Malaysian Applied Biology (2023) 52(4): 153-160
https://doi.org/10.55230/mabjournal.v52i4.d180

Research

A Study on an Active Functional Group and Antimicrobial Properties From *Rhizophora apiculata* Extracts Used in Traditional Malay as Medicine

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ABSTRACT

A mangrove plant known as *Rhizophora apiculata* is employed by Malay for treating skin diseases, diarrhea, vomiting, and nausea, as an antiseptic, for tanning, and also as fuelwood and fodder. Its large-scale use can be attributed to its high-quality timber, availability as well and the presence of a chemical named tannin that is employed for reinforcing fishing lines, nets, and ropes. The tannin content of *R. apiculata*'s roots, bark, and leaves is regarded to be a natural inhibitor of fungal infections. This study is focused on determining the different kinds of functional groups, as well as individual phenolic compounds present in *R. apiculata* for identifying new bioactive compounds via decoding of the traditional values of Malay remedies. There is a high demand for such natural bioactive compounds, particularly in the healthcare and pharmaceutical markets. Alkaline fractional extracts were employed to design an analytical extraction method for *R. apiculata*. As per the HPLC results, there were three phenolic acids detected namely Caffeic acid, 4-Hydroxybenzoic acid, and Vanillic acid. Meanwhile, ten volatile compounds were identified by the GCTOF-MS. With regards to antibacterial activity, *S. aureus*, *S. epidermidis*, and *E. coli* were inhibited by *R. apiculata* leaf extract, while *C. albicans* and *Fusarium* sp. were inhibited by their antifungal activity.

Key words: Bioactive ingredients, ethnoscience, GCTOF-MS, HPLC, phenolic compound, *Rhizophora apiculata*

Article History
Accepted: 24 July 2023
First version online: 31 October 2023

Cite This Article:
Ramya, R., Kamoona, S., Mohd Hatta, F.A., Wan Sulaiman., W.S.H., Mohd Latiff, N.H., Othman, R. 2023. A study on an active functional group and antimicrobial properties from *Rhizophora apiculata* extracts used in traditional Malay as medicine. Malaysian Applied Biology, 52(4): 153-160. https://doi.org/10.55230/mabjournal.v52i4.d180

INTRODUCTION

For hundreds of years, the Asia-Pacific (APAC) communities living in coastal, estuarine, and riverine have been dependent on coastal resources, like the mangrove swamp ecosystem. Malaysian mangroves are the third largest when it comes to mangrove areas in the Asia-Pacific Countries, after Indonesia and Australia (Azian *et al*., 2014). In Malaysia, the mangrove area dominates Sabah (60%), followed by Sarawak (22%) and Peninsular Malaysia (18%) (Hamdan & Muhamad Afizzul, 2020). The mangrove habitats are seen to range from shallow coastal areas to deltas, estuaries, and lagoons, with differing tides, substrates, and water salinity. Even if these conditions are limited to the local boundary, they would differ about adaptations and formations (Muta Harah & Japar Sidik, 2020). Global climate changes like sea level rise in precipitation, tempest, and increase in temperature largely impact mangrove survival (Hamdan, 2020). Should deforestation of 1 ha of mangroves occur, it would result in the discharging of 112 - 392 Mg ha^{-1} of carbon (Donato *et al*., 2011). In the coastal area, the rapid expansion of aquaculture ponds is regarded to be the key reason for mangrove loss. This is comparable to the emission of almost 5 ha of tropical forest as well as 11.5 ha of tropical dry forests when 1 ha of mangrove land is cleared for aquaculture ponds (Kauffman

et al., 2014).

Rhizophora apiculata belongs to the family Rhizophoraceae, also called red mangroves or 'bakau minyak' by the Malay community, and it includes nearly 16 genera and 120 species, including 7 genera and 17 species in Peninsular Malaysia (Noraini *et al*., 2017). Rhizophoraceae includes *Bruguiera* sp. (tumu, berus dan lenggadai), *Ceriops* sp. (tengar), *Rhizophora* sp. (bakau) and *Kandelia* (berus-berus). Wan Juliana *et al*. (2014) reported *R. apiculata* to be the most predominant species amongst Rhizophoraceae in Peninsular Malaysia, followed by *R. mucronata,* which are normally distributed in Perlis, Kedah, Perak, Melaka, Negeri Sembilan, Terengganu, Johor, Selangor, and Kelantan. *Rhizophora apiculata*'s intertidal wetland communities could also reach 30 - 40 m in terms of height with stem diameters reaching 15 - 35 cm just beyond the prop root that is the highest. In the southern hemisphere, *R. apiculata*'s flowering season generally occurs between August to December and in the northern hemisphere, it is between February to June (Duke, 2006). The stilt roots of this mangrove perhaps play a key environmental role in protecting the coastal areas, promoting marine food chains, enhancing water quality, and also in helping to maintain a balanced ecosystem (Bandaranayake, 2002; Othman *et al*., 2015).

Traditionally, charcoal, firewood, housing, tannins for dyeing and leather production, boats, furniture, fishing gear, medicine, food, cosmetics, and many other traditional artifacts and products primarily employ mangroves. Usually, the traditional knowledge about plant usage can be inherited based on verbal exchanges and experiences (Styawan *et al*., 2016). It was found that the community for medicinal, dye, timber, aromatic, food, and craft were the predominant users of the Rhizophoraceae family. Amongst mangrove parts, the most commonly used parts were leaves (38%) and fruits (26%), and then the stems, roots, seeds, flowers, barks, and finally tubers (Muflihati *et al*., 2018). On top of that, traditional medicine plays a key role in indigenous human health systems, particularly in developing countries where there is limited access to professionals and allopathic medicines (Nabatanzi *et al*., 2020). Globally, 80% of the population heavily relies on plants from forests as a medicinal source for their health (Dossou-Yovo *et al*., 2017). *Rhizophora apiculata* can also treat diarrhea, vomiting, nausea, hepatitis, and typhoid as well and it can be employed as an insecticide and antiseptic (Kumar *et al*., 2011; Pullaiah *et al*., 2016). However, these assertions have been verified by a handful of controlled experiments. Also, as per various studies conducted by Bandaranayake (2002), Abdul Halim *et al*. (2013), Ravikumar *et al*. (2010) and Kumar *et al*. (2011), *R. apiculata* includes secondary products, such as glycosides, alkaloids, aliphatic alcohols aldehydes, essential oils, phenolic compounds, condensed and hydrolyzable tannins, carboxylic acids, benzoquinones, n-alkanes, carotenoids, lipids, minerals, terpenoids, polysaccharides, steroids, triterpenes as well as tannins for the treatment of both non-infectious and infectious diseases by many communities in Africa and Asia.

Besides, various techniques are available to consume the leaves of the plants for medicinal purposes, including boiling in water (*Melastoma candidum* & *Acanthus* sp.), and grinding and rubbing for external use (*Pluchea indica* & *Acrostichum* sp.), while the fruits could be squeezed for drinking or eaten directly (*Morinda citrifolia* & *Acanthus ilicifolis*) (Muflihati *et al*., 2018). As per Bandaranayake (2002), mangrove leaves are rich sources of vitamins, minerals, and amino acids, which are crucial for livestock growth and marine life. It is thus important to know about the chemical constituents in these plants, which would also help to discover new therapeutic agents, especially for those keen on 'decoding' the value of folklore remedies.

Thus, this study is focused on determining the different kinds of functional groups as well as individual phenolic compounds in *R. apiculata* (bakau minyak) to discover new bioactive compounds based on the traditional medicinal values of Malaysia. We have assessed the phenolic compositions, i.e., total phenolic and phenolic acids contents, to identify the relationships that exist between inhibition of antimicrobial activities and phenolic composition profiles. Interestingly, this research study is also aimed at exploring how the polarity of solvents employed for extraction could cast an impact on the phenolic acids content based on four solvents, in ascending order with regards to the polarity.

MATERIALS AND METHODS

Plant material and sample preparation

The leaves of *Rhizophora apiculata* (bakau minyak) were collected from a mangrove forest in Bagan Lalang, Selangor, Malaysia. After cleaning the leaves, they were freeze-dried before grinding into fine powder and then stored at –20 °C for further analysis.

Plant alkaline extraction

To prepare alkaline extraction, in 100 mL NaOH (2 M) solution, soaking of 10 g of freeze-dried powdered material was done. Heat was applied to the sample at a temperature of 60 °C for 12 h in an oven, after that, it was cooled to a temperature of 20 °C. Then, hydrochloric acid (HCl) was used to treat the alkaline extract to reach pH 2 so that precipitation of hemicellulose would occur. Then, the final leaves extracts were re-extracted with hexane, butanol, ethyl acetate, and ethanol extracts as described by Bertin *et al*. (2003) with some minor adjustments by employing a funnel separator. Next,

the extracts were evaporated to dryness by using a rotary evaporator at a temperature of 45 °C. The crude extract of *R. apiculata* was then resuspended by using 5 mL of methanol and then kept at –25 °C for subsequent analysis.

Determination of volatile compounds by Gas Chromatography Time-of-Flight Mass Spectrometry (GCTOF-MS)

The leaves extract from *R. apiculata* was qualitatively performed by GCTOF-MS (Agilent 7890 system) equipped with a capillary column (30 m × 0.25 mm, 0.25 µm) based on the method reported by Portóles *et al*. (2009) with some modification. Split-less injection of 1 µL sample was performed with a purge time of 1.0 min. The solvent delay was set at 4 min. The carrier gas used was helium at the flow rate of 1.0 mL min^{-1}. The column temperature was initially maintained at 80 °C for 2 min, then programmed at 5°C min^{-1} to 80 °C min^{-1} and then at 10 °C min^{-1} to 250 °C. The inlet temperature and detector sets were 220 °C and 340 °C respectively. The time-of-flight mass spectrometer was operated at 1 spectrum/s acquiring the mass range m/z 50-1000. The identification of the peaks was based on mass spectra based on >90% similarity index with the National Institute of Standards and Technology library (NIST 14) and by comparison with published data.

Determination of Total Phenolic Content (TPC)

The Folin-Ciocalteau assay procedure was employed to determine the overall phenolic presence (Singleton & Rossi, 1965). 20% v/v deionized water was used to prepare a solution comprising 90 µL Folin-Ciocalteu reagent. The sample was divided into wells using a flat-bottom microplate comprising 96 wells. Subsequently, 1.0 mg/g DW of distilled water diluted (1000 µg/mL) specimen was subjected to incubation at room temperature for five minutes. Further, 7.5% w/v deionized water was used to dilute 90 µL sodium carbonate; this solution was subjected to incubation at room temperature for 2 h. The TECAN microplate reader determined extract absorption and standards set at λmax = 725 nm corresponding to a blank.

Quantification of phenolic acids content with High-Performance Liquid Chromatography (HPLC)

LC rapid resolution apparatus Agilent 1200 series (Agilent Technologies, Palo Alto, CA, USA) was used for phenolic acid HPLC assessment. The setup had a specimen auto-injection system-based binary pump, thermostat-controlled column area, micro vacuum degassing chambers, and a diode array detector (DAD); this setup was adapted from Zhao *et al*. (2008) with specific changes. The column used was a Zorbax SB-C18 column (Eclipse 100 × 2.1 mm, 1.8 µm) with a diode array detector. For the analysis, a linear gradient elution was used, with the two mobile phases consisting of 1% formic acid in water/ acetonitrile 90:10 v/v (phase A) and acetonitrile (phase B) using the following gradient: 0 - 20 min, linear gradient from 0% B to 40% B; 20 - 25 min, linear gradient from 40% B to 60% B; 25.10 - 35 min, linear gradient from 100% B to 100% B and 35.10 - 40 min, isocratic of 0% B. The temperature of the column was set at 25 °C. The injection volume was 20 µL, and the flow rate was set at 0.4 mL/min. Phenolic acids standards; Caffeic acid, Ferulic acid, *trans-p*-Coumaric acid, 2-Coumaric acid, 3-Coumaric acid, 4-Hydroxybenzoic acid, and Vanillic acid were purchased from Sigma-Aldrich.

Determination of antibacterial activity assay

Rhizophora apiculata activity against bacteria was assessed based on five gram-negative bacterial strains *Staphylococcus epidermidis, Staphylococcus aureus, Pseudomonas aeruginosa, Methicillin-resistant Staphylococcus aureus* (MRSA), and *Escherichia coli*. The Microbiology Lab at the International Islamic University, Malaysia provided the test strains. Muller Hinton (MH) and broth medium were mixed to act as a nutrient source for inoculum preparation to provide bacterial growth. The agar well diffusion technique was employed to delicately swipe the strains on the MH agar medium (Biruhalem *et al*., 2011). Extracts demonstrating growth inhibition areas of more than 7 mm were selected, and the minimum inhibitory concentration (MIC) was determined. These zones were incubated at 37 °C for 24 h, and the MIC was evaluated again. The lowest concentration restricting growth was recorded as the MIC value for the sample.

Determination of antifungal activity assay

The antifungal activity of *R. apiculata* was evaluated using the test pathogens (*Candida albicans*, *Fusarium* sp., *Microsporum gypseum*, *Phanerochaete chrysosporium,* and *Aspergillus niger*) were swabbed in Potato Dextrose Agar (PDA) plates and all clinical isolates were obtained from the Microbiology Laboratory, International Islamic University Malaysia.

Statistical analysis

Analysis was based on mean ± standard deviation values concerning phenolic acid extract concentration conducted in triplicate. One-way variance assessment (ANOVA) and Turkey's validation were performed using XLSTAT-Pro (2014) software (Addinsoft, Paris, France). The tests indicated that the mean differences were significant at 99% confidence levels (p<0.0001).

RESULTS

Analysis of volatile compounds of *R. apiculata*

Rhizophora apiculata leaves were assessed using GCTOF-MS analysis, which indicated ten constituent substances from distinct chemical groups. The mass values and formulae were obtained using data from the National Institute Standard and Technology (NIST) library. Table 1 lists a volatile substance detected in hexane, ethyl acetate, and butanol extracts. The outcomes were 90% or more similar to data obtained from the NIST library. However, none of the volatile compounds was detected in the ethanol extract.

Table 1. Volatile compounds identified in different solvents of sequential alkaline extract of *R. apiculata* leaves

Solvents	No	Volatile compounds	Exact mass/ Formula
Hexane	1	Hexadecane	226.448 / $C_{16}H_{34}$
	2	2,4-Di-tert-butylphenol	206.329 / $C_{14}H_{22}O$
	3	Eicosane	282.556 / $C_{20}H_{42}$
	4	Pentadecane	212.421 / $C_{15}H_{32}$
	5	Tridecane	184.367 / $C_{13}H_{28}$
Ethyl acetate	1	2,4-Di-tert-butylphenol	206.329 / $C_{14}H_{22}O$
Butanol	1	Isobutylamine	73.139 / $C_4H_{11}N$
	2	15-Methylhexadecanoic acid methyl ester	284.484 / $C_{18}H_{36}O_2$
	3	2,4-Di-tert-butylphenol	206.329 / $C_{14}H_{22}O$
	4	4-[1,3]Dioxolan-2-yl-3,4-dimethyl-cyclohex-2-enone	196.246 / $C_{11}H_{16}O_3$

Analysis of total phenolic and phenolic acids content of *R. apiculata*

Rhizophora apiculata extracts were treated with Folin-Ciocalteu reagent, and the overall phenolic presence was described in the form of gallic acid equivalent. Overall, the phenolic concentration was 977.90 ± 2.13 µg GAE/g DW. *R. apiculata* plant extracts comprise phenolic acids with varying polarity depending on the extraction solvent. In this research, the phenolic acids were expressed in µg/g DW. The concentration of the phenolic acids in plant extracts from *R. apiculata* was ranged from 0.23-13.13 µg/g DW (Figure 1). Caffeic acid, Vanillic acid, and 4-Hydroxybenzoic acid were the only phenolic acids detected in all extracted samples. Phenolic acid concentration showed decreases in this order: ethanol, butanol, ethyl acetate, and hexane.

Analysis of antimicrobial activity of *R. apiculata*

Rhizophora apiculata leaves have been confirmed as a rich source of phenolic compounds, but only a few reports on the antimicrobial activity of *R. apiculata* leaves or the comparison with the efficiency of fractional extracts (hexane, ethyl acetate & butanol). The leaves were assessed for antimicrobial properties through several fractional extracts tested using agar and the diffusion technique. Antimicrobial properties were detected against *S. epidermis, E. coli,* and *S. aureus*. Nevertheless, there were no antimicrobial characteristics against *P. aeruginosa* and MRSA (Table 3). Further, fractional extracts from *R. apiculata* leaves demonstrated antifungal activity against *Fusarium* sp. and *C. albicans*; however, *A. niger, P. chrysosporium,* and *M. gypseum* were unaffected (Table 2).

Plants are significant food and medicine sources. Recent data suggests that over 45% of all medicines released between 1981 and 2019 comprise natural substances or synthetic analogs (Newman & Cragg, 2012). Recent studies indicate that phenolics can be used for enhancing food safety, stability, and quality (Katalinic *et al*., 2013). Plants with medicinal effects are vital for human health. Local communities exchange such products primarily for their therapeutic properties rather than economic value (Cahyaningsih *et al*., 2021). This research work demonstrated the quantitative and qualitative aspects of phenolic substances present in *R. apiculata*. HPLC assessment indicated that caffeic acid (0.23 – 2.09 µg/g DW), vanillic acid (1.88 – 8.40 µg/g DW), and 4-Hydroxybenzoic acid (13.13 µg/g DW) were present in all extracts. Kumar *et al*. (2011) asserted the need to fractionally extract substances using sequential solvents and column chromatography to isolate an active compound possessing therapeutic properties for phytomedicine. Also, phenols are highly soluble in polar solvents; hence, such solvents can help obtain extract with significant concentrations (Stankovi, 2011). The

present study re-extracted *R. apiculata* constituent compounds using butanol, ethanol, hexane, and ethyl acetate. Observations indicate that different solvent categories lead to distinct phenolic extracts. As reported by Karim *et al*. (2020), The level of solvent polarity determines the chemical structures of total phenolic compounds. Therefore, it is vital to use column chromatography to isolate a concentrated form of one phenolic acid. Karim *et al*. (2020) studied phytochemical assessment techniques; they presented a minor (25 mM) or moderate (50 mM) antioxidant concentration increase for flavonoids and phenolic compounds. Nevertheless, extreme salinity levels, i.e., 75 and 100 mM, impacted extract concentrations. Hence, it may be understood that saline characteristics of mangrove soils affect plant physiology significantly, thereby affecting biosynthesis. Kostić *et al*. (2012) specified a 0.23 - 2.85 mg GAE g^{-1} phenol concentration range for therapeutic plants. Additionally, nutritive species have a concentration range of 0.26 and 17.51 mg GAE g^{-1}. *R. apiculata* plant extracts indicated 0.978 mg GAE/g DW levels. However, interfering substances like aromatic amines, sugar, vitamin C, organic acids, iron (II), sulfur dioxides, and other non-polyphenolic compounds might cause interference, and inadequate representation of overall polyphene compounds detected using the Folin-Ciocalteu technique (Kostić *et al*., 2012).

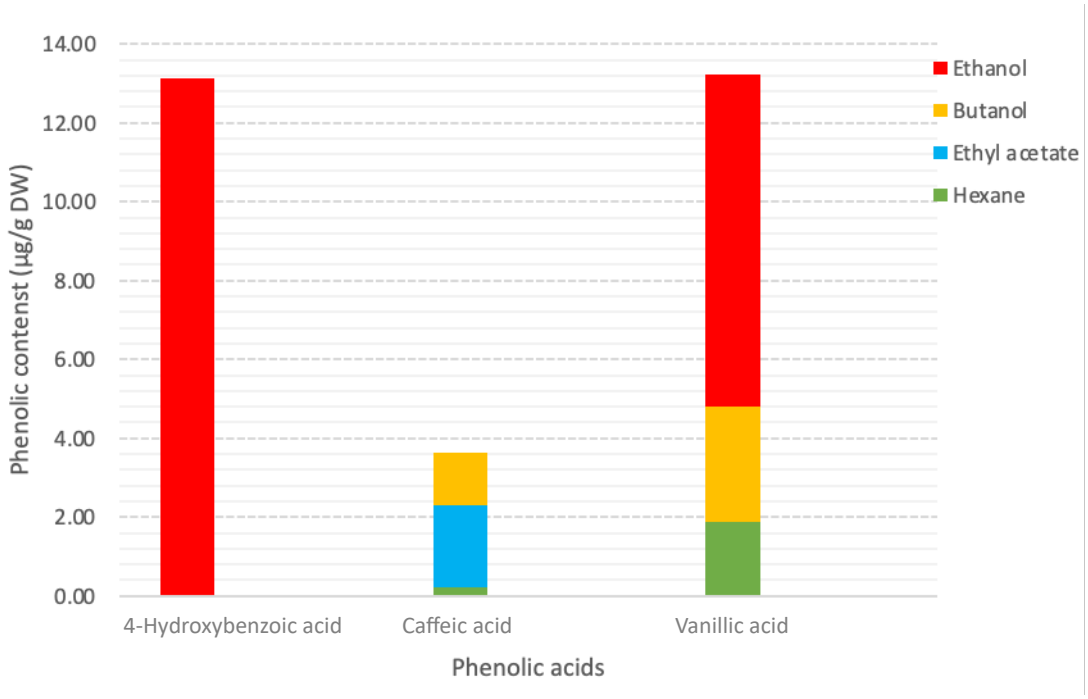

Fig. 1. Phenolic acids content of *R. apiculata* by different extracts (ethanol, butanol, ethyl acetate & hexane).

Table 2. Antimicrobial activities from *R. apiculata* extract

	Plant extracts	Hexane	Ethyl acetate	Butanol
Antibacterial	*S. aureus*	~	+	~
	S. epidermidis	-	~	~
	E. coli	-	~	~
	Methicillin-resistant Staphylococcus aureus (MRSA)	-	-	-
	P. aeruginosa	-	-	-
Antifungal	*C. albicans*	~	+	+
	Fusarium sp.	-	-	~
	M. gypseum	-	-	-
	P. chrysosporium	-	-	-
	A. niger	-	-	-

[#] Note as referred to Rauha *et al*. (2000):

a) - : No antimicrobial activity, inhibition zone of sample < inhibition zone of ethanol +1 mm

b) ~ : Slight antimicrobial activity, inhibition zone of sample 1 - 3 mm > inhibition zone of ethanol

c) + : Moderate antimicrobial activity, inhibition zone of sample 3 - 4 mm > inhibition zone of methanol

d) ++ : Clear antimicrobial activity, inhibition zone of sample 4 - 10 mm > inhibition zone of ethanol

e) +++ : Strong antimicrobial activity, inhibition zone of sample +10 mm > inhibition zone of ethanol

DISCUSSIONS

Recent research indicates that mangrove and mangrove-dependent-plant extracts demonstrate antipathogenic activity against human and animal pathogens; however, research concerning bioactive metabolite identification concerning bioactivities is limited. Mangroves have distinct morphological or physiological systems such as photosynthetic pathways, glucose metabolism, and polyphenol synthesis to survive in adverse settings caused by biotic or abiotic influences (Resmi *et al*., 2021). Phenolics, terpenoids, alkaloids, and steroids undergo secondary metabolism, having significant ecological, toxicological, and pharmacological characteristics (Bandaranayake, 2002). *Cynometra ramiflora, Kandelia candel, A. marima, Amoora cuculata, Sonneratia griffithii, Lumnitzera racemosa* and *R. apiculata* are examples of mangroves with antihyperglycemic properties (Tiwari *et al*., 2008). Medicinal plants derive their therapeutic aspects from flavonoids, phenolics, alkaloids, and tannins. Flavonoids and phenols exert a preventive influence on cancer and cardiac ailments (Karim *et al*., 2020). As a result of the medicinal properties of these secondary metabolites, mangrove plants are used in traditional medicine.

Polyphenols' hydroxyl group is important for scavenging free radicals, allowing for quick testing of antioxidant efficacy (Karim *et al*., 2020). Mangrove tannins possess noteworthy inhibitory characteristics because of the presence of hydroxyl groups (Tan & Kassim, 2011). Tannins facilitate the synthesis of recalcitrant protein complexes that reduce the activity of extracellular microbial enzymes (Kraus *et al*., 2003). Polyols and phenolic acids undergo esterification to produce hydrolyzable tannins (Bandaranayake, 2002). Phytochemicals and their interactions, such as the anthelmintic activity of *A. ilicifolius* aerial parts extract, may have synergistically improved therapeutic efficacy (Sardar *et al*., 2018). Due to the past success of natural goods, multinational pharmaceutical companies have invested in this traditional arena, with natural products accounting for around 60% of small-molecule authorized medications (Abdelghani *et al*., 2021).

In the antimicrobial properties context, *Avicennia* demonstrated broad-spectrum activity against *Mycobacterium fortuitum, Staphylococcus aureum, Mycobacterium vaccae, Mycobacterium aurum, Mycobacterium smegmatis* and *Candida albicans* (Kumar *et al*., 2011). A triterpene ester extracted using *H. littoralis* demonstrated potent antifungal characteristics (Bandaranayake, 2002). *Candida albicans* is the most common species to cause candidemia and disseminated candidiasis in humans (Palla *et al*., 2020). Interestingly in the present study, all fractional extracts of *R. apiculata* leaves showed a presence of antifungal activity against *Candida albicans*.

CONCLUSION

This research was conducted to identify phenolic substances and functional groups present in *R. apiculata* (bakau minyak). HPLC assessment confirmed the presence of Vanillic, Caffeic, and 4-Hydroxybenzoic acids. The phenolic substances extracted during this research demonstrated antibacterial characteristics against *S. epidermis, E. coli,* and *S. aureus*. Simultaneously, these substances have antifungal effects against *Fusarium* sp. and *C. albicans* except for ethanol extract. Further, solvent types used for the extraction process lead to varying phenolic acid concentrations specified in an increasing polarity order. However, *R. apiculata* remains to be evaluated from ethnomedicinal and traditional perspectives. Researchers have recently discovered the potential uses of *R. apiculata*. Extensive effort is required to gather and distribute information to the pharmaceutical sector to identify novel therapeutic agents.

ACKNOWLEDGEMENTS

The authors are grateful to the Geran Galakan Penyelidik Muda (GGPM), Universiti Kebangsaan Malaysia (UKM), grant number GGPM-2022-061, Ministry of Education (MOE) and the International Islamic University Malaysia (IIUM) for the Research Initiative Grant Scheme SRCG 20-031-0031.

ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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