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Original Article

Evaluation of the Effect of Lemongrass Essential Oil on *Candida Albicans* Adhesion on Heat Cured Acrylic Based Soft Lining Material

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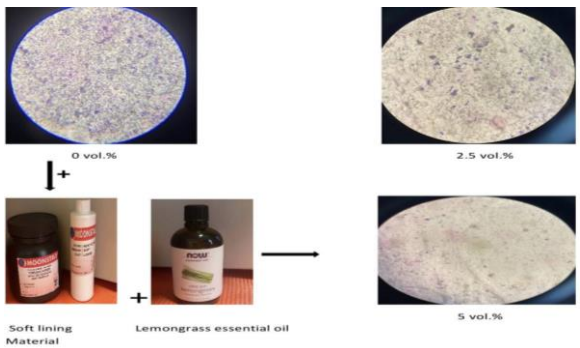
ABSTRACT

Background: Microorganism buildup is one of the most critical issues encountered with soft denture liner materials. The buildup of the fungus *Candida albicans* results in a disease known as denture-induced stomatitis. Therefore, creating a soft liner material with a medication delivery mechanism is required. This study aims to discover the best concentrations of lemongrass essential oil (LGEO) that are effective for inhibiting *C. albicans* growth for addition to the heat-cured soft denture liner materials.
Materials and methods: For pilot study, three specimens were fabricated for each of the three concentrations (2.5, 5, and 7.5 vol%) of LGEO additives. For the main study, 40 specimens were prepared and divided into four groups (negative control, 2.5 vol.% LGEO additive, 5 vol.% LGEO additive, and positive control, which contained 1.4 wt.% nystatin additive) in accordance to the results of the pilot study. Each group consisted of 10 specimens.
Results: In the pilot study, 2.5 and 5 vol.% LGEO were identified as the best two concentrations with good antifungal activity in the selected heat-cured soft-liner material. Meanwhile, the *C. albicans* adherence test showed that the number of *C. albicans* cells adhering to the soft liner specimens incorporating 2.5 and 5 vol.% LEGO additive (experimental groups) significantly decreased relative to that to specimens in the control negative and control positive groups ($P < 0.05$). The best concentration of LGEO additive that decreased the number of adherent *C. albicans* was 5 vol.%.
Conclusion: The results of this work show that LGEO is a potent antifungal substance that may be effectively incorporated into soft liners to create materials that are effective against *C. albicans* fungi.

GRAPHICAL ABSTRACT



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Introduction

Complete and partial removable dentures employ soft denture liners to distribute functional loads throughout the supporting oral tissues appropriately. Soft liners should be utilized in patients with uneven bone resorption, thin atrophic mucosa, immediate dentures, bony undercuts, xerostomia, and bruxism and in the recovery period following implant implantation. Soft liners are applied to treat inflammatory, hypertrophic, and wounded denture-bearing tissue, restoring them to a healthy state. They serve as a cushion layer between the oral mucosa and rigid denture base. Liners distribute mastication forces equally to the underlying oral tissue [1].

Soft liners have several clinical advantages, including the ability to absorb stress, maintain an even load distribution on denture-bearing tissue, and reduce discomfort in patients with severely resorbed and sharp residual ridges and sensitive mucosa [2]. Denture liners increase the tolerance of patients to regular denture bases [3]. They can also improve the dentures retention by the underlying tissue such that the fitness of the dentures is increased [4]. These materials are indicated in the cases of dentures against natural dentition, atrophic ridges or resorption, bruxism, xerostomia, bony undercuts, and sore mouths due to constant denture use [5]. Soft liners can be used to treat patients who require obturators due to postoperative complications and can be employed during the recovery period in the case of implant-supported overdentures [4]. Moreover, they can be utilized in mandibular dentures with deficiencies in the denture-bearing area [6].

The microbial colonization of soft liners is a severe problem that compromises material durability. *Candida albicans* is principally responsible for denture-induced stomatitis, the most frequent clinical symptom linked to this problem [7, 8]. Denture-induced stomatitis is an inflammatory condition that affects the mucosa beneath complete or partial removable dentures. It is most common in total denture wearers, with the maxillary arch being the most commonly affected site. Patients with cleft palates who wear obturators and those who wear orthodontic appliances are frequently affected by this disease. Males and females are susceptible to denture stomatitis, which is more prevalent in the latter than in the former [9].

Topical antifungal medicines are the most frequently prescribed treatment for denture-induced stomatitis. Nevertheless, it has considerable disadvantages, such as the loss of motor dexterity that prevents the administration of the correct medication amount in the elderly. Adopting a medicine delivery method including denture materials may help solve this issue [10]. Herbal medications provide a potent alternative therapy for oral microbial infections with few or no side effects and have thus motivated a global search for physiologically safe herbal-based therapies with efficient antifungal and antibacterial capabilities [11-13]. Recent research has shown that plant oils with excellent antifungal qualities are a promising therapeutic option for denture-induced stomatitis [14-16]. Oil plants are any of the numerous cultivated or wild plants that are used as sources of oil. They include trees such as palm, herbaceous plants such as flax, and even fungi. Plant essential oils are a possible source of natural antimicrobials [17]. Essential oils and plant extracts have lately attracted widespread appeal and scientific curiosity. Plant oils possess antibacterial, antifungal, antiviral, antiparasitic, and antidermatophytic properties [18].

Plant oils are a rich source of natural ingredients for the development of treatments against various diseases and for food production.

In this study, lemongrass essential oil (LGEO) was used. LGEO is a medicinal plant extract obtained from fresh lemongrass herb and known for its anti-inflammatory, antipyretic, and antimicrobial properties [19]. It has been utilized in folk medicine since ancient times as a remedy to improve circulation, stabilize menstrual cycles, promote digestion, or increase immunity. It is also used to produce perfumes, flavors, detergents, and pharmaceuticals [19]. LGEO contains chains of citronellal and geranyl acetate, which have been proven to be active against fungi, particularly *C. albicans* [19, 20].

This study was conducted to determine the LGEO effect on *C. albicans* adhesion on soft liner material.

Materials and Methods

The pilot study was conducted to determine the optimum two concentrations of LGEO (NOW, USA) that need to be added to heat cure soft lining material (Moonstar, Turkey) that inhibits *C. albicans* growth, different concentrations of LGEO were used; 0% negative (-ve) control, 2.5%, 5.0% and 7.5% (v/v), and then mixed with the liquid of heat cured soft liner material, and thereafter subdivided into the following:

1-For control group (-ve control) 0 vol% additive 3 specimens.

2-For experimental group for each concentration (including 2.5%, 5.0%, and 7.5% (v/v) of LGEO additive) 3 specimens.

Then three standardized fields for each specimen were used for reading through the inverted light microscope, and the mean of these readings was calculated, as listed in Table 1.

Specimen model preparation and mold preparation

For plastic specimen model preparation, computer numerical control equipment was employed, and soft-liner models were prepared with a diameter of 10 mm and a thickness of 2 mm [21]. For mold preparation separating medium (BMS Dendal, Italy) was applied to the plastic models and allowed to dry. Type IV extremely hard dental die stone (Zhermach, Italy) was mixed in accordance with the manufacturer's recommendations (P:W ratio of 100 g of polymer to 20 ml water) and placed into the lower half of a flask, and then vibrated to remove any air bubbles. Half of the plastic models were deeply implanted, whereas the other half of the models were left above the stone level to prevent their immersion in the stone, which would make their extraction after opening the flask impossible. After the lower half had set, it was covered with separating media and left to dry. The flask was then placed on top of its counterpart, filled with stone, and vibrated before being securely closed with its lid and left for 1 hour to ensure that the stone had set completely. When the stone had set completely, the two parts of the flask were split, and the plastic models were removed to produce molds for the heat curing of acrylic soft-liner specimens. Subsequently, the stone molds were painted with separating media and left to dry before being packed with soft liners.

Ratios for the heat curing and mixing of acrylic soft liners

For the negative control specimens, the proportions of soft liner powder and monomer were calculated, and the materials were combined in a no moisturized, clean container made of glass sealed with a lid to avoid the evaporation of the monomer in accordance with the manufacturer's specifications (polymer: monomer ratio of 10 g:7.8 mL). The separating medium was used to cover the top and bottom portions of the flask to prevent the adhesion of the soft liner adhesion to the stone. When the mixture reached the dough stage, it was shaped with a finger and placed in the mold area of the stone, while for the positive control specimens and in accordance to the instructions provided by the manufacturer (P:L ratio of 10 g:7.8 mL), the necessary quantity of nystatin powder was weighed, subtracted from the total weight of the acrylic powder, and then combined with the soft liner powder using a mini electric hand mixer for 1 minute. Subsequently, the required quantity of the monomer was added to the mixture and combined in the same manner as described for the control negative specimens. Likewise, for the experimental specimens LGEO was measured using a micropipette, and then added and mixed with the soft-liner liquid by using an electronic mixer. Subsequently, it was added immediately to the powder of the soft liner to prevent the separation of oil from the liner liquid. The amount of the oil was subtracted from that of the soft liner liquid to maintain the P:L ratio in accordance with the manufacturer's recommendations. The powder was weighed using an electronic balance (KERN, Germany) with an accuracy of 0.001, and the monomer was measured by using a micropipette.

Packing

When the soft liner achieved the correct dough-like consistency in accordance to the manufacturer's recommendations, it was placed into a prepared mold. Its top was coated with polyethylene (JIAO JIE, China) and the lid. A hydraulic press was then used to apply 100 kg/cm^2 of continuous pressure to distribute the soft-liner material uniformly within the mold and expel any surplus. Then, the flask was opened. The polyethylene film was removed, excess material was cut away using a wax knife, and the stone was coated with separating media and allowed to cure. Finally, the flasks were joined, secured, and pressed at 100 kg/cm^2 for 5 min before being clamped for curing [22].

Curing and finishing

The packed dental flask was placed in a digitally thermostatically controlled water bath to complete the operation. The flask was placed in a water bath with water at room temperature (15-25 °C), and then the heat was increased to 100 °C and kept at 100 °C for 20 minutes in accordance to the manufacturer's recommendations [23]. After the curing was completed, the flask was taken from the water bath and allowed to cool at room temperature for 30 minutes before being cooled under running water for 15 minutes. The flask was opened after it had cooled completely, and the specimens were removed from the mold [13]. Following the removal of excess material from the specimens with a sharp blade and the finishing of the specimens with a fine grit silicon polishing bur and fine grit sandpaper, the specimens were cleaned with distilled water, positioned, and sterilized by using UV light (NÛVE MN 120, Germany) [24]. The final specimens were kept in test tubes with distilled water for 24 hours.

Isolation of *C. albicans*

C. albicans was isolated from the oral cavity of three male patients aged 60, 62, and 65 years old who were complete denture wearers seeking treatment at the prosthodontics clinic with signs and symptoms of denture stomatitis, including dysphagia, redness, changes in color, changes in mucosal permeability and texture, and soft tissue dryness [25]. A sterile cotton swab was used carefully to scrub the oral lesions, and then inoculated into Sabouraud dextrose agar (SDA) (Oxoid, United Kingdom), which was prepared as the primary isolation medium [26]. The swabs were cultured, incubated aerobically at 37 °C for 48 hours, and then stored at 4 °C until they were utilized in the subsequent tests [27].

Preparation of SDA

In accordance with the recommendations provided by the manufacturer, 62 g of SDA was measured and completely dissolved in 1000 ml of distilled water. Next, the mixture was placed in an autoclave at 121 °C and 15 psi for15 minutes. Finally, it was allowed to cool down to 47 °C to prevent Petri dishes from deteriorating. A total of 0.05 g of the broad-spectrum antibiotic chloramphenicol was added to each 1000 ml of medium to prevent bacteria from growing in the prepared SDA. The SDA was then placed in Petri dishes and allowed to cool and harden before being stored in a cold area (4 °C) [22].

Identification of *C. albicans*

Morphological and microscopic examination

For the morphological examination a candida colony with a texture that was described as smooth, creamy, and pasty can be seen on the SDA medium [28], Figure 1, while for the microscopic examination a suspension was made on a glass slide by emulsifying a little amount of a single isolated colony in a drop of normal saline. Following distribution, this suspension was allowed to dry at room temperature before being fixed by periodically passing the slide over a bunsen burners flame. According to Gram's method [29], this slide was stained as mentioned in the following:

1-Firstly, the glass slide was stained with crystal violet stain for one minute and rinsed with distilled water.

2-Add the second stain (Gram's iodine) for one minute, and then wash it.

3-Eliminate the dark blue color of the slide by rinsing with acetone alcohol and again rinse with distilled water.

Table 1: Results of *Candida albicans* adherence test

Specimen	-ve Control	2.5 vol.%	5 vol.%	7.5 vol.%
1	44.8	23.6	20.4	24
2	48.2	21.2	17	23.4
3	42.4	19.2	19.4	20.6
Mean	45.13	21.3	18.9	22.6

Figure 1: Candidal colonies on sabouraud dextrose agar media

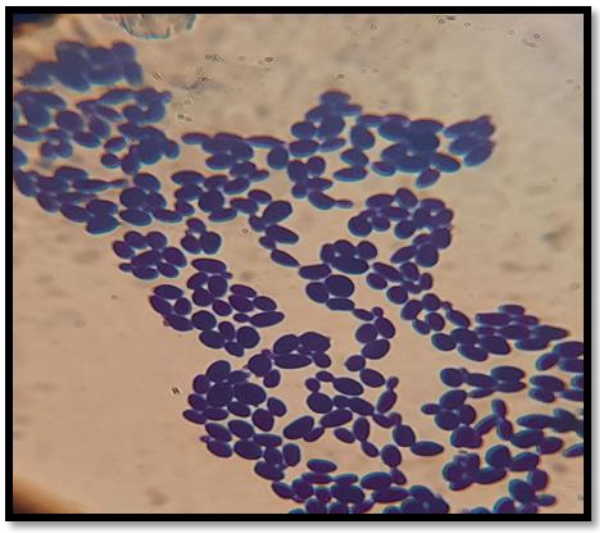

Figure 2: Candida yeast under the light microscope

4-Safranin was applied to the counter for one minute before being washed off and dried.

The slide was inspected under a light microscope, and candida appeared in the form of round or oval cells, as displayed in Figure 2.

Germ tube formation

A single colony was its beginning. The presence of germ tubes was determined by extracting yeast cells. The cells were placed in 0.5 mL of serum and incubated at 37 °C for 3 hours. A drop of the resulting solution was then placed on a glass slide and inspected under a light microscope [30].

Biochemical identification

VITEK 2 (BioMérieux, France) is a completely automated microorganism identification tool that uses fluorescence-based technology. The yeast

suspension was automatically added to the VITEK ID-YST card, which was then sealed and incubated for 18 hours at 35.5 °C in the VITEK 2 apparatus. The optical density readings were recorded every 15 minutes. The identification of the unknown microorganism was established by using the database [31]. The laboratory report's final classification ranked from excellent, very good, good, acceptable, and poor and was judged as acceptable.

Preparation of Sabouraud dextrose broth

A total of 30 g of broth powder (DIREVO, Germany) was melted in distilled water (1000 ml) and autoclave sterilized in accordance with the instructions of the manufacturer (121 °C/15 pressure for 15 minutes), cooled to 47 °C, and added with 0.05 g of chloramphenicol to inhibit bacterial development [22].

Evaluating the effect of LGEO additives on adherence of *C. albicans* on the control and experimental specimens under an inverted light microscope.

A small amount of the yeast that had been isolated was suspended in Sabouraud dextrose broth in sterile tubes. The suspension's concentration was measured by using a McFarland densitometer (BioMérieux, France) and adjusted to 0.5 McFarland standards [32]. The sterilized soft liner specimens were inoculated into sterilized tubes containing previously prepared medium and incubated for 1 hour at room temperature. The specimens were withdrawn from the suspension and washed for 1 minute with phosphate-buffered saline (PBS) to eliminate yeast cells that had not been attached to the filter paper [22].

The adherent *C. albicans* cells on the soft liner specimens were fixed with methanol, stained with crystal violet for 60 seconds, rinsed with PBS for 30 s, dried with filter paper, and stored in sterile test tubes, and then inspected under an inverted light microscope [33]. The adherent *C. albicans* cells were counted in three standardized fields under an inverted light microscope. The means of these fields were taken for each specimen.

Ethical approval

The research was carried out in conformity with the ethical standards set out in the Helsinki Declaration. Before a sample was obtained, it was done with the patient's verbal and analytical consent. A local ethics committee evaluated and approved the research protocol, subject information, and permission form in accordance with document number 645, which also included the date 18.8.2022.

Results and Discussion

The statistical analyses were classified into two categories:

1-Descriptive analysis: Mean, minimum, maximum, standard error, and standard deviation, frequency, and percentage.

2-Inferential analysis: Levene test and One-way analysis of variance (ANOVA).

The negative control group exhibits the highest mean value (44.900) cells when each group's stained specimens had been examined using an inverted light microscope, while the experimental group (5% by volume of LGEO additive) exhibits the lowest mean value (19.300), as depicted in Figure 3.

Descriptive statistics of candida adherence test results for all the studied groups were presented in Table 2.

Using a one-way ANOVA test, a comparison of the group means yielded a significant result ($P< 0.05$) Table 3.

The comparisons of adherent *C. albicans* between groups based on Tukey's Honestly Significant Difference are reported in Table 4.

No statistically significant difference ($P > 0.05$) was found between the 2.5 vol.% LGEO additive and 1.4 wt.% nystatin additive groups. However, the data demonstrated a significant difference ($P < 0.05$) between groups.

The incorporation of 2.5 and 5 vol.% LGEO resulted in a statistically significant reduction in the mean values of adherent *C. albicans* compared with that of 0 vol.% LGEO.

The ability of LGEO to interact with the mitochondrial membrane leads to cidal effects [34].

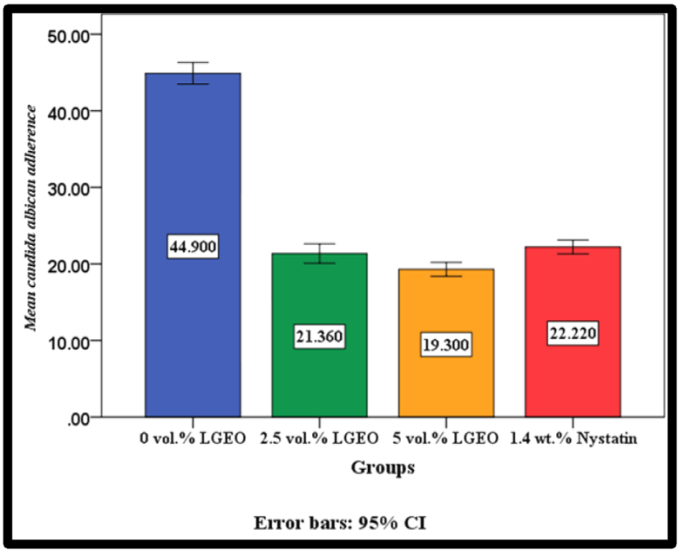

Figure 3: The mean and standard deviation values of Candida albicans adherence test for all tested groups

Table 2. Descriptive statistics of *Candida albicans* adherence test among groups

Group	N	Mean	±SD	±SE	Minimum	Maximum
0 vol.% LGEO	10	44.900	1.971	0.623	42.000	48.200
2.5 vol.% LGEO	10	21.360	1.771	0.560	19.000	23.800
5 vol.% LGEO	10	19.300	1.283	0.406	17.000	20.800
1.4 wt.% Nystatin	10	22.220	1.273	0.403	20.000	23.800

Table 3: *Candida albicans* adherence test statistical analysis among groups using one-way analysis of variance (ANOVA)

	Sum of Squares	Df	Mean Square	F	*P*-value
Between Groups	4343.459	3	1447.820	562.746	0.000 *.
Within Groups	92.620	36	2.573		
Total	4436.079	39			

*Levene P-value= 0.444 **, **= not significant at P > 0.05, and *=significant at P <0.05.*

Table 4: Multiple comparisons of Candida albicans adherence between each two groups using Tukey's Honestly Significant Difference (Tukey's HSD)

(M) Groups	(G) Groups	Mean Difference (M-G)	*P*-value
0 vol.% LGEO	2.5 vol.% LGEO	23.540	0.000*
	5 vol.% LGEO	25.600	0.000*
	1.4 wt.% Nystatin	22.680	0.000*
2.5 vol.% LGEO	5 vol.% LGEO	2.060	0.032*
	1.4 wt.% Nystatin	-0.860	0.631**
5 vol.% LGEO	1.4 wt.% Nystatin	-2.920	0.001*

**= significant at P <0.05 and **= not significant at P> 0.05.*

Essential oils can act against *C. albicans* through the inhibition of ergosterol synthesis [35], thus altering the morphology of the cell wall [36] and inhibiting the enzymes that are involved in cell wall synthesis [37] and reactive oxygen species production [38].

The increase in membrane permeability caused by LGEO ultimately results in cell death as reflected by the loss of potassium, protein, genetic material (DNA and RNA), and membrane potential [39].

This antifungal activity of LGEO against *C. albicans* can be explained by hydrophobicity, an essential characteristic of essential oils that enables essential oils to accumulate in cell

membranes, leading to an increase in permeability until cells die [40].

The mean values of adherent *C. albicans* significantly decreased under treatment with 5 vol.% LGEO relative to those under treatment with 2.5 vol.% LGEO. The mean values of adherent *C. albicans* in the experimental groups (2.5 and 5 vol.% LGEO additive) decreased compared with those of the negative control group likely because the surface roughness of the soft liner material decreased with the increase in the concentration of LGEO [41].

The mean values of adherent *C. albicans* nonsignificantly decreased between the 1.4 wt.% nystatin and 2.5 vol.% LGEO additive groups and significantly decreased between the 1.4 wt.% nystatin additive and 5 vol.% LGEO additive groups because increasing the LGEO concentration enhanced fungal cell permeability, thus increasing oil penetration into cells [39].

This finding was proven by a 2010 research by Tyagi and Malik on the influence of LGEO and other essential oils on *C. albicans*, which showed that LGEO exhibited the most potent antifungal effect [42].

Consistent with the findings of Nakahara's 2013 experiment on the antifungal efficacy of LGEO, the results of this study indicated that citronellal completely suppressed the development of all tested fungi [34].

In agreement with this work, Muttagi's 2016 study demonstrated that seed oil inhibited the growth of *C. albicans* when it was incorporated into soft liner material [43].

Given that LGEO inhibits candida growth and biofilm formation to a significant extent, it has the potential to be employed in clinical situations to preserve silicone rubbers for silicone prostheses or medical equipment [2].

LGEO eliminated = the *C. albicans* biofilm that had already formed on medical-grade silicones. In agreement with the results of the present work, the findings of a previous study demonstrated that LGEO exerted a dose-dependent fungicidal effect against fungal biofilms on silicone specimens [40].

This work was consistent with Godil's 2021 study, which showed that incorporating *Ocimum sanctum* oil into soft denture liners decreased the number of *C. albicans* adhering to soft liner material [44]. It also agreed with the 2022 study by Patil *et al.*, who discovered that LGEO can act as an antifungal agent and may be utilized in combination with denture cleaners [45].

In agreement with this study, Songsang's 2022 experiment revealed that the incorporation of 10% and 30% v/v *Litsea cubeba* essential oil could inhibit the growth of *C. albicans* [46]. Similarly, in 2021, Choonharuangdej discovered that the addition of 0.04% and 0.01% v/v LGEO to acrylic resin reduced the number of *C. albicans* that adhered to acrylic resin [47].

Conclusion

LGEO has a potent antifungal effect against *C. albicans* when it is incorporated into a heat-curing soft lining material. Specimens with 5 vol.% LGEO incorporation demonstrated a larger decrease in *C. albicans* than those with negative control, positive control, and 2.5 vol.% LGEO incorporation.

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Authors' contributions

All authors contributed to data analysis, drafting, and revising of the paper and agreed to be responsible for all the aspects of this work.

Conflict of Interest

The author declared that they have no conflict of interest.

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