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Antimicrobial and antioxidant activities of condensed tannin from *Rhizophora apiculata* barks

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ABSTRACT

Condensed tannin from Rhizophora apiculata barks was subjected to antimicrobial screening test. The extract was tested in vitro against 18 bacteria species, 12 filamentous fungal species, and 4 yeast by agar diffusion and broth dilution methods. Condensed tannin demonstrated good antimicrobial activity against certain bacterial and yeasts tested ($P<0.05$) but not on filamentous fungal species. The MIC (minimum inhibitory concentration), MBC (minimum bacteriocidal concentration) and MYC (minimum yeastocidal concentration) were ranged from 3.13 mg/ml to 12.50 mg/ml. The antioxidant activity of the extract was evaluated by using DPPH (2,2-diphenyl-1-picrylhydrazyl) and hydrogen peroxide scavenging methods with (±)-catechin, butylated hydroxytoluene (BHT), α-tocopherol and L-(+)-ascorbic acid as standards. In the DPPH assay, the condensed tannin showed higher percentage of DPPH scavenging activity compared to other standards. The condensed tannin also showed stronger hydrogen peroxide scavenging activity than the α-tocopherol and BHT standards. Scavenging activity in both DPPH assay and hydrogen scavenging activities increased as the tannin concentration increased. The results presented here suggested that condensed tannin extracted from R. apiculata barks can be used to discover bioactive natural product that may serve as leads in the development of new pharmaceuticals in food and also as antimicrobial agent against certain food spoilage organisms.

Key words: *Rhizophora apiculata*, condensed tannin, antimicrobial activity, antioxidant activity, food spoilage organisms.

INTRODUCTION

It has long been recognized that naturally occuring substances in higher plants have antimicrobial and antioxidant activities. The extracted plants were classified as natural compounds, which were the secondary metabolites. These metabolites have demonstrated

biological activities and received particular attention as potential natural agents for food preservation. According to the current consumers, more natural and with fewer synthetic additives but increase safety and shelf-life are needed [1]. Resulting from those demands, plants have emerged as popular ingredients and have a tendency of replacing synthetic antimicrobial and antioxidant agents [2].

Rhizophora apiculata (family: Rhizophoraceae) is one of the species of mangrove tree. It is a tropical plant and widely distributed in the tropical countries, like Malaysia[3]. Tannins are known to exist in *R. apiculata* [4-5] and *R. Mangle* [6] but their chemical, biological and pharmacological properties have not yet been determined. The tannins extracted from *R. apiculata* have been reported to possess some medicinal value, especially to overcome the bacterial and viral infections [7]. Nevertheless, this extract has also been reported to be used in many areas, such as in leather trade, painting and pharmacy [8]. Haggerman et al., [9] reported that tannins were 15-30 times more effective in the quenching of peroxyl radicals than simple phenolics. There are several reports on the use of tannins in treating various ailments in humans, including diarrhoea, gastric ulcers, snake bites and wounds [10].

R. apiculata tannins consist primarily of condensed tannins or proanthocyanidins [5]. Condensed tannins are oligomers and polymer of flavan-3-ol units which are most frequently linked via $C_4 - C_6$ or $C_4 - C_8$ bonds. The most widely distributed condensed tannin in the plant tissues are procyanidins, which derived from catechin or epicatechin and may contain gallic acid esters [11]. The reactivity of condensed tannin with molecules of biological significance has important nutritional and physiological consequences [12].

In Malaysia, *R. apiculata* is a plant widely used in charcoal industry. It has been reported the barks of this plant able to produce high yields of tannins [13]. In charcoal making, the barks are normally scraped out from the log and left to rot in the field. Therefore, the aim of this study was to investigate the antimicrobial and antioxidant properties of condensed tannin obtained from *R. apiculata* barks (as agrowaste), which could be used to against a wide range of pathogenic and food spoilage microorganisms.

EXPERIMENTAL SECTION

2.1. Chemicals

(±)-Catechin, ascorbic acid, α-tocopherol, butylated hydroxytoluene (BHT) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Company (St. Louis, MO). Folin-Ciocalteu's reagent was from Merck (Darmstadt-Germany). All other chemicals were of analytical grades.

2.2. Plant materials and condensed tannin isolation

The barks of *R. apiculata* were collected from Kuala Sepetang, Daerah Larut Matang, Perak, Malaysia. The barks were washed with running tap water, dried under the sun and finely ground to pass a 1 mm siever and stored at 4ºC prior to use. 100 g of the powder formed barks were macerated in 300 ml of 70% aqueous acetone for 3 consecutive days at room temperature (30±2ºC). The acetone was removed using a rotary evaporator under pressure and the resultant extract was then concentrated to dryness and then freezed–dried. The 70% aqueous acetone extract of the mangrove barks yielded between 27–29% (by weight) dark brown tannin powder. The tannin powder (1.5 g) was defatted with hexane (50 ml), followed by extraction with ethyl acetate (50 ml). A fraction of the aqueous phase was concentrated to dryness in a rotary evaporator and freeze-dried. 10 g of the powder was dissolved in methanol: water (1:1), loaded

into a Sephadex LH 20 column (30 x 2.0 cm), and eluted with acetone:water (1:1) with a flow rate of 10 ml/ minutes to produce condensed tannins. The resultant eluents were then concentrated to dryness in a rotary evaporator and then freeze-dried. The condensed tannin obtained was kept in the dark at 4°C until further used.

2.3. Microorganisms

Eighteen bacteria, four yeasts and twelve fungal species were used as test microorganisms. They were obtained from the stock cultures of Industrial Biotechnology Research Laboratory (IBRL), School of Biological Sciences, Universiti Sains Malaysia. Bacterial cultures were maintained on nutrient agar slants (Difco, US) at 37ºC while yeast and fungal cultures were maintained on Sabouraud dextrose agar (Difco, US) at 37ºC for yeast and 30ºC for fungi. All the cultures were kept in appropriate media slants and stored at 4ºC until used.

2.4. Antimicrobial activity of condensed tannin on test microorganisms

The antibacterial, antiyeast and antifungal activities were tested by the disc-diffusion agar method. 1 mL of inoculum $1x10^6$ bacterial cells/ml from 24 hour old culture or $4x10^5$ yeast cells (from 24 hour old culture) or fungal spores/ml from a 5 days old culture were placed in sterile Petri dishes and then 15 ml of molten nutrient agar for bacteria or molten Sabouraud agar for yeast and fungi were poured into the respective plates. The plates were sharked gently to allow evenly mixing of inoculum and agar. Once the seeded agars were solidified, the extract impregnated antibiotic discs (6.00 mm diameter disc, Whatman) were placed on it and incubated either at 37ºC for 24 hours for bacteria and yeast or at 30ºC for 3 days for fungi. 1000 mg of condensed tannin was dissolved in 10 ml of 70% methanol to obtained 100 mg/ml of the extract. Chloramphenicol (30µg/ml) and miconazole (30µg/ml) were used as positive control for bacteria and yeast/fungi, respectively, while discs impregnated with 70% methanol were used as negative control. The volume used for each disc was 20 µl. The experiments were performed in triplicates and the means of the diameter of the inhibition zones were calculated.

Clear inhibition zones that formed around the discs indicated the presence of antimicrobial activity. However, inhibition zone formed around the discs with the lowest concentration of extracts were considered for determination of minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC) and minimal yeastocidal concentration (MYC) values.

2.5. Determinition of minimum inhibitory concentration (MIC)

MIC was determined by broth dilution method [14]. Two-fold serial dilutions (0.3125 – 100.000 mg/mL) of the extracts, with the appropriate antibiotics, were prepared as positive controls in Nutrient broth for bacteria and Sabouraud dextrose broth for yeasts. For broth dillution tests, 0.1 mL of standardized suspension of bacteria (1×10^6 CFU/ mL) and yeasts cells (4×10^5 CFU/ mL) was added to each tube (containing fractions of 5 extracts at a final concentration of 0.3125 to 100 mg/mL) and incubated at 37°C for 24 hours for bacteria and yeasts. MICs were taken as the average of the lowest concentration showing no growth of the organism and the highest concentration showing visible growth by macroscopic evaluation [15].

2.6. Determinition of minimum bacteriocidal/ yeastocidal concentration (MBC/ MYC)

A loop full of microbial cultures was taken from each test tube and inoculated onto nutrient agar plates for bacteria and Sabouraud dextrose agar plates for yeasts. Then, the plates were incubated at 37°C for 24 hours. The MBC or MYC was recorded as the lowest concentration of the extract that gave complete inhibition of colony formation of the test microorganisms.

2.7. Antioxidant-activity assays

2.7.1. Free radical scavenging activity of extracts using DPPH assay

The method for estimating free radical scavenging of the extracts of *R. apiculata* barks was adapted from that of Burits & Baucar [16] with some modifications. 5.0 ml of methanolic solution of DPPH (0.004%; w/v) were mixed with 50 µl of tannin extract (concentration between 0.0 – 1.0 mg/ml). After 30 minutes of incubation period at room temperature (30±2ºC) the absorbance was read against a blank at 517 nm. Inhibition of free radical DPPH in percent (I%) was calculated in following way:

$$I\% = [(A_c - A_s) / A_c] \text{ X } 100$$

Where A_c is the absorbance of the control reaction (containing all reagents except the test compound) and A_s is the absorbance of the tested sample. Synthetic antioxidant reagent butylated hydroxytoluene (BHT), α-tocopherol and ascorbic acid were used as positive controls and all tests were carried out in triplicates.

2.7.2. Scavenging of hidrogen peroxide

The ability of the condensed tannin extract to scavenge hydrogen peroxide was determined according to the method of Shon et al., [17]. A solution of hydrogen peroxide (43 mM) was prepared in phosphate buffer (pH 7.4). Then, 3.4 ml of condensed tannin extract (concentration between 0.0 – 50.0 µg/ml) in phosphate buffer (pH 7.4) were added to a hydrogen peroxide solution (0.6 ml, 43 mM). Absorbance of hydrogen peroxide at 230 nm was determined 10 min later against a blank solution containing the phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging of both the extracts and standard compounds was calculated:

$$\% \text{ Scavenged } [H_2O_2] = [(A_o - A_I) / A_o] \text{ X } 100$$

where A_o is the absorbance of the control, and A_I is the absorbance in the presence of the sample of condensed tannin extract or standards.

2.8. Statistical analysis

All analyses were run in triplicates. The average value and standard deviation were calculated using Excel. One-way analysis of variance (ANOVA) was used to evaluate the significant differences between activity and concentration of samples used with the criterion of P values < 0.05.

RESULTS AND DISCUSSION

In this study, 70% acetone (v/v) was used to derive crude tannin extract from *Rhizophora apiculata* barks. Djipa et al., [18] reported that an acetone-water mixture was believed to be a better solvent for tannin extraction in comparison with other extractants. The crude extract was then further separated to hydrolysable and condensed tannins. The extraction of tannin from *Rhizophora apiculata* barks using acetone gave practically about 23.85% of raw tannin, 9.00% of hydrolysable tannin and 3.69% of condensed tannin. The condensed tannin extract was tested on a panel of Gram positive and negative bacteria, yeasts and fungi. The agar disc susceptibility test of condensed tannin from *R. apiculata* barks is shown in Table A.1. It was found that the condensed tannin exhibited very good antibacterial against the all the Gram-positive organisms as compared to Gram-negative bacteria. The highest zone of inhibition of 14.0 mm was observed with *Bacillus cereus*, followed by *Bacillus subtilis* and *Acinobacter calcoaticus*; and

the least was found with *Staphylococcus saprophyticus* which produced 9.0 mm diameter inhibition zone. It was observed that out of eleven Gram-negative bacteria tested, the condensed tannin demonstrated inhibition against all the Gram-negative bacteria excepted for *Citrobacter freundi, E. coli, Salmonella paratyphoid B and Proteus mirabilis.* Farag et al., [19] have reported plant extracts have little effect against Gram-negative bacteria. It is possible that less susceptibility of Gram-negative organisms to the action of antibacterial due to the outer membrane surrounding their cell wall [20].

Table A.1 Antimicrobial activities of condensed tannin extracted from *R. apiculata* barks

Test microorganisms	Zone of inhibition (mm)[a]		
	Condensed Tannin	Miconazole	Chloramphenicol
Bacteria			
Bacillus subtilis	13.0	ND	18.5
Bacillus cereus	14.0	ND	18.0
Bacillus licheniformis	10.0	ND	20.0
Staphylococcus aureus	11.0	ND	22.0
Staphylococcus saprophyticus	9.0	ND	16.0
Staphylococcus epidermdis	11.5	ND	20.0
Acitobacter anitritus	11.0	ND	22.0
Acinobacter calcoaticus	13.0	ND	20.0
Citrobacter freundii	-	ND	20.5
Micrococcus sp.	10.5	ND	22.0
Serratia marcescens	11.0	ND	20.5
Erwinia sp.	11.0	ND	18.0
Escherichia coli	-	ND	16.0
Klebsiella sp.	11.0	ND	22.0
Pseudomonas aeruginosa	12.0	ND	18.0
Salmonella paratyphii	-	ND	18.0
Enterobacter aerogenes	10.0	ND	16.0
Proteus mirabilis	-	ND	21.0
Yeast			
Candida albicans	12.0	24.5	ND
Rhodotorula rubra	14.0	22.0	ND
Cryptococcus neoformans.	-	22.0	ND
Saccharomyces cerevisiae	10.0	25.0	ND
Fungi			
Aspergillus niger	-	18.5	ND
Aspergillus flavus	-	20.0	ND
Fusarium oxysporium	-	18.0	ND
Fusarium sp.	-	18.0	ND
Mucor sp.	-	18.0	ND
Penicillium sp.	-	21.0	ND
Rhizopus sp.	-	22.5	ND
Microsporum gypseum	-	20.0	ND
Microsporum canis	-	20.0	ND
Trichopyton mentagrophytes	-	20.0	ND
Trichophyton rubrum	-	19.0	ND
Trichoderma viride	-	22.0	ND

Antimicrobial activities were determine based on diameter of zone inhibition (mm);
- No inhibition, ND = Not determined, [a] Mean values from triplicate results .

Additionally, the condensed tannin demonstrated inhibition against three of the yeast tested while no inhibition was observed with fungi. The results obtained suggested that the condensed tannin was effective against prokariotic compared to eukariotic cells. It is clear that bacteria and

yeast are more sensitive to condensed tannin than fungi. The resistance of fungal species against condensed tannin could be due to their morphological structure where fungi have thicker walls and contain high percentage of chitin [21]. Even though *C. neoformans* is yeast, but it was resistant to the extract. This condition could be due to the capsule that it possessed. Marino et al., [22] reported that plant extracts generally have more inhibitory effect against Gram positive than Gram negative bacteria. This condition could be due to the characteristic of the outer membrane layer surrounding their cell wall. Gram negative bacterial cell wall is very rich with lipopolysaccharide and it may restrict diffusion of the hydrophobic compounds through the lipopolysaccharide layer.

Based on the antimicrobial activities, those microorganisms that showed positive results were selected for further studies to determination their minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum yeastocidal concentration (MYC) values. Table A.2 shows the MIC values of the bacteria (*Acinetobacter calcoaticus, Klebsiella pneumoniae, Bacillus subtilis, B. licheniformis, Staphylococcus aureus, S. epidermidis, B. cereus, Serratia marcescens, Erwinia* sp. and *Pseudomonas aeruginosa*) which ranged from 3.13 to 6.25 mg/ml, whereas the MIC for yeast species (*Candida albicans, Rhodotorula rubra* and *Saccharomyces* cerevisiae were ranged from 3.13 to 6.25 mg/ml. The MBC for those bacteria were ranged from 6.25 to 12.50 mg/ml the MYC values were ranged from 6.25 to 12.50 mg/ml.

Table A. 2 Minimum inhibition concentration, minimum bacteriocidal concentration and minimum yeastocidal concentration of condensed tannin extract

Test microorganisms	Condensed Tannin (mg/ml)		Miconazole (µg/ml)		Chloramphenicol (µg/ml)	
	MIC	MBC/ MYC	MIC	MBC/ MYC	MIC	MBC/ MYC
Bakteria:						
Acinobacter calcoaticus	3.13	6.25	>1000	>1000	ND	ND
Klebsiella sp.	6.25	12.50	125	250	ND	ND
Bacillus subtilis	6.25	12.50	>1000	>1000	ND	ND
Bacillus licheniformis	3.13	6.25	>1000	>1000	ND	ND
Staphylococcus aureus	6.25	12.50	>1000	>1000	ND	ND
Staphylococcus epidermidis	6.25	12.50	250	500	ND	ND
Bacillus cereus	6.25	12.50	>1000	>1000	ND	ND
Serratia marcescens	6.25	6.25	>1000	>1000	ND	ND
Erwinia sp.	6.25	6.25	>1000	>1000	ND	ND
Pseudomonas aeruginosa	6.25	6.25	>1000	>1000	ND	ND
Yis :						
Candida albicans	6.25	12.50	ND	ND	500	1000
Rhodotorula rubra	3.13	6.25	ND	ND	ND	ND
Saccharomyces cerevisiae	12.50	12.50	ND	ND	ND	ND

Result was the average of records determined by both agar and broth dilution methods;
ND = not determined

The purple-colured DPPH is a stable free radical, which is reduced to α,α-diphenyl-β-picrylhydrazine (yellow coloured) by reacting with an antioxidant. Free radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation [23]. Antioxidant interrupt free radical chain oxidation by donating hydrogen from hydroxyl groups to form a stable end product, which does not initiate or propagate further oxidation of lipid [24]. Fig. A.1 shows the percentage inhibition of free radical by condensed tannin extract due to hydrogen donation from the antioxidant. There was significant different (P<0.05) on the effect of radical scavenging activity by different concentrations used in this study. The colour of the DPPH reagent was significantly reduced from purple to yellow. Natural antioxidants may have the potential to prevent the oxidation of food. The antioxidant activity of tannin extracts showed effective antioxidant activity when compared to artificial antioxidants. The order of antioxidant

activity of condensed tannin extract was: condensed tannin > catechin > ascorbic acid > BHT > α-tocopherol. The results indicated that the condensed tannin was able to act as free radical inhibitor and primary antioxidant that reacts with free radicals.

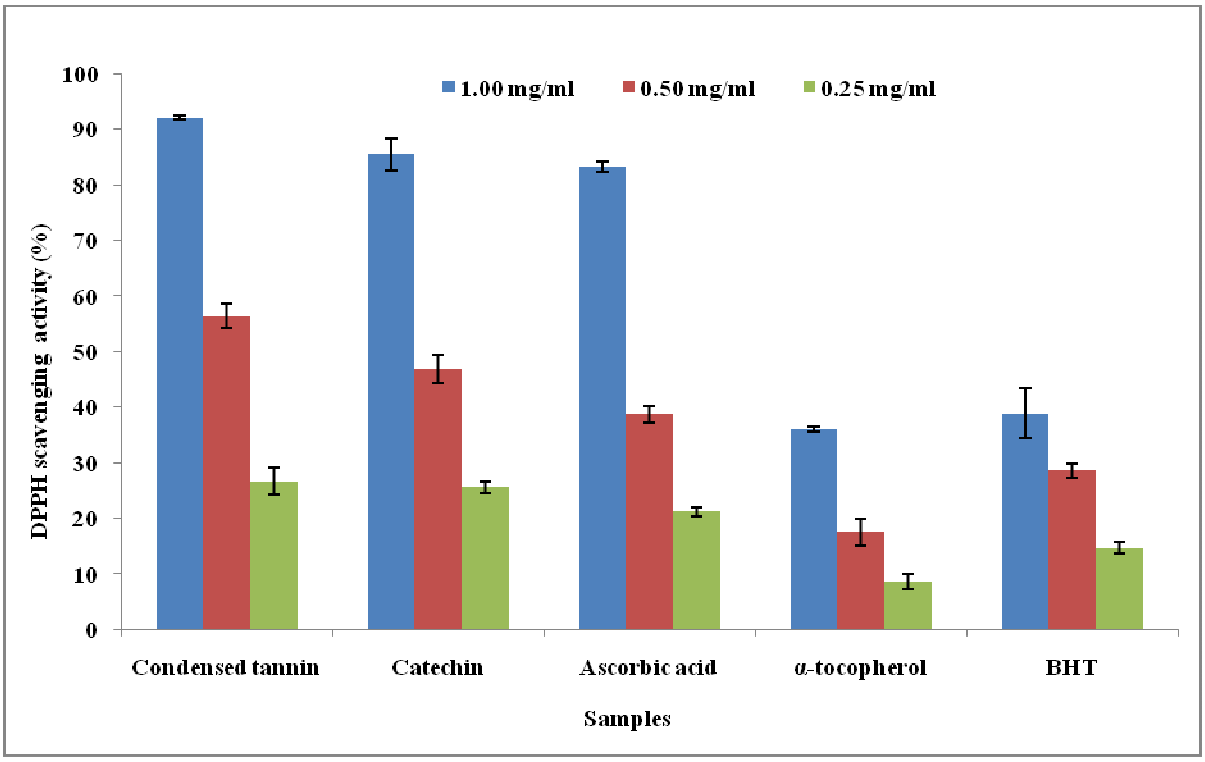

Fig. A.1 Free radical-scavenging activity of the condensed tannin extracts using different concentrations

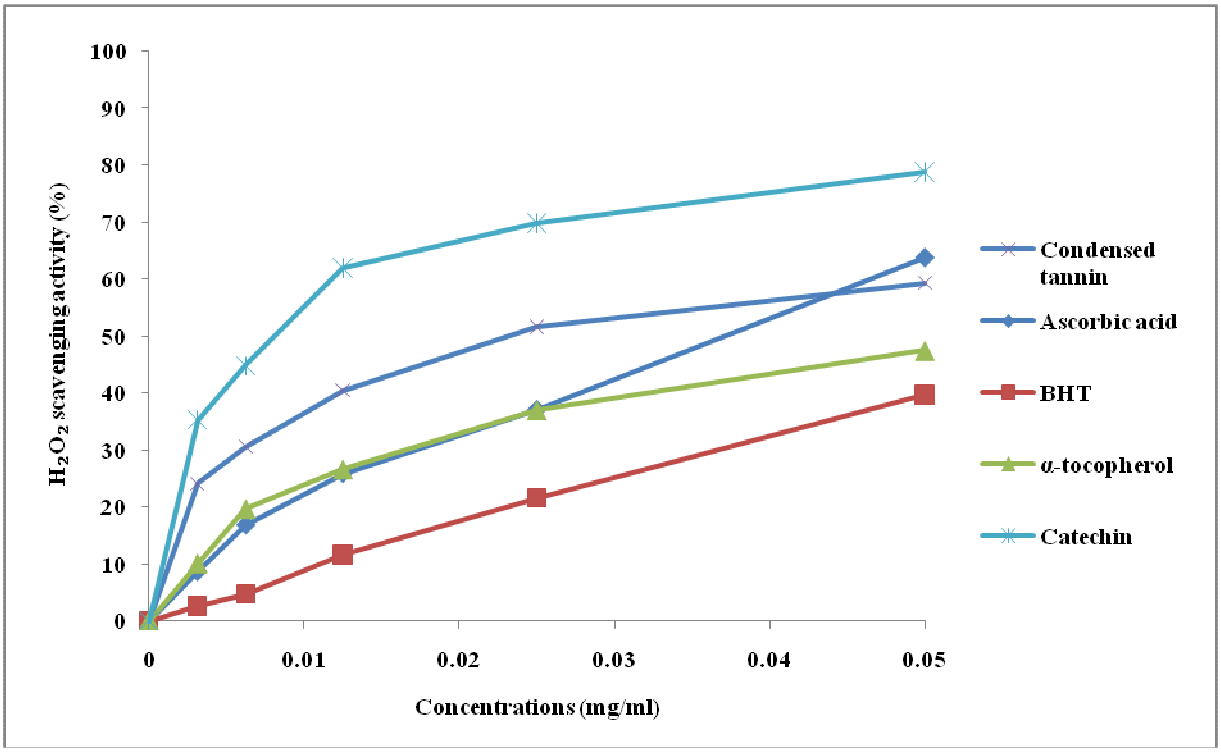

Fig. A.2 H_2O_2 scavenging activity of the condensed tannin extracts using different concentrations

Although hydrogen peroxide is not a free radical, it has a great physiological relevance because of its ability to penetrate biological membranes and to act like an intermediate in the production of more reactive oxygen species, namely hydroxyl radical and hypochlorous acid [25]. The ability of the condensed tannin extract to scavenge hydrogen peroxide is shown in Fig. A.2 and was compared with those of catechin, ascorbic acid, BHT and α-tocopherol as standards. Condensed tannin extract was capable of scavenging hydrogen peroxide in a concentration-dependent manner. A 50 μg/ml of condensed tannin extract exhibited 56.2% scavenging activity on hydrogen peroxide. On the other hand, hydrogen peroxide scavenging activity of catechin, ascorbic acid, BHT, and α-tocopherol at the same dose were exhibited 78.76%, 63.74%, 39.66% and 47.43%, respectively. These results showed that condensed tannin extract had stronger hydrogen peroxide scavenging activity compared to BHT and α-tocopherol. Those values are close to that of ascorbic acid, but lower than that of catechin. There was statistically significant different (P<0.05) on the effect of hydrogen peroxide scavenging activity by different concentrations used in this study. The hydrogen peroxide scavenging effect of 50 μg/ml concentration of condensed tannin extract and standards decreased in the order of catechin > ascorbic acid > condensed tannin > α-tocopherol > BHT. Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells [26]. Thus, removing H_2O_2 is very important for protection of food systems.

Rahim et al., [27] have reported HPLC analyses of condensed tannin from *R. apiculata* barks and identified the four terminal units, namely catechin, epicatechin, epigallocatechin and epicatechin gallate. Among that, one extender unit and catechin were found to be the most predominant constituent of mangrove tannins. It is possible that this compound was mainly responsible for the observed antimicrobial and antioxidative activities in this study.

CONCLUSION

The data obtained show the inhibition potency on condensed tannin that can be considered as preservative agent for antibacterial, antiyeast and antioxidant activities. In recent year the interest to evaluate plants possessing antimicrobial activity to serve as additives or to replace synthetic preservatives in food is growing. This study provides the important baseline information for the use of condensed tannin extracted from *R. apiculata* as well as the preservative agent for some types of food. However, *in vivo* experiment should be conducted for further study to enhance the scientific findings.

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