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Antifungal activity of alpha-mangostin against *Candida albicans*

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Abstract: This study was conducted to examine the activity of alpha-mangostin against *Candida albicans*, the most important microorganism implicated in oral candidiasis. Its activity was compared to Clotrimazole and Nystatin. Results showed that alpha-mangostin was effective against *C. albicans*, the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were 1,000 and 2,000 µg/ml, respectively. The *C. albicans* killing activity of alpha-mangostin was more effective than Clotrimazole and Nystatin. The cytotoxicity of alpha-mangostin was determined and it was found that alpha-mangostin at 4,000 µg/ml was not toxic to human gingival fibroblast for 480 min. The strong antifungal activity and low toxicity of alpha-mangostin make it a promising agent for treatment of oral candidiasis. (J Oral Sci 51, 401-406, 2009)

Keywords: *Garcinia mangostana* Linn; alpha-mangostin; antifungal activity; *Candida albicans*.

Introduction

Oral candidiasis is a common oral lesion caused by overgrowth of fungal species in the genus *Candida*. Among the many species, *Candida albicans* is the most important microorganism implicated in fungal infection (1). These microorganisms reside in the oral cavity as part of the normal microflora, however, they can cause disease when the host is predisposed. Several local and systemic host factors, particularly impaired immunity, can facilitate opportunistic infection by *Candida* species (2). Therefore, the rising number of people with immunocompromising conditions including human immunodeficiency virus (HIV) infection, old age, endocrine disorders, radiotherapy, malignant diseases and other critical illnesses may have led to a gradual increase in the prevalence of oral candidiasis over the past decade (3). The lesions may cause burning sensation and interfere with food intake, thus adversely affecting the patient's general health and quality of life (4). Several groups of antifungal drugs are currently available. The most efficient ones for the treatment of oral candidiasis are polyenes and azoles (5). However, the choices are still rather limited, especially when there is a rise in antifungal resistance due to an increase in the use of these drugs for the treatment and prevention of opportunistic fungal infection in HIV-infected and immunocompromised individuals (6). For these reasons, it is necessary to identify new antifungal agents and research into new antifungal drug discovery is of great importance in the fight against fungal infections in the future.

The pericarps of mangosteen, *Garcinia mangostana* Linn., have been used as a traditional medicine for treatment of diarrhea, skin infection and chronic wounds in Southeast Asia for many years (7). Extract from its pericarp has demonstrated antibacterial activity against a wide variety of microorganisms (8-9). Moreover, antifungal activity of the extract from *G. mangostana* against three phytopathogenic fungi, *Fusarium oxysporum vasinfectum*, *Alternaria tenuis*, and *Dreschlera oryzae*, has been described (10). Phytochemical studies have shown that mangosteen pericarp extract contains several active components belonging to a group of xanthone derivatives

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such as alpha-, beta-, gamma-mangostin, garcinone, mangostanol and gartinin (11). Among these, alpha-mangostin has been shown by several studies to exert the most potent antibacterial activity (8-9). Previous studies, both *in vitro* and *in vivo*, have demonstrated low toxicity of the mangosteen pericarp extract and its active components (12-15).

Owing to its antifungal activity and low toxicity, the alpha-mangostin may have potential for oral candidiasis therapy. The purpose of this study was to determine the minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC) and kinetics of killing of alpha-mangostin against *C. albicans*. Its activity was also compared to Clotrimazole and Nystatin, antifungal agents commonly used in oral candidiasis treatment.

Materials and Methods

Preparation of alpha-mangostin

Pericarps of mangosteen were collected from Thewate market, Bangkok, Thailand. Crude extract and purified alpha-mangostin were prepared. Briefly, dried and ground pericarps were macerated in hexane for 24 h to remove non-polar substances. The resulting macerate was subsequently macerated in ethyl acetate for 24 h. The ethyl acetate extract was then recrystallized, and ground into powder. The yield of mangosteen crude extract from the dried pericarp was approximately 3% (w/w). To obtain alpha-mangostin, the crude extract was chromatographed on a silica gel column, and eluted with increasing percentages of ethyl acetate in hexane (0-25%). A hexane-ethyl acetate (4:1) elute was selected based on the thin layer chromatography profile. The selected fraction was further identified as alpha-mangostin by using mass spectrometry, nuclear magnetic resonance spectroscopy and a Gallenkamp melting point apparatus. The yield of alpha-mangostin from the dried pericarp was approximately 0.4% (w/w) and the concentrated stock solution was prepared in dimethyl sulfoxide (DMSO).

Antifungal agents and Candidal culture

Both Clotrimazole powder (Nida Pharma Co. Ltd., Bangkok, Thailand) and Nystatin powder (Continental-Pharm Co. Ltd., Bangkok, Thailand) from concentrated stock solutions were prepared in DMSO. *Candida* strain used in this study was *C. albicans* ATCC 90028 and was cultured in Sabouraud dextrose broth and agar (Becton, Dickinson and Company, Sparks, MD, USA) at 37°C.

Susceptibility testing

A broth dilution technique was employed to determine the susceptibility of the *C. albicans* to alpha-mangostin, clotrimazole and nystatin (16-17). Susceptibility was expressed as minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC). The stock solutions of three agents were dissolved in DMSO. clotrimazole and nystatin were used as positive controls. Solvent and media controls were used for reference. The Sabouraud dextrose broth containing varying amounts (serially and two-fold diluted) of alpha-mangostin was inoculated with actively dividing *C. albicans*. The initial density of *Candida* was approximately $2\text{-}5 \times 10^6$ colony forming units (CFU)/ml. After 24 h incubation, the growth was monitored both visually and colorimetrically (at 600 nm). The MIC was defined as the lowest concentration required to arrest the growth of the fungi at the end of 24 h incubation. For determination of MFC, a 0.01-ml aliquot of the medium drawn from the culture tubes showing no macroscopic growth at the end of the 24-h culture was sub-cultured on Sabouraud dextrose agar plates to determine the number of vital organisms and incubated further for appearance of yeast-like growth. The MFC was defined as the lowest concentration of the agent that reduced the number of viable organisms by 99.9% (18). All MIC and MFC experiments were repeated three times.

Time-kill assay

Measuring the rate of fungicidal activity by time-kill assay can assess the speed with which killing may occur at a given drug concentration (19-20). *C. albicans* in the log growth phase were used to prepare suspensions of 10^4 CFU/ml in Sabouraud dextrose broth. The agents (alpha-mangostin, Clotrimazole or Nystatin) at the concentrations of $2 \times$ MFC and $4 \times$ MFC were added to the suspensions. Aliquots were removed at 10, 20, and 30 min after the start of the experiment and plated onto Sabouraud dextrose agar. The numbers of CFU were counted after incubation for 24-48 h. The broth without any agents was used as the control for *Candida* growth at each time point. Relationship between the treatment time and the viable cell count (CFU/ml) was performed by the time-kill curve. Tests were performed three times.

Cytotoxicity test

Human gingival fibroblasts were obtained from explants of gingival tissue attached to non-carious, freshly extracted third molars. All patients gave informed consent for tissue collection. Ethical approval for the study had been obtained from Naresuan University. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 5 µg/ml amphotericin B at 37°C in a humidified

atmosphere of 95% air and 5% CO_2. Medium and supplements were purchased from GibcoBRL (Grand Island, NY, USA). Cells were seeded at 5×10^4 cells per well of 24-well plate, and then cells were treated with alpha-mangostin at 2 × MFC in serum free medium (SFM) for 240 and 480 min for determining the cytotoxic effect of the alpha-mangostin. The cytotoxicity of alpha-mangostin was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. This assay is based on the reduction of the yellow tetrazolium salt to purple formazan crystals by dehydrogenase enzymes secreted from the mitochondria of metabolically active cells. The amount of purple formazan crystal formation is proportional to the number of viable cells. Before terminating the experiment, the culture medium was aspirated, replaced with 0.5 μg/ml MTT solution and incubated for 30 min in a CO_2 incubator. The solution was then aspirated and 1,000 μl DMSO was added to dissolve the formazan crystals. After 30 min of rotary agitation, the absorbance of the solution at 540 nm was measured using Genesis10 UV-vis spectrophotometer (Thermo Spectronic, NY, USA). The viable cell number was calculated from the standard curve of cell number by plotting a scattergram of the absorbance value against the known number of cells.

Statistical analysis

All statistical computations were performed by SPSS for Windows (version 13.0; SPSS, Inc., Chicago, IL, USA). Data from time-kill kinetics were presented as means ± standard deviations. Differences in viable *Candida* count at each time point were analyzed by one-way analysis of variance (ANOVA). To compare the differences between groups, a post-hoc test was used. Statistical significance was defined as $P < 0.05$.

Results

The MIC and MFC values obtained for alpha-mangostin, Clotrimazole and Nystatin susceptibility testing determined by broth dilution technique are summarized in Table 1. The alpha-mangostin was active against *C. albicans*, with the MIC and MFC at 1,000 and 2,000 μg/ml, respectively. clotrimazole and nystatin, antifungal drugs used as positive control in this study, were strongly active against *C. albicans*, with MIC and MFC values lower than those for alpha-mangostin. The time-kill assay was conducted to determine the rates at which *C. albicans* were killed exposed to alpha-mangostin, clotrimazole, or nystatin at the concentrations of 2 × MFC and 4 × MFC for 10, 20 and 30 min (Fig. 1). The employed concentrations were 4,000 and 8,000 μg/ml for alpha-mangostin, 40 and 80 μg/ml for clotrimazole and 640 and 1,280 μg/ml for nystatin. At 10 min, the group treated with the alpha-mangostin at 2 × MFC and 4 × MFC showed a dramatic decrease in viable cell count, while the group treated with clotrimazole at 2 × MFC or 4 × MFC or nystatin at 2 × MFC was unchanged in viable cell count, and the group treated with Nystatin at 4 × MFC showed a significant decrease in viable cell count. At 20 min, the alpha-mangostin at both concentrations completely killed (99.9% reduction of the starting inoculums) the *C. albicans*, the group treated with clotrimazole at 2 × MFC or 4 × MFC was still unchanged in viable cell count, while nystatin at 2 × MFC or 4 × MFC decreased. At 30 min, the group treated with clotrimazole at 2 × MFC or 4 × MFC showed a slight decrease in viable cell count, while nystatin at 2 × MFC or 4 × MFC continued to decrease. The time-kill assay that demonstrates the rate of *Candida* killing showed alpha-mangostin to be more effective than clotrimazole and nystatin. When compared at the same concentration (2 × MFC and 4 × MFC) and at the same time point (10, 20, 30 min), significant differences in the number of remaining viable *C. albicans* were observed between the alpha-mangostin-treated group and antifungal drugs-treated groups. Next, the cytotoxic effect of the alpha-mangostin on human gingival fibroblast was investigated (Fig. 2). Human gingival fibroblast was incubated with the alpha-mangostin at 2 × MFC (4,000 μg/ml) for 240 and 480 min,

Table 1 The MIC and MFC (μg/ml) of alpha-mangostin against *C. albicans* compared to those of clotrimazole and nystatin

Pathogen	Alpha-mangostin		Clotrimazole		Nystatin	
	MIC	MFC	MIC	MFC	MIC	MFC
Candida albicans	1000	2000	10	20	160	320

MIC, the minimum inhibitory concentration; MFC, the minimum fungicidal concentration

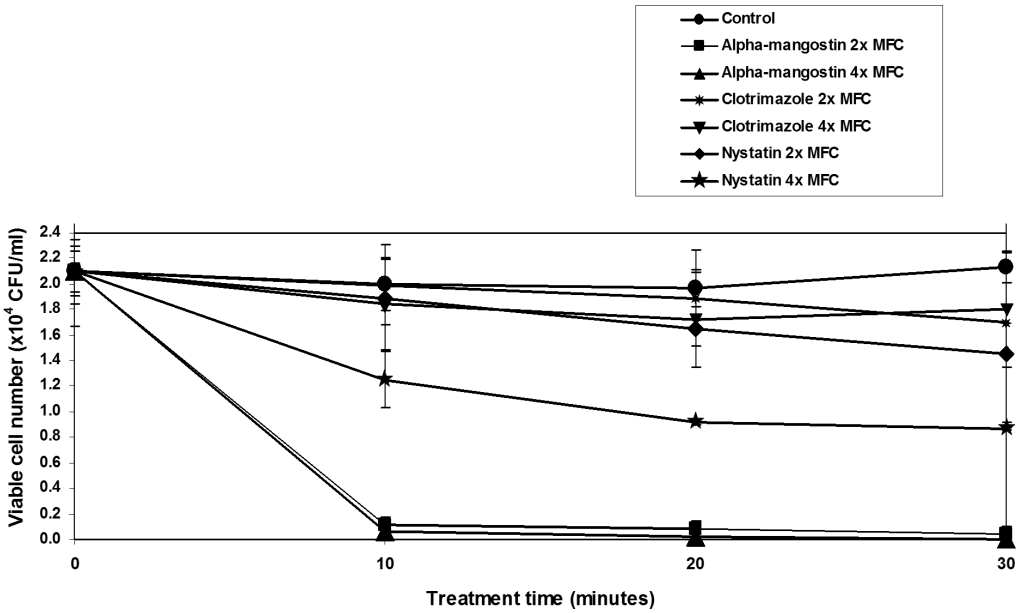

Fig. 1 Time-kill assay. Time-kill curve for *C. albicans* plotted as the number of remaining viable cells (CFU/ml) against time. The *Candida* was treated with alpha-mangostin, or clotrimazole, or nystatin at the concentrations of 2 × MFC and 4 × MFC for 10, 20 and 30 min. The employed concentrations were 4,000 and 8,000 μg/ml for alpha-mangostin, 40 and 80 μg/ml for clotrimazole and 640 and 1,280 μg/ml for nystatin. The results are presented as means ± standard deviations of three independent experiments.

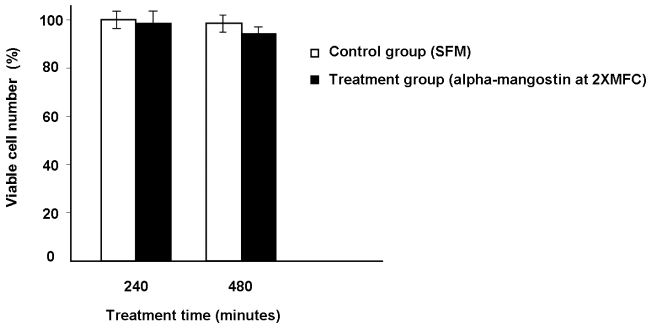

Fig. 2 The cytotoxicity of alpha-mangostin on human gingival fibroblast cells. Cells were treated with alpha-mangostin at 2 × MFC (4,000 μg/ml) for 240 and 480 min and the viable cell number was determined by MTT assay. Histograms represent average percentage of cell number from three independent experiments which were performed in triplicate.

and then the cytotoxicity of the alpha-mangostin was determined by the MTT assay. No difference in cell number was observed between the alpha-mangostin-treated group and control group in both 240 and 480 min of the treatment.

Discussion

In the 1990s, there has been a dramatic increase in the prevalence of fungal infections, which is probably the result of alterations in immune status. At the same time, drug resistance has become an important issue in a variety of fungal infections, which will have profound effects on human health. Oropharyngeal candidiasis due to drug-resistant fungi is a major problem for patients infected with HIV (3,6). The rise in prevalence of fungal infections and drug resistance has exacerbated the need for new antifungal agents. The xanthone compound is present in many natural products, including some compounds that have activity against pathogenic fungi (21-24). The pericarp of mangosteen is a concentrated source of xanthones. Xanthones in the pericarp are composed of alpha-, beta-, gamma-mangostin, garcinone, mangostanol and gartinin (11). Furthermore, a previous study showed that extract from mangosteen pericarp has demonstrated antifungal activity against phytopathogenic fungi, *Fusarium oxysporum vasinfectum*, *Alternaria tenuis*, and *Dreschlera oryzae* (10). The present study also demonstrated the antifungal activity of alpha-mangostin against *C. albicans*. For the time-kill assay, we compared the antifungal activity of alpha-mangostin with that of clotrimazole and nystatin in order to find out a new natural inexpensive agent that might be used as adjunct therapy in the management of oral candidiasis. The results showed that the rate of *Candida*

killing of alpha-mangostin was more than clotrimazole and nystatin. However, we found that clotrimazole and nystatin showed a slight decrease in viable cell count in 30 min. This might be due to the onset of action of these agents, which was longer than 30 min. On the other hand, alpha-mangostin exerts rapid antifungal activity against *C. albicans* within 20 min. This property makes it a promising new agent for treatment of oral candidiasis.

Regarding the cytotoxicity test, our results indicate that the alpha-mangostin at 2 × MFC (4,000 μg/ml) was not toxic to human gingival fibroblast for 480 min. Previous studies, both *in vitro* and *in vivo*, have also demonstrated low toxicity of the mangosteen pericarp extract and its active components (12-15). The extract from mangosteen pericarp was not toxic to gingival fibroblast for up to 48 hours at the concentration 200 μg/ml (12). Alpha-mangostin, an active component of the mangosteen pericarp, was administered orally to rats at a high dose (1.5 g/kg body weight) to test its hepatotoxicity. After 12 h, increases in serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) activities were found to be much less than those of paracetamol given at the same dose (13). Another study which used alpha-mangostin given to rats at an oral dose of 200 mg/kg body weight per day did not observe any toxicity after 6 days of treatment (14). In human clinical trials, herbal mouth-wash containing the pericarp extract of *Garcinia mangostana* was used in subjects with chronic gingivitis up to 2 weeks and no local irritation or side effects were observed (15). However, the antifungal mechanisms of alpha-mangostin have not been evaluated. Alpha-mangostin may act by attacking the structure and function of the fungal cell especially ergosterol, the main lipid component in membranes and the most important for the life of fungi that is not present in animals (25). Thus, further studies are required to clarify its mechanism against *C. albicans*.

In conclusion, this study showed that alpha-mangostin was effective against *C. albicans*. Regarding cytotoxicity, the alpha-mangostin at 2 × MFC (4,000 μg/ml) was not toxic to human gingival fibroblast for 480 min. Thus, the strong, rapid antifungal activity and low toxicity of alpha-mangostin may make it a good candidate for further development as an antifungal agent for oral candidiasis therapy. However, the clinical significance of these *in vitro* results should be verified by clinical trials.

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