Songklanakarin J. Sci. Technol.
42 (2), 299-304, Mar. - Apr. 2020

http://www.sjst.psu.ac.th

Original Article

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

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Received: 7 May 2018; Revised: 27 October 2018; Accepted: 12 December 2018

Abstract

Candidiasis is the most prevalent fungal infection found in the 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. A lemongrass gel was formulated and its antifungal potential was evaluated in the rat model. The results from the *in vitro* study revealed that lemongrass oil possesses potent antifungal activity. In the *in vivo* study, 15 Sprague-Dawley rats were divided into 3 groups and treated with gel base, lemongrass gel, and Daktarin® gel. After 2 weeks, the quantity of *C. albicans* in the 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 treated with Daktarin® gel. The data indicated that lemongrass and Daktarin® gel achieved comparable efficacy against *C. albicans* in a rat model which suggests the 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, i.e. transforms 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

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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, & Pahlevan, 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, & Khunkitti, 2012a). Moreover, limited exposure of yeasts to lemongrass oil at subcidal concentrations 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 humans, 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). It was dissolved in 95% ethanol to an initial concentration of 900 µL/mL and further diluted with a solution that contained 5% ethanol and 5% Tween 80 to a concentration of 64 µL/mL before use. The 1% (v/w) lemongrass gel 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

An analysis of the lemongrass oil components at a concentration of 10 µL/mL lemongrass oil in dichloromethane was performed by gas chromatography (Model CN 10402086, Agilent, China) coupled 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 ºC and the ion source at 230 ºC. The column temperature started at 70 ºC for 5 min and increased to 120 ºC at the rate of 3 ºC/min (hold for 2 min), then the temperature was raised to 270 ºC at the rate 5 ºC/min. Helium was used as the carrier gas at 1 mL/min flow rate. The injection volume 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. Identification of the lemongrass oil components was analyzed by MSD ChemStation software based on the comparison of their mass spectra with Wiley 7n.l MS Search library. Confirmation was done by referring to the linear retention index (LRI) data generated from a series of *n*-alkane (C_{10}–C_{23}) standards.

2.3 Microorganisms

C. albicans ATCC 10231 and *C. albicans* from a clinical isolate were maintained on Sabouraud dextrose agar (BBL Microbiology Systems, Cockeysville, MD, USA) 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 the oral inoculum, *C. albicans* ATCC 10 231 was streaked to Sabouraud dextrose agar and incubated at 37 ºC for 24 h. *C. albicans* was harvested and suspended in phosphate buffered saline (PBS) to 1×10^8 cell/mL determined by a hemocytometer.

2.4 Determination of antifungal activities of the lemongrass oil

The antifungal activities of the lemongrass oil towards *C. albicans* ATCC 10231 and *C. albicans* clinical isolate were determined by the broth dilution method (National Committee for Clinical Laboratory Standards [NCCLS], 20 02). 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). 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 microorganisms 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).

The 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 1×10^5 to 5×10^5 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

Fifteen male Sprague-Dawley (SD) rats that were 6 to 7 weeks old were obtained from the National Animal Centre, Salaya, Nakornpatom, Thailand. 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*

The 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 the oral route to simulate the oral environment of an immunocompromised host. To prepare prednisolone, 5 mg tablets of prednisolone were dissolved in 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. To inoculate the rats with *C. albicans*, the rats were initially anesthetized with pentobarbital. Then a small cotton pad soaked with 200 µL of *C. albicans* ATCC 10231 cell suspension (1×10^8 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. Groups 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 the excised buccal mucosa, palatal mucosa, and tongue tissues. The protocols were approved by the Animal Ethics Committee of Khon Kaen University (AEKKU 29/2556).

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

For enumeration of *C. albicans* ATCC 10231 in the 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 mm^3 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. The 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 shown in Table 1 and Figure 1. The major component of lemongrass oil is citral which is divided into two isomers, neral (beta citrals) and geranial (alpha citral) which were found in percentages of 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 *C. albicans* ATCC 10231 and *C. albicans* from the clinical isolate by the broth microdilution method was 0.5 µL/mL while the MFC values 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 *C. albicans* ATCC 10231 within 1 min (Figure 2).

Table 1. Chemical components of lemongrass oil.

Components	Retention time (min)	Percent area	LRI
6-methyl-5-heptan-2-one	6.78	1.32	NI
Beta-myrcene	6.88	8.03	NI
Linalool	11.19	0.70	1086
1-tert-butyl-3,3-dimethylcyclopropene	13.99	0.49	1148
ethenyl-cyclohexane	14.81	0.86	1166
Beta-citral (neral)	17.38	34.79	1221
Geraniol	18.10	3.75	1237
Alpha-citral (geranial)	18.71	48.13	1251
Geranyl acetate	23.81	1.92	1359

Wiley 7n.1 MS Search library was used to identify chemical components of lemongrass oil
LRI, linear retention index (from a series of *n*-alkanes C_{10}–C_{23})
NI: not identified

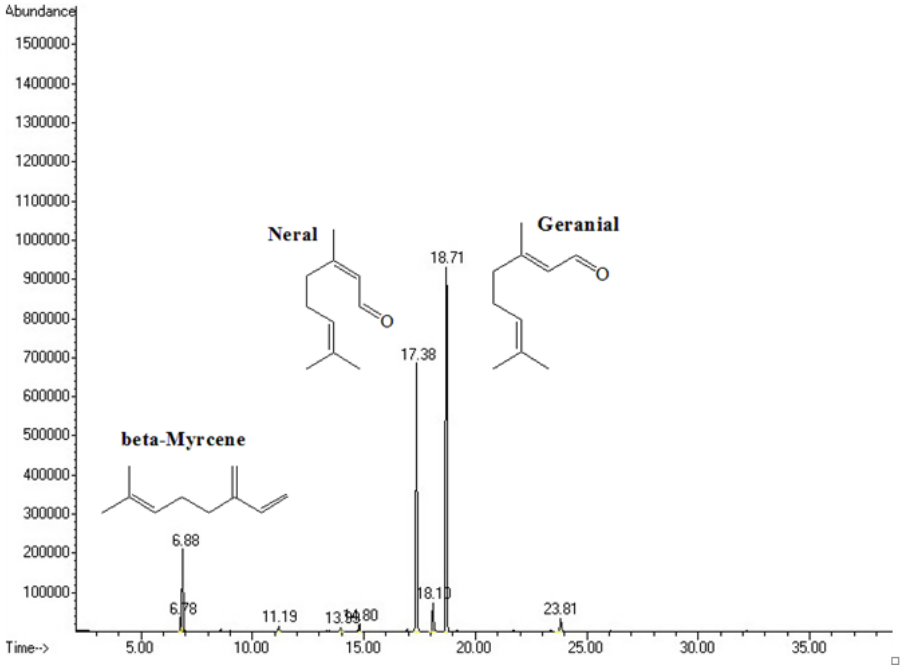

Figure 1. GC/MS chromatogram of lemongrass oil.

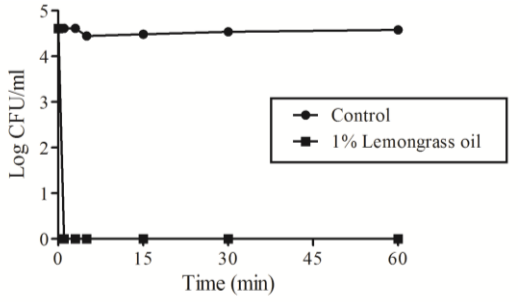

Figure 2. Killing kinetics of lemongrass oil against *C. albicans* ATCC 10231. Fungal suspensions were incubated with lemongrass oil and samples were taken at the indicated time points (0, 1, 3, 5, 15, 30, and 60 min). The colonies were counted and a fungicidal effect was defined as a ≥3 $\log_{10}$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

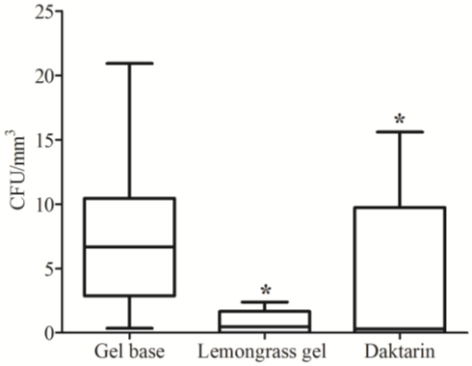

Figure 3. Effect of lemongrass gel in a rat model of oral candidiasis. Rats (n=5 per group) were challenged with oral application of pelleted 10^8 CFU *C. albicans* ATCC 10231 and administration of gel base, lemongrass gel, and Daktarin® three times daily which was started 7 days later and continued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

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 *C. albicans* CFU in oral tissues compared with the gel base group (P<0.001 for lemongrass gel and P=0.019 for Daktarin®), but the difference between the 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 the 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 *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, Takizawa, & Yamaguchi, 2001; Tawee-

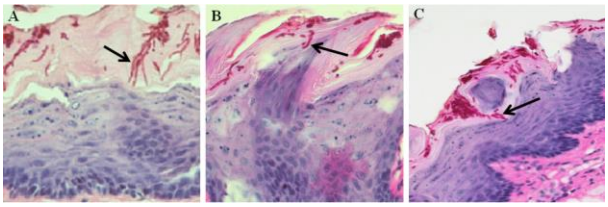

Figure 4. Histological appearance of periodic acid-Schiff (PAS)-stained tongues of rats that received gel base as a control (A), lemongrass gel (B), and Daktarin® (C). Tissues were collected from rats on day 14 after treatment. Black arrows indicate fungal elements that were positive in PAS staining.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

chaisupapong *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 showed that lemongrass oil possessed the same MIC and MFC on planktonic cells of *C. albicans* clinical isolate as our previous observations (Taweechaisupapong *et al*., 2012a). However, *C. albicans* ATCC 10 231 was found to be more resistant to lemongrass oil than *C. albicans* clinical isolate because the MFC of lemongrass oil against *C. albicans* ATCC 10231 was higher than the *C. albicans* clinical isolate. Therefore, *C. albicans* ATCC 10231 was selected to determine the killing kinetics of lemongrass oil by time-kill assay and for further study in a rat model of oral candidiasis.

Rats are used far more often than mice as hosts for experimental oral *Candida* infections, especially the SD rat because it 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 administered orally to all rats every day (5 mg/kg/d) throughout the experiment because it was demonstrated in both mice (Lacasse, Fortier, Chakir, Cote, & Deslauriers, 1993) and rats (Jones & Russell, 1973a) that without the use of an 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 10 231 within 1 min (Figure 2). Therefore, 1% lemongrass gel was developed and its therapeutic effect on oral candidiasis was tested in a rat model. 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 the gel base group ($P<0.001$), but the difference between the lemongrass gel and Daktarin®-treated rats was not statistically significant (Figure 3). The histopathological observation in this study was consistent with several previous reports that the SD rat that succumbed to infection showed histologic changes similar to chronic candidiasis of the posterior dorsum of the human tongue (Jones & Russell, 1973b) and *C. albicans* hyphae infiltrated 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.

5. Conclusions

Our results demonstrated that lemongrass gel could decrease the size of *C. albicans* hyphae in the oral tissues of infected rats and an overall decrease in *C. albicans* CFU was observed. A striking feature of *C. albicans* which is relevant to its pathogenesis is its ability to switch between different morphological forms. Since the hyphal form of *C. albicans* is often considered the virulent form (Bastidas & Heitman, 2009; Sanchez-Martinez & Perez-Martin, 2001; Sudbery, 2011; Sudbery, Gow, & Berman, 2004), 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 anticandidal agent for the prevention and treatment of oral candidiasis in humans.

Acknowledgements

This work was supported by grants from Biofilm Research group and Faculty of Dentistry, Khon Kaen University. We thank Mr. Bryan Hamman for assistance with the English language presentation.

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