ANTIMICROBIAL ACTIVITIES OF TANNINS EXTRACTED FROM *RHIZOPHORA APICULATA* BARKS

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LIM, S. H., DARAH, I. & JAIN, K. 2006. Antimicrobial activities of tannins extracted from *Rhizophora apiculata* barks. Tannin which was extracted from barks of *Rhizophora apiculata* was further separated into hydrolysable and condensed tannin. The results of the study revealed that only hydrolysable tannin showed significant antibacterial and antiyeast activities but not antifungal activity. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of the tannin against bacteria and yeasts were also determined. Special attention was given to hydrolysable tannin which exhibited a significant antiyeast activity. The hydrolysable tannin was further tested *in vitro* for antifungal activity against *Candida albicans*, a pathogenic yeast which sometimes causes infection in human and animals. SEM and TEM microscopy studies revealed that the treated cells of *C. albicans* demonstrated severe morphological changes especially on its cell wall and cell membrane.

Keywords: Hydrolysable tannin, condensed tannin, antimicrobial activity, fungi, minimum inhibitory concentration, bacteria, yeast

LIM, S. H., DARAH, I. & JAIN, K. 2006. Aktiviti antimikrob oleh tanin yang diekstrak daripada kulit kayu *Rhizophora apiculata*. Tanin yang diekstrak daripada kulit kayu *Rhizophora apiculata* telah dipisahkan lagi kepada tanin terhidrolisis dan tanin terkondensasi. Hasil daripada kajian ini menunjukkan hanya tanin terhidrolisis mempamerkan aktiviti antibakteria dan antiyis yang berkesan, tetapi bukan aktiviti antikulat. Nilai kepekatan perencatan minimum (MIC) dan kepekatan bakterisid minimum (MBC) untuk tanin terhadap bakteria dan yis juga ditentukan. Perhatian khusus diberikan kepada tanin terhidrolisis yang menunjukkan aktiviti antiyis yang berkesan. Tanin terhidrolisis telah diuji secara *in vitro* untuk aktiviti antiyis terhadap sel *Candida albicans*, iaitu sejenis yis patogen yang kadangkala menyebabkan jangkitan kepada manusia dan haiwan. Kajian mikroskopi SEM dan TEM membuktikan sel *C. albicans* yang dirawat dengan tanin terhidrolisis menunjukkan perubahan morfologi yang ketara terutama pada dinding dan membran selnya.

Introduction

Higher plants produce hundreds to thousands of diverse chemical compounds with different biological activities and important ecological roles. They can be chemical defenses against insects, herbivores and microorganisms (Harborne 1990). Antimicrobial compounds produced by plants are active against plant and human pathogens. There are several reports published on antimicrobial activity of crude plant extracts and the bioassay-guided fractionation of them to yield active principles (Cowan 1999, Panizzi *et al.* 2002, Voda, *et al.* 2003). It is estimated that there are about 2.5 million species of higher plants and the majority of these have not yet been examined for their pharmacological activities (Ram *et al.* 2003).

Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives. In many cases these substances serve as plant defense mechanism against predation by micro- and macroorganisms. In fact, certain substances such as terpenoids, give plant their smells while others, such as quinones and tannins, are responsible for plant pigmentation.

Tannins are naturally occurring plant polyphenols which combine with protein and other polymers to form stable complexes. They are fairly large molecules having molecular weights of 500–3000 kD. Several phenolic hydroxyl groups located on the surface of tannin molecules are believed to participate strongly in the properties and biological activities of the tannins. There are several reports

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on the use of tannins in treating various ailments in humans, including diarrhoea, gastric ulcers, snake bites and wounds (e.g. Perera *et al.* 2001). However, reports on antimicrobial activity of tannins are scarce.

In Malaysia, *Rhizophora apiculata* (family Rhizophoraceae), or locally known as bakau minyak, is a famous mangrove plant widely used in charcoal industry. It has been reported to produce high yields of tannins (Jain *et al.* 2002). In charcoal making, the barks are normally scraped out from the log and left to rot in the field. The objective of the present study was to assess the antimicrobial activity of tannins extracted from barks of *R. apiculata*, which are considered as waste from charcoal industry.

Materials and methods

Preparation of extract

Barks of *R. apiculata* were collected from Kuala Sepetang in Perak, Malaysia. About 150 g of coarsely powdered dry barks were successively extracted in 70% acetone, at 30 °C for three consecutive days. The resultant extract was then concentrated to dryness in a rotary evaporator before freeze-drying. The crude extract obtained was mixed tannin and was purified by the method described by Jain *et al.* (2002). Two separate compounds, namely, hydrolysable and condensed tannin were obtained. The stock solution of hydrolysable tannin containing 100 mg ml^{-1} (w/v) was prepared in methanol and then further diluted in distilled water.

Test microorganisms

The test microorganisms used in this study consisted of 23 bacterial, 4 yeasts and 12 fungal species, which were obtained from stock cultures of the Fermentation and Enzyme Technology Laboratory, School of Biological Sciences, Universiti Sains Malaysia, Penang. The bacterial strains were grown and maintained on nutrient agar (NA) slants, while yeast and fungi on Sabouraud dextrose agar (SDA) slants. The inoculated agar slants were incubated at 37 °C for bacteria and yeasts, and 30 °C for fungi.

Antimicrobial activity test

Tests were performed by disc diffusion method, using 1 ml of inoculum containing 1×10^5 bacterial cells or 4×10^5 yeast cells or fungal spore per ml. Extract-impregnated discs (20 µl per disc) at the concentration of 100 mg ml^{-1} were placed on the seeded agar and incubated either at 30 °C for three days for fungi, or at 37 °C overnight for bacteria and yeasts. Antimicrobial activity was indicated by clear zone of growth inhibition formed around the disc. Complete inhibition was indicated by a clear zone formed around the disc, while partial inhibition, by a semi-clear zone.

Determination of minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC)

Hydrolysable tannin which showed significant results in the antimicrobial activity test was chosen for this study. For the test microorganisms, only microorganisms which showed positive results in antimicrobial sensitivity test were chosen for this study. The MIC value was determined by liquid dilution method. About 1.0 ml of Sabouraud dextrose broth containing 4×10^5 yeast cells per ml and 1.0 ml of nutrient broth containing 1×10^5 bacterial cells per ml were mixed separately with the extract to give cell concentrations of 0.78–100 mg ml^{-1}. Incubation for yeasts and bacteria was carried out at 30 °C for 24 hours. The lowest concentration, which did not show any growth of the test microorganism after macroscopic evaluation was taken as the minimum inhibitory concentration (MIC).

The viability of treated cells was confirmed by incubating the broth on SDA plate for yeast and NA plate for bacteria. In this test, the lowest concentration of extract that did not yield any growth following the second inoculation, i.e. subculturing, was the minimum bactericidal concentration (MBC).

Structural and morphological studies of the yeast cells after exposure to hydrolysable tannin

One ml of 1×10^6 cells per ml of yeast cell suspension was inoculated on SDA plate and then incubated at 30 °C for 12 hours. Two ml of hydrolysable tannin (100 mg ml^{-1}) was added to the plate and further incubated for 12, 24, and 36 hours at the same incubation temperature.

At the end of the incubation period a small block of agar containing yeasts was taken and fixed for scanning (SEM, Borgers *et al.* 1989) and transmission (TEM, Mares 1989) electron microscopy works.

Results and discussion

The results of the antimicrobial activity tests of crude (mixed tannin), condensed and hydrolysable tannins are shown in Table 1. It was found that the three types of tannins tested exhibited various antibacterial and antiyeast activities but no antifungal activity. *Proteus mirabilis, Acinetobacter calcoaceticus, Micrococcus* sp., *Staphylococcus epidermidis, Yersinia enterocolitica, S. aureus, Erwinia* sp., *Pseudomonas aeruginosa, Bacillus cereus, Klebsiella* sp. and *Saccharomyces cerevisiae* showed complete inhibitions, whereas *Escherichia coli, Bacillus subtilis, Candida albicans* and *Cryptococcus neoformans* showed partial inhibition by the mixed tannin. *Acinetobacter calcoaceticus, S. saprophyticus, Micrococcus* sp., *S. aureus, Erwinia* sp., *P. aeruginosa, Klebsiella* sp. and *S. cerevisiae* exhibited complete inhibitions, while *Proteus mirabilis, Serratia marcescens, A. anitratus, Salmonella paratyphi, B. licheniformis, S. epidermidis, B. cereus, Enterobacter aerogenes, C. albicans* and *C. neoformans* exhibited partial inhibition when exposed to condensed tannin. When exposed to the hydrolysable tannin, *P. mirabilis, A. calcoaceticus, S. saprophyticus, A. anitratus, B. licheniformis, Micrococcus* sp., *S. epidermidis, S. typhi, S. aureus, Erwinia* sp., *Klebsiella* sp., *C. albicans* and *S. cerevisiae* ex hibited complete inhibitions, whereas *P. aeruginosa, B. cereus, E. aerogenes, Rhodotorula rubra* and *C. neoformans* showed partial inhibition. Differences in antimicrobial activities of various plant extracts against microorganisms are expected as the activities are based not only on different structures of microorganisms but also on their susceptibilities. The resistance of fungal species against the three types of tannins could be due to their morphological structure; fungi have thicker cell walls and contain higher percentage of chitin (Madigan & Martinko 2006).

The results of this study also showed that hydrolysable tannin was found to possess better antibacterial and antiyeast activities compared with mixed and condensed tannins (Table 1). The MIC values of the hydrolysable tannin were determined against bacterial and yeast species that exhibited positive results in the antimicrobial activity tests and are presented in Table 2. The hydrolysable tannin showed significant MIC values for bacteria and yeasts ($p \leq 0.1$). The MIC values for *B. cereus, Erwinia* sp., *P. mirabilis* and *S. saprophyticus* were 3.13 mg ml^{-1}, *A. calcoaceticus* and *B. licheniformis* were 1.56 mg ml^{-1}, *Klebsiella* sp., *Micrococcus* sp., *P. aeruginosa, S. aureus* and *S. epidermidis* were 6.25 mg ml^{-1}, whereas the MIC value for *A. anitratus* and *S. typhi* were 12.5 mg ml^{-1}. The MIC values for *C. albicans* and *S. cerevisiae* were 6.25 and 12.5 mg ml^{-1} respectively. The inhibition or reduction in growth was possibly due to interferences by the active principles of the extracts (Richards *et al.* 1994, Saxena *et al.* 1994).

Test microorganisms that had low MIC values also showed low concentrations of MBC. The results showed that the extract exhibited bacteriostatic and yeastostatic activities at lower concentrations and bactericidal or yeastocidal activities at higher concentrations. Therefore, the MIC and MBC values are useful as guideline to the choice of appropriate and effective concentrations for therapeutic substances.

From the SEM study, it was noted that the cells had undergone some distinct morphological and cytological alterations. Figure 1 shows the SEM micrographs of the untreated and extract-treated cells of *C. albicans* at various times of exposure to hydrolysable tannin extracted from *R. apiculata*. Control cells showed typical cells with budding and smooth surface (Figure 1a). After 12 hours of

Table 1 Antimicrobial activities of the tannins extracted from *Rhizophora apiculata* barks

	Zone of inhibition		
Test microorganism	MT	CT	HT
Bacteria			
Proteus mirabilis	+	p	+
Acinetobacter calcoaceticus	+	+	+
Staphylococcus saprophyticus	-	+	+
Serratia marcescens	-	p	-
Klebsiella pneumoniae	-	-	-
Acinetobacter anitratus	-	p	+
Escherichia coli (enteropathogenic)	p	-	-
Salmonella paratyphi B	-	p	-
Bacillus licheniformis	-	p	+
Micrococcus sp.	+	+	+
Staphylococcus epidermidis	+	p	+
Citrobacter freundii	-	-	-
E. coli	-	-	-
Yersinia enterocolitica	+	-	-
Salmonella typhi	-	-	+
Burkholderia pseudomallei	-	-	-
Bacillus subtilis	p	-	-
Staphylococcus aureus	+	+	+
Erwinia sp.	+	+	+
Pseudomonas aeruginosa	+	+	p
Bacillus cereus	+	p	p
Enterobacter aerogenes	-	p	p
Klebsiella sp.	+	+	+
Yeasts			
Candida albicans	p	p	+
Rhodotorula rubra	-	-	p
Cryptococcus neoformans	p	p	p
Saccharomyces cerevisiae	+	+	+
Fungi			
Trichoderma viride	-	-	-
Rhizopus sp.	-	-	-
Mucor sp.	-	-	-
Penicillium sp.	-	-	-
Fusarium solani	-	-	-
Tricophyton rubrum	-	-	-
Microsporum canis	-	-	-
Tricophyton mentagrophytes	-	-	-
Fusarium oxysporium	-	-	-
Aspergillus niger	-	-	-
Aspergillus flavus	-	-	-
Microsporum gypseum	-	-	-

+ = complete inhibition, - = no inhibition, p = partial inhibition, MT = mixed tannin, HT = hydrolysable tannin, CT = condensed tannin

Table 2 Minimum inhibitory (MIC) and minimum bactericidal (MBC) concentration values of hydrolysable tannin from *R. apiculata*

Test microorganism	MIC (mg ml^{-1})	MBC (mg ml^{-1})
Bacteria		
Acinetobacter anitratus	12.5	25.0
Acinetobacter calcoaceticus	1.56	3.13
Bacillus cereus	3.12	6.25
Bacillus licheniformis	1.56	3.13
Erwinia sp.	3.13	6.25
Klebsiella sp.	6.25	12.5
Micrococcus sp.	6.25	12.5
Proteus mirabilis	3.13	6.25
Pseudomonas aeruginosa	6.25	12.5
Salmonella typhi	12.5	25.0
Staphylococcus aureus	6.25	12.5
Staphylococcus epidermidis	6.25	12.5
Staphylococcus saprophyticus	3.13	6.25
Yeasts		
Candida albicans	6.25	12.5
Saccharomyces cerevisiae	12.5	25.0

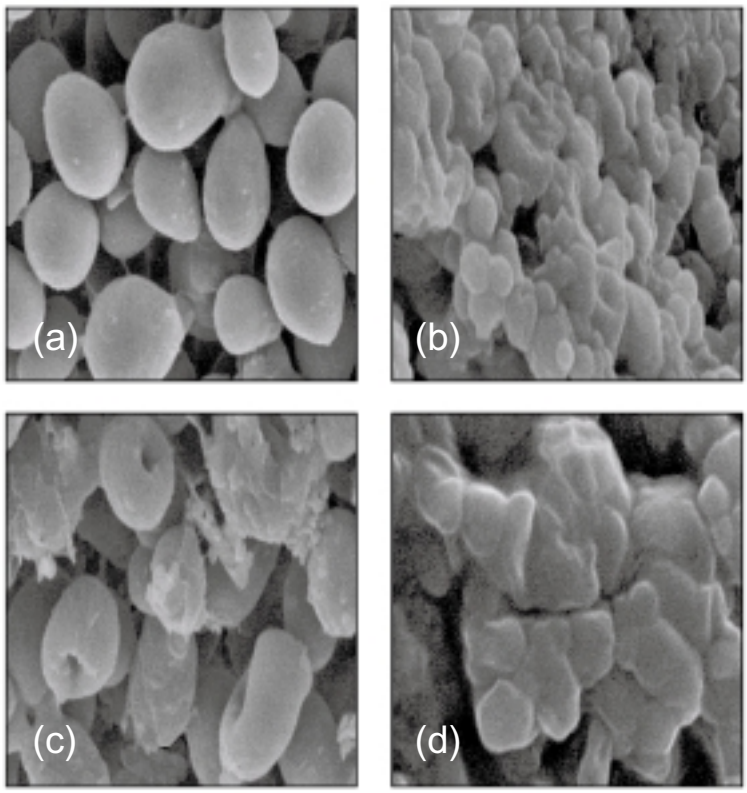

Figure 1 SEM photomicrographs showing the morphological changes of *Candida albicans* cells after exposure to hydrosable tannin extracted from *Rhizophora apiculata*. (a) Control (10 500×), (b) 12 hours (7500×), (c) 24 hours (10 500×) and (d) 36 hours (8500×)

exposure the microbial effect of the extract was evident (Figure 1b). Treated cells had sticky surface which caused the cells to bind together leading to alteration and distortion. The condition of the cells became worse after 24 hours exposure (Figure 1c). The cells showed severe alteration of cell walls with formation of invaginations, cavitated cells and eventually collapsed cells. After 36 hours of exposure (Figure 1d), the yeast cells completely collapsed and lysed. Based on the results obtained, it is believed that at this stage, the cells had lost its metabolic function (Park *et al.* 2003).

The TEM study gave further evidence of cell alteration (Figure 2). Untreated yeast cells showed typical and normal cells with buddings (Figure 2a). After 12 hours of treatment the cytoplasm of the cells shrunk and the cell wall started to collapse (Figure 2b). Cells incubated for 24 hours of exposure exhibited notable alteration in the cell membrane and cell wall (Figure 2c). Collapsed cell membrane caused the cytoplasm to flow out of the cell. After 36 hours of exposure most cells had collapsed and lysed (Figure 2d).

In view of these data, it appeared that the hydrolysable tannin extracted from *R. apiculata* could be a potential agent to be used especially against yeast. The antimicrobial properties of hydrolysable tannin could be associated with the hydrolysis of ester linkage between gallic acid, usually as multiple esters with D-glucose (Cowan 1999), which eventually affects the biosynthesis steps in the syntheses of cell wall and cell membrane. Changes in the permeability of cell membrane could cause a decrease in cell volume, evidenced by the disjunction of cell membrane from the cell wall. Cell wall abnormalities also may be attributable to cell membrane alterations (Suraya & Darah 2002).

The present investigation confirms that there are antibacterial and antiyeast properties in hydrolysable tannin extracted from *R. apiculata*. However, it is important to point out that hydrolysable tannin such as this, needs to be further processed to give pure compound(s) that can then be tested for antibacterial and antiyeast activities.

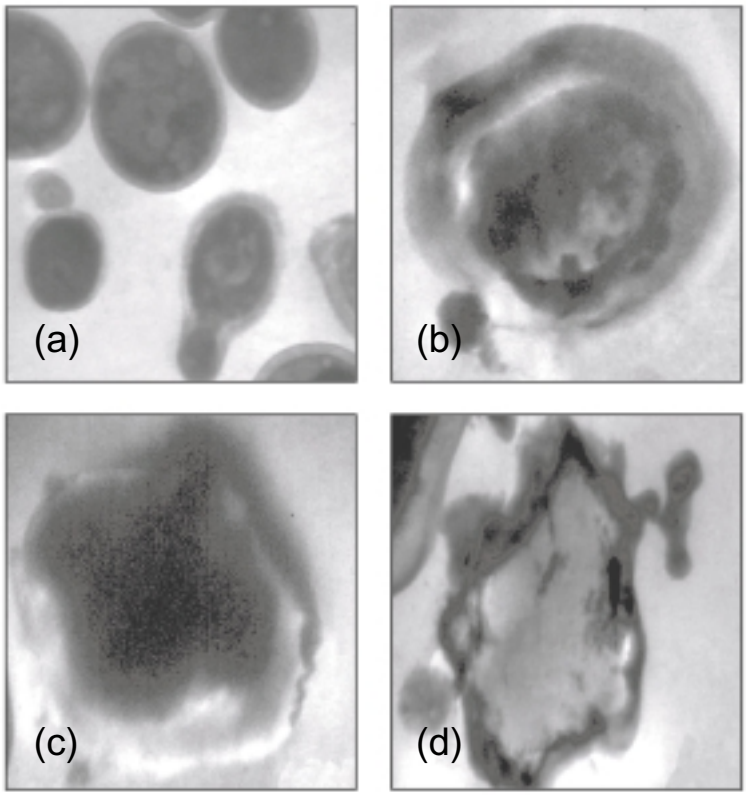

Figure 2 TEM photomicrographs of *Candida albicans* cells after exposure to the hydrosable tannin extracted from *Rhizophora apiculata*. (a) Control (8300×), (b) 12 hours (17500×), (c) 24 hours (17500×) and (d) 36 hours (17500×)

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References

Borgers, M., Van De Ven, M. A. & Van Cutsen, J. 1989. Structural degeneration of *Aspergillus fumigatus* after exposure to saperconazole. *Journal of Medical and Veterinary Mycology* 27: 381–389.

Cowan M. M. 1999. Plant products as antimicrobial agents. *Clinical Microbiology Review* 12(4): 564–582.

Harborne, J. B. 1990. Role of secondary metabolites in chemical defense mechanisms in plants. Pp. 126–139 in *Bioactive Compounds from Plants*. Ciba Foundation Symposium 154. Wiley, Chichester.

Jain, K., Afidah, A. R. & Mohd Azman, I. 2002. Anti-corrosive performance of wash primer based on mangrove tannin. Pp: 323–327 in *Proceedings of the 15th Symposium of Malaysian Chemical Engineering*. 11–12 September 2001. Universiti Teknologi Malaysia, Skudai.

Madigan, M. T. & Martinko, J. M. 2006. *Brock Biology of Microorganisms*. 11th edition. Pearson-Prentice Hall, Upper Saddle River.

Mares, D. 1989. Electron microscopy of *Microsporum* cookie after *in vitro* treatment with protoanemonin: a combined SEM and TEM study. *Mycopathologia* 108: 37–46.

Panizzi, L., Caponi, C., Catalano, S., Cioni, P. L. & Morelli, I. 2002. *In vitro* antimicrobial activity of extracts and isolated constituents of *Rubus ulmifolius*. *Journal of Ethnopharmacology* 79: 165–168.

Park, K. M., You, J. S., Lee, H. Y., Baek, N. I. & Hwang, J. K. 2003. Kuwanon G: an antibacterial agent from the root bark of *Morus alba* against oral pathogens. *Journal of Ethnopharmacology* 84: 181–185.

Perera, L. M. S., Ruedas, D. & Gomez, B. C. 2001. Gastric antiulcer effect of *Rhizophora mangle* L. *Journal of Ethnopharmacology* 77: 1–3

Ram, A. J., Bhakshu, L. M. & Raju, R. R. V. 2003. *In vitro* antimicrobial activity of certain medicinal plants from Eastern Ghats, India, used for skin diseases. *Journal of Ethnopharmacology* 90: 353–357.

Richards, R. M. E., Durham, D. G. & Liu, X. 1994. Antibacterial activity of compounds from *Rubus pinfaensis*. *Planta Medica* 60: 471–473.

Saxena, G., Mccutcheon, A. R., Farmer, S., Towers, G. H. N. & Hancock, R. E. W. 1994. Antimicrobial constituents of *Rhus glabra*. *Journal of Ethnopharmacology* 42: 95–99.

Suraya, S. & Darah, I. 2002. SEM and TEM studies of the structural modifications of *Candida albicans* cells after treatment with extract from *Cuculigo latifolia* Dryand. Pp. 203–205 in *Proceeding of the Fourth Regional IMT-GT UNINET Conference*. 15–17 October 2002, Penang.

Voda, K., Boh, B., Vrta-Nik, M. & Pohleven, F. 2003. Effect of the antifungal activity of oxygenated aromatic essential oil compounds on the white-rot *Trametes versicolor* and the brown-rot *Coniophora puteana*. *International Biodeterioration and Biodegradation* 51(1): 51–59.