ผลของตัวทำละลายในการวิเคราะห์พฤกษเคมีและฤทธิ์ต้านเชื้อแบคทีเรียของสารสกัดจากใบและเปลือกของโกงกางใบเล็ก

Effect of Solvents on Phytochemical Analysis and Antibacterial Activity of Leaf and Bark Extracts from *Rhizophora apiculata*

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บทคัดย่อ

โกงกางใบเล็ก (*Rhizophora apiculate*) เป็นพืชป่าชายเลนในวงศ์ Rhizophoraceae การศึกษาครั้งนี้นำส่วนใบและเปลือกของโกงกางใบเล็กมาสกัดด้วยตัวทำละลายตามลำดับขั้วคือ เฮกเซน เอทิลอะซีเตทและเมทานอลศึกษาฤทธิ์ต้านแบคทีเรียของสารสกัดหยาบทั้งหมดกับเชื้อสี่ชนิดคือ *Bacillus cereus* TISTR 687, *Staphylococcus aureus* TISTR 1466, *Escherichia coli* TISTR 780 และ *Samolnella typhi* TISTR 292 ด้วยวิธี disc diffusion พบว่าสารสกัดหยาบเมทานอลจากเปลือกของโกงกางใบเล็กสามารถออกฤทธิ์ต้านเชื้อแบคทีเรียได้ดีที่สุด ทดสอบพฤกษเคมีเบื้องต้นพบว่ามีสารกลุ่มเทอร์พีนอยฟลาวานอยด์ ซาโปนินแทนนินและแอนทราควิโนน ทั้งในใบและเปลือก แต่พบสารแอนทราควิโนนเฉพาะในเปลือกที่สกัดจากตัวทำละลายเมทานอลการศึกษาวิจัยนี้ชี้ให้เห็นว่าตัวทำละลายเมทานอลสามารถสกัดสารทุติยภูมิจากใบและเปลือกได้ดีที่สุด

คำสำคัญ: โกงกางใบเล็ก, ป่าชายเลน, ฤทธิ์ต้านแบคทีเรีย, สารพฤกษเคมี

ABSTRACT

Rhizophora apiculata is a traditional mangrove plant belonging to RHIZOPHORACEAE family. Leaves and bark of this plant were extracted with sequential increasing polarity of organic solvents: hexane, ethyl acetate, and methanol, respectively. All six extracted were studied on antibacterial activity with four bacterial strains (*Bacillus cereus* TISTR 687, *Staphylococcus aureus*

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TISTR 1466; *Escherichia coli* TISTR 780 and *Samolnella typhi* TISTR 292) by using disc diffusion method. It was found that the methanolic bark extracted show the best antibacterial activity. Phytochemical screening method was employed and the results revealed the presence of terpenoids, flavonoids, saponins, tannin and anthraquinone in both of leaves and bark. However, anthraquinone was only found in bark extracted with methanol. This study suggested that the best solvent which extracted secondary metabolites from leaves and bark was methanol.

Key words: *Rhizophora apicualta*, mangrove, antibacterial activity, phytochemical agents

INTRODUCTION

Mangrove forest plays role as an important natural source which founded in tropical and subtropical areas. Extracted mangrove plants contain various biological actives antiviral, antibacterial, antifungal and antioxidant compounds which provide rich sources of a wide variety of phytochemicals such as; terpenoid, flavonoid, saponin, tannin and alkaloid (Shelar *et al.*, 2012). In addition, it has been reported that mangrove leave extracts were nontoxic to human and were environmentally friendly because of less pollutant produced (Opara and Wokocha, 2008). *Rhizophora apiculata*, locally known as bakau minyak, belongs to Rhizophoraceae family which is one of the most common mangrove plants in case of containing an abundance of biologically active compounds. The crude extracts from different parts of this plant were reported to possess diverse medicinal properties such as polysaccharide from leaves showed anti- HIV activity (Premanathan *et al.*, 1999), the barks of *R. apiculata* found its property to treat anticandadal activity (Hong *et al.*, 2011), and antioxidant from bark and twig (Vijayavel *et al.*, 2006; Loo *et al.*, 2007; Loo *et al.*, 2008; Rahim *et al.*, 2008; Gao and Xiao, 2012) as well as mosquito larvicidal activity from root (Shelar *et al.*, 2012). The previous studies reported that several extracts from some organic solvents of *R. apiculata* leaves and bark provided diverse medicinal properties (Abeysinghe, 2010; Ravikumar *et al.*, 2010). Additionally, methanolic and water extracted from *R. apiculata* leaves (Shamsuddin *et al.*, 2013) and petroleum ether, ethyl acetate, ethanol, and water from *R. apiculata* bark (Sulaiman *et al.*, 2011; Lim *et al.*, 2006) significantly exhibited against a series of test bacteria, but 70% aqueous acetone bark extract have little effect against Gram-negative bacteria (Sulaiman *et al.*, 2011). However, the explanations about correlation between antibacterial activities with phytochemical property which extracted from different solvents appears in only a few studies. For further study of the additional information about *R. apiculata* leaves and bark, the screening of this plant

finding for any antibacterial activity in order to find the new source of valuable materials as well as the second metabolites with new therapeutic agents is important to work. Expanding the same research (instead of using petroleum ether, ethanol, and water extracts), the aim of this study was to evaluate the antibacterial activity of medicinal *R. apiculata* by using different solvents including hexane, ethyl acetate, and methanol extracted leaves and inner barks in order to gain the phytochemical correlation and preliminary them *in vitro* for antibacterial activity which could be used to against a wide range of pathogenic and food spoilage microorganisms. Correlation between secondary metabolite by phytochemical study and antibacterial agent will be discussed.

MATERIALS AND METHODS

Extraction of *R. apiculata'* leaves and bark

The leaves and barks of *R. apiculata* were collected during September-October 2011 from Rajamangala beach, Rajamangala University of Technology Srivijaya, Trang province which located in the southern part of Thailand. One thousand grams of green leaves and 1000 g of reddish brown inner barks from *R. apiculata* were dried at 50^{o}C and were successively extracted by sequential increasing polarity of organic solvents such as hexane, ethyl acetate, and methanol over a period of seven days at room temperature. Crude extracts were acquired by concentrating the extract under the reduced pressure by using the rotary evaporator to give the crude extracts of leaves and bark as green and pale-yellow, green and green, and green and reddish-brown from hexane, ethyl acetate and methanol, respectively.

Phytochemical analysis

Six various extracts of *R. apiculata* extracted by hexane, ethyl acetate, and methanol were subjected to the qualitative chemical analyses in order to detect the presence of anthraquinone, terpenoid, saponin, tannin, and alkaloid by the modified following procedure (Tukiran, 2013; Bhatt and Dhyani, 2012; Mouafi *et al.*, 2014):

Alkaloid

Two grams of leaf and bark extracts were shaken in 15 mL of 2% H_2SO_4 and warmed. Few drops of Dragendroff's reagent were added to each filtrate and subsequently observed for the formation of orange brown precipitate which may indicate the presence of alkaloids.

Anthraquinone

Two hundred milligrams of leaf and bark extracts were boiled with10 mL of 10% H_2SO_4 in the test tube for 5 minutes, filtered and extracted with chloroform. Then, 3% ammonia solution was added to give rose pink color which appeared in the ammonia layer indicating the presence of anthraquinone.

Flavonoid

Two hundred milligrams of leaf and bark extracts were treated with 3 mL of 95% ethanol. The solution was warmed, and fragment of magnesium metal was added to this solution.

Then 2-3 drops of concentrated hydrochloric acid was added. Cherry color would indicate the presence of flavonoids.

Saponin

Two hundred milligrams of leaf and bark extracts were treated with water in a microtube separately shaken well. Foam that appeared could indicate the presence of saponin.

Terpenoid

Two hundred milligrams of leaf and bark extracts was extracted with 3 mL of petroleum ether for three times and 2mL of chloroform was later added. Then concentrated solution of H_2SO_4 was slowly added. This test is called "Salkoski test". Appearing of brown ring at the edge joint of two layers indicated the presence of terpenoid.

Tannin

Two hundred milligrams of leaf and bark extracts were separately shaken in water and warmed. A few drop of 1% $FeCl_3$ solution was consequently added and observed for the formation of brownish green or blue black colors which may indicate the presence of tannin.

Antimicrobial activity

Two gram-positive (*Bacillus cereus* TISTR 687 and *Staphylococcus aureus* TISTR 1466) and two gram- negative (*Escherichia coli* TISTR 780 and *Salmonella typhi* TISTR 292) bacterial strains were used for antibacterial screening which were obtained from the National Center for Genetic Engineering and Biotechnology, Thailand. The *in vitro* antibacterial activities of leaves and bark from *R. apiculata* were determined by using the paper disc diffusion method which modified from NCCLS, 1993 (Villanova, 1993). For the disc diffusion assay, the bacteria which were incubated in Tryptic soy broth (TSB) at 35°C for 24 h was measured for the turbidity at 0.5 McFarland standards (10^8 CFU/ml) before swabbing over the surface of media (Tryptic soy agar, TSA) which allowed to solidify by using a sterile cotton swab. All crude extracts were dissolved in the dimethyl sulfoxide (DMSO) to give stock solutions of 100 mg mL^{-1} and were used as 10 μL of tested crude extracts on sterile filter paper discs (6 mm in diameter of Whatman No. 1 filter paper). After overnight incubation at 35°C for 24 h, the zones of inhibition were measured in the millimeter. Gentamicin and penicillin were used as the positive controls whereas DMSO was the negative controls. All experiments were repeated three times and the average values are presented.

Statistical analysis

The result were presented as the mean ± standard deviation of three independent experiments (n = 3). Statistical analyses were done by one-way ANOVA followed by the Duncan's Multiple Range Test (DMRT) with $P < 0.05$ as a limit of significant.

RESULTS AND DISCUSSIONS

In this work, three solvents (hexane, ethyl acetate, and methanol) were used to prepare *R.*

apiculata leaves and bark extracts. All crude extracts were tested for the antibacterial activity (*S. aureus*, *B. cereus*, *E. coli* and *Samonella typhi*) which showed in Table 1 and Figure 1 by the paper disc diffusion method. The results showed that almostcrude extracts were active against *B. cereus*, *S. aureus*, and *Samonella typhi.* For *B. cereus* strain, all leaf extract showed diameter inhibition zone in 7.81-9.38 mm range which found the highest in hexane extract (9.38 mm). Meanwhile, *S. aureus* and *Samonella typhi* occurred at same range of 8.38-8.85 and 8.17-8.93 mm, respectively. However, the crude extract from barks with methanol showed the highest antibacterial activity with diameter inhibition zone at 9.75, 10.44, and 9.75 mm in *B. cereus*, *S. aureus*, and *Samonella typhi*, respectively. None part of this plant showed any

Table 1 Antimicrobial activities of crude extracts *Rhizophora apiculata* leaf and bark extract

plant	Part used	Solvent used	Zone of inhibition diameter (mm)			
			Gram positive		Gram negative	
			B. cereus	*S. aureus*	*E. coli*	Salmonella
R. apiculata	leaves	H	9.38±0.48[a]	8.85±0.34[a]	0.00±000[c]	8.17±0.09[b]
		E	7.81±0.30[b]	8.74±0.17[a]	0.00±000[c]	8.39±0.45[a]
		M	8.77±0.50[a]	8.38±0.58[a]	0.00±000[b]	8.93±0.74[a]
	bark	H	6.37±0.37[a]	0.00±000[b]	0.00±000[b]	0.00±000[b]
		E	8.09±0.46[b]	8.85±0.31[a]	0.00±000[c]	0.00±000[c]
		M	9.75±0.30[b]	10.44±0.40[a]	0.00±000[c]	9.75±0.36[b]

Remark : Values are express as mean±SD (n = 3) (Conc. 0.1 mg/disc); H = hexane, E = ethyl acetate, M = methanol

[a – c] Mean within a row with same letters are not significantly different at the 95% confidence level by the Duncan's Multiple Range Test (DMRT)

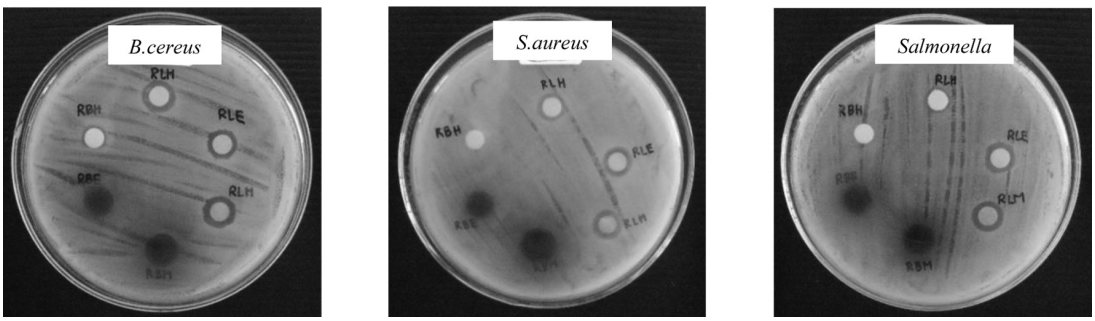

Figure 1 Antimicrobial activity of crude extracts to *Bacillus cereus* (left) *Staphylococcus aureus* (Middle) and *Salmonella typhi* (Right) RBH, RBE and RBM are bark of *R. apiculata* extracted by hexane, ethyl acetate and methanol, respectively. RLH, RLE and RLM are leaf of *R. apiculata* extracted by hexane, ethyl acetate and methanol, respectively. The technique was performed by disc diffusion method.

antibacterial activity to *E. coli* and two parts of hexane and ethyl acetate bark extracts also showed none antibacterial active for *Salmonella*. It could be explained that Gram-negative bacteria has thicker cell wall than Gram-positive bacteria. It has an outer membrance comprising high content of lipid-polysaccharide sheet, which may protect against the passage of hydrophobic groups (Abed *et al.*, 2013).

The result of phytochemical screening (Table 2) of hexane, ethyl acetate, and methanol *R. apiculata* leaf and bark extracts showed the presence of bioactive compounds such as terpenoids, flavonoids, saponins, tannin, and anthraquinone. None of extracts showed positive results for alkaloid. The positive results provided from terpeniod test in almost extracts except methanolic bark extract. Flavonoid, saponinand, and tannin were founded in only methanolic extract. In addition, anthraquinone was only founded in methanolic bark extract. As a phytochemical screening result, *R. apiculata* contains these secondary metabolites which may show antibacterial activity. Moreover, the difference in the antibacterial activity of extracts may be attributed to the difference in the composition of those bioactive phytochemicals. These results could suggest that the presence of anthraquinones from methanolic bark extract plays an important role to antibacterial activity with the highest zone of inhibition value 10.44 mm for *S. aureus*. Hence, more studied are required to isolate the bioactive compounds and to determine the action of bioactive substances in this part.

Table 2 Phytochemical constitution of *Rhizophora apiculata* extracts.

Part used	Phytoconstituents	*R. apiculata* extract		
		Hexane extract	Ethyl acetate extract	Methanol extract
leaves	Anthraquinone	-	-	-
	Terpenoid	+	+	+
	Flavonoid	-	-	+
	Saponin	-	-	+
	Tannin	-	-	+
	Alkaliod	-	-	-
bark	Anthraquinone	-	-	+
	Terpenoid	+	+	-
	Flavonoid	-	-	+
	Saponin	-	-	+
	Tannin	-	-	+
	Alkaliod	-	-	-

Remark: "+" means presence and "-" means absence

CONCLUSION

The leaves and bark of *R. apiculata* on the successive extraction with hexane, ethyl acetate, and methanol gave six fractions exhibiting the bioactive substances i.e. anthraquinone, terphenoid, flavonoid, saponin, and tannin by preliminary phytochemical analysis. Both parts of *R. apiculata* on various crude extracts gave fraction which demonstrated a varied level of broad spectrum antibacterial activity. Good activity was exhibited by methanol fraction by barks against *R. apiculata* to *S. aureus*, *B. cereus*, and *Salmonella typhi*, respectively. However, none parts of this plant showed antibacterial activity against the *E. coli*. Therefore, it could be concluded that methanol was determined to the best solvent for isolation of bioactive secondary metabolites. The finding of the present study provide further evidence which was consistent to the antimicrobial property corresponding to phytochemical studies which showing the active ingredients in *R. apiculata*.

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REFERENCES

Abed, S.A., Sirat, H.M. and Taher, M. 2013. Total phenolic, antioxidant, antimicrobial activities and toxicity study of *Gynotroches axillaris* Blume (Rhizophoraceae). **EXCLI Journal** 12: 404-412.

Abeysinghe, P.D. 2010. Antibacterial activity of some medicinal mangroves against antibiotic resistant pathogenic bacteria. **Indian Journal of Pharmaceutical Sciences** 72(2): 167-172.

Bhatt, S. and Dhyani, S. 2012. Preliminary phytochemical screening of *Ailanthus excels* Roxb. **International Journal of Current Pharmaceutical Research** 4(1): 87-89.

Gao, M. and Xiao, H. 2012. Activity-guided isolation of antioxidant compounds from *Rhizophora apiculata*. **Molecules** 17: 10675-10682.

Hong, L.S., Ibrahim, D., Kassim, J. and Sulaiman, S. 2011. Gallic acid: An anticandidal compound in hydrolysable tannin extracted from the barks of *Rhizophora apiculata* Blume. **Journal of Applied Pharmaceutical Science** 01(06): 75-79.

Lim, S.H, Darah, I. and Jain, K. 2006. Antimicrobial activities of tannins extracted from *Rhizophora apiculata* barks. **Journal of Tropical Forest Science** 18(1): 59-65.

Loo, A.Y., Jain, K. and Darah, I. 2007. Antioxidant and radical scavenging activities of the pyroligneous acid from a mangrove plant, *Rhizophora apiculata*. **Food Chemistry** 104: 300-307.

Loo, A.Y., Jain, K. and Darah, I. 2008.

Antioxidant activity of compounds isolated from the pyroligneous acid, *Rhizophora apiculata*. **Food Chemistry** 107: 1151-1160.

Mouafi, F.E., Abdel-Aziz, S.M., Bashir, A.A. and Fyiad, A.A. 2014. Phytochemical analysis and antimicrobial activity of mangrove leaves (Avicenna marina and *Rhizophora stylosa*) against some pathogens. **Journal World Applied Sciences** 29 (4): 547-554.

Opara, E.U. and Wokocha, R.C. 2008. Efficacy of some plant extracts on the *in vitro* and *in vivo* control of *Xanthomonas campestris* Pv. *Vesicatoria*. **Agricultural Journal** 3(3): 163-170.

Premanathan, M., Arakaki, R., Izumi, H., Kathiresan, K., Nakano, M., Yamamoto, N. and Nakashima, H. 1999. Antiviral properties of a mangrove plant, *Rhizophora apiculata* Blume, against human immunodeficiency virus. **Antiviral Research** 44: 113-122.

Rahim, A.A., Rocca, E., Steinmetz, J., Kassim, M.J., Ibrahim, M.S. and Osman, H. 2008. Antioxidant activities of mangrove *Rhizophora apiculata* bark extracts. **Food Chemistry** 107: 200-207.

Ravikumar, S., Gnanadesigan, M., Suganthi, P. and Ramalakshmi, A. 2010. Antibacterial potential of chosen mangrove plants against isolated urinary tract infectious bacterial pathogens. **International Journal of Medicine and Medicinal Science**s 2(3): 94-99.

Shamsuddin, A.A., Najiah, M., Suvik, A., Azariyah, M.N., Kamaruzzman, B.Y., Effendy, A.W. and John, B.A. 2013. Antibacterial properties of selected Mangrove plants against *Vibrio* species and Its cytotoxicity against *Artemia salina*. **World Applied Sciences Journal** 25(2): 333-340.

Shelar, P.S., Reddy, S., Shelar, G.S. and Reddy, G.V.S. 2012. Medicinal value of mangroves and its antimicrobial properties-A review. **Continental Journal Fisheries and Aquatic Science** 6(1): 26-37.

Sulaiman, S., Ibrahim, D., Kassim, J. and Sheh-Hong, L. 2011. Antimicrobial and antioxidant activities of condensed tannin from *Rhizophora apiculata* barks. **Journal of Chemical and Pharmaceutical Research** 3(4): 436-444.

Tukiran. 2013. Phytochemical analysis of some plants in Indonesia. **Journal of Biology, Agriculture and Healthcare** 3(4): 6-10.

Vijayavel, K., Anbuselvam, C. and Balasubramanian, M.P. 2006. Free radical scavenging activity of the marine mangrove *Rhizophora apiculata* bark extract with reference to naphthalene induced mitochondrial dysfunction. **Chemico-Biological Interactions** 163: 170-175.

Villanova, P.A. 1993. Method for antimicrobial disk susceptibility tests for bacteria that grow aerobically; approved standard, **Clinical Laboratory Standards NCCLS Document M2-A5.** Clinical and Laboratory Standards Institute, 5th Edition.