Effect of lemongrass gel against Candida albicans in rat model of oral candidiasis

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Effect of lemongrass gel against *Candida albicans* in rat model of oral candidiasis

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Abstract

Candidiasis is the most prevalent fungal infection found in oral cavity. Current interventions for oral fungal infections are not very successful and require a long treatment period. This research aims to study the in vitro and in vivo effects of lemongrass oil on Candida albicans. The antifungal activities of lemongrass oil towards C. albicans were determined by broth microdilution and time-kill assay. The lemongrass gel was formulated and its antifungal potential was evaluated in the rat model. The results from in vitro study revealed that lemongrass oil possesses potent antifungal activity. In the in vivo study, 15 Sprague-Dawley rats (divided into 3 groups) were treated with gel base, lemongrass gel and Daktarin® gel. After 2 weeks, the quantity of C. albicans in oral tissues of lemongrass gel- and Daktarin®-treated rats was significantly lower than the gel base group (P < 0.05). The C. albicans colony-forming units of the lemongrass-treated rats was insignificantly different from the rats those treated with Daktarin® gel. These data indicate that lemongrass and Daktarin® gel achieved comparable efficacy against C. albicans in a rat model, suggesting a potential use of lemongrass gel for oral candidiasis.

Keywords: Candidiasis, Candida albicans, lemongrass gel, rat model
1. Introduction

Oral candidiasis is the most frequent mucocutaneous mycosis present in the oral cavity which is produced by yeast of the genus Candida, with *Candida albicans* being the most common species (Bensadoun, Patton, Lalla, & Epstein, 2011; Zadik *et al*., 2010). *C. albicans* is a fungal pathogen that undergoes dimorphism (transformation from a yeast form to a hyphal form) and the ability to transition between different morphologies is strongly correlated with its ability to cause both disseminated and mucosal infections (Jacobsen *et al*., 2012; Saville, Lazzell, Monteagudo, & Lopez-Ribot, 2003). Mucosal infections involve the formation of a biofilm at the site of infection. In severely immunocompromised patients, disseminated infections often result in death. Although numerous antifungal agents are available, failure of therapy is not uncommon (Giannini & Shetty, 2011; Vazquez, 2010).

In the last few decades, medicinal plants have been the subject of intense pharmacological studies as new sources of antimicrobial agents because of increasing awareness of hazards associated with the use of antibiotic and chemical agents. The wide variety of plant products have been studied as treatment of oral candidiasis (Amanlou, Beitollahi, Abdollahzadeh, & Tohidast-Ekrad, 2006; Bakhshi, Taheri, Shabestari, Tanik, & Pahleven, 2012; Pinelli, Montandon, Corbi, Moraes, & Fais, 2013; Vazquez & Zawawi, 2002; Wright, Maree, & Sibanyoni, 2009). Essential oils are odorous, volatile products of plant secondary metabolism, found on many leaves and stems. A large number of essential oils and their constituents have been investigated for their antimicrobial properties against some bacteria and fungi in more than 500 reports (Kalemba & Kunicka, 2003). Our previous studies reported that lemongrass (*Cymbopogon citratus* DC) oil possessed the strongest antifungal and inhibitory effect on *Candida* biofilm formation in vitro compared to another seven essential oils (Taweechaisupapong, Aieamsaard, Chitropas, 

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Khunkitti, 2012a). Moreover, limited exposure of yeasts to lemongrass oil at subcidal concentration can suppress growth for more than 24 h (Taweechaisupapong, Ngaonee, Patsuk, Pitiphat, & Khunkitti, 2012b). In order to provide more information about lemongrass oil for its potential development as a new potential therapeutic agent which may help in the treatment of oral candidiasis in human, lemongrass gel was developed and its antifungal effect against C. albicans was evaluated in a rat model.

2. Materials and methods

2.1 Preparation of lemongrass oil and gel

Lemongrass oil was purchased from Thai China Flavours & Fragrances Industry Co. (Thailand). They were dissolved in 95% ethanol to an initial concentration of 900 µl/ml and further diluted with the solution contained 5% ethanol and 5% Tween 80 to a concentration of 64 µl/ml before used.

The lemongrass gel (1% v/w) and gel base were manufactured by the Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Khon Kaen University as described in Thai Petty Patent No. 9929 (date of announcement : 19 May 2015).

2.2 Determination of lemongrass oil components using GC/MS spectrophotometry

Analysis of lemongrass oil components at a concentration of 10 µl/ml lemongrass oil in dichloromethane was performed by gas chromatography (Model CN 10402086, Agilent, China) couple with mass spectrometry (Model US 35120381, Agilent, USA). A DB-5ms capillary column (30 m × 0.25 mm i.d.) coated with 0.25 µm film thickness of 5%-phenyl-methylpolysiloxane was used for separation. The GC transfer line temperature was at 280 °C and the ion source at 230 °C. The column temperature started at 70 °C for 5 min and increased to 120
°C at the rate 3 °C/min (hold for 2 min), then the temperature was raised to 270 °C at the rate 5 °C/min. Helium was used as carrier gas at 1 ml/min flow rate. The injection volume of was 1 µl in a purged split mode (1:100). The scan range was 35-550 m/z and the scan rate was 1388.2 amu/s. The identification of lemongrass oil components was analyzed by MSD ChemStation software based on the comparison their mass spectra with Wiley 7n.l MS Search library. The confirmation was done by referring to linear retention index (LRI) data generated from a series of n-alkane (C_{10-23}).

2.3 Microorganisms

*C. albicans* ATCC 10231 and *C. albicans* from clinical isolate were maintained on Sabouraud dextrose agar (BBL Microbiology Systems, Cockeysville, MD) and grown in the yeast phase in Sabouraud dextrose broth (Pronadisa, Hispanlab, S.A.) for 18 h. The organisms were adjusted to give a final absorbance at 600 nm = 0.1 (~1x10^6 colony forming unit (CFU/ml) and used as inoculums in the broth dilution and time-kill assay.

To prepare oral inoculum, *C. albicans* ATCC 10231 was streaked to Sabouraud dextrose agar and incubated at 37°C for 24 h. *C. albicans* was harvested and suspended in phosphate buffered saline (PBS) to 1x10^8 cell/ml determined by hemocytometer.

2.4 Determination of antifungal activities of the lemongrass oil

Antifungal activities of the lemongrass oil towards *C. albicans* ATCC 10231 and *C. albicans* clinical isolate were determined by the broth dilution method (NCCLS, 2002). Briefly, 50 µl of the lemongrass oils (64 µl/ml) was two-fold serially diluted with Sabouraud dextrose broth in a microtiter plate. An equal volume of the Candida suspension was added and mixed with the oils. The plates were incubated for 24 h, at 37 °C. The Candida growth was examined by eyes and
the lowest concentration of the oil which inhibited the visible growth of the Candida was recorded as the minimum growth inhibitory concentration (MIC). The positive growth of each microorganism cultured in the broth without oils served as a positive control and the negative growth found in the mixture of broth and oil without microorganism served as a negative control.

Aliquots of the mixture of oils and the Candida suspension which showed negative-visible growth after the first 24 h of incubation, were inoculated onto the surface of Sabouraud dextrose agar. The lowest concentration of the oil giving negative growth of the Candida was recorded as the minimum fungicidal concentration (MFC).

Time-kill procedure was conducted as previously described with modification (Klepser, Wolfe, Jones, Nightingale, & Pfaller, 1997). The fungal suspension was adjusted according to spectrophotometric methods to give an optical density (OD) at 600 nm of 0.1. Then a 1:10 dilution of this suspension was made. This dilution yielded a starting inoculum of approximately $1 \times 10^5$ to $5 \times 10^5$ CFU/ml. The lemongrass oil was added to the fungal suspension to a final concentration of 1% (10 µl/ml) and was incubated at 37°C. At the indicated times (0, 1, 3, 5, 15, 30 and 60 min), samples were taken, serially diluted, plated in triplicate on Sabouraud dextrose agar and incubated at 37°C for 24 h to allow colony counting. A given concentration of the lemongrass oil was considered fungicidal if it reduced the inoculums viable count by $\geq 3 \log_{10}$ CFU/ml, or fungistatic if it reduced the inoculum viable count by $< 3 \log_{10}$ CFU/ml.

All experiments were repeated on three separate occasions, with triplicate determinations on each occasion.

2.5 Rat experimental infection

2.5.1 Animals
A total of 15 male Sprague-Dawley (SD) rats, 6 to 7 weeks old, obtained from the National Animal Centre, Salaya, Nakornpatom, Thailand, was used. The rats were housed in groups of five in polypropylene cages equipped with hepa-filtered covers and fed commercial pellet diet and water ad libitum. The cages were maintained in an isolated animal room.

2.5.2 Infection of rats with *C. albicans*

Oral cavities of all rats were swabbed and examined to rule out indigenous candidal growth, before experimental inoculation with the respective *C. albicans* isolate. All rats were immunosuppressed with prednisolone (INPAC Pharma, Thailand) via oral route to simulate the oral environment of an immunocompromised host. To prepare prednisolone, 5 mg tablets of prednisolone were dissolved with absolute ethanol and then mixed into drinking water (Juasook et al., 2013). This was orally administered to all rats every day (5 mg/kg/d) throughout the experiment. For infection, rats were anesthetized with Nembutal and a small cotton pad soaked with 200 µl of *C. albicans* ATCC 10231 cell suspension (1x10⁸ cells) was used to swab the entire oral cavity. The swab was left for 30 min in the oral cavity and was removed before the rats awoke. Following inoculation, the establishment of *C. albicans* infection was evaluated by swabbing the inoculated oral cavity with a sterile cotton applicator every 2 days, followed by plating on CHROMagar candida plates (CHROMagar, Paris, France). After 7 days of exposure to *C. albicans*, the animals were divided into three groups of five rats each: Group 1 received gel base orally via topical application using a cotton pad as a control, and Group 2 and 3 received lemongrass gel and Daktarin® gel (JANSSEN-CILAG Ltd, UK), respectively, orally at doses of 20xMIC using a cotton pad three times a day for two weeks. After two weeks of treatments, rats were sacrificed under anesthesia for enumeration of *C. albicans* in excised buccal mucosa, palatal
mucosa and tongue tissues. The protocols were approved by the Animal Ethics Committee of Khon Kaen University (AEKCU 29/2556).

2.5.3 Quantification of *C. albicans* CFU in oral tissues

For enumeration of *C. albicans* ATCC 10231 in excised oral tissues, the buccal mucosa, palatal mucosa and longitudinally half-cut tongue tissues were digested with collagenase (SIGMA-ALDRICH, USA) for 30 min at 37°C and serial dilutions of the homogenate were plated on CHROMagar candida plates and incubated for 48 h at 37°C. The number of *C. albicans* colonies formed on each plate was counted and the total CFU value per mm³ was calculated.

2.5.4 Histopathological observation

The other longitudinal half-cut tongue tissue was fixed in 10% formalin and embedded in paraffin wax. Sections of 4 µm thickness were subjected to periodic acid-Schiff (PAS) staining for histological observation.

2.6 Statistical analysis

The effect of the tested agents on the number of *C. albicans* in oral tissues was analyzed using Kruskal Wallis with Mann–Whitney U test to evaluate the differences between the test and control groups. Bonferroni method was used to adjust for multiple comparisons. P-values < 0.05 were considered as statistically significant.

3. Results

3.1 Chemical components of lemongrass oil
The chemical components of lemongrass oil are showed in Table 1 and Figure 1. The major component of lemongrass oil is citral which divided to two isomers, neral (beta citrals) and geranial (alpha citral), found in 34.79% and 48.13%, respectively. The total citral of this lemongrass was 82.92 %. Moreover, beta-myrcene (8.03%), geraniol (3.75%) and geranyl acetate (1.92%) were also found.

3.2 Antifungal activities of the lemongrass oil

The MIC of lemongrass oil on \textit{C. albicans} ATCC 10231 and \textit{C. albicans} from clinical isolate by the broth microdilution method was 0.5 μl/ml while the MFC were 2 μl/ml and 1 μl/ml, respectively. The results from time-kill assay showed that 1% (10 μl/ml or 20xMIC) lemongrass oil killed all $10^5$ CFU/ml \textit{C. albicans} ATCC 10231 within 1 min (Figure 2).

3.3 Therapeutic effect of the lemongrass gel in a rat model of oral candidiasis

Administration of lemongrass gel and Daktarin® for two weeks significantly reduced the \textit{C. albicans} CFU in oral tissues compared with gel base group ($P < 0.001$ for lemongrass gel and $P = 0.019$ for Daktarin®), but the difference between lemongrass gel and Daktarin®-treated rats was not statistically significant (Figure 3). After two weeks of treatments, histological studies were conducted and representative images are shown in Figure 4. Large hyphae that stained positively with PAS reagent were found in the cornified layer of the oral epithelium in the tongue tissues of rats in gel base group (Figure 4A). In contrast, rats that received lemongrass gel and Daktarin® at a dose of 20xMIC showed a decrease in the size of \textit{C. albicans} hyphae in the infected tongue tissues (Figure 4B-C).

4. Discussion
The chemical components of lemongrass oil in this study were similar to the reports of previous studies which found 32.5 - 33.7% neral, 37.8 - 43.4% geranial, 9.5 - 10.3% beta myrcene and 4.4 - 4.6% geraniol (Aiemsaard, Aiumlamai, Taweechaisupapong, Aromdee, & Khunkitti, 2010; Inouye, Takizawa, & Yamaguchi, 2001; Taweechaisupapong et al., 2012a; Taweechaisupapong et al., 2012b). The total citral of lemongrass oil used in this study was 82.92% which indicated that this lemongrass oil met the requirement of ISO 3217:1974. The present study shows that lemongrass oil possessed the same MIC and MFC on planktonic cells of \textit{C. albicans} clinical isolate as our previous observations (Taweechaisupapong et al., 2012a). However, \textit{C. albicans} ATCC 10231 was found to be more resistant to lemongrass oil than \textit{C. albicans} clinical isolate because the MFC of lemongrass oil against \textit{C. albicans} ATCC 10231 was higher than that of \textit{C. albicans} clinical isolate. Therefore, \textit{C. albicans} ATCC 10231 was selected to determine the killing kinetic of lemongrass oil by time-kill assay and for further study in a rat model of oral candidiasis.

The rat was used far more often than the mouse as a host for experimental oral Candida infections, especially the SD rat appears to be the most popular model by far for the study of mucosal candidiasis (Allen, 1994; Samaranayake & Samaranayake, 2001). Therefore, the SD rat was used in this study. The two main advantages of the rat model are the low maintenance cost and the sufficient size of the oral cavity, which easily permits inoculation and sample collection (Costa, Pereira, Junqueira, & Jorge, 2013; Samaranayake & Samaranayake, 2001). In the present study, prednisolone was orally administered to all rats every day (5 mg/kg/d) throughout the experiment because it has been demonstrated in both mice (Lacasse, Fortier, Chakir, Cote, & Deslauriers, 1993) and rats (Jones & Russell, 1973a) that without the use of immunosuppressive agent, oral fungal burdens in mice and rats are variable and often decline rapidly.
The results from time-kill assay showed that 1% lemongrass oil killed all $10^5$ CFU/ml *C. albicans* ATCC 10231 within 1 min (Figure 2). Therefore, 1% lemongrass gel was developed and tested its therapeutic effect in a rat model of oral candidiasis. The dose based on the active constituent (citral) in 1% lemongrass gel was 0.83%. The results revealed that administration of lemongrass gel for two weeks significantly reduced the *C. albicans* CFU in oral tissues compared with gel base group ($P < 0.001$), but the difference between lemongrass gel and Daktarin®-treated rats was not statistically significant (Figure 3). Histopathological observation in this study is consistent with several previous reports that the SD rat that succumb to infection show histologic changes similar to chronic candidiasis of the posterior dorsum of the human tongue (Jones & Russell, 1973b) and *C. albicans* hyphae infiltrating the cornified layer of the rat lingual epithelium as in humans (Russell & Jones, 1973; Samaranayake, Wu, Samaranayake, & Ho, 1998). However, the rats that received 1% lemongrass gel showed a decrease in the size of *C. albicans* hyphae in the infected tongue tissues. We recently reported that *C. albicans* cells treated with lemongrass oil and its major constituents had a reduced ability for germ tube formation (Taweechaisupapong *et al*., 2012a). Moreover, lemongrass oil could induce transition to yeast of *C. albicans* hyphae (unpublished data). Therefore, a decrease in the size of *C. albicans* hyphae in the infected tongue tissues observed in this study may be related to those effects of lemongrass oil.

In conclusion, our results demonstrated that lemongrass gel could decrease the size of *C. albicans* hyphae in the oral tissues of infected rats and overall decrease in *C. albicans* CFU. Since a striking feature of *C. albicans*, relevant to its pathogenesis, is its ability to switch between different morphological forms and the hyphal form of *C. albicans* is often considered the essentially virulent form (Bastidas & Heitman, 2009; Sanchez-Martinez & Perez-Martin, 2001; Sudbery, Gow, & Berman, 2004; Sudbery, 2011), inhibition of hyphal growth of *C. albicans* by
lemongrass oil may be one of the effects by which lemongrass oil prevents candidiasis. In addition, lemongrass oil possessed potent in vitro activity in inhibiting biofilm formation and against preformed biofilm of *C. albicans* (Taweechaisupapong *et al.*, 2012a). Therefore, our findings suggest the potential use of lemongrass gel as an anti-candidal agent for the prevention and treatment of oral candidiasis in human.

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**References**


Figure legends

**Figure 1** GC/MS chromatogram of lemongrass oil

**Figure 2** Killing kinetics of lemongrass oil against *C. albicans* ATCC 10231. Fungal suspensions were incubated with lemongrass oil and samples were taken at the indicated time points (0, 1, 3, 5, 15, 30 and 60 min). The colonies were counted and a fungicidal effect was defined as a ≥3 log_{10} reduction in CFU/ml compared with the initial inoculum. Data are the mean value of three independent experiments performed in triplicate.

**Figure 3** Effect of lemongrass gel in a rat model of oral candidiasis. Rats (n=5 per group) were challenged with oral application of pelleted 10^8 CFU *C. albicans* ATCC 10231 and administration of gel base, lemongrass gel and Daktarin® three times daily was started 7 days later and continued for two weeks. Numbers of viable *C. albicans* CFU isolated from oral tissues are plotted. *P* < 0.05 compared to gel base group.

**Figure 4** Histological appearance of periodic acid-Schiff (PAS)-stained tongues of rats that received gel base as a control (A), lemongrass gel (B) and Daktarin® (C). Tissues were collected from rats on day 14 after treatment. Black arrows indicate fungal elements that were positive in PAS staining. The histological examination showed decreases in the size of *C. albicans* hyphae in tongue tissues of lemongrass gel and Daktarin® -treated rats. Original magnification, 400x.
Table 1 Chemical components of lemongrass oil

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<td>1.32</td>
<td>NI</td>
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<tr>
<td>Beta-Myrcene</td>
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<td>8.03</td>
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<td>Geranyl acetate</td>
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Wiley 7n1 MS Search library was used to identify chemical components of lemongrass oil.

LRI: linear retention index (from a series of n-alkanes C\textsubscript{10} – C\textsubscript{23})

NI: not identified
Figure 1 GC/MS chromatogram of lemongrass oil
Figure 2 Killing kinetics of lemongrass oil against \textit{C. albicans} ATCC 10231. Fungal suspensions were incubated with lemongrass oil and samples were taken at the indicated time points (0, 1, 3, 5, 15, 30 and 60 min). The colonies were counted and a fungicidal effect was defined as a $\geq 3 \log_{10}$ reduction in CFU/ml compared with the initial inoculum. Data are the mean value of three independent experiments performed in triplicate.
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