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Lemongrass oil disrupts the biofilm of *Candida albicans* MTCC 1637T on soft denture reliners at lower concentrations compared to thyme and tea tree oils

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ABSTRACT

The study aimed to investigate the concentration-dependent effect of different essential oils on *Candida albicans* biofilm growth on denture reliners and their effect on the hardness of soft denture reliners. Soft liner specimens (n = 160) were fabricated and seeded with *C. albicans*. The specimens were distributed into four groups: one control group (n = 40) and three experimental groups (n = 40), namely lemongrass essential oil (LGO), tea tree essential oil (TTO), and thyme essential oil (ThO). The specimens treated with essential oil for 1–5 minutes were subsequently analyzed by biofilm adhesion assay, MTT assay, and scanning electron microscopy. Shore A hardness of the soft denture reliners was recorded after immersion. Inhibition activity of the oils in the order LGO > ThO > TTO was observed with a minimum of 5 minutes of treatment. LGO reduced biofilm formation at a concentration as low as 0.03125%. There was a significant linear increase in mean hardness values in all groups from baseline to day 60 with a statistically lesser increase rate in the TTO group. Disinfection with LGO and ThO was effective to remove the biofilm of soft reliners at very low concentrations without altering the hardness. Hence, LGO and ThO can be used as effective denture cleansers.

1. INTRODUCTION

As age advances, the issues associated with the denture-bearing area also increases, and hence these individuals are prone to residual ridge resorption. Soft denture reliners are the materials used to provide a cushioning layer between the oral mucosa and the hard denture base. They are used not only to enhance the comfort of denture wearers and provide support to the prosthodontic treatment, but also to overcome the problems associated with resorption and abused tissue [1]. The soft reliners are broadly categorized as semi-permanent or permanent, and, in turn, are classified as silicone rubbers or plasticized acrylics that could be either auto, heat, or visible light polymerized [2]. Silicones that are utilized as soft liners are categorized into autopolymerized (room temperature vulcanized) or heat-cured [3].

Aggregation of microorganisms occurs when there is a separation between the denture base and reliner, increased rough surfaces, and changes in hardness that in turn hamper the liner's durability and can steer oral health conditions, such as denture stomatitis, which in turn compromise the restorative prosthodontic treatment's success and in general quality of life [4].

In the oral cavity, *Candida* frequently grows as biofilms, which are "three-dimensional complex polymicrobial communities of microorganisms attached to surfaces including human tissue or abiotic substrates (e.g., denture surface)" [5]. Chronic erythematous candidiasis (*Candida*-associated denture stomatitis) is a common infection of the oral cavity that influences nearly 60% of denture

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wearers and is accompanied by biofilms containing fungi of the genus *Candida*.

Dentures can be properly maintained with the use of several methods, including mechanical cleansing methods, such as brushing with a brush; chemical cleansing methods, like soaking in commercial denture cleansers; ultrasonic cleaning methods; and combinations of each [6,7]. Soaking in disinfectant solutions with chemical reagents manifests to be an effective procedure to reduce the number of contaminating organisms, but some chemical agents used for denture cleaning are known to damage acrylic resin and metal alloy materials [8]. An ideal denture cleanser should be "biocompatible, bactericidal, fungicidal, harmless, and nontoxic to the structure of denture; should effectively remove deposits, and should be easy to use" [9].

Synthetic chemical substances are substituted by natural products due to the growing curiosity in medicinal plants as a source of antimicrobial agents. Natural products, such as essential oils, are propitious curative tools for oral infection [9–11]. Plant essential oils are known to produce analgesic, anti-inflammatory, and antimicrobial effects [12]. Amidst such products, tea tree oil (TTO) and lemongrass oil (LGO) have been tested against several different bacteria and *Candida* species, which showed their antibacterial activities [6,13].

The hardness of the soft denture liners might be influenced by the exposure of essential oils, and thereby the quality of the material after exposure should be appropriate for use as a soft denture liner [14]. Hence, in this study, the antimicrobial efficacy of different essential oils as denture cleansers and their effect on the hardness of soft denture reliners are evaluated and compared.

2. MATERIALS AND METHODS

2.1. Essential Oils

Three commercially available standardized essential oils were selected, i.e., tea tree, lemongrass, and thyme, which were procured from Veda oils, India, for the study. Preparation of stock solutions of oils was made by mixing the essential oil in dimethyl sulfoxide [dimethylsulfoxide (DMSO), HiMedia Laboratories Pvt. Ltd., Mumbai, Maharashtra] to get a 50% (v/v) aqueous solution prior to carrying out the experiments.

2.2. Determination of Minimum Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFC)

Candida albicans MTCC 1637^T was utilized for the study, which is equivalent to *C. albicans* ATCC 18804, a standard reference strain with a moderate to strong biofilm capacity that is most commonly used for the analysis of disinfectants and antifungal agents. *Candida albicans* was grown in Sabouraud dextrose broth (SDB) (HiMedia Laboratories Pvt. Ltd., Mumbai, Maharashtra) at 37°C for 24 hours. The liquid dilution method was used to determine the MIC value that can inhibit *C. albicans*, for which 4 ml of SDB was mixed with 80 µl of 50% (v/v) essential oil. The concentration of the first suspension was kept at 1.0% per ml. From the first tube, 2 ml of media was transferred to another tube containing SDB, and this procedure was subsequently repeated for five tubes to obtain the final concentrations in each tube as 1.0, 0.5, 0.25, 0.125, 0.0625, and 0.03125. An aliquot of overnight grown *Candida* culture (100 µl) was inoculated to the tubes and incubated at 37°C for 48 hours. MIC is the minimum concentration of the oil that will inhibit the microorganism's growth, which is basically indicated by "clear fluid with no development of turbidity and without visible growth." Each liquid sample (50 µl) was seeded into Sabouraud agar plates and incubated for 24 hours at 37°C. MFC is the lowest concentration without any colony formation. The MIC and MFC values obtained were used in the biofilm adhesion assay [6].

2.3. Effects on the Removal of Biofilm Adhesion Assays

2.3.1. Specimen fabrication

A circular metal mold of dimensions 6 mm diameter and 3 mm thickness was used to fabricate specimens of silicone soft liner material, following the manufacturer's instructions. The resilient liner was transferred into the metal mold and the cartridge mixed material was expressed directly into the metal mold. The specimens were allowed to autopolymerize at room temperature for 20 minutes. Before testing, the specimens were cleaned and sterilized in a UV sterilizer. For the biofilm adhesion assay, 80 disk specimens were prepared using GC soft liner (GC India) of dimensions 6 mm diameter and 3 mm thickness. Before starting the experiment, each specimen sterilization was carried out under UV radiation for 30 minutes [14].

2.3.2. Biofilm formation

Primary biofilm inoculum was prepared by adding an appropriate volume of *Candida* cell suspension in phosphate buffered saline (PBS) to obtain a cell density of 1×10^7 cells per ml. To each well of the 24-well plate, containing soft liner disk specimens, the primary biofilm inoculum (2 ml) was added. The plate(s) were carefully placed for 90 minutes in the incubator (with no shaking) at 37°C. This stage represents the "adhesion phase" of biofilm formation. In a fresh, sterile 24-well plate, 2 ml of sabouraud dextrose agar (SDA) medium was added to each well. The soft liner disk specimens were then carefully removed with forceps and placed gently in each well of the 24-well plate with 2 ml of fresh SDA medium. The plate with adhered cells on soft liner disk specimens was incubated at 37°C. This step represents the initiation of biofilm formation. The plates containing soft liner disk specimens with adherent *Candida* cells were incubated for 72 hours at 37°C, representing the "growth phase" of biofilm formation [15].

2.3.3. Soaking treatment with essential oils

After 72 hours of incubation, the media were removed and the disks specimens were gently cleaned with PBS. The soft liner disks specimens were soaked in essential oils based on the MIC values in 2 ml of fresh culture media. The soaking time in each solution of essential oil was 1 and 5 minutes [16].

2.3.4. Quantification of biofilms

After soaking, each soft liner disk specimen was kept in a new well of the 24-well plate containing 2 ml of PBS in each well. Then, 50 µl of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (1 mg/ml) was added to each well containing biofilm formed on the soft liner disk specimens. To each well, 4 µl of menadione (1 mM) was added and was mixed gently so as not to disturb the biofilm. The plate containing biofilm was then covered with aluminum foil after adding MTT solution and menadione, and was incubated for 6 hours at 37°C. The biofilms were then scraped into the wells using sterile cell scalpels. The mixture containing the biofilm and formazan product was then transferred to microcentrifuge tubes and was centrifuged at 6,000 rpm for 5 minutes. The supernatant obtained was transferred to one well of a 96-well culture plate and measured at 550 nm in a spectrophotometer [17]. Readings (n = 5) were recorded and expressed as percentage reduction of growth, using the following equation:

$$\text{Percentage reduction} = \left(\frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{experimental}}}{\text{Abs}_{\text{control}}} \right) \times 100$$

Percentage reduction is the reduction in *C. albicans* growth in percent values.

2.4. Evaluation of the Antibiofilm Activity of the Essential Oils by Scanning Electron Microscopy (SEM)

The inhibition effect of all the three essential oils on the biofilm formed on the surfaces of the denture soft liner specimens was visualized using a SEM. The biofilm was similarly formed on the specimens as the biofilm adhesion assay, followed by essential oil immersion treatments for all the specimens except for the specimens of the control group. The fixation of specimens was carried out with the use of 4% formaldehyde, which was added to each well of a 24-well plate containing the specimens. Then, the plate was sealed with aluminum foil and kept overnight in the refrigerator. This step was followed by dehydration of the specimens with increasing concentrations of ethanol (25%, 50%, 75%, 99%, and 100%). Following the dehydration of specimens, the coating was applied; stub mounting was carried out; and then the samples were observed under a SEM [18].

2.5. Hardness Testing

Acrylic specimens of diameter 44 and 3 mm thickness were fabricated. For the bonding of the resilient soft liner, these acrylic specimens imitated the denture base. To fabricate soft liner specimens, a metal ring whose diameter (internal) was lesser than the diameter (internal) of acrylic specimens was used. A groove in the shape "V" was made on the metal ring which allowed the excess material to flow. A total of 80 specimens were fabricated. The hardness test was carried out using Shore A durometer and the hardness readings were made before immersion (baseline) and after 24, 48 hours, 4, 7,15, 30, 45, and 60 days. For the rest of the time, the specimens were kept in artificial saliva at 37°C ± 1°C before each test, with the intent to simulate the oral cavity [19].

The test groups were as follows: Group A: Control (C) group (20 samples); Group B: lemongrass essential oil (LGO) (20 samples); Group C: tea tree essential oil (TTO) (20 samples); and Group D: thyme essential oil (ThO) (20 samples). Exposure of liner specimens to disinfectant solutions/cleanser solution showed that the immersion time was 2, 6, and 10 minutes for Group B, Group C, and Group D, respectively.

2.6. Statistical Methods

The data were entered into MS Excel followed by analysis using Statistical Package for the Social Sciences (version 22). The biofilm reduction assay was carried out in replicates of five (n = 5), and all the readings have been mentioned as mean ± standard deviation. The statistical analysis was carried out using paired *t*-test. For definitive statistics, in the present study, a comparison of Shore A hardness among the control (Group A), LGO (Group B), TTO (Group C), and ThO (Group D) was carried out. Descriptive statistics such as mean and standard deviation were calculated for all the groups. For inferential statistics, one-way analysis of variance (ANOVA) was applied to find out the significance of the difference between different groups in their mean hardness value. The *post-hoc* test (Scheffe's) was used to find out the difference between each pair of hardness' means at an alpha level of 0.05. "*t*"-test was applied for intragroup comparisons for mean hardness value.

3. RESULTS

3.1. MIC and MFC

All the three essential oils used in the study showed antifungal effects toward the MTCC 1637^{T} *C. albicans* strain (Fig 1). It is noteworthy that the MIC value of LGO was 0.03125% with an MFC value of 0.125%, whereas the MIC value of ThO was 0.0625% with an MFC value of 0.125%. The MIC and MFC values of TTO were 0.25%. The MIC and MFC values were considered for the biofilm adhesion assay.

3.2. Effects on the Removal of Biofilm Adhesion Assays

LGO was shown to reduce the biofilm formation of *C. albicans* by half with a concentration as low as 0.03125%. With an increase in immersion time from 1 to 5 minutes, a 1.78-fold reduction of the biofilm was noted. However, ThO showed a reduction of around 30%. Reduction in the growth of *C. albicans* is seen in the order LGO (0.03125%) > ThO (0.0625%) > TTO (0.25%) (Fig 2).

3.3. Evaluation of the Antibiofilm Activity of the Essential Oils by SEM

The SEM images of the control group show biofilm morphology formed on the surface of a soft liner with the smooth cell membrane of *Candida*. In the case of the treated groups with an immersion time of 1 minute, there is a considerable amount of reduction in *Candida* and disruption of the biofilm. However, among all the groups, LGO has shown a significant reduction in the biofilm, with no clumping of cells and disruption of the cell-to-cell adhesion. This is found consistent with the biofilm reduction.

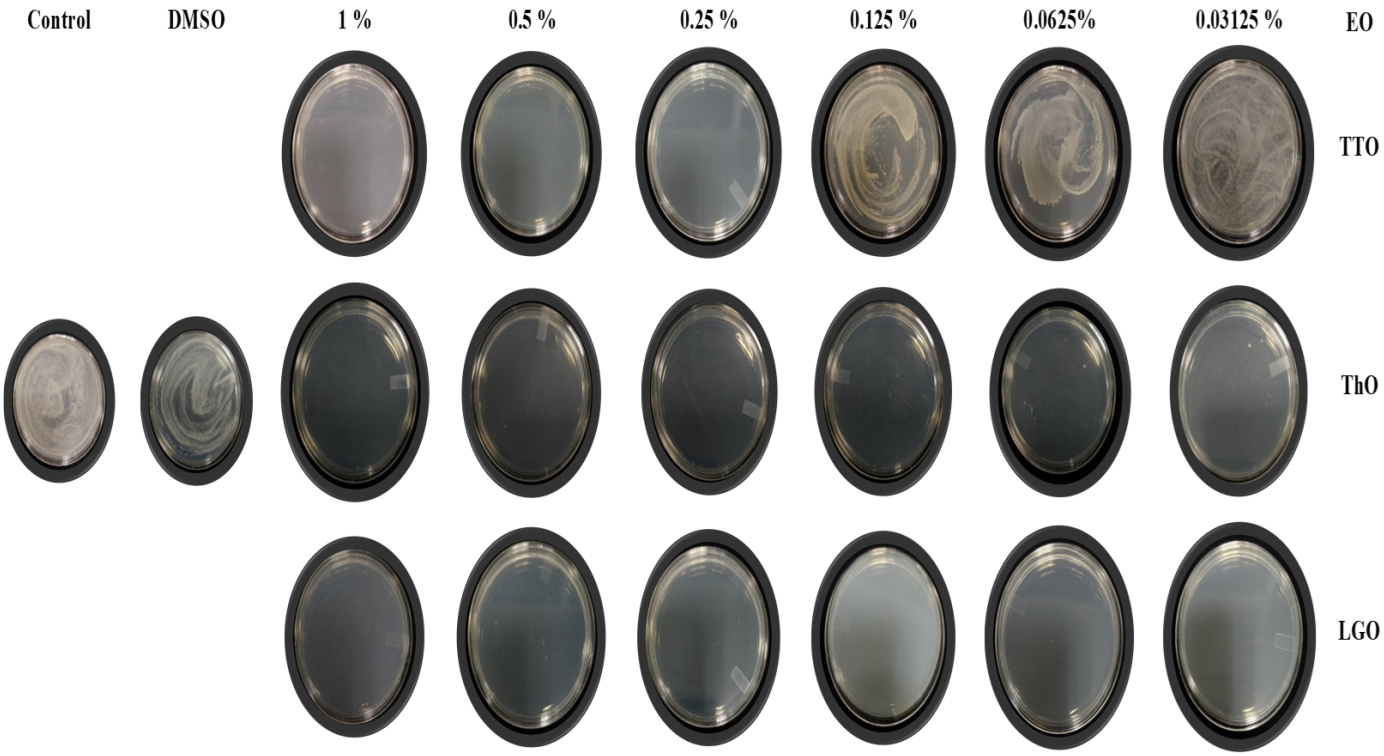

Figure 1: MIC and MFC of LGO, ThO, and TTO against *C. albicans* determined using broth dilution method. The MIC values were determined from the turbidity (OD at 600 nm) as the lowest concentration which inhibited visible growth of the organism and the MFC values were determined by absence of visible colony growth on SD agar plate. The final concentration of oils obtained after dilution were 1%, 0.5%, 0.25%, 0.125%, 0.0625, and 0.03125%. *Candida albicans* culture without any treatment was kept as positive control and culture treated with DMSO was kept as carrier control. LGO: Lemon grass oil, ThO: Thyme oil, and TTO: Tea tree oil.

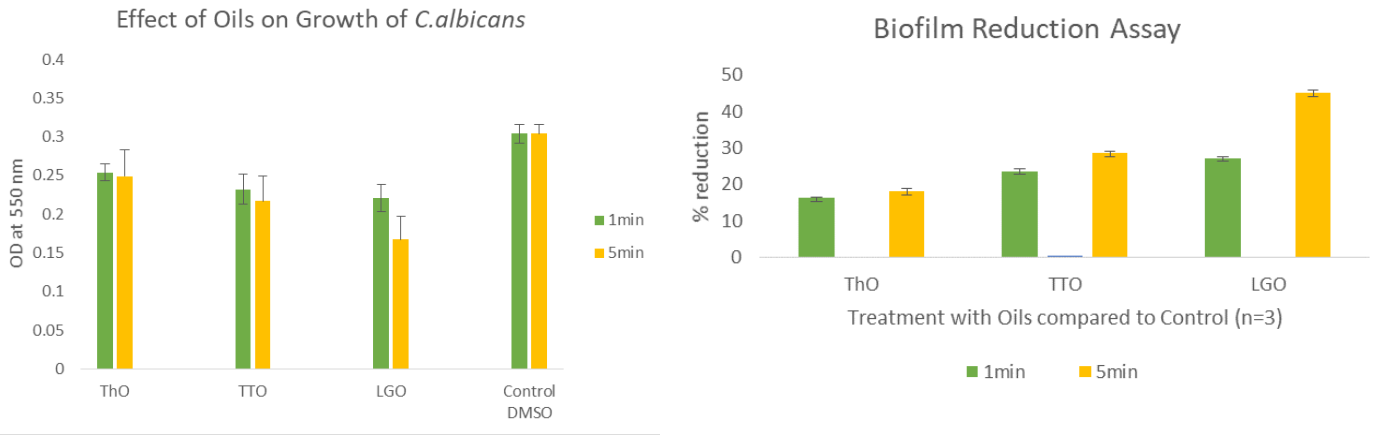

Figure 2: (A) Effects of essential oils on biofilm growth of *C. albicans* (comparison between the absorbance value at 550 nm of all the groups at 1 and 5 minutes time interval). LGO has the least absorbance value at 550 nm for both 1 and 5 minutes, followed by TTO and then ThO as compared to the control group. LGO and ThO were the most potent essential oils among the three essential oils to bring about a reduction in the number of *Candida* cells; (B) biofilm reduction expressed in terms of percentage. There is a reduction in the growth of *C. albicans* in the order LGO > TTO > ThO. A minimum of 5 minutes of immersion in the essential oil was found to be effective in inhibiting the growth of *C. albicans*.

3.4. Hardness Testing

Irrespective of the groups, a significant increase was observed from baseline to day 60 (p = 0.001) (Table 1 and Fig. 3). At baseline, the mean hardness was 48.60, which significantly increased to 56.95 on day 60. Furthermore, the table shows that the increase with reference to groups was verified, and repeated measures ANOVA revealed a significant value (p = 0.001) where we found that the TTO group had a minimal increase compared to control, LGO, and ThO groups. The rate of increase was statistically lesser in the TTO group as compared to the rest of the groups from baseline to day 60.

4. DISCUSSION

Previously, the findings of the study carried out revealed that all three essential oils have antifungal activity against *Candida*. The MIC and MFC values obtained in this study are comparatively

Table 1: Descriptive statistics—mean Shore A hardness values for silicone liner as a function of different time intervals. Groups tested: control, lemongrass group (LGO), tea tree group (TTO), and thyme group (ThO).

Duration	Groups	Mean	SD	Duration	Groups	Mean	SD
	Control	48.566	1.117		Control	57.983	1.082
	LGO	48.332	0.572		LGO	56.233	0.405
Baseline	TTO	48.566	0.953	Day 15	TTO	51.934	0.829
	ThO	48.950	0.690		ThO	56.783	0.678
	Total	48.604	0.873		Total	55.733	2.424
	Control	50.600	0.951		Control	58.066	0.676
	LGO	50.115	0.842		LGO	56.385	0.277
24 hours	TTO	50.150	0.700	Day 30	TTO	52.150	0.509
	ThO	49.833	0.809		ThO	56.865	0.600
	Total	50.175	0.860		Total	55.867	2.306
	Control	51.565	0.622		Control	58.216	0.316
	LGO	52.965	0.587		LGO	56.517	0.377
48 hours	TTO	50.350	1.435	Day 45	TTO	52.301	0.474
	ThO	53.117	1.033		ThO	58.033	0.708
	Total	51.999	1.489		Total	56.267	2.446
	Control	53.952	1.028		Control	59.099	0.728
	LGO	54.632	0.593		LGO	57.183	0.414
Day 4	TTO	50.933	1.043	Day 60	TTO	52.586	0.810
	ThO	55.048	0.672		ThO	58.918	0.650
	Total	53.641	1.828		Total	56.947	2.723
	Control	55.617	0.596				
	LGO	55.651	0.579		Test statistics		
Day 7	TTO	51.316	0.678		F (Overall) = 1,345.652; p = 0.001		
	ThO	56.050	0.601		F (Change × groups) = 61.187; p = 0.001		
	Total	54.659	2.041				

lower than the values obtained by Koseki *et al.* [6] in which TTO had 0.5% MIC and minimum fungicidal concentration (MFC) values, and LGO had a 0.0625% MIC value and a 0.125% MFC value.

In a study by Taweechaisupapong *et al.* [20], LGO showed the strongest inhibitory effect on biofilm formation of *Candida* isolates. It exhibited more than 90% inhibition on biofilm formation of *C. albicans* and at concentrations of 0.8 mg/ml. In addition, LGO at concentrations less than the MIC values against the isolates (0.06–0.25 µl/ml) also possessed an inhibitory effect on biofilm formation.

Although several experiments have demonstrated that natural antifungal compounds act by interacting with cytoplasmic membranes of *Candida*, the precise mechanisms involved in their mode of action are not completely known. The major sterol portion of the fungal cell membrane responsible for sustaining cell structure and integrity is ergosterol, which is unique to fungi [21]. Altering the permeability of *C. albicans* cells is the mechanism of antifungal activity provided by TTO [22]. The values of MIC and MFC observed in the present study were lower than those reported by others. Due to the proportion of chemical constituents found in the synthesis of all three essential oils, the disparity in MIC and MFC values can be attributable. The difference between this study's findings and other studies can be mainly due to the variations in the composition of each plant ecotype and the difference in the study design [23].

According to the ISO standard, "long-term soft lining materials are categorized into type A (soft type) for 25 < durometer shore hardness (SH) ≤ 50 and type B (extra soft type) for durometer SH ≤ 25 when measured at 24 hours after specimen preparation." The standard specifies that the specimens that are aged for 28 days should exhibit "durometer SH ≤ 55 for type A and durometer SH ≤

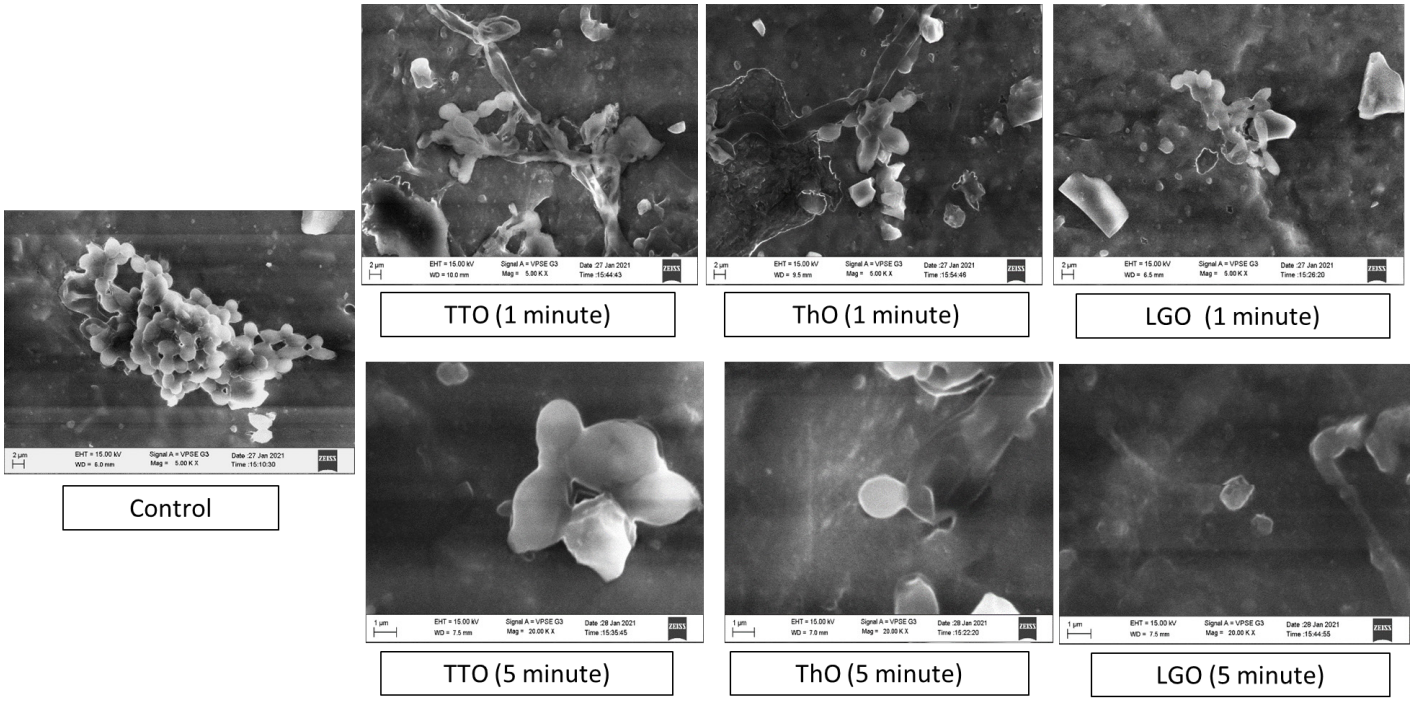

Figure 3: SEM images of *C. albicans* biofilm inhibition on the surface of soft reliners treated with essential oils for 1 minute (scale = 2 µm, magnification 5×) and 5 minute (scale = 1 µm, magnification 20×). The control group shows an aggregation of cells typical of biofilm formation, while treatment with essential oils resulted in morphological damage to the cell surface, disruption of cell to cell adhesion, and clumping of cells.

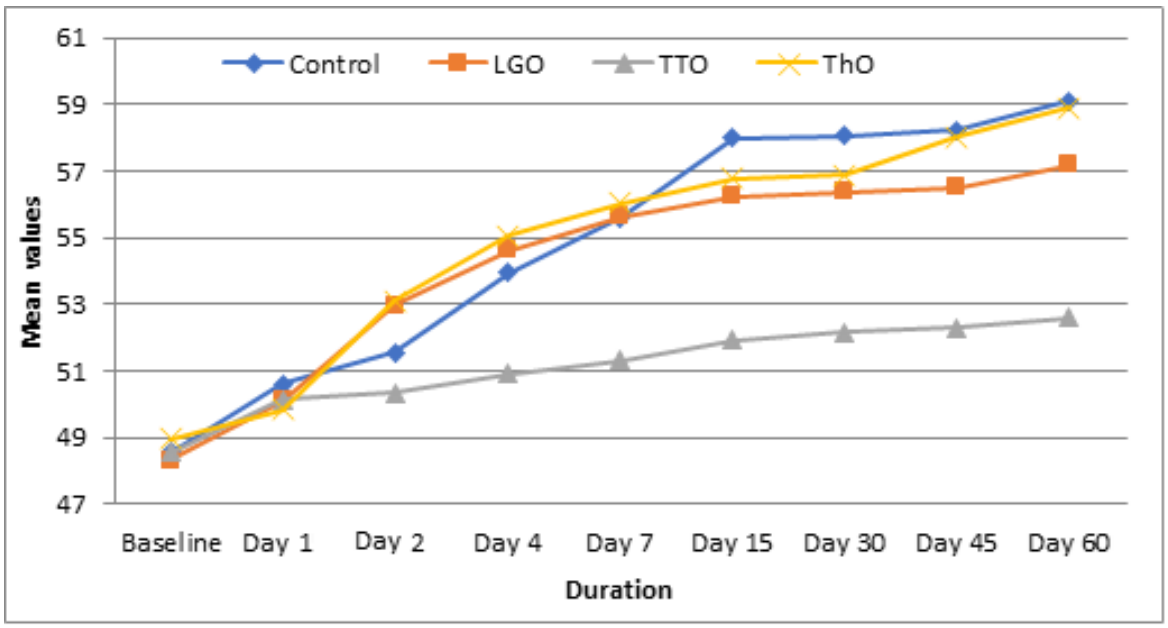

Figure 4: Comparison between mean Shore A hardness values for silicone liner as a function of different time intervals. Groups tested: control, lemongrass group (LGO), tea tree group (TTO), and thyme group (ThO).

35 for type B materials," after an extended period of use because the soft denture reliner should not harden extremely [19].

A minor change in the Shore A hardness for long-term soft lining material is considered desirable. Nonetheless, Shore A hardness value for the 28-day elevated value unquestionably correlated with the 24-hour Shore A hardness value for all the groups of essential oil, and this result is in agreement with studies conducted by Kim *et al.,* [19]. In a study by Kim *et al.* [19], "GC Reline Soft showed the mean durometer Shore A hardness of 50.13 ± 0.48 for 24 hours and 57.20 ± 0.28 for 28 days [23]."

In the present study, there is a linear increase in mean hardness values in all groups from baseline, which are all significant (Fig. 4). Polymerization of autopolymerized lining materials continues over some time and usually happens at room temperature. This may be one of the many reasons for the increase in the hardness of the soft lining material during its lifetime. As it does not have a plasticizer in its composition, the silicone soft lining material as compared to acrylic soft lining material would result in less water absorption and less increase in hardness as there was no plasticizer loss [24].

In this study, the determination of the initial hardness of the specimens (baseline value) was carried out, and they were kept in artificial saliva at 37°C for the simulation of the oral conditions. Weekly, the artificial saliva solution was changed. In one of the studies, where they evaluated the hardness of different soft liners after immersion in artificial saliva at various time intervals, it was seen that "GC Reline Soft was the hardest material initially and during the whole experiment (48.3–58.3 SH A degrees). After 30 days, its hardness no longer increased [25]." Additionally, it was concluded that storage of the soft liner specimens in artificial saliva at mouth temperature can be an added factor for changes in the hardness of the soft liner material over a period of 60 days. The current study is the only study where essential oils were used as denture cleansers rather than incorporating the oil into the liner, as the amount of leaching of the oil into the oral environment can occur with time and can cause toxic effects to the mucosal cells.

In the present study, despite the increased hardness of the soft liner over the days of evaluation, "Shore A hardness values fall within the ISO specification for this category of materials." Furthermore, previously carried out research states that it is possible to advocate the alterations found in an increased amount of Shore A hardness that would not bargain the clinical use of reliner immersed in an essential oil denture cleanser, within the tested time, which provides crucial data regarding the potential use of essential oil as cleansers for the relined denture for the clinical treatment of denture stomatitis. Based on the results of the present study, an increased amount of Shore A hardness would not hamper the clinical use of reliner immersed in essential oil as a cleansing solution. Furthermore, it is necessary to evaluate the performance and properties of these denture liners in clinical use.

5. CONCLUSION

For the present study, it is recorded that the reduction in the growth of *C. albicans* is in the order LGO > ThO > TTO. A minimum of 5 minutes of immersion in the essential oil is required to inhibit the growth of *C. albicans*. Based on MIC results, LGO was potent enough to reduce biofilm formation at 0.03125%, followed by ThO and TTO. There is a linear increase in mean hardness values which was observed in all the groups from baseline, which are all significant. The rate of increase in the Shore A hardness was statistically lesser in the TTO group as compared to the rest of the groups from baseline to day 60. However, this increase is within the clinical parameters of the ISO standard, and the clinical significance remains to be evaluated. Therefore, it can be concluded that all the three essential oils, i.e., LGO, ThO, and TTOs, in the same order have a potential antifungal effect and can be used as denture cleansers.

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7. CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

8. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

9. DATA AVAILABILITY

All data generated and analyzed are included within this research article.

10. PUBLISHER'S NOTE

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