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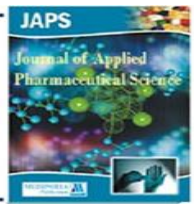

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Gallic acid: An anticandidal compound in hydrolysable tannin extracted from the barks of *Rhizophora apiculata* Blume

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ABSTRACT

A research was conducted to study the anticandidal compound of tannin extracted from the barks of a mangrove tree, *Rhizophora apiculata* Blume. Tannin obtained from the barks of *Rhizophora apiculata* Blume was further separated into condensed and hydrolysable tannins. A strong anticandidal activity was detected in the hydrolysable tannin, which exhibited minimal inhibitory concentration (MIC) of 6.25 mg/ml, and it was found to have yeastostatic activity at lower concentration (below MIC value) and yeastocidal activity at higher concentration (more than the MIC value). Furthermore, the isolation of the bioactive compound in hydrolysable tannin that responsible for the anticandidal activity was also determined using thin layer chromatography and high-performance liquid chromatography (HPLC). The results obtained confirmed that gallic acid was the bioactive compound that plays role in inhibiting and killing the *Candida albicans* cells.

Key words: Hydrolysable tannin, *Rhizophora apiculata*, anticandidal activity, gallic acid.

INTRODUCTION

There is a continuous and need to discover new antimicrobial compounds with diverse chemical structures and novel mechanism of action because there has been an alarming increase in the incidence of new and re-emerging infectious diseases. Another main concern is the development of resistance to the antibiotics in current clinical use. Furthermore, the incidence of infection caused by opportunistic fungal pathogens such as *Candida albicans* has increased markedly with increase in frequency of immunocompromised and immunosuppressive patients as well as patients who are undergoing organ transplantation, cancer chemotherapy and also a number of patients with human immunodeficiency viral infection (Lamagni et al. 2001; Blot et al. 2003; Kibbler et al. 2003; Barnes 2008). Amphotericin B and azole group of drug such as fluconazole are two important agents that have been used to treat diseases caused by human pathogenic fungi and yeasts. However, there are reports on the side effects as well as toxic effects especially if in a prolonged usage (Viscoli et al. 1991). Thus, there is the need for better, novel antifungal agents against these pathogens.

Among all known natural drugs, those originating from plants have been chosen by many people as an apparently limitless source of novel antimicrobial bioactive compounds. Among them, hydrolysable tannin which is a secondary metabolite belonging to the family of vegetable tannin has been reported to possess antimicrobial activity and property. Our previous studies demonstrated that hydrolysable tannin extracted from *Rhizophora apiculata* barks exhibited significant activities against a series of test bacteria and yeasts, but not on filamentous fungi (Darah et al. 2006; Lim et al. 2006). Therefore, this study was carried out to isolate the bioactive

compound(s) of hydrolysable tannin extracted from barks of *R. apiculata* that was responsible against *Candida* albicans, which can cause candidiasis in human.

MATERIAL AND METHODS

Extraction

The barks of *Rhizophora apiculata* Blume were collected from Kuala Sepetang, Daerah Larut Matang, Perak, Malaysia. About 150 grams of coarsely powdered and dried barks was successively extracted using a Soxhlet apparatus with 70 % acetone as a solvent, at 30°C for 3 days. The mixture was then filtered using a cheese cloth followed by Whatman no. 1 filter paper. The resultant extract was concentrated to dryness in a rotary evaporator before freeze-drying it. The crude extract obtained was mixed tannin or called raw tannin, which was further purified by the method of Jain et al. (2002), into two separated groups of tannin namely hydrolysable and condensed tannins.

Culture and Maintenance of *Candida albicans*

A clinical isolate of *C. albicans* was used as the test microorganism and was obtained from the Industrial Biotechnology Research Laboratory, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia. The yeast was cultured on Sabouraud dextrose agar at 30°C for 24 hours. The cultures were then kept at 4°C until further used.

Anticandidal Activity Test Using Disk Diffusion Technique

The 24 hours old *C. albicans* culture was transferred aseptically to a sterile test tube containing 5 ml sterile distilled water. The suspension was adjusted to 4 x 10^5 yeast cells/ml. The yeast suspension was then streaked evenly with a sterile cotton swab on Sabouraud dextrose agar plate (9 cm in diameter). Aseptically, the 20 µl hydrolysable tannin (100 mg/ml) and its fractions impregnated discs (commercial antibiotic discs, Whatman) were placed on the seeded agar plate. Ketoconazole (30 µg/ml) and gallic acid were used as positive controls, whereas sterile distilled water was used as a negative control. The discs were placed on the surface of the seeded agar plates and incubated at 30ºC for 24 hours. Anticandidal activity was indicated by clear zone of growth inhibition.

Determination of Minimum Inhibitory Concentration (MIC)

MIC values of the extracts were determined by broth dilution assay method (Nurul et al. 2010). Briefly, extracts were subjected to a serial dilution to give final concentrations between 0.78 to 100.00 mg/per ml. Extracts (100.00 mg/ml) with different concentration were added aseptically in to different labeled test tubes containing 1.5 ml sterile Sabouraud dextrose broth (SDB). Then, 0.5 ml of the yeast suspension was inoculated in to the respective test tubes.

The test tubes were vortex and incubated at 30ºC for 24 hours. The MIC value was measured by comparing the turbidity of the whole series of the test tubes with a negative control (SDB inoculated with the yeast) and two positive controls (SDB with extract and SDB only). MIC value was stated as the highest concentration that showed no turbidity which indicates no growth. Each test was performed in triplicate.

Times-Kill Study

In order to assess the anticandidal activity with MIC, ½ MIC and 2MIC concentrations over time, growth profile curves were plotted. The 16 hour yeast cells were harvested by centrifugation, washed twice with distilled water and resuspended in sterile distilled water. The suspension was adjusted to OD (540 nm) equivalent to 0.2. The extract was added to aliquots of 25 ml SDB in a 50 ml Erlenmeyer flask to achieve a concentration of 0 (control) and 6.25 mg/ml (MIC), 3.13 mg/ml (1/2 MIC) and 12.5mg/ml (2MIC) after addition of the inoculum. Then, 1.0 ml inoculum was added to all Erlenmeyer flasks. Finally, 1.0 ml portion was removed and the growth of *C. albicans* was monitored spectrophotometrically at 540 nm. The growth of *C. albicans* was measured every 4 hours for 48 hours continuously by the above method.

Isolation of Bioactive Compound

The hydrolysable tannin was separated on silica gel thin layer chromatography (TLC) plate. The eluting solvent systems used are shown in Table 1. The eluting solvent systems that showed the highest number of fractions on TLC plate under UV-light were selected as a mobile phase in a column chromatography. Then, 1.0 g of hydrolysable tannin was chromatographed over silica gel in a column (30.0 cm x 2.0 cm) using the chosen mobile phase. Some 30 fractions of 10 ml each were collected and evaporated to about 1.0 ml. All of the fractions obtained were tested for its anticandidal activity.

Determination of Anticandidal Compound

Purification of the fraction that showed the biggest diameter inhibition zone in anticandidal test was chosen for further separation techniques using HPLC according to the methods described by Kuzuya et al. (2001). The HPLC system for the determination of the compound of interest consisted of L-6200 Intelligent pump and Chromato-Integrator, D-2500. The system was connected to an Intersil ODS Science Inc, 10 µm, 4.6 x 300 mm (Hitachi) column with the flow rate of 1 ml/ minute. Then, 20 µl of samples were injected with sample valve equipped with a 100 µl loop. Chromatograms of HPLC were recorded with Hitachi, L-4250 UV-VIS Detector. Seventy percent of methanol (HPLC grade) was used as a mobile-phase and gallic acid was used as a control in this HPLC system. Two chromatograms were obtained, it was a chromatogram which only showed a fraction and the other chromatogram showed a fraction and gallic acid. The height-peaks of the both chromatograms were compared later.

Statistical Analysis

The data were analyzed by student *t*-test for comparing the extract on the *C. albicans* vs ketoconazole using Statistical Package for the Social Sciences (SPSS version 12.0) software

(SPSS, Chicago, IL, USA). Statistical significance was assumed at the 0.05 levels ($p<0.05$).

RESULTS

MIC Value of the Hydrolysable Tannin on *Candida albicans* Cells

The MIC value obtained was 6.25 mg/ml, whereas the concentrations for 1/2MIC and 2MIC were 3.13 mg/ml and 12.50 mg/ml, respectively.

Times-Kill Study

Time kill-studies were carried-out over a period of 48 hours with the yeast cells being exposed to the MIC value (MIC), half of the MIC value (1/2MIC) and twice of the MIC value (2MIC) of the hydrolysable tannin. The results of the time-kill curves for *C. albicans* are shown in Figure 1. At ½MIC (3.13 mg/ml) value, the hydrolysable tannin exhibited a large drop in OD after 20 hours of exposure, which leads to the stationary phase of the yeast growth compared to the control. At the MIC (6.25 mg/ml) and 2MIC (12.50 mg/ml) values, the hydrolysable tannin produced absolute yeast eradication after 4 hours of exposure. The time-kill curves described above show that the hydrolysable tannin has potency to inhibit the yeast cells (yeastostatic) at lower concentration (in this case at the 1/2MIC value of 3.13 mg/ml) and to kill the cells (yeastocidal) at concentrations of MIC (6.25 mg/ml) and above.

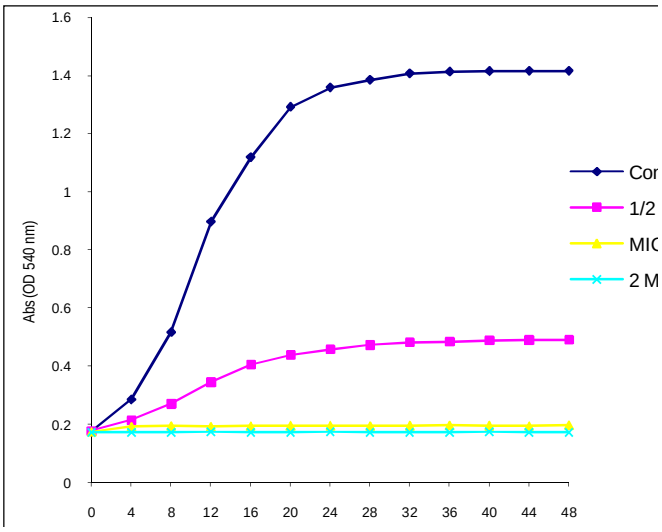

Fig 1: Effects of the hydrolysable tannin on *Candida albicans*. Control (SDB + inoculums); MIC, 6.25 mg/ml; ½ MIC, 3.13 mg/ml; 2MIC, 12.5 mg/ml.

Isolation of the Bioactive Compound with Anticandidal Properties

The results of the separation systems of hydrolysable tannin on TLC plate are shown in Table 1. It was found that methanol: water (7:3) showed a better result than the other eluting solvent systems. Three distinct bands were separated well on TLC plate and it can be seen clearly under visible UV-light. Therefore, methanol: water (7:3) was selected as the mobile phase to be used for column chromatography.

Table 1: Fraction of hydrolysable tannin on TLC plate counted under UV-light.

Eluting solvents	Hydrolysable tannin
	Total number of fractions
Methanol 100%	2
Methanol : water (9:1)	2
Methanol : water (8:2)	2
Methanol : water (7:3)	3
Methanol : water (6:4)	1
Hexane : chloroform (3:2)	0
Benzene : butane : acetic acid (4:1:5)	0
Chloroform : acetone (4:1)	0
Chloroform : methanol (17:3)	0
Hexane : acetic acid (1:1)	1
Hexane : acetone (1:4)	2
Chloroform 100%	0
Butane : water (1:1)	0
Benzene : chloroform (10:1)	0
Chloroform : methanol (2:8)	1
Chloroform : methanol: water (13:7:1)	1

About thirty fractions were obtained from the elution of the hydrolysable tannin in the column chromatography (Table 2).

Table 2: Fractions of hydrolysable tannin against *Candida albicans*

Fraction of hydrolysable tannin	Inhibition zone (mm)
Hydrolysable tannin	19
Gallic acid (positive control)	25
Water (negative control)	-
Fraction 1	-
Fraction 2	14
Fraction 3	14
Fraction 4	13
Fraction 5	-
Fraction 6	17
Fraction 7	17
Fraction 8	18
Fraction 9	15
Fraction 10	17
Fraction 11	-
Fraction 12	19
Fraction 13	18
Fraction 14	12
Fraction 15	-
Fraction 16	19
Fraction 17	15
Fraction 18	19
Fraction 19	19
Fraction 20	20
Fraction 21	15
Fraction 22	23
Fraction 23	21
Fraction 24	16
Fraction 25	14
Fraction 26	-
Fraction 27	-
Fraction 28	-
Fraction 29	-
Fraction 30	-

Key: - no inhibition zone occurred

All the fractions obtained were tested against *Candida albicans* for their anticandidal activity. Fraction-22 was the most effective fraction and produced about 23.0 mm of zone of inhibition ($P\leq0.05$). Other fractions except for fractions 1, 5, 11, 15, 26, 27, 28, 29 and 30, showed significant diameter of zones of inhibition. The results indicated the present of anticandidal compounds in the hydrolysable tannin.

Figure 2 shows the chromatogram of HPLC for fraction-22 of hydrolysable tannin of *Rhizophora apiculata* Blume. Figure 2A shows that there were three peaks exhibited with retention time of 2.04, 2.96 and 3.28 minutes. However, the second peak with a retention time of around 2.96 minutes showed the highest peak. Figure 2B showed a chromatogram from the combination of fraction-22 and gallic acid, when pure gallic acid was used as a positive control. The result showed that the retention time for both, fraction-22 and gallic acid exhibited were about 2.91 minutes. After doing a detailed analytical studies, it seems that gallic acid was the key component in the hydrolysable tannin which posses a significant anticandidal activity.

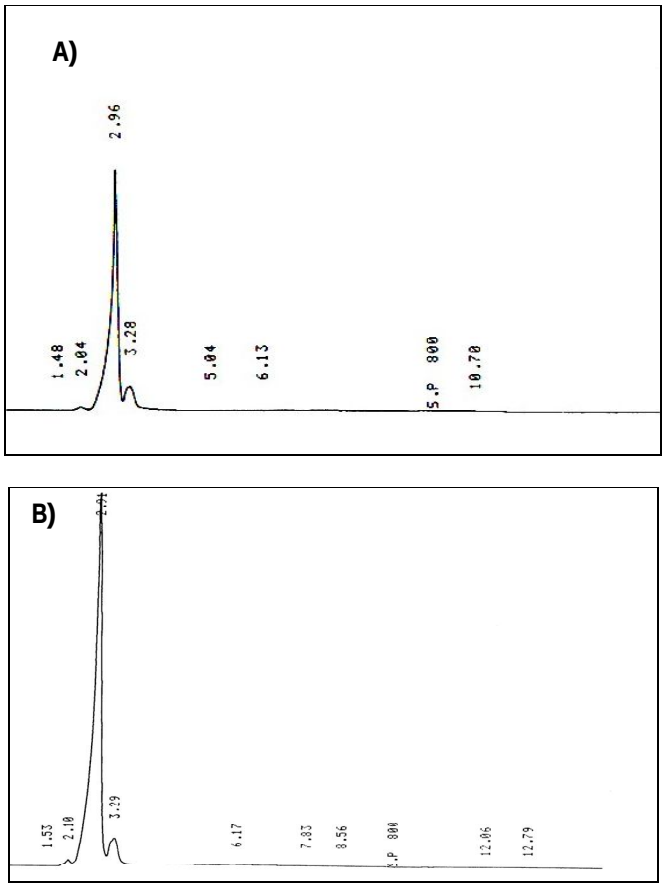

Fig 2: Chromatogram of HPLC for fraction-22 hydrolysable tannin of *Rhizophora apiculata* Blume. (a) Fraction -22, (b) Fraction-22 added with gallic acid.

DISCUSSION

In the last three decades, research has focused on using of plant components as antimicrobial, antiviral and antifungal agents which are cheaper, safer and less side effects. Thus plant components are preferred in the cure of the infections.

Tannins are water-soluble polyphenols that are commonly found in higher herbaceous and woody plants and can be classified into two groups: hydrolysable and condensed tannins (Romani et al. 2006; Buzzini et al. 2008). Hydrolysable tannins are based on gallic acid, usually as multiple esters with D-glucose, while the more numerous condensed tannins (often called proanthocyanidins) are derived from flavonoid monomers. Hydrolysable tannins are characterized by being water soluble and by having molecular weight between 500 to ≥5000 Da. An important group of hydrolysable tannins are esters of gallic acid with glucose, the products of their oxidation and polymerization, as well as other oxidized and polymerized derivatives. Hydrolysable tannin is different from condensed tannin that are derivatives of catechins (Karonen et al. 2004). The results obtained from this study proved that gallic acid that present in the hydrolysable tannin extracted from barks of *R. apiculata* is responsible as the antifungal or specifically as antiyeast against *C. albicans*. Panizzi et al. (2002) also found that gallic acid isolated from extract of *Rubus ulmifolius* was one of the compounds that showed anticandidal activity.

Even though, the actual mechanism of action of the hydrolysable tannin (in this case gallic acid) on yeast cells has not been widely studied, but it can be postulated that its antifungal activity is by disrupting the structure of the cell membrane and inhibiting the normal budding process due to the destruction of the membrane integrity (Kim et al. 2009). Endo et al. (2010) had found that a compound extracted from pomegranate showed an irregular budding pattern and pseudohyphae formation of *C. albicans* cells. Nevertheless, its mechanism of action also could be due to the inhibition on the biosynthesis of ergosterol, the main sterol in membranes of fungi including yeasts (Ghannoum and Rice 1999). Sterols are essential structural and regulatory components of eukaryotic cell membranes (Iwaki et al. 2008). Ergosterol is the end product of the sterol biosynthetic pathway and is the major sterol in yeasts. Like cholesterol in mammalian cells, it is responsible for membrane fluidity and permeability (Parks and Casey 1996).

CONCLUTION

In this study, the gallic acid from hydrolysable tannin extracted from the barks of *R. apiculata* possessed a significant antiyeast (anticandidal) activity towards some yeast species of medically importance. In conclusion, it is anticipated that galic acid from *R. apiculata* can find its potential as a novel of antiyeast agent might be useful to cure particularly candidiasis.

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