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Inhibitory Activities of Herbal Based Toothpaste on Germ Tube and Adhesion of *Candida albicans*

Laina Benlatef[1], Metawee Malinee[2], Benja Norrapong[2], Suwanna Cowawintaweewat[2] and Anek Pootong[2]

[1]Medical Technology Laboratory, Yala Regional Hospital, Yala, 95000, Thailand.
[2]Department of Medical Technology, Faculty of Allied Health Sciences, Thammasat University, Pathumthani, 12121, Thailand.

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Oral candidiasis caused by *Candida albicans* is most often seen in immunocompromised patients. The good practice of oral hygiene is essential to prevent or reduce the risk for the disease. In the study, we evaluated the anticandidal activity of toothpastes available in Thailand and determined the inhibitory effect of herbal formulated toothpaste on the virulence factors of *C. albicans*. The inhibition activity of toothpaste supernatant on growth of *C. albicans* ATCC 10231 was tested by agar diffusion assay, and minimal inhibition concentration (MIC) and minimal fungicidal concentration (MFC) were determined by broth microdilution technique. The inhibition activity of herbal toothpaste supernatant on germ tube formation was tested using crystal based assay. The effect of herbal toothpaste on adherence of *C. albicans* to buccal epithelial cells (BEC) *in vitro* was also determined. In our study, all toothpastes (n=6) inhibited the growth of *C. albicans* with mean inhibition zone range between 8.00 and 16.92 mm. The MIC and MFC of six toothpastes were 0.78 to 1.56% (w/v). Herbal toothpaste derived supernatant at subinhibitory concentration reduced germ tube formation and adhesion of *C. albicans* to BEC with dose dependent manner ($p<0.01$). These results may provide the considerable information for individuals who need to maintain a healthy oral hygiene.

Keywords: *Candida albicans*, Herbal toothpaste, Germ tube formation, Adhesion.

In human oral cavity, *Candida* species are yeast like fungi which colonize from 20 to 80% of adults without evidences of infection[1,2]. Among them, *Candida albicans* is the predominant species occurring in human oral cavity[3]. However, under compromised conditions, *Candida* may cause a different pathologic oral infection involved psuedomembranous, erythematous and hyperplastic candidiasis. Many predisposing factors for oral candidiasis are diabetic mellitus[4], cigarette smoke[5], receipt of broad spectrum antibiotic[6], and especially, HIV infection and AIDS[7]. Adherence of *Candida* to oral epithelial cells is an initial step of candidiasis. It is importance for colonization and subsequent invasion of oral mucosal tissue. Additional, colonization of mucosal surfaces is a risk factor for systemic candidiasis in immunocompromised people[8].

The aim of maintaining an oral hygiene is to reduce pathological agent in the mount[9]. Correlation between colonization of *Candida* species and poor oral hygiene was proved[10]. Tooth brushing is the important part of personnel oral hygiene practices to keep the mouth clean and healthy[11]. It is able to improve oral hygiene, reduce plaque, gingival inflammation or dental caries[12,13,14]. Nowadays, many brands of toothpastes containing different formulations have become available. Several studies showed that toothpaste possessed antimicrobial activity on oral

* To whom all correspondence should be addressed.
Tel.: +662 986 9213-9 ext 7209; Fax: +662 986 9057
E-mail: anek.p@allied.tu.ac.th

pathogenic bacterias and inhibited the growth of *Candida* sp[15, 16,17,18]. However, the study on the inhibition effect of toothpaste on *C. albicans* virulence factor was limited[19]. In this study, we evaluated the *in vitro* antifungal efficacy against *C. albicans* of six different toothpastes available in Thailand and also determined the antifungal activities of Thai herbal toothpaste on morphogenesis and adhesion of *C. albicans* to oral epithelial cell.

MATERIALS AND METHODS

C. albicans

C. albicans ATCC 10231 was used in this study. It was cultured on Saboraud dextrose agar (Difco™, France) at 37°C for 48 hours before used.

Preparation of toothpaste supernatant

Six different brands of toothpastes were purchased from the local market in Pathumthani province, Thailand. The formulations of each brand were shown in Table 1. The toothpastes were suspended in sterile 0.85% Normal saline solution (NSS) or RPMI 1640 medium to final concentration at 50% w/v, and centrifuged at 2,800 × g for 5 minutes. Then, supernatant was used for further antifungal activity assay.

Determination of antifungal activity of toothpaste supernatant against *Candida albicans* ATCC 10231 by agar well diffusion

The antifungal activity of toothpastes against *C. albicans* were determined using agar well diffusion as described previously with minor modification[18]. A 0.5 McFarland suspension of *C. albicans* in sterile NSS was spread in three directions on SDA. These inoculated plates were dry for 5 minutes and a sterile 6 mm cork borer was used to cut wells in each plates. Then, 10 µl of the 50% (w/v) toothpaste supernatant prepared with NSS was added into the well. The plate was incubated at 37°C for 48 hours. Diameter of complete inhibition zone was determined. All experiments were repeated on three independent experiments with duplicate determinations.

Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of toothpaste

The MIC and MFC of toothpaste against *C. albicans* were determined by broth microdilution assay as described in the M27-A3 protocol of the Clinical and Laboratory Standards Institute[20] with some modifications. Briefly, supernatant of toothpaste were two-fold serially diluted with RPMI 1640 medium. An equal volume (100 µl) of yeast suspension (1×10^6 cfu/ml) prepared in RPMI was added, resulting in 5×10^5 cfu/ml in each well. The microwell plate was incubated at 37°C for 48 hours. The minimum inhibitory concentration (MIC) was the lowest concentration of the toothpaste that inhibits growth of yeast cells, as indicated by the absence of turbidity. Culturing from the optically clear tubes was performed to determine the MFC. All experiments were repeated on three independent experiments with duplicate determinations.

Germ tube inhibition assay

C. albicans at 5×10^5 cfu/ml were incubated in 0.85% NSS containing 10% fetal bovine serum (FBS) and toothpaste supernatant at 0, 0.25 and 0.5 ×MIC at 37°C for 2 hours. The total of 300 yeast cell and germ tube forming cell were counted under light microscope at ×400 magnification. The counting criteria comprised: (1) yeast cells with a germ tube, without any constriction at the junction between the cell and the elongation were included, while (2) clumped cells with germ tubes, and (3) pseudohyphae-forming yeast cells were excluded[21]. The experiments were repeated on two separate occasions with duplicate.

Buccal epithelial cell (BEC) adhesion inhibition assay of toothpaste supernatant against *C. albicans*

BEC adhesion of *C. albicans* was tested by method of Taweechaisupapong *et al.*[21] with some modification. Briefly, *C. albicans* ATCC 10231 were washed three times in sterile NSS by centrifugation at 3,000 × g for 15 minutes and resuspended in NSS to a concentration of 1×10^7 cfu/ml. BEC were collected from three healthy human subjects by gently rubbing the inside of the cheeks with sterile swabs and pooled in sterile NSS. To eliminate debris and loosely attached microorganism, The pooled BEC suspension was washed three times in NSS by centrifugation at 3,000 × g for 15 minutes. The BEC were then re-suspended in NSS to a concentration of 1×10^5 cell/ml and used for adhesion assay. A 100 µl of *C. albicans* and BEC suspensions were added to 0.85% NSS (200 µl) containing various

concentrations of herbal toothpaste supernatants. The final concentration of herbal toothpaste supernatants was 0, 0.5 and 1×MIC. The mixtures were incubated at 37°C with gentle mix at 160 rpm for 1 hour. The cells suspension were filtered through a 12 µm pore size polycarbonate filters and washed with 50 ml of 0.85% NSS to remove unattached yeast. Each filter was removed and placed on a glass slide. The slides were air-dried, fixed with methanol and stained with Gram's stain. The number of yeast cells adhering to 100 BEC was quantified by light microscopy. The following criteria were used for quantification of adherent yeasts: 1) Yeasts with daughter cells smaller than the mother cell were counted as one unit, 2) Only single BEC was included, but 3) Overlapping and folded BEC were excluded[21]. The experiments were repeated on three independent experiments with duplicate determinations.

Statistical Analysis

Germ tube formation and BEC adhesion of *C. albicans* in the present of herbal toothpaste

Table 1. Ingredients of six different bands of toothpaste

Toothpaste	Ingredient
Toothpaste 1Darly®	Dicalcium phosphate dihydrate, Water, Sorbitol, Glycerin, Dicalcium phosphate, Sodium lauryl sulphate, Flavor, Carrageenan, Sodium monofluorophosphate, Tetrasodium pyrophosphate, Sodium saccharin, Sodium lauryl sarcosinate
Toothpaste 2Close up®	Sorbital, Hydrated silica, Water, PEG-32, Sodium lauryl sulfate, Flavor, Cellulose gum, Sodium fluoride, Sodium saccharin, Titanium dioxide, Menthol, Cl 19140, Cl 42090, Thymol
Toothpaste 3Systema®	Water, Sorbital, Hydrated silica, PEG-400, Sodium lauryl sulfate, Flavor, Xanthan gum, Titanium dioxide, *Chondrus cripus* (Carrageenan), Sodium saccharin, Methylparaben, Sodium fluoride, o-Cymen-5-ol, Dipotassium glycyrrhizate, Buttylparaben
Toothpaste 4Herbal Twin Lotus®	Sorbital, Calcium carbonate, Cuttlefish bone, Toothbrush tree, *Clinacanthus nutans*, Sodium lauryl sulfate, Silicon dioxide, Orange jessamine, Peppermint oil, Menthol crystal, Eucalyptus oil, Sodium benzoate
Toothpaste 5Salz®	Water, Sorbitol, Hydrated Silica, Sodium chloride, Sodium lauryl sulfate, Propylene glycol, PEG-20, Hydrogenated castor oil, Cellulose gum, Flavor, Titanium dioxide, Sodium hydroxide, Sodium fluoride, Dipotassium glycyrrhizate, Sodium saccharin, Ethylparaben, Buthylparaben, Ubiquinone
Toothpaste 6Colgate®	Triclosan, Lauryl sulfate, Flavor, Cellulose gum, Sodium monofluorophosphate, Tetrasodium pyrophosphate, Sodium saccharin

Table 2. Antifungal activity of supernatant of toothpaste against *C. albicans* ATCC 10231 by agar well diffusion assay and broth dilution assay

Toothpaste	Inhibition zone (mm)(Mean ± SD)	MIC(%)	MFC(%)
Toothpaste 1	15.17 ± 0.41	0.78	0.78
Toothpaste 2	14.00 ± 0.32	0.78	0.78
Toothpaste 3	14.42 ± 0.58	1.56	1.56
Toothpaste 4	16.92 ± 0.49	0.78	0.78
Toothpaste 5	8.00 ± 0.55	12.5	12.5
Toothpaste 6	13.08 ± 0.49	1.56	1.56

mm, millimeter; SD, Standard deviation; MIC, Minimal inhibition concentration; MIC, Minimal fungicidal concentration

supernatant at 0, 0.25 and 0.5× MIC were analyzed using Mann-Whitney U test. A probability values < 0.05 was considered significant.

RESULTS AND DISCUSSION

C. albicans was a causative agent of opportunistic oral candidiasis. In recent years, a number of toothpaste preparations containing active ingredients that may be beneficial for oral health have been developed. Several studies focus on their potential for growth inhibition of *C. albicans*[15,16,17,18]. Little information concerning the effect of toothpaste on the virulence factor of *C. albicans* was found[19]. In this study, we demonstrated the supernatant derived from different toothpaste formulation available in Thailand possessed an antifungal activity on the growth and virulence factors of *C. albicans.*

In this study, we have determined the antifungal activity of toothpaste derived supernatants by using agar well diffusion assay and broth microdilution technique. All toothpaste exhibited the inhibition activity on growth of *C. albicans* with the varying activity as shown in Table 2. The MIC and MFC of tested toothpastes ranged between 0.78 to 12.5% (w/v). It could be due to the different type and concentration of the active ingredients incorporated in each formulation. A detergent like sodium lauryl sulphate (SLS) was presence in all brands of toothpaste. SLS exert their antimicrobial action via destabilization of microbial cell membrane[16]. Sodium monofluorophosphate and sodium fluoride in non-herbal toothpastes were effective caries-preventive agents. Our results are supported by a previous study which found that toothpastes containing sodium monofluorophosphate and sodium fluoride inhibited the growth of *C. albicans*[18]. The active ingredients incorporated in toothpaste 6 are triclosan (0.3%), sodium lauryl sulphate and monofluorophosphate. Triclosan is a small hydrophobic bisphenolic compound that commonly used as an anti-plaque agent and displays a high level of oral retention in plaque and on tooth surfaces for several days following administration[22]. It also exhibits a broad spectrum of antimicrobial activity. The investigation of its activity against *Candida* sp. revealed that triclosan was fungicidal at a concentration of 16 mg/l[23]. However, the mechanisms of antifungal action of these active ingredients of toothpaste on *Candida* were still unknown.

Agar well diffusion technique is widely used to screen the antifungal activity of toothpaste[15,16,17,18]. In our study, the results obtained from agar well diffusion method of toothpaste 3 was not in accordance with those obtained in MIC test. Since, herbal toothpaste 4 showed highest mean inhibition zone of 16.92 mm, but the MIC and MFC of herbal toothpastes were 0.7% (w/v) appeared to be equal to those of non-herbal toothpaste 1 and 2. It might due to poor solubility or poor diffusion rate of antifungal active agent through the agar used.

In present study, herbal toothpast 4 was further assayed for its inhibition activity on germ tube formation, or mycelial conversion, and oral epithelium adhesion of *C. albicans*. *C. albicans* incubated in fetal bovine serum containing herbal toothpaste supernatant resulted in a significant decrease of germ tube formation from 49.5% for

Table 3. Effect of herbal toothpaste supernatant on germ tube formation of *C. albicans* ATCC 10231

Concentration (% w/v)	Germ tube formation (%)	Reduction (%)
Control	49.5 ± 2.5	-
0.25 × MIC	41.0 ± 2.5**	17.2
0.5 × MIC	13.3 ± 2.4**	73.2

**p<0.01

SD, standard deviation; w/v, weight by volume; MIC, Minimal inhibition concentration

Table 4. Effect of herbal toothpaste supernatant on the adhesion of *C. albicans* ATCC 10231 to BEC

Concentration (% w/v)	Number of yeast / 100 BEC (Mean ± SD)	Reduction (%)
Control	386.3 ± 6.2	-
0.25 × MIC	322.5 ± 6.0**	16.6
0.5 × MIC	260.3 ± 5.5**	32.6

**p<0.01

w/v, weight/volume; BEC, buccal epithelial cell; SD, standard deviation

untreated control group to 41.0% for 0.25×MIC and 13.3% for 0.5×MIC ($p< 0.01$) (Table 3). To test the inhibition effect of herbal toothpaste on BEC adhesion of *C. albicans*, yeast cells were incubated with BEC in present of toothpaste supernatant. The mean number of yeast cells adhering to 100 BEC of unexposed group was 386 cells. While, numbers of yeast cell exposed to 0.25 and 0.5×MIC were reduced to 322 and 260 cells, respectively ($p< 0.01$) (Table 4). It revealed that herbal toothpaste supernatant reduced germ tube formation and BEC adherence of *C. albicans* significantly ($p< 0.01$). The effective ingredients of herbal toothpaste 4 are sodium lauryl sulphate and herbal extracts derived from *Streblus asper* (toothbrush tree) leaf extract, *Clinacanthus nutans* (Phaya Yo), *Murraya paniculata* (Orange jessamine) and essential oils of peppermint and eucalyptus. Most herbal constituents in this formulation were found to have inhibition activity on growth of *C. albicans*, except *M. paniculata*[24]. *Clinacanthus nutans*, also known as snake grass, was used in traditional herbal medicine for treating skin rashes, insects and snake bites and lesions caused by herpes simplex virus in China and Southeast Asian country[25]. The study of Choonharuangdej demonstrated the inhibitory activity of *C. nutans* aqueous extract against *C. albicans* ATCC 10231 with MIC and MFC of 12.5 mg/ml[26]. Oils obtained from peppermint and eucalyptus were effective against *C. albicans* at very low concentration (0.05 and 0.08% v/v, respectively)[27]. However, the inhibition effects of *C. nutans*, peppermint and eucalyptus extract on germ tube and BEC adhesion of *C. albicans* have not been determined. Other herbal preparations such as *Streblus asper* leaf extract was able to inhibit *C. albicans* germination and the pre-treatment of *Candida albicans* and human buccal epithelial cells (HBEC) at sub-inhibitory concentration significantly reduced the adherence ability of *Candida albicans* to HBEC[21]. It has been suggested that *Streblus asper* leaf extract may affect the cell wall structure, thereby suppressing germ tube formation, and alter to cell surface that could mask the adhesions present on the yeast or on the receptors present on the buccal cells. But these required further clarification.

Since, candidal adhesion is considered as an important virulence attribute once it represents the first step for persistent colonization, biofilm formation and establishment of disease[28]. While, germ tube was an initial projection occurring when *Candida* morphologically switch from yeast to hyphal form[29]. Germ tube and hyphae form of *C. albicans* adhered to epithelial cells more efficiently than yeast form[30]. Thus, the agents inhibiting adhesion to the oral mucosa might be of considerable benefit in managing oral candidasis[21]. In our study, it revealed that the supernatant of different formula toothpaste are effective in inhibiting the growth of *C. albicans*. The toothpaste containing effective herbal ingredients was able to reduce germ tube formation and adhesion of *C. albicans* to BEC *in vitro*. These results may provide the substantial information for individual who need to maintain a healthy oral hygiene and was interested in natural based products.

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