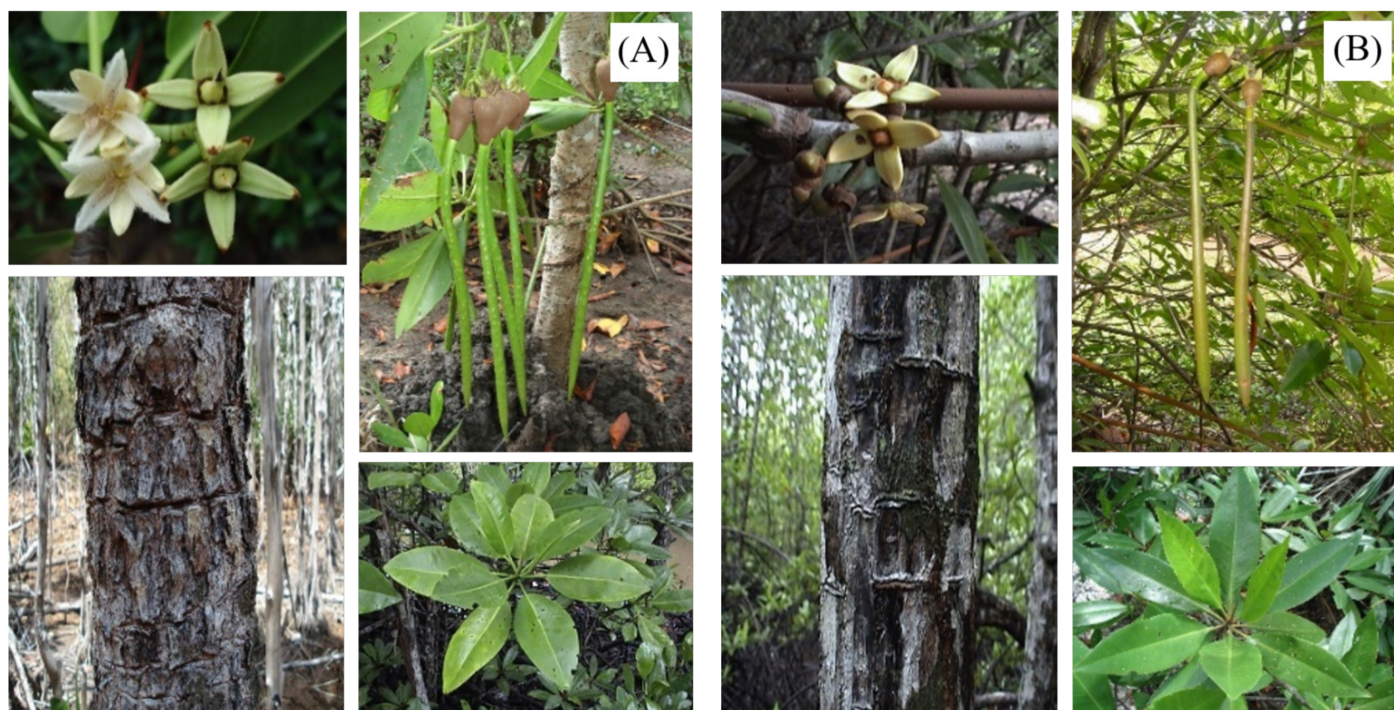


Figure 1. Each part of *R. mucronata* (A) and *R. apiculata* (B).

(w/v) (Vittaya *et al*., 2020b). The resulting extracts were filtered and concentrated using a rotary evaporator at 45°C. All extracts were refrigerated until used for analysis, and the qualitative and quantitative analyses of the major contents of phytochemical compounds and antibacterial activity were studied.

Phytochemical analysis

Qualitative analysis

The major components of the secondary metabolite were searched in the crude extract (CE) using qualitative analysis based on precipitation and coloring reactions. All procedures were examined using the standard method described by Vittaya *et al*. (2020a). Briefly, saponin was determined by the formation of a stable form (fourth test). The appearance of the dark green extract after the addition of ferric chloride indicated the existence of phenolic composition. Flavonoid was indicated by the reaction of metal and the appearance of yellow. Borntrager's test was used to determine anthraquinone, the rose-pink color that occurred in the ammonia layer. Terpenoid and alkaloid were tested by using Salkowski's test and Dragendroff's solution, respectively. The reddish-brown ring occurred at the junction of two layers, indicating the presence of terpenoid. The appearance of orange-yellow precipitates confirmed the presence of alkaloid.

Quantitative analysis

Determination of saponin content

The saponin content (SC) of each *R. mucronata* and *R. apiculata* extract was determined according to the method described by Senguttuvan *et al*. (2014), with a modification to the procedure that reduced the volume of the extract solution. Briefly, 0.2 mL of extract was mixed with 0.5 ml of a 0.8% (w/v) vanillin solution, 5 ml of 72% (v/v) sulphuric acid was added, and the mixture was allowed to stand for a 1 min. After standing, the mixture was incubated at 70°C for 10 minutes and rapidly cooled with ice water to room temperature. The absorbance of the mixture was measured at 560 nm using UV-vis spectroscopy (U-1800 Spectrophotometer, Hitachi High-Tech Science Corp., Tokyo, Japan). Methanol and escin were used as a control and standard, respectively. SC was expressed as milligram of EE/g CE through the calculation curve of escin ($y = 0.0006x + 0.0284$). The experiment was performed in triplicate, and the obtained data were expressed as the means ± standard deviation with linearity R^2 equal to 0.9931.

Determination of phenolic content

The total phenolic content was determined using the Folin–Ciocalteu reagent by the method described by Vittaya *et al*. (2019). Briefly, a volume of 0.2 ml of eight methanolic extracts or gallic acid was added to a centrifuge tube, followed by 2.5 ml of distilled water. After 0.2 ml of the Folin–Ciocalteu reagent was added, 2.0 ml of a 7% sodium carbonate solution was added. Immediately after that, each sample was vigorously shaken by a vortex mixer. It was kept away from light for 60 minutes. The absorbance of the standard and all extracts was measured at 765 nm against the blank. Total phenolic content (TPC) was expressed as milligram of gallic acid equivalent per gram CE through the calibration curve of gallic acid ($y = 0.004x + 0.0028$) with linearity R^2 equal to 0.9995.

Determination of flavonoid content

The total flavonoid content of all extracts of *R. mucronata* and *R. apiculata* was measured by the colorimetric method of aluminum chloride, based on the method described previously by Vittaya *et al*. (2019). Briefly, a volume of 0.2 ml of each sample or rutin was added to a centrifuge tube. After 0.5 ml of a 5% sodium nitrite solution was added, the reaction mixture was left to stand at room temperature for 6 minutes. Two hundred microliters of aluminum chloride (10%) was then added, followed by 0.5 ml of 1 M sodium hydroxide. The total volume was made up to 1.5 ml with distilled water. The reaction mixture was mixed well again, and the absorbance was recorded against a blank at 510 nm. Total flavonoid content (TFC) was expressed as milligram of rutin equivalent per gram of CE through the calibration curve of gallic acid ($y = 0.001x - 0.004$) with linearity R^2 equal to 0.9990.

Antioxidant activity

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical free radical scavenging

The DPPH method was conducted using spectroscopy to determine activity based on the method described by Vittaya *et al*. (2020b). Briefly, the stock solution of extracts or standard 1 mg/mL was prepared, and 0.5 ml was then mixed with 0.5 ml of a 0.15 M DPPH solution. The reaction mixture was incubated to stand at room temperature away from light for 30 minutes. Their absorbance was measured at 517 nm against the blank. The percentage of free radical scavenging activity of the sample was calculated as follows:

$$\text{DPPH radical scavenging (\%)} = [1-(A_{sample}-A_{sample\ blank})/A_{control}] \times 100, \quad (1)$$

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 $Abs_{control}$ is the absorbance of the DPPH solution.

2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical free radical scavenging

The ABTS assay was examined according to the method of Vittaya *et al*. (2020(b)). Briefly, the dilution of the $ABTS^+$ working solution that gave an absorbance of 0.700 ± 0.025 units at 734 nm was prepared. The 0.1 ml of standard or sample extract was added to a centrifuge tube, and then 0.9 ml of the diluted $ABTS^+$ working solution was added to each test tube. The reaction mixture was incubated for 6 min at room temperature. After incubation time, the absorbance at 734 nm was measured. The percentage of free radical scavenging activity of the sample tested was calculated as follows:

$$\text{ABTS radical scavenging (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100, \quad (2)$$

where A_{contro}l is the absorbance of the extract without $ABTS^+$ solution and A_{sample} is the absorbance of the extract with $ABTS^+$ solution.

Antibacterial investigation

The antibacterial activities of the pod, leaf, twig, and bark extracts ($n = 4$) of *R. mucronata* and *R. apiculata* were tested against the Gram-positive strain *S. agalactiae* SAAQ001 (de-

rived from Kasetsart University) and the Gram-negative strains *A. hydrophila* AHAQ001 and *V. harveyi* VHAQ001 (derived from Kasetsart University) and *V. parahemolyticus* (derived from Songkhla Aquatic Animal Health Center, Thailand). The hole-plate diffusion method was conducted according to the method of Brantner *et al*. (1994), with slight modification. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts were determined using the standard method (Eloff, 1998; National Committee for Clinical Laboratory Standard, 2000) as outlined by Vittaya *et al*. (2020b).

Statistical analysis

The results were expressed as mean ± standard deviation in triplicate for chemical analysis and quadruplet for antibacterial activity. Statistical analysis was performed by one-way analysis of variance, followed by Duncan's comparison. A two-way analysis of variance was used to evaluate the interaction between the two plants (*R. mucronata* and *R. apiculata*) with four parts (pod, leaf, twig, and bark). The correlation between the phytochemical composition and free radical scavenging activity was carried out using Pearson's analysis.

RESULTS AND DISCUSSION

Yield and phytochemical analysis

Yield and phytochemical screening

The yields of the pod, leaf, twig, and bark of *R. mucronata* were obtained as 14.42% (216.25 g), 29.95% (299.53 g), 15.37% (153.73 g), and 19.15% (191.52 g), respectively. For *R. apiculata*, the yields of the pod, leaf, twig, and bark were 12.57% (125.74 g), 16.81% (168.06 g), 9.81% (98.14 g), and 17.24% (172.39 g), respectively. Phytochemical screening of the pod, leaf, twig, and bark of *R. mucronata* and *R. apiculata* was carried out by using a qualitative characterization reaction. These reactions were based on the appearance of color or precipitation by specific reagents. The results of this phytochemical screening are reported in Table 1. All parts of these plants have revealed the presence of saponin, phenolic, flavonoid, anthraquinone, terpenoid, and alkaloid contents differently. For *R. mucronata*, all phytochemical components were found in the leaf and bark, whereas anthraquinone and terpenoid were not detected in the pod, and anthraquinone and alkaloid were not found in the twig. In contrast to *R. apiculata*, no parts of this plant contain all kinds of phytochemicals. However, the bark extract of *R. apiculata* showed the most phytochemicals except terpenoid. Anthraquinone was not detected in the pod and leaf. Terpenoid was not found in the twig and pod, and alkaloid was not observed in the leaf and twig. Interestingly, the high levels of three chemical compositions, like saponin, phenolic, and flavonoid, were observed in both plants, especially in *R. mucronata.* The part of the plant may be related to the phytochemical composition. The current research in this area has focused on the content of phenolic compounds because they are known to provide therapeutic properties (Adebayo and Ishola, 2009; Gajula *et al*., 2020). Studies also showed that mangrove plants contain significant levels of saponins, which have anti-inflammatory, antioxidant, and anticancer properties (Sobolewska *et al*., 2020; Verma *et al*., 2013). Since they were also found to exercise antimicrobial activity against a wide range of microorganisms *in vitro* (Saad *et al*., 2011), the SCs, as well as phenolic and flavonoid levels, may contribute to explain their antioxidant and antibacterial activities.

Determination of the saponin, phenolic and flavonoid contents

In the present study, the SCs of the extracts of both plants were calculated from the equation: $y = 0.0006x + 0.0284$ ($R^2 = 0.9931$), as escin equivalents [escin equivalent (EE)/g CE], and the data are presented in Table 2. The calculations produced values from 0.46 to 8.05 mg EE/g CE. All extracts of the *R. mucronata* part used had higher contents of saponins (2.94 and 8.05 mg EE/ g CE) than those of *R. apiculata* (0.46 and 4.57 mg EE/ g CE). Two-way analysis of variance clearly indicated that the main effects (species and plant parts) had a significant influence on the content of saponin with $p < 0.001$. Additionally, the phenolic contents of the extracts of both plants were calculated from the following equation: $y = 0.0040x + 0.0028$ ($R^2 = 0.9995$), as gallic acid equivalents [gallic acid equivalent (GAE)/g CE], and the data are presented in Table 2. The calculations

Table 1. Phytochemical screening performed on aerial parts of *R. mucronata* and *R. apiculata*.

Species	Phytoconstituents	Verification methods	Parts			
			Pod	Leaf	Twig	Bark
R. macronata	Saponin	Forth test	+++	+++	+++	+++
	Phenolic	Ferric chloride test	+++	+++	+++	+++
	Flavonoid	Reaction of metal	+	+	+	+
	Anthraquinone	Borntrager's test	−	+	−	+
	Terpenoid	Salkowski's test	−	++	++	+
	Alkaloid	Dragendroff's test	+++	+	−	+
R. apiculata	Saponin	Forth test	+	+	+	+++
	Phenolic	Ferric chloride test	++	++	+++	+
	Flavonoid	Reaction of metal	+	+	+	+
	Anthraquinone	Borntrager's test	−	−	+	+
	Terpenoid	Salkowski's test	−	+	−	−
	Alkaloid	Dragendroff's test	++	−	−	++

+++: very abundant; ++: moderately abundant; +: present; −: absent.

Table 2. Chemical compositions and free radical scavenging activity in four parts of *R. mucronata* and *R. apiculata.* Two-way analysis of variance of effectors SP and PP.

Species	Plant parts	Total saponin content (TSC: mg EE/g CE)	Total phenolic content (TPC: mg GAE/g CE)	Total flavonoid content (TFC: mg RU/g CE)	DPPH (% inhibition)	ABTS (%inhibition)
R. mucronata	Pod	8.05 ± 0.50[a]	1.96 ± 0.10[b]	6.10 ± 0.23[b]	93.75 ± 2.19[a]	99.69 ± 0.16[a]
	Leaf	2.94 ± 0.14[d]	1.05 ± 0.04[e]	4.30 ± 0.27[c]	93.21 ± 3.96[a]	92.04 ± 6.53[b]
	Twig	4.87 ± 0.32[c]	1.39 ± 0.09[d]	4.51 ± 0.19[c]	93.39 ± 0.98[a]	99.79 ± 0.24[a]
	Bark	6.84 ± 0.24[b]	2.21 ± 0.11[a]	6.73 ± 0.25[a]	93.17 ± 2.21[a]	99.43 ± 0.50[a]
R. apiculata	Pod	0.46 ± 0.01[f]	0.41 ± 0.03[f]	1.10 ± 0.07[g]	75.33 ± 1.06[b]	40.70 ± 2.18[d]
	Leaf	2.44 ± 0.04[e]	0.22 ± 0.01[g]	2.40 ± 0.07[f]	92.75 ± 0.97[a]	61.40 ± 8.69[c]
	Twig	2.47 ± 0.04[e]	1.01 ± 0.06[e]	2.73 ± 0.05[e]	94.48 ± 2.07[a]	99.22 ± 0.46[a]
	Bark	4.57 ± 0.19[c]	1.56 ± 0.07[c]	3.71 ± 0.23[d]	95.16 ± 0.71[a]	99.37 ± 0.16[a]

Two-way analysis of variance.

Variable	df	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
SP	1	1,029.529	<0.001	815.209	<0.001	1,410.558	<0.001	22.583	<0.001	197.756	<0.001
PP	3	160.493	<0.001	293.653	<0.001	120.884	<0.001	30.682	<0.001	90.263	<0.001
SP × PP	3	235.993	<0.001	71.503	<0.001	91.973	<0.001	34.029	<0.001	77.111	<0.001
Error	16										

DPPH = 2,2-diphenyl-1-picrylhydrazyl (% inhibition); ABTS = 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (% inhibition).
Data are presented as mean ± SD from triplicate analysis. TSC = Total saponin content; TPC = Total phenolic content; TFC = Total flavonoid content.
The lowercase superscripts (a–g) in each column denote significant ($p < 0.05$) differences in each plant.

produced values from 0.22 to 2.21 mg GAE/g CE. All extracts of the *R. mucronata* part used had higher phenolic contents (1.05 and 2.21 mg GAE/g CE) than those of *R. apiculata* (0.22 and 1.56 mg EE/g CE). Two-way analysis of variance clearly indicated that the main effects (species and plant parts) had a significant influence on the phenolic content with $p < 0.001$. Moreover, the flavonoid contents of the extracts of both plants were calculated from the equation $y = 0.0010x - 0.0040$ ($R^2 = 0.9990$), as rutin equivalents (rutin equivalent (RU)/g of CE), and the data are presented in Table 2. The calculations produced values from 1.10 to 6.73 mg RU/g CE. All extracts of the *R. mucronata* part used had higher contents of flavonoid (4.30 and 6.73 mg RU/g CE) than those of *R. apiculata* (1.10 and 3.71 mg RU/g CE). Two-way analysis of variance revealed that the main effects (species and plant parts) had significant effect on the content of saponin with $p < 0.001$. Based on a two-way analysis of variance, all phytochemical compositions (saponin, phenolic, and flavonoid) were affected by species (*R. mucronata* and *R. apiculata*) and plant parts (pod, leaf, twig, and bark) ($p < 0.01$). Interestingly, species × plant parts had a highly significant effect, as shown in the interaction in Figure 2. The findings from the current study showed that the pod of *R. mucronata* had the highest saponin and flavonoid, while the bark showed phenolic content at its highest. In addition, the SC was significantly correlated with the phenolic and flavonoid contents at $r = 0.893$ and $r = 0.828$ for *R. mucronata* and $r = 0.775$ and $r = 0.975$ for *R. apiculata* ($p < 0.01$, Fig. 3). In the same way, the phenolic and flavonoid contents were correlated significantly with $r = 0.961$ and $r = 0.798$ for *R. mucronata* and *R. apiculata* ($p < 0.01$), respectively. From the above, rich sources of phytochemicals (saponin, phenolic, and flavonoid) from both plants might be related directly to their biological activities, especially antioxidant and antibacterial activities further studied in the next section.

Antioxidant activity

The free radical scavenging activity was measured *in vitro* using two methods, DPPH and ABTS, for each of the eight extracts, and the data are shown in Table 2. The percentage of free radical scavenging of DPPH was determined by spectroscopic analysis at 517 nm of quenched DPPH after reaction with samples. Free radical scavenging by *R. mucronata* extracts ranged between 93.17% and 93.75% and between 75.33% and 95.16% for *R. apiculata* extract. All the extracts of *R. mucronata* and *R. apiculata* showed very high levels of inhibitory activity (>90%(except the pod of the latter. The ABTS method is a good tool for determining free radical scavenging, and $ABTS^+$ is a synthetic compound widely used to evaluate the antioxidant activity. This radical is produced by the oxidation of ABTS with potassium persulfate. This radical converts to a nonradical form, when exposed to antioxidants. The percentage of free radical scavenging of ABTS ranged between 92.69% and 99.79% for *R. mucronata* and between 40.70% and 99.37% for *R. apiculata* (Table 2). Interestingly, the percentage of free radical scavenging activity of the *R. apiculata* leaf has the trend to decrease slightly. It may be due to the least total phenolic content of the *R. apiculata* leaf, which affected the trend of free radical scavenging activity. Two-way analysis of variance revealed that the main effect of species and plant parts had an influence on antioxidant capacity as shown in Figure 3. Additionally, species × plant parts have a highly significant effect ($F = 34.029$, $p < 0.001$) as shown in the interaction in Figure 4. Furthermore, there was good agreement between the results of DPPH and ABTS in our study with the correlation coefficient ($r = 0.834$; $p < 0.01$ for only *R. apiculata*), presented in Figure 3. It is promising that a significant linear correlation was found between the free radical scavenging activity determined and phytochemical composition at a significant level of 0.01 in only *R. apiculata*. However, *R. mucronata* had only SC correlated with ABTS ($r = 0.623$; $p < 0.05$). It is possible that saponin has more effect on free radical scavenging activity than the phenolic and flavonoid contents in this plant. Moreover, it was observed in this study that other phytochemical compounds such as anthraquinone and alkaloid exist in *R. mucronata* and they are assumed to have

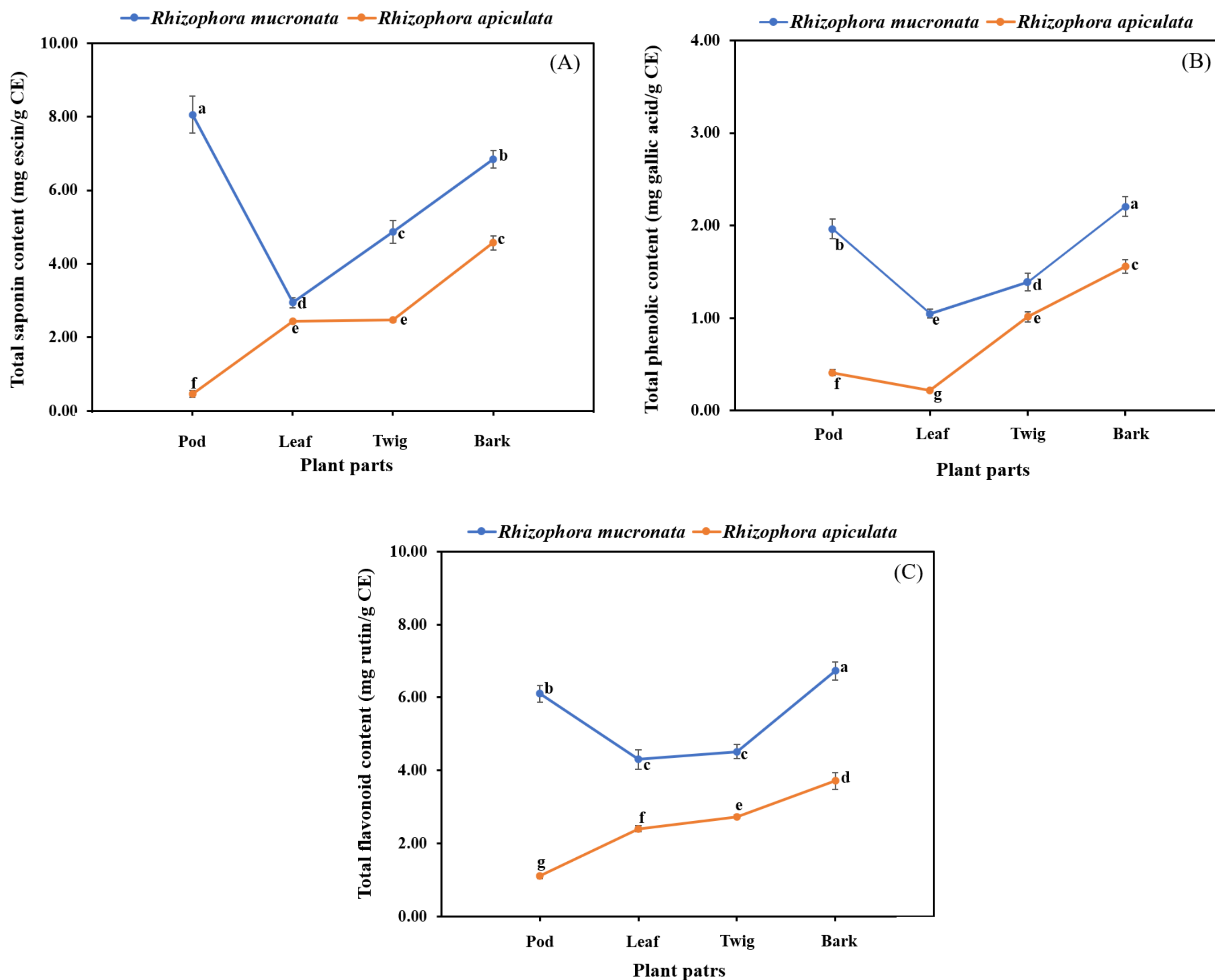


Figure 2. Interaction effect of species and plant parts on quantity of total saponin content (A), total phenolic content (B), and total flavonoid content (C) in *R. macronata* and *R. apiculata* extracts, where different lowercase letters indicate significant ($p < 0.05$) difference among mean and error bars represent ± SD.

a role in free radical scavenging activity, which is observed in previous research. According to the report of Gurudeeban *et al*. (2013), the alkaloid-rich extracts from *Rhizophora mucronate* displayed a potential application in antibacterials and antioxidants. Also, Kremer *et al*. (2012) reported that most derivatives of anthraquinone extracts from *Frangula rupestris and Frangula alnus* bark had high antioxidant and antimicrobial activities. The determination of the bioactive constituents in *R. mucronata* would be recommended for further study.

Antibacterial screening

The antibacterial activities of the pod, leaf, twig, and bark extracts of *R. mucronata* and *R. apiculata* were determined with four bacteria strains, *S. agalactiae*, *A. hydrophila*, *V. harveyi*, and *V. parahemolyticus*. Antibacterial potency was evaluated by measuring the diameter of the zone of inhibition in millimeters (mm) and determining MIC and MBC values. The results of antibacterial activity by the hole-plate diffusion method are reported in Table 3. The antibacterial activities of various part extracts of *R. mucronata* and *R. apiculata* were compared with the activity of the antibiotic agent oxolinic acid. The area of inhibition produced by each extract against the four pathogenic bacterial strains has a wide spectrum of the zone of inhibition, which varies depending on the strain tested, species, and the part extracted. According to Table 3, all extracts were active against all tested strains of the diameters of the inhibition zones ranging from 6.00 to 13.31 mm. It is tempting to explore or explain the effect of species (SP) and plant parts (PP) to inhibit pathogenic bacteria. Therefore, a two-way analysis of variance was used to measure the antibacterial activity of the two plants (*R. mucronata* and *R. apiculata*) with various parts (pod, leaf, twig, and bark), to inhibit pathogenic bacteria (Table 3). The results showed that the species of the plant and parts used influenced antibacterial activity ($p < 0.001$), except for *V. harveyi*. The observed antibacterial activity resulted from active compounds like phenolics, flavonoids, and saponins, which were more significantly found in *R. mucronata* than *R. apiculata* in all parts, as shown in Figure 2. For the above reason, more antibacterial activity was observed in *R. mucronata*

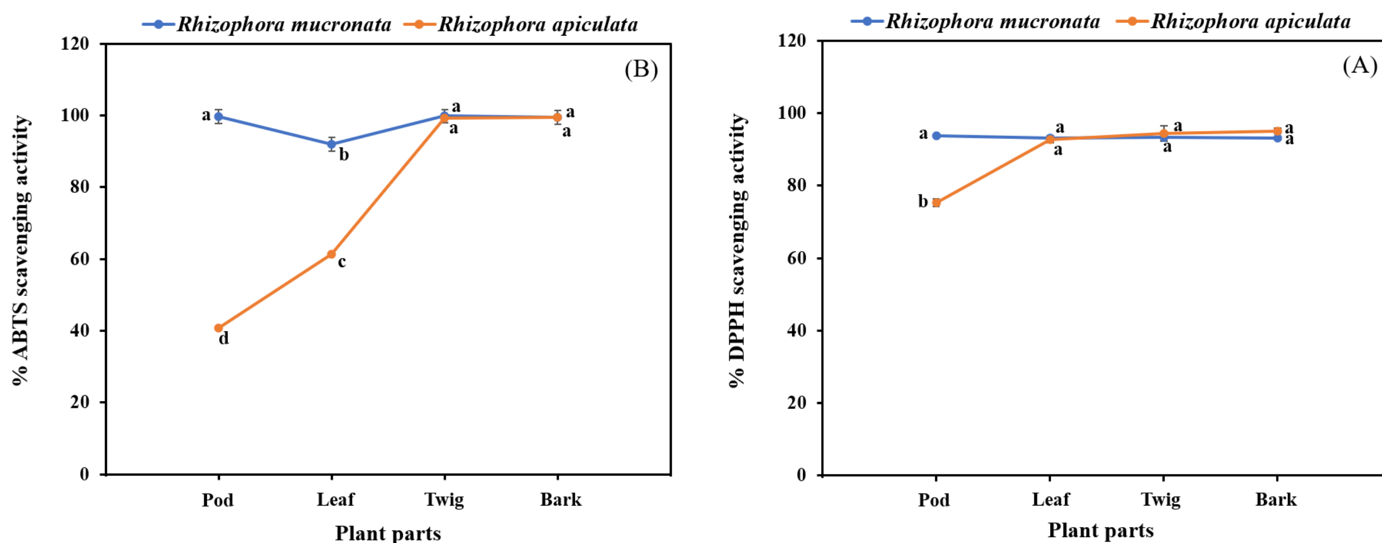
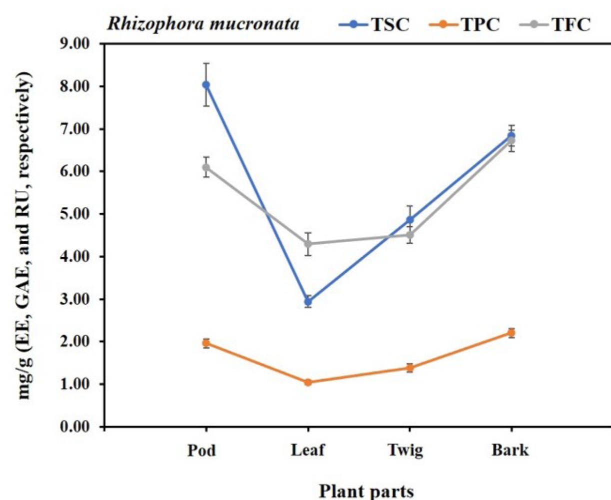
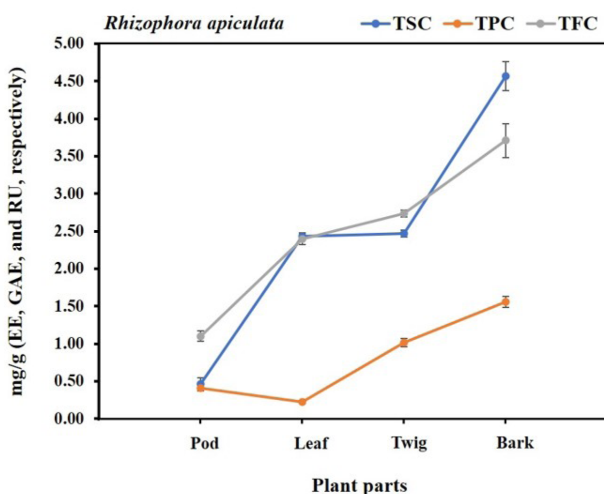


Figure 3. Interaction effect of species and plant parts on quantity of DPPH assay (A) and ABTS assay (B) in *R. macronata* and *R. apiculata* extracts, where different lowercase letters indicate significant ($p < 0.05$) difference among mean and error bars represent ± SD.



Correlation : TSC, TPC, and TFC (*Rhizophora mucronata*)

Assay	TSC	TPC	TFC	DPPH
TPC	0.893			
	0.000**			
TFC	0.828	0.961		
	0.001**	0.000**		
DPPH	0.061	0.000	0.006	
	0.851	0.999	0.989	
ABTS	0.623	0.540	0.398	0.132
	0.031*	0.070	0.200	0.681



Correlation : TSC, TPC, and TFC (*Rhizophora apiculata*)

Assay	TSC	TPC	TFC	DPPH
TPC	0.775			
	0.003**			
TFC	0.975	0.798		
	0.000**	0.002**		
DPPH	0.835	0.519	0.889	
	0.001**	0.084	0.000**	
ABTS	0.813	0.836	0.880	0.834
	0.001**	0.001**	0.000**	0.001**

Figure 4. TSC, TPC, and TFC are positively correlated with antioxidant activity [2,2-diphenyl-1-picrylhydrazyl (% inhibition): DPPH and 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (% inhibition): ABTS] in pod, leaf, twig, and bark of *R. mucronata* and *R. apiculata*. Data shown are mean ± SD. Pearson's correlation coefficient (r) between each pair of plants (*R. macronata* and *R. apiculata*) and chemical compositions ($n = 12$). * $p < 0.05$, ** $p < 0.01$.

Table 3. Zones of inhibition from methanolic extracts of *R. mucronata* and *R. apiculata* against four pathogenic bacteria (*S. agalactiae*, *A. hydrophila*, *V. harveyi*, and *V.parahaemolyticus*) that cause infectious diseases in aquatic conditions. Two-way analysis of variance of effectors plant species (SP) and plant parts (PP).

Species		Zone of inhibition diameter (mm)			
		S. agalactiae	*A. hydrophila*	*V. harveyi*	*V. parahaemolyticus*
R.mucronata	Pod	12.88 ± 0.75[a]	13.31 ± 0.72[b]	11.56 ± 3.00	10.75 ± 0.50[bc]
	Leaf	11.50 ± 1.29[ab]	10.88 ± 0.43[c]	12.19 ± 2.73	10.38 ± 0.48[c]
	Twig	11.25 ± 0.50[bc]	10.44 ± 0.31[cd]	12.75 ± 2.84	11.00 ± 0.71[abc]
	Bark	12.50 ± 0.41[ab]	11.81 ± 0.68[c]	12.75 ± 1.37	11.88 ± 0.25[a]
R. apiculata	Pod	10.00 ± 0.82[c]	8.56 ± 1.81[e]	10.75 ± 3.12	6.31 ± 0.47[d]
	Leaf	7.94 ± 2.08[d]	6.63 ± 1.25[f]	10.13 ± 2.09	6.00 ± 0.00[d]
	Twig	9.88 ± 0.25[c]	8.12 ± 0.75[e]	12.00 ± 3.02	10.00 ± 0.00[c]
	Bark	12.88 ± 1.44[a]	9.25 ± 1.50[de]	12.75 ± 1.40	11.75 ± 0.64[ab]
	Oxolinic acid	11.63 ± 1.59[ab]	29.50 ± 0.68[a]	11.63 ± 1.59	11.62 ± 1.59[ab]

Two-way analysis of variance.

Variable	df	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Species (SP)	1	64.655	<0.001	87.177	<0.001	1.019	0.323	234.808	<0.001
Plant parts (PP)	3	32.226	<0.001	7.675	0.001	0.847	0.482	110.870	<0.001
SP × PP	3	18.218	<0.001	2.663	0.071	0.227	0.877	48.059	<0.001
Error	24								

Values for zones of inhibition are expressed as mean (mm) ± SD ($n = 4$).
The lowercase superscripts (a–f) in each column denote significant ($p < 0.05$) differences in each plant.

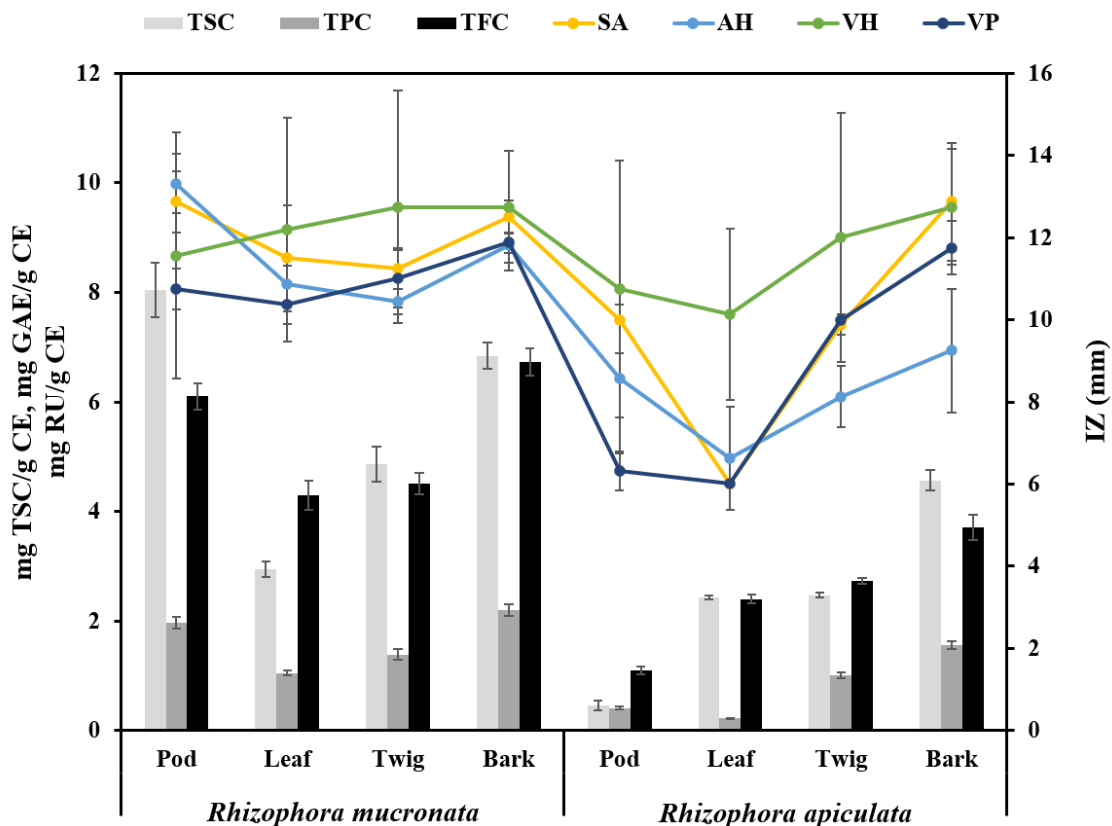


Figure 5. The relative between quantitative contents with antibacterial activity of species and plant parts in *R. macronata* and *R. apiculata* extracts (TSC: Total saponin content, TPC: Total phenolic content, TFC: Total flavonoid content; IZ: Zone of inhibition; SA: *Streptococcus agalactiae*, AH: *Aeromonas hydrophila*, VH: *Vibrio harveyi,* and VP: *Vibrio parahemolyticus*).

Table 4. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of extracts of *R. mucronata* and *R. apiculata* against four pathogenic bacteria that cause infectious diseases in aquatic conditions.

Species	MIC, MBC (mg/mL)											
	S. agalactiae			*A. hydrophila*			*V. harveyi*			*V. parahaemolyticus*		
	MIC	MBC	R	MIC	MBC	R	MIC	MBC	R	MIC	MBC	R
R. mucronata												
Pod	0.78	0.78	1	0.39	0.39	1	3.12	12.50	4	6.25	25.00	4
Leaf	0.78	0.78	1	0.78	0.78	1	1.56	12.5	8	6.25	25.00	4
Twig	0.78	1.56	2	0.78	0.78	1	1.56	6.25	4	6.25	25.00	4
Bark	0.39	1.56	4	0.39	0.39	1	1.56	12.50	8	3.12	25.00	8
R. apiculata												
Pod	6.25	6.25	1	3.12	3.12	1	6.25	25.00	4	12.50	25.00	2
Leaf	12.50	12.50	1	12.50	25.00	2	1.56	12.50	4	12.50	25.00	2
Twig	6.25	12.50	2	1.56	1.56	1	6.25	12.50	2	6.25	25.00	4
Bark	3.12	12.50	4	0.78	3.12	4	3.12	25.00	8	1.56	25.00	16
Oxolinic acid	0.19	3.12	16	0.19	3.12	16	0.19	3.12	16	0.19	3.12	16

Values are expressed as mean from quadruplicate determination ($n = 4$).
R-values are calculated from MBC/MIC ($R < 4.00$ indicates bactericidal extract; $R > 4.00$ indicates bacteriostatic extract).

than in *R. apiculata*. Interestingly, all parts of *R. mucronata* have more antibacterial activity than in *R. apiculata* as shown in Figure 5. Some of these phytochemical compounds have already been reported to have antibacterial properties (Krishnavignesh *et al*., 2012). In agreement with Santhi and Sengottuve (2016), they found that the potential of the extracts such as flavonoids, phenol, and saponin was an important source of useful drugs.

MIC and MBC were used to determine the lowest concentration of antibacterial agent to inhibit the growth of the microorganism and the minimum bactericidal concentration of extracts. Table 4 presents the results obtained from the minimum inhibitory and bactericidal concentrations of the extracts of *R. mucronata* and *R. apiculata*. All tested extracts of antibacterial activity gave MIC values between 0.39 and 25.00 mg/ml. It was found that the pod and bark of *R. mucronata* extracts were the most active with an MIC value of 0.39 mg/mL against *A. hydrophila* and *S. agalactiae*. MIC values of 0.78 mg/mL were found in the leaf and twig extracts, which performed less well against *A. hydrophila* and *S. agalactiae*. On the contrary, only the bark extract of *R. apiculata* showed a MIC of 0.78 mg/ml and worked against *A. hydrophila*. These results showed that the extracts of *R. mucronata* were more active against the tested pathogens than the extracts of *R. apiculata*, especially against *S. agalactiae* and *A. hydrophila.* Therefore, the bark extract of *R. mucronata* strongly inhibited both strains with a MIC of 0.39 mg/ml while the pod extract of *R. mucronata* showed strong activity against only *A. hydrophila* with a MIC of 0.39 mg/ml. Data from the quantitative analysis of the phytochemical compositions (Table 2) show that the *R. mucronata* extracts contained higher amounts of saponins, phenolic, and flavonoid contents than the equivalent extracts of *R. apiculata* almost part of which is supported by Figure 4. This difference may play an important role in the antibacterial activity of the two plants.

The MBC/MIC values examined the nature of the antibacterial effect of the extracts tested. The extract is considered a bactericidal extract when this ratio is lower than 4, and when it is higher than 4, it is considered a bacteriostatic extract (Boulfia *et al*., 2021). In Table 4, it can be seen that almost all extracts tested have a bactericidal effect on *S. agalactiae* and *A. hydrophila*. In contrast to *harveyi*, it is found that the pod and twig of *R. mucronata* and the pod, leaf, and twig of *R. apiculata* have a bactericidal effect. For *V. parahemolyticus*, only the bark of *R. mucronata* and *R. apiculata* has a bacteriostatic effect and the other extracts have a bactericidal effect.

Phytochemical constituents were effective in inhibiting the growth of these pathogenic strains, even in the face of this barrier (Ravikumar *et al*., 2011). Consisting of a hydrophobic triterpene or steroid skeleton and hydrophilic carbohydrate molecules, the structure of saponins gave rise to amphipathic properties (Them *et al*., 2019). It is possible that both properties enable the active substance to pass through the cell membrane to destroy the cell, which is in excellent agreement with the previous theoretical reports (Sam and Anne, 2012). Additionally, the phenolic and flavonoid compounds confirmed by the phytochemical analysis of the extracts could inhibit the cell protein synthesis of the bacteria (Ravikumar and Kathiresan, 1993; Scalbert, 1991). Based on the results, it is possible to conclude that the methanolic extract of *R. mucronata* is a potential source of antibacterial agents against pathogenic aquatic bacteria.

CONCLUSION

From the present study, it was concluded that both plants *Rhizophora mucronata* and *Rhizophora apiculata* have the potential to act as a source of useful drugs because of their antibacterial activity against especially *S. agalactiae* and *A. hydrophila.* The determination of biologically active compounds, such as predominant saponin, phenolic, and flavonoid contents in the extracts, provided evidence of the presence in these plants of antimicrobial phytochemicals. These species could provide natural bioactive agents to replace the synthetic compounds that are currently used to treat the diseases caused by aquatic pathogens.

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DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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ETHICAL APPROVAL

This study does not involve experiments on animals or human subjects.

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