Volatile Constituents of Mangrove Vinegar from *Rhizophora apiculata* and Their Antioxidant Activity

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The chemical constituents, phytochemical screening, antioxidant activity, and total phenolic content of mangrove (*Rhizophora apiculata)* vinegar were identified and evaluated. Eleven compounds representing 79.56 % of the *n*-hexane extract of mangrove vinegar (MV) were determined using gas chromatography-flame ionization detector (GC-FID) and gas chromatography-mass spectrometry (GC-MS) techniques. From these analyses, the major constituents were identified as 2,6-dimethoxyphenol (28.80 %), guaiacol (18.14 %), p-cresol (7.82 %) and 1,2,4-trimethoxybenzene (7.00 %). In addition, phytochemical screening of the MV extract indicated the presence of several active glycosides, saponins, coumarins, and flavonoids. However, steroids, tannins, alkaloids and cardiac glycosides were absent in the extract. The total phenolic content (162.07 ± 5.55 GAE/g) of the MV extract was determined using the Folin-Ciocalteu method, while its antioxidant activity was evaluated using ferric reducing antioxidant power assays (FRAP) and compared with the ascorbic acid standard curve.

Keywords: *Rhizophora apiculata*; mangrove vinegar; volatile component analysis; antioxidant

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Rhizophora apiculata (R. apiculata) belongs to the Rhizophoraceae family, and is a mangrove species locally known as *bakau* in Malaysia [1]. Mangrove plants are endemic to coastal areas of Southeast Asia from Malaysia to New Guinea. They can be found in 80 % of Indo-Pacific shores, 9 % of East Africa, 6 % of West Africa, 5 % of South America and 5 % of the Caribbean [2]. In Southeast Asia, mangroves play a significant role in supporting sustainable coastal and marine ecosystems. They protect the shorelines and prevent coastal erosion [3].

Traditionally, mangroves have been used in firewood and charcoal production. They are also utilised for the construction of dwellings, furniture, boats and fishing equipment, while their tannins are used for leather dyeing. The chemical extracts of mangroves are used as insecticides and piscicides [4]. In addition, previous studies reported that the bark, roots and leaves of *R. apiculata* showed interesting biological activities such as anticancer, antidiabetic and antimicrobial properties [5,6]. Mangrove vinegar (MV) or pyroligneous acid can be defined as the crude condensate produced from the distillation of smoke generated in the process of charcoal making. This MV is categorized as acidic (pH 2-3) and has mild corrosive properties as it consists of 5.5 % acetic acid, 3.4 % methanol and 6.5 % of wood tar [7]. In Malaysia, the MV of *R. apiculata* is exploited as a sterilizing agent, deodorizer and fertilizer, as well as anti-microbial, antifungal, growth-promoting agents and as a mosquito repellent [6, 9-12]. Another study reported that MV and its dichloromethane extract had a high total phenolic content and good antioxidant properties due to the presence of polyphenols and other beneficial compounds such as organic acetic acids, methanol, ketones and aldehydes [8]. Figure 1 shows the charcoal kiln at a charcoal factory in Matang, Perak, Malaysia and the MV produced from this factory.

There is little information available on the volatile components of the *R. apiculata* MV from Matang, Perak. Recently, there has been considerable interest in exploring MV for its antioxidant activity and chemical constituents [7,13]. Therefore, the present study was designed to determine the chemical components of the *n*-hexane extract MV of *R. apiculata* and to evaluate their antioxidant activity via a ferric reducing antioxidant power assay (FRAP) in combination with the determination of total phenolic content.

Figure 1. Mangrove charcoal kiln (left) and collected mangrove vinegar (right).

MATERIALS AND METHODS

Chemicals and Solvents

Analytical reagent grade *n*-hexane, methanol, dichloromethane, sodium carbonate, sodium dihydrogen phosphate and disodium hydrogen phosphate were obtained from QRec Chemical (Malaysia). The *n*-hexane for gas chromatography ECD and FID (SupraSolv®), potassium ferricyanide, trichloroacetic acid, iron (III) chloride hexahydrate, gallic acid standard (Fluka) and the C_7–C_{30} saturated alkane standard were purchased from Sigma Aldrich GmbH (Germany).

Material

Mangrove vinegar from *R. apiculata* was collected from the Matang Mangrove factory in Perak, Malaysia (4°50′N, 100°35′E). It is a liquid by-product of charcoal manufacturing, that had a light to dark brown colour and a strong smoky aroma, with a pH value of 3. The sample was collected in a 5-litre bottle and kept in the dark at 4 °C before use.

Extraction of Volatile Components

The extraction methodology of the mangrove's volatile components was slightly modified from Loo et. al. [8], which used liquid-liquid extraction. Initially, the sample was filtered using Whatman® filter paper (0.45 µm pore size) to remove debris. Then, 25 mL of the sample was extracted with 25 mL of *n*-hexane solvent (a ratio of 1:1). The mixture was left standing for 36 hours. This extraction was performed in a separating funnel and the *n*-hexane extract was concentrated using a rotary evaporator under pressure at a temperature < 40 °C. These procedures were repeated three times. The crude extract (5.55 g/mL) was stored at 4 °C for further use.

Gas Chromatography - Flame Ionization Detector (GC-FID)

GC-FID analysis was performed with an Agilent 7890A instrument (Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was performed with an Agilent J&W HP-5 capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness) under the following instrumental conditions: nitrogen as carrier gas at a constant flow of 1.0 mL min^{-1}, injector temperature 220 ºC; the initial oven temperature was 80 ºC which increased to 220 ºC at a rate of 10 ºCmin^{-1}. The total run time was 16 min. The injection volume was 1.0 µL [8].

Gas Chromatography - Mass Spectrometry (GC-MS)

GC-MS analysis was performed using a Shimadzu GC-2010 system (Shimadzu Corporation, Kyoto, Japan) coupled to a quadruple mass spectrometer, MS-QP2010 Plus. The capillary column used was Rtx-5 MS (30 m × 0.25 mm i.d.; 0.25 µm film thickness), with helium as carrier gas at a constant flow of 1.0 mL min^{-1}, and an electron ionization voltage of 70 eV. The instrumental conditions were the same as for the GC-FID analysis [8]. The scanning range of the mass to charge ratio was 35 – 400 m/z.

Compound Identification

Identification of the mangrove vinegar's volatile components was based on the comparison of their mass spectra with those in the NIST 08 database and confirmed by comparison of their retention indexes (RIs) with published values. RIs were calculated using the retention data of a solution of linear alkanes (C_7–C_{30}) in hexane, and the formula is provided below. Data were analysed as a mean of three replicates (n = 3).

$$RI = 100(n) + 100\,(m - n)\left(\frac{tri - trn}{trm - trn}\right)$$

Where

- RI: retention time of compound
- i: constituent that is being analysed
- n: carbon number of the alkane which elutes before 'i'
- m: number of carbons of the alkane which elutes after 'i'
- tri: retention time of 'i'
- trn: retention time of the alkane which elutes before 'i'
- trm: retention time of the alkane which elutes after 'i'

Phytochemical Screening Tests

Tannins
A few drops of 5 % ferric chloride were added to 2.0 mL of the aqueous extract. A dark green colour appeared, indicating the presence of tannins [13].

Glycosides
Liebermann's test. The aqueous extract was added to 2.0 mL acetic acid and 2.0 mL chloroform. The mixture was cooled and a few drops of concentrated sulphuric acid (H_2SO_4, 12 M) was added. The presence of aglycone, a steroidal part of glycoside, caused the mixture to turn green [13].

Salkowski's test. The aqueous extract was mixed with concentrated H_2SO_4 and the presence of steroidal glycosides was confirmed by the formation of a reddish brown colour [14].

Steroids
3.0 mL of the aqueous extract was mixed with 2.0 mL chloroform and a few drops of 12 M H_2SO_4. The denser chloroform layer turned red, indicating the presence of steroids [13].

Saponins
2.0 mL of distilled water was added to 2.0 mL of the crude extract (1:1). The mixture was shaken vigorously until the formation of a stable froth that indicated the presence of saponins [15].

Alkaloids
2.0 mL of the crude extract was added to 2.0 mL of 1 % HCl and the mixture was heated in a water bath for 20 mins and then cooled before being submitted to Dragendorff's test. A few drops of Dragendorff's reagent were added to the mixture and the presence of alkaloids could be observed by the formation of a reddish-brown precipitate [16].

Coumarins
2.0 mL of the aqueous extract and 3.0 mL of 10 % NaOH were mixed. The presence of coumarins were detected by the formation of a yellow mixture [16].

Flavonoids
An alkaline reagent test was conducted for this screening. A deep yellow colour was formed after 3.0 mL of the aqueous extract was added to 1.0 mL of 10% NaOH solution, confirming the presence of flavonoids [14].

Cardiac glycosides
Keller-Killani test. One drop of $FeCl_3$ was added to 2.0 mL glacial acetic acid and mixed with 2.0 mL of the aqueous extract. The presence of cardiac glycosides could be determined by the formation of a brown ring or two layers of greenish brown and deep brown violet [17].

Antioxidant Activity

Ferric reducing antioxidant power assay (FRAP)
The ferric reducing power of the sample was determined by the reduction of Fe^{3+} ions to Fe^{2+}. The antioxidant activity of the MV extract was determined with slight modifications [18]. 1.0 mL of aqueous extract or ascorbic acid standard solution (20, 40, 60, 80, 100 ppm) was added to a 0.2 M phosphate buffer (0.2 M sodium dihydrogen phosphate and 0.2 M disodium hydrogen phosphate) with pH 6.6 and 2.5 mL 1% (w/v) potassium ferricyanide. The mixture was incubated for 20 mins at 50 °C in a water bath. Next, 2.5 mL of 10 % trichloro-acetic acid was added to the mixture and centrifuged for 10 min at 3000 rpm. After that, 2.5 mL of the upper layer of the mixture was mixed with 2.5 mL distilled water (1:1). 0.5 ml of 0.1 % ferric chloride solution was added and the mixture was left for 10 mins at room temperature before measuring the absorbance at 700 nm using a UV-Vis spectrophotometer.

Total Phenolic Content (TPC)

The total phenolic content of MV was determined by the Folin-Ciocalteu method with a gallic acid (GA) standard curve [19]. 0.5 mL (1:10, m/v) of the GA standard or crude extract was mixed with 5 mL diluted Folin Ciocalteu reagent (1:10, v/v) and 4.0 mL aqueous Na_2CO_3, 1.0 M. The GA standard curve was prepared by analysing concentrations of 20, 40, 60, 80 and 100 ppm. The mixture was kept in the dark for 2 hours, and then the absorbance was measured with a UV-Vis spectrophotometer at 765 nm. The total phenolic content was expressed as GA equivalents, GAE (mg g^{-1}).

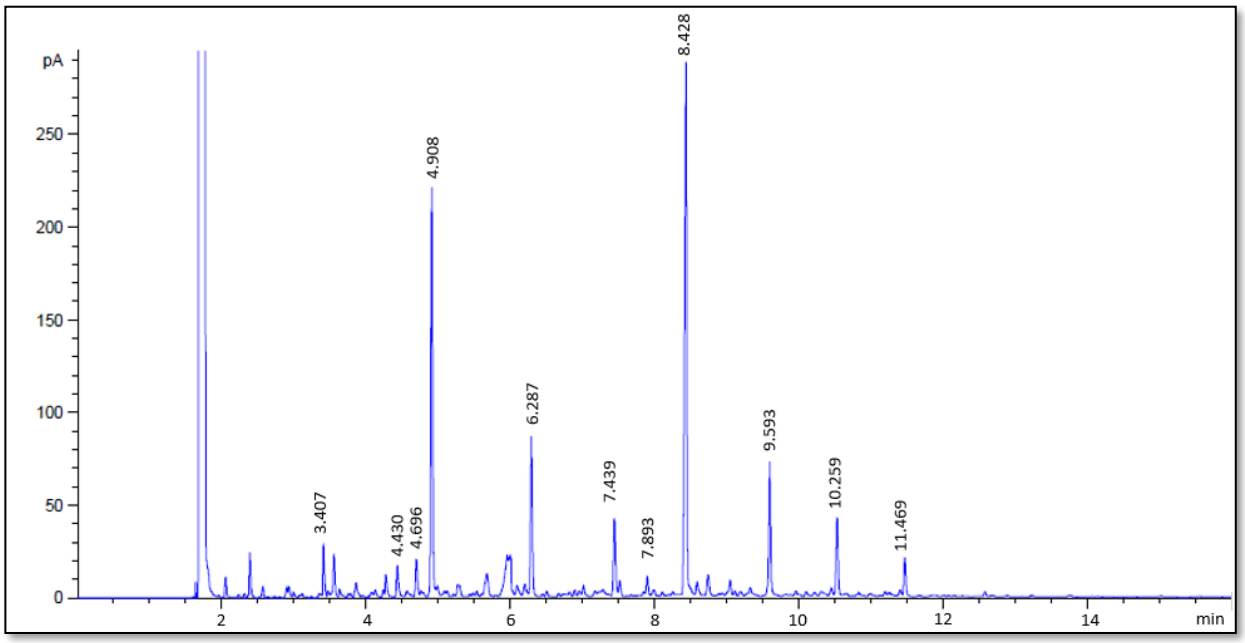

Figure 2. GC-FID chromatogram of the MV extract.

RESULTS AND DISCUSSION

The percentage yield of the extract was 0.011 % w/w (kg L^{-1}) and its chemical composition is listed in Table 1. GC-FID and GC-MS analyses successfully detected the presence of eleven compounds comprising 79.56 % of the extract (Figure 2). The main constituents were phenolic compounds such as 2,6-dimethoxyphenol (28.80 %), guaiacol (18.14 %), *p*-cresol (7.82 %) and 1,2,4-trimethoxybenzene (7.00 %) (Figure 2). Other constituents included 4-ethylguaiacol (4.95 v%), 3,4,5-trimethoxytoluene (3.99 v%) and 5-methylfurfural (2.38 v%). There were four compounds detected below 2 %, namely *m*-cresol (1.95 %), 4-nitro-2,6-xylenol (1.93 %), *o*-cresol (1.69 %) and 1,2,3-trimethoxybenzene (0.92 %).

2,6-dimethoxyphenol or syringol was found to be the major compound at 28.80%, and it is responsible for the MV's smoky odour. A previous study of the wood vinegar of *R. apiculata* also reported that this constituent was the primary compound detected [19]. Other studies reported that syringol was the primary compound of wood vinegar from other plants such as *Pinus densiflora* – 25.32% [20], *Litchi chinensis* – 29.54% [21] and *Toona sinensis* – 2.41% [22].

Table 1. Volatile constituents identified from the MV extract.

No.	Component	Rt (min)	RI[a]	RI[b]	Methods	Area (%)[c]	Ref.
1	5-Methylfurfural	3.41	973	962	MS, KI	2.38	[23]
2	*o*-Cresol	4.43	1063	1056	MS, KI	1.69	[23]
3	*m*-Cresol	4.70	1085	1077	MS, KI	1.95	[23]
4	Guaiacol	4.91	1102	1086	MS, KI	18.14	[23]
5	*p*-Cresol	6.29	1205	1190	MS, KI	7.82	[23]
6	4-Ethylguaiacol	7.44	1291	1279	MS, KI	4.95	[24]
7	1,2,3-Trimethoxybenzene	7.90	1326	1315	MS, KI	0.92	[25]
8	2,6-Dimethoxyphenol	8.43	1368	1348	MS, KI	28.80	[23]
9	1,2,4-Trimethoxybenzene	9.60	1463	1384	MS	7.00	[26]
10	3,4,5-Trimethoxytoluene	10.53	1542	1405	MS	3.99	[27]
11	4-Nitro-2,6-xylenol	11.47	1626	1694	MS, KI	1.93	[28]
	Identified components (%)					**79.56**	

Note:
[a]Retention indices (RI[a]) were obtained by calculation
[b]Retention indices (RI[b]) were obtained from the NIST 08 database.
[c]Percentage of total FID area obtained using the HP-5 column.

Table 2. Phytochemical screening tests performed on the MV extract.

No.	Compounds	Result
1.	Tannins	-
2.	Glycosides	
	• Liebernmann's test	-
	• Salkowski's test	+
3.	Steroids	-
4.	Saponins	+
5.	Alkaloids (Dragendorff's reagent)	-
6.	Coumarins	+
7.	Flavonoids (Alkaline reagent)	+
8.	Cardiac glycosides	-

Note: (+) = present; (–) = absent

The results of the phytochemical screening tests on the MV extract are presented in Table 2. It can be observed that the MV extract showed the presence of a variety of phytochemicals such as glycosides, saponins, coumarins and flavonoids. The presence of glycosides in the extract was due to syringol, *o*-, *m*-, *p*-cresol and guaiacol, volatile phenols resulting from exposure to the smoke from charcoal production [29]. The flavonoids in the extract also influenced the phenolic content of mangrove vinegar [30]. The ethanol and methanol extracts of mangrove leaves have alsobeen reported to be rich in tannins, saponins, glycosides, flavonoids, phenols and volatile oils, making them excellent antibacterial agents [5].

The antioxidant activity of the extract was measured using a ferric reducing power assay (FRAP) and the total phenolic content was determined using the Folin-Ciocalteu method. Figure 3 below shows the reducing power of the MV extract and the ascorbic acid standard against concentration (mg mL^{-1}). The reduction of Fe^{3+} to Fe^{2+} was observed by the series of colour changes in the assay solution, from yellow to different shades of green or blue [31].

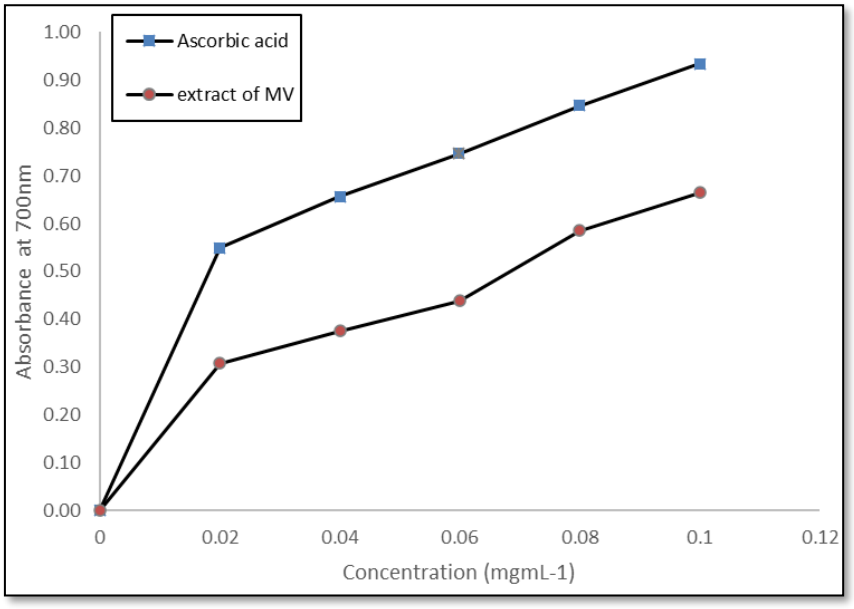

Figure 3. Antioxidant activity of ascorbic acid and the *n-hexane* extract of MV.

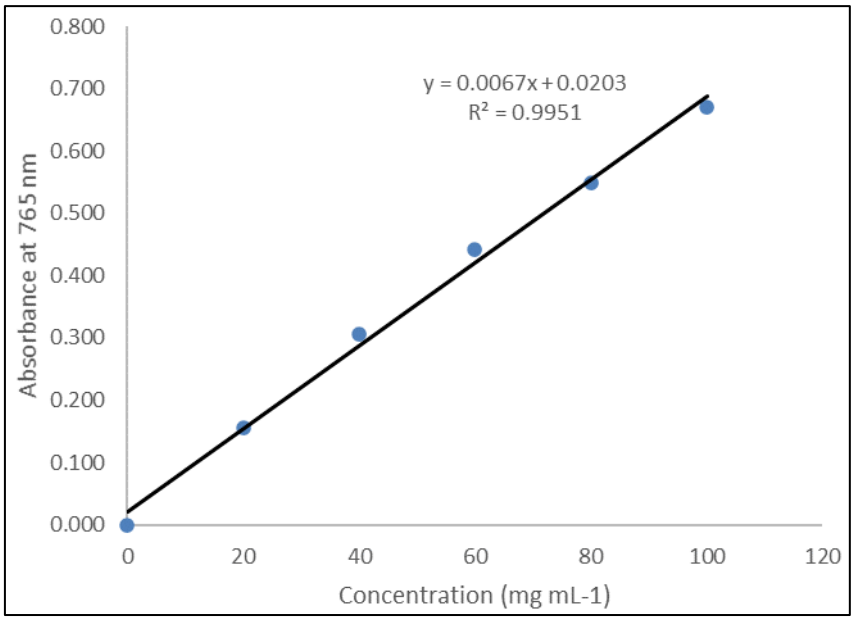

Figure 4. Gallic acid calibration curve

The reducing power of MV was compared to the ascorbic acid standard curve. The higher absorbance values at 700 nm indicate higher reducing power, and the absorbance values of both the ascorbic acid standard and the MV extract increased with concentration. Hydrogen-donating ability influences the reducing power of certain chemical compounds [31]. The presence of volatile phenols (syringol, *o*-, *m*-, *p*-cresol) from the extract may be the cause of its reducing power. At 0.1 mg mL^{-1}, the reducing power of MV was (A_{700} = 0.664), while that of ascorbic acid was (A_{700} = 0.934). In addition, at 0.02 mg mL^{-1}, the reducing power of MV was (A_{700} = 0.308), while that of ascorbic acid was (A_{700} = 0.549). Thus, the MV possessed a lower reducing power than the ascorbic acid standard. These findings contrasted with a previous study, where the reducing power of concentrated mangrove vinegar was found to be higher than ascorbic acid [8]. However, the previous study was conducted on a dichloromethane extract, while our study focussed on the non-polar *n*-hexane extract of mangrove vinegar. In conclusion, the polarity of the solvent used for the extraction significantly affects the antioxidant activity.

The total phenolic content of MV was determined by the Folic-Ciocalteu (F-C) method where GA served as the standard [32]. A calibration curve of the GA standard was constructed (Figure 4). The principle of the F-C method was that the solution turned blue with the reduction of the alkaline medium from phenolic compounds to a phosphotungstic acid complex, $(PMoW_{11}O_{40})^{-4}$ [33]. The absorbance was determined at 765 nm. The regression equation of the calibration curve was obtained (y = 0.0067x + 0.00203; R^2 = 0.9951) and expressed in mg gallic acid equivalents (GAE) per gram sample (mg g^{-1})**.** The total phenolic content of the MV extract was 162.07 mg g^{-1} with two times dilution and this value was fit to the calibration curve, indicating the presence of phenolic compounds in the extract. It was highly influenced by the solvent used for the extraction [13]. The low polarity of the *n*-hexane extract exhibited a low phenolic content compared to the more polar methanol crude extract [35]. In an earlier study on mangrove leaves, a higher level of polyphenols was found in the methanol crude extract in contrast to the water and *n*-hexane crude extracts [34].

CONCLUSION

In conclusion, the *n*-hexane extract of *R. apiculata* was found to contain eleven volatile compounds, with 2,6-dimethoxyphenol (28.80 %), guaiacol (18.14 %), *p*-cresol (7.82 %) and 1,2,4 trimethoxybenzene (7.00 %) as the major constituents. The presence of active compounds in the *n*-hexane extract was limited, and the MV components generally displayed antioxidant properties with a lower potential than the ascorbic acid standard. Additionally, the total phenolic content was 162.07 ± 5.55 mg g^{-1}, expressed as mg gallic acid equivalents (GAE).

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