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Inhibitory Effect of Alpha-Mangostin on Adhesion of *Candida albicans* to Denture Acrylic

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Abstract: *Objective*: *Candida*-associated denture stomatitis is a very common disease affecting denture wearers. It is characterized by the presence of yeast biofilm on the denture, primarily associated with *C. albicans*. The investigation of agents that can reduce *C. albicans* adhesion may represent a significant advancement in the prevention and treatment of this disease. This study aims to investigate the effect of alpha-mangostin on the *in vitro* adhesion of *C. albicans* to denture acrylic and germ tube formation by *C. albicans* and to compare its activity with clotrimazole which is a topical antifungal agent commonly used for the treatment of *Candida*-associated denture stomatitis. *Materials and Methodology*: Alpha-mangostin was extracted by thin layer chromatography. The effect of alpha-mangostin on adhesion of *C. albicans* to denture acrylic was determined by using a colorimetric tetrazolium assay and germ tube formation by *C. albicans* was determined by using the counting chamber. *Results*: A significant reduction of *C. albicans* adhesion to denture acrylic was evident after exposure to 2,000 µg/ml of alpha-mangostin for only 15 min. In addition, the 2,000 µg/ml of the alpha-mangostin-treated *C. albicans* had a reduced ability for germ tube formation. These inhibitory effects of alpha-mangostin were as effective as clotrimazole. *Conclusion*: Alpha-mangostin has antifungal property against *C. albicans* by inhibiting the adhesion to denture acrylic and germ tube formation *in vitro*. These results suggest the potential application of alpha-mangostin as a topical medication or a natural oral hygiene product for treatment of *Candida*-associated denture stomatitis.

Keywords: Adhesion, alpha-mangostin, *Candida albicans*, denture acrylic, denture stomatitis, germ tube formation.

INTRODUCTION

Candida-associated denture stomatitis is a very common disease affecting denture wearers, which occurs in up to 65-70% of denture wearers [1, 2]. It usually manifests as an erythematous and edematous area of the mucosa in contact with the fitting surface of the denture [3]. This disease is characterized by the presence of yeast biofilm on the denture, primarily associated with *C. albicans*, which has been shown to be the principal *Candida* species accountable for inflammatory pathology [4, 5]. Clinical studies have shown that *C. albicans* is not only able to adhere to mucosal surface, but also acrylic resin denture and produce a complex biofilm [6, 7]. The presence of the biofilm on the denture is critical to the development of the observed clinical entity of *Candida*-associated denture stomatitis [8]. Despite treating with antifungal therapy, infection can be re-established soon after treatment ceases, suggesting that the denture act as a reservoir for *C. albicans* to continually re-infect the mucosal surface [7]. As the adhesion of *C. albicans* to the denture acrylic is a prerequisite for colonization and biofilm formation [9], the investigation of novel antifungal agents, which can reduce *C. albicans* adhesion, will represent a significant advance in the prevention and treatment of *Candida*-associated denture stomatitis.

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Natural products have been proved to be an alternative to synthetic chemical substances and many plant extracts exert antifungal activity [10-12], including alpha-mangostin [13]. Alpha-mangostin is an antimicrobial xanthone isolated from *Garcinia mangostana* Linn. (mangosteen). The pericarp of mangosteen fruit has been used as traditional medicine for treatment of skin infections, wounds, and diarrhea by Southeast Asians for many years [14, 15]. The results from our previous study revealed that alpha-mangostin was active against *C. albicans*, with the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) at 1 and 2 mg/ml, respectively [13].

In order to provide the information on the use of alpha-mangostin as a topical medication or a natural oral hygiene product, we determined the effect of alpha-mangostin on the *in vitro* adhesion of *C. albicans* to denture acrylic and germ tube formation by *C. albicans*. Its activity was compared with clotrimazole, which is a topical antifungal agent commonly used for treatment of *Candida*-associated denture stomatitis.

MATERIALS AND METHODOLOGY

Preparation of Alpha-Mangostin

Crude extract and purified alpha-mangostin were prepared based on methods in previous articles [13, 16, 17]. Briefly, dried and ground mangosteen pericarps were macerated in hexane for 24 h to remove non-polar substances. The resulting marc was subsequently macerated in ethyl acetate

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for 24 h. The ethyl acetate extract obtained was then recrystallized, and ground into power. 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). The stock solution of alpha-mangostin was dissolved in dimethysulfoxide (DMSO) and sterilized by filtration through 0.2 μm disc filters.

Antifungal Agent and *C. albicans* Culture

Clotrimazole powder (Continental-Pharm Co. LTD., Bangkok, Thailand) was prepared as concentrated stock solution in DMSO. The final concentration of prepared clotrimazole stock solution was 48 μg/ml. *C. albicans* strain ATCC 90028 used in this study was obtained from the microbiology laboratory of the Department of Medical Sciences, Ministry of Public Health, Thailand. The working stock cultures of *C. albicans* were maintained on Sabouraud dextrose agar slants (Becton, Dickinson and Company, Sparks, MD, USA) at 4°C until use. *Candida* cells to be tested were grown in Sabouraud dextrose broth for 24 h at 37°C.

Preparation of Denture Acrylic

The acrylic strips were prepared based on the method in a previous report [18]. Self-polymerizing acrylic powder and monomer liquid were mixed according to the manufacturer's instruction (Leone Spa, Sesto Fiorentino, Italy). The mixture was placed between two glass slides (2.5 cm × 7.5 cm), which were fixed at both ends with two clips. Then the acrylic was polymerized in a hydroflask at 50°C for 5 min afterwards, the formed transparent acrylic sheet was stripped from the slides and cut into strips (5 mm × 5 mm square and 0.4 mm thick). These strips were immersed in distilled water for 1 week to leach excess monomer then dipped into 70% alcohol for 1 min and washed three times in distilled water. After that the strips were ultrasonicated for 20 min at 60°C. Finally, the strips were sealed in the petri dishes for sterilization in autoclave for 15 min at 1.2 bar, 121°C, then the strips were dried overnight in a hot air oven at 50°C prior to use in adhesion assay.

Effect of Alpha-Mangostin on Adhesion of *C. albicans* to Denture Acrylic

The adhesion assay was modified from the previous reports [18, 19]. 0.2 ml of different alpha-mangostin and clotrimazole concentrations (1×MFC, 2×MFC and 4×MFC) was placed in 48-well plates (Thermo Fisher Scientific, Roskilde, Denmark). Wells containing 0.2 ml of normal saline solution (NSS) served as the negative control. *Candida* suspension were spectrophotometrically standardized to 10^8 cells/ml. Then the acrylic strips were placed in the 48-well plates and 0.2 ml of *Candida* suspension in Sabouraud dextrose broth was added to each well, which completely soaked the acrylic strips, and incubated at 37°C for 3 h with agitation at 120 rpm/min. After incubation, the acrylic strips were removed from the wells and washed three times with phosphate buffered saline (PBS) to remove the unattached *Candida* cells. Then the acrylic strips were placed in the new 48-well plates. The fungal cell viability was determined using a 2,3-bis (2-methoxy-4-nitro-5-sulfo-phenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium hydroxide (XTT) (Sigma, St. Louis, MO, USA) reduction assay that measures the activity of mitochondrial dehydrogenase. Briefly, XTT solution (1 mg/ml) was prepared by dissolving XTT powder in PBS, and the solution was filter-sterilized (0.22 μm pore size filter). XTT solution was mixed with freshly prepared menadione solution (0.4 mM) at 20:1 (v/v) immediately prior to the assay. XTT-menadione solution 200 μl was transferred to each well, and incubated in the dark for 2 h at 37°C. After the incubation, the colored supernatant (100 μl) was transferred to new microtiter plates, and the optical density of the supernatant was measured at 490 nm with a microtiter plate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA). All experiments were performed on three separate occasions with triplicate determinations on each occasion.

To determine the minimum duration of alpha-mangostin in reducing *C. albicans* adhesion to denture acrylic, the alpha-mangostin at the lowest concentration capable of reducing candidal adhesion to denture acrylic as effective as 1xMFC (20 μg/ml) of clotrimazole was chosen. Adhesion of *C. albicans* to denture acrylic was determined after exposure to alpha-mangostin or clotrimazole for 1, 15, 30, 60, 120 and 180 min. All experiments were performed on three separate occasions with triplicate determinations on each occasion.

Effect of Alpha-Mangostin on Germ Tube Formation

Effect of alpha-mangostin on germ tube formation was performed based on the method in a previous report [20]. The germ tube formation was induced in a medium containing fetal bovine serum (10% v/v) (Gibco-BRL, Gland Island, NY, USA) as an inducer of germ tube formation. Aliquots of 100 μl from *Candida* suspension (1-5 x 10^7 viable cell/ml) were incubated in sterile tubes containing Sabouraud dextrose broth medium (2 ml) in the presence of 10% fetal bovine serum and different alpha-mangostin and clotrimazole concentrations (1×MFC, 2×MFC and 4×MFC). The contents of the tubes were vigorously mixed and incubated at 37°C. After 3 h of incubation, aliquots were taken to count the cell number microscopically. The total cell number and germ tube formation were counted using the counting chamber, and the germ tube formation was expressed as a percentage of cells forming germ tube from the total cell number. All experiments were performed on three separate occasions with triplicate determinations on each occasion.

Statistical Analysis

All statistical computations were performed by SPSS for Windows software (version 15.0; SPSS Inc., Chicago, IL, USA). Data from adhesion assay and germ tube formation were presented as means and standard error. Differences in the adhesion of *C. albicans* to the denture acrylic and germ tube formation at each group were analyzed by one-way

Table 1. Adhesion of *Candida albicans* to denture acrylic following a 3-h exposure to various concentrations of alpha-mangostin and clotrimazole.

	Control	Alpha-mangostin (μg/ml)			Clotrimazole (μg/ml)		
		2,000	4,000	8,000	20	40	80
OD_{490} (mean ± S.E.) (n=9)	0.507±0.034	0.010±0.029*	0.087±0.008*	0.035±0.006*	0.071±0.009*	0.058±0.015*	0.024±0.004*
Reduction in adherence (%)	0	80.28	82.68	93.16	85.86	88.56	95.27

* $P<0.05$ compared with control

Table 2. Adhesion of *Candida albicans* to denture acrylic after exposure to 2,000 μg/ml of alpha-mangostin and 20 μg/ml of clotrimazole at various time intervals.

Time (min)	OD_{490} (mean ± S.E.) (n=9)			Reduction in adherence (%)	
	Control (*Candida*+acrylic)	Alpha-mangostin (2,000 μg/ml)	Clotimazole (20 μg/ml)	Alpha-mangostin (2,000 μg/ml)	Clotimazole (20 μg/ml)
1	0.035±0.008	0.034±0.010	0.026±0.006	2.93	25.71
15	0.319±0.029	0.200±0.021*	0.126±0.025*	37.30	60.45
30	0.331±0.017	0.157±0.046*	0.097±0.005*	52.56	70.56
60	0.351±0.021	0.138±0.013*	0.087±0.012*	60.48	75.18
120	0.386±0.023	0.127±0.010*	0.065±0.007*	66.98	83.17
180	0.507±0.034	0.010±0.029*	0.071±0.009*	80.28	85.86

* $P<0.05$ compared with control

analysis of variance (ANOVA). Statistical significance was defined as $P < 0.05$.

RESULTS

The adhesion values and the percentage reduction in the adhesion of *C. albicans* to denture acrylic following a 3-h exposure to various concentrations of alpha-mangostin and clotrimazole were presented in Table **1**. Compared with the control, a significant reduction of *C. albicans* adhesion to denture acrylic was observed after exposure to alpha-mangostin at concentration of ≥ 2,000 μg/ml ($P< 0.05$). Clotrimazole, an antifungal drug used as positive control in this study, strongly inhibited the adhesion of *C. albicans* to denture acrylic. A significant reduction of *C. albicans* adhesion to denture acrylic was observed after exposure to clotrimazole at concentration of ≥ 20 μg/ml when compared with control ($P< 0.05$).

The alpha-mangostin at a concentration of 2,000 μg/ml was capable of significantly reducing candida adhesion to denture acrylic as effective as 20 μg/ml of clotrimazole. Thus, 2,000 μg/ml of alpha-mangostin was used to determine the minimum duration of alpha-mangostin in reducing *C. albicans* adhesion to denture acrylic. The adhesion and the percentage reduction in the adhesion of *C. albicans* to denture acrylic after exposure to 2,000 μg/ml of alpha-mangostin and 20 μg/ml of clotrimazole at various time intervals are presented in Table **2**. Compared to the control, a significant reduction of *C. albicans* adhesion to denture acrylic was observed after exposure to 2,000 μg/ml of alpha-mangostin for only 15 min ($P<0.05$). The effect of alpha-mangostin and clotrimazole on germ tube formation by *C. albicans* was also investigated. As shown in Table **3**, the suppression of germ tube formation was significant in comparison with that of the control ($P< 0.05$).

DISCUSSION

The most prevalent oral infection involving *Candida* is *Candida*-associated denture stomatitis [21]. Denture wearing is the major predisposing factor, especially in cases the denture is not sufficiently cleansed or is retained overnight in the oral cavity. Under these conditions, the dormant area above the upper fitting surface of the denture provides an ideal environment for growth of *Candida*, which is adept at adhering to denture acrylic. Adherence of *Candida* to denture acrylic is a crucial first step in the initiation and propagation of *Candida*-associated denture stomatitis [22].

Several groups of antifungal drugs are currently available for treatment of *Candida*-associated denture stomatitis. However, the increasing prevalence of drug-resistant *C. albicans* recovered from patients is a major concern worldwide. Therefore, it is necessary to develop new classes of antifungal agents. Among a number of candidate therapeutic agents, plant extracts have recently received an increasing attention. The effects of the medicinal plants claimed to be useful as antifungal agents in the treatment of *Candida*-associated denture stomatitis were reported [12]. Our previous study revealed that alpha-mangostin was active against *C. albicans* [13]. The present study also demonstrated the antifungal

Table 3. Germ tube formation by *Candida albicans* following a 3-h exposure to various concentrations of alpha-mangostin and clotrimazole.

	Control	Alpha-mangostin (μg/ml)			Clotrimazole (μg/ml)		
		2,000	4,000	8,000	20	40	80
Number of germ tubes formed/300 yeast (mean ± S.E.) (n=9)	218±40	120±30*	108±50*	71±20*	120±60*	101±30*	78±70*
Reduction in germ tube formation (%)	0	45.03	50.27	67.56	44.67	53.53	64.32

* P<0.05 compared with control

effect of alpha-mangostin on the *in vitro* adhesion of *C. albicans* to denture acrylic and germ tube formation by *C. albicans*. The results indicated that a significant reduction of *C. albicans* adhesion to denture acrylic was evident after exposure to 2,000 μg/ml of alpha-mangostin for only 15 min. In addition, the 2,000 μg/ml of the alpha-mangostin-treated *C. albicans* had a decreased ability for germ tube formation. These inhibitory effects of alpha-mangostin were as effective as clotrimazole. These properties makes it a promising alternative antifungal agent for treatment of *Candida*-associated denture stomatitis. However, this study design was limited to the examination of one *C. albicans* strain, drug concentrations at 1×MFC, 2×MFC and 4×MFC, and at 6 time intervals. Therefore, it is possible that differences in antifungal activity might be evident if the experiment was conducted using different strains, concentrations, and time intervals. Further studies are required to obtain more understanding on antifungal properties of alpha-mangostin.

The effect of alpha-mangostin on adhesion of *C. albicans* to denture acrylic was determined by using a colorimetric tetrazolium assay (XTT assay). The XTT assay and other colorimetric methods are considered as valuable tool for examining the behavior of yeast, whether in planktonic or biofilm form. Due to their ease of use, the colorimetric tetrazolium assays are increasingly used to make direct comparisons between *Candida* isolates, often in the absence of other numerical methods. However, limitations of the XTT assay in studies of *Candida* growth and metabolism have been reported. First, although tetrazolium assays are valuable for quantitation within a yeast strain, it cannot be assumed that there is necessarily a linear relationship between organism number and colorimetric signal. Second, interstrain comparisons cannot be measured in the absence of detailed standardization, since different strains metabolize substrate with different capabilities. Third, the relationship between the XTT concentration used and the resultant colorimetric signal is not necessarily proportional; valid quantitation can only be performed after the creation of appropriate standard curves for each amount of tetrazolium used. Fourth, while the XTT formazan product readily appears in solution, there can be in some strains a significant amount of retained intracellular product, which only becomes soluble after cell treatment with DMSO. The amount of retained product may vary between different cellular states, *e.g.*, planktonic and biofilm [23].

C. albicans is able to form biofilm on the surfaces of denture acrylic. The formation of *Candida* biofilms are more resistant to antifungal drugs, resulting in treatment failure [18, 21]. The process of *Candidal* adhesion to denture acrylic is complex and involving a number of factors including poor oral and denture hygiene, low pH under dentures, high intake of dietary carbohydrates, *Candida* cell surface mannoprotein, surface structure, properties and composition of denture materials, cell surface hydrophobicity, surface free energy and roughness of the denture acrylic [18, 22].

The mechanism of alpha-mangostin for inhibition of *Candida* adhesion remains undetermined, but it is suggested that it might alter cell surface structures and integrity, resulting in masking of adhesins on *Candida* cell [24]. In addition, alpha-mangostin-treated *C. albicans* also demonstrated a decreased capacity to produce germ tube, which might be related to the effect of alpha-mangostin on *Candida* cell wall structure that it may interfere with any changes in cell surface hydrophobicity. These alterations might result in a shift in the free energy of interaction of the cell with the denture acrylic surface, thereby reducing *Candida* adhesion [18]. However, further *in vitro* and *in vivo* studies are required to clarify its mechanism.

Regarding the toxic effects of alpha-mangostin, previous *in vitro* and *in vivo* studies have demonstrated low toxicity of the mangosteen pericarp extract and its active components [13, 25-28]. The extract from mangosteen pericarp was not toxic to human gingival fibroblast culture for up to 48 h at the concentration 200 μg/ml [25]. In addition, alpha-mangostin at 4000 μg/ml was not toxic to human gingival fibroblast culture for 480 min [13]. Furthermore, in animal study, alpha-mangostin was administered orally to rats at a high dose (1.5 g/kg body weight) to test its hepatotoxicity. It was found that after 12 h, level of serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) activities in study groups were less than those of paracetamol given at the same dose [26]. Another study used alpha-mangostin given to rats at an oral dose of 200 mg/kg body weight per day, and did not observe any toxicity after 6 days of treatment [27]. In human clinical trials, herbal mouthwash 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 [28]. According to its antifungal activity and low toxicity, alpha-mangostin might be beneficial for *Candida*-associated denture stomatitis therapy. However, because the effective concentration of alpha-mangostin against *C. albicans* in this study was relatively high, it might be toxic in a clinical use. Therefore, we suggest a new potential application of alpha-mangostin as a denture cleanser or disinfecting agent against *C. albicans* and the clinical significance of these *in vitro* results should be determined by clinical trials. In conclusion, this study indicated that alpha-mangostin has antifungal property against *C. albicans* by inhibiting *Candida* adhesion to denture acrylic and germ

tube formation *in vitro*. These results suggest the potential application of alpha-mangostin as promising antifungal agent for treatment of *Candida*-associated denture stomatitis.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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