Dental Materials Journal 2018; 37(4): 659–666

Development of antibacterial denture cleaner for brushing containing tea tree and lemongrass essential oils

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We evaluated effectiveness of tea tree oil (TO) and lemongrass oil (LO) for removal of *Candida* biofilm from denture base resin and their influence on that surface. Biofilm of *C. albicans* was formed on resins, and immersed in various concentrations of each oil and distilled water (DW). The biofilm removal effect was determined by incubating specimens in RPMI medium containing Alamar blue (AB) and measuring absorbance. Wear test was also conducted, and surface condition of resins was determined using laser scanning microscope and digital microscope. Specimens immersed in the TO and LO solutions tended to have a lower AB value at higher concentrations and longer soaking times. Use of these agents resulted in less surface roughness as compared to DW. Our results suggest that TO and LO were valid to remove biofilm attached to resin with lower levels of abrasion, and these are effective for use in denture cleaner.

Keywords: Denture cleaner, *C. albicans*, Tea tree oil, Lemongrass oil, Wear test

INTRODUCTION

Debris attached to a denture appliance is generally considered to be denture plaque and typically includes various kinds of microbes, such as *Candida* species, mainly *C. albicans* causing denture stomatitis and oral candidiasis, and bacteria that cause oral diseases, and are related to caries and periodontitis development[1]. A recent epidemiological study revealed that denture stomatitis among denture wearers ranges from 15 to over 70%[2]. Many who use a denture appliance are elderly, whose oral functions and resistance to infection are reduced. Use of a contaminated denture can cause aspiration-related pneumonia or gastrointestinal fungal infection from infected denture plaque, thus seriously threatening the health of the wearer[3]. Furthermore, deterioration of denture materials can lead to a worsened disease state. Thus, denture care in addition to oral care is necessary to maintain the health of patients.

Proper denture care is attained with various methods, such as mechanical cleaning method such as brushing with a denture brush, chemical washing method such as soaking in commercial denture cleansers, ultra-sonic cleaning method, and combinations of each[4,5]. However, it is sometimes difficult to completely eliminate *Candida* with these methods[6], as the acrylic resins used for fabrication of complete dentures are composed of PMMA, a material with a low level of hardness[7]. Elderly individuals may damage the denture surface by inappropriate brushing and it has been shown that small scratches on the denture surface can become a breeding ground for oral pathogens[8,9]. In addition, Tanaka *et al.* reported damage to a resin surface cause not only by a denture dentifrice containing abrasives but also distilled water (DW)[10]. Therefore, a novel denture cleaning method that does not cause damage to resin surfaces is needed.

Plant essential oils are used widely for aromatherapy and known to produce relaxation, analgesia, anti-inflammation, and antimicrobial effects, as well as others. Among such products, tea tree oil (TO) and lemongrass oil (LO) have been tested against several different bacteria and *Candida* species, which showed their antibacterial activities[11,12]. TO, also known as *Melaleuca alternifolia* oil, has been used as a panacea with safe antiseptic effects for more than 100 years in Australia, and is now incorporated in many pharmaceutical and cosmetic products[11]. In recent years, clinical studies have been performed to investigate its antibacterial effects as an alternative medicine for dermatophytosis and vaginal candidiasis[13,14]. In addition, application of essential oils in the field of dentistry has also received attention, as methods for mouth rinsing with peppermint, TO, and LO with water have been introduced in aromatherapy textbooks. Antimicrobial effects of such oils against oral microorganisms have also been reported, including the caries inducing bacterium *Streptococcus mutans*, periodontal disease-inducing bacteria *Porphyromonas gingivalis* and *Fusobacterium nucleatum*, and oral candidiasis-inducing microorganism *C. albicans*[15]. LO is known to have antifungal properties, with the east India type, extracted from *Cymbopogon flexuosus,* commonly available. LO has also been considered for application as a mouth rinse for preventing oral candidiasis[16].

Furthermore, several studies have reported that both TO and LO reduced biofilm formation on denture surface resin, and suggested their use for denture cleaning[17-19]. However, in clinical situations, many dentures with attached biofilm already developed are washed, though few reports have focused on removal of surface biofilm by use of an essential oil. Therefore, further study is necessary on applying essential oil to cleaning materials for dentures.

Received Aug 18, 2017: Accepted Nov 13, 2017
doi:10.4012/dmj.2017-295 JOI JST.JSTAGE/dmj/2017-295

The purpose of this study was to examine whether essential oils are suitable for denture cleaner. The abilities of TO and LO to remove biofilm and influence the denture surface were investigated, as they were considered capable to destroy mature biofilm formed by *C. albicans* on denture base resin surface. In addition, we speculated that damage to resin surface would be reduced by mechanical cleaning with these essential oils.

MATERIALS AND METHODS

Essential oils

For the present study, we selected 2 oils that TO and LO (Tree of life, Tokyo, Japan) for our examinations (Table 1). Each essential oil was dissolved in dimethyl sulfoxide (DMSO) and adjusted to a 50% (v/v) aqueous solution prior to performing the experiments.

Determination of minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

This study utilized *Candida albicans* ATCC 18804, which is typically used as a reference strain for analyses of disinfectants and antifungal agents. *C. albicans* was seeded into Sabouraud agar (Nissui, Tokyo, Japan) at 37°C for 24 h, then simple colonies were transferred to tryptic soy broth medium (TSB; Becton, Dickinson and Company, NJ, USA) at 37°C with shaking for 24 h. The number of cells was adjusted to approximately 1×10^6 to 1×10^7 cells/mL with PBS.

The MIC value to inhibit *C. albicans* was determined using the CLSI M27 liquid dilution method[20], for which 80 μL of 50% (v/v) essential oil was added to a tube with 4 mL of TSB and mixed by inversion. The concentration of the first standard was 1.0% per mL. Five tubes were prepared and 2.0 mL of TSB was added to each tube, then 2.0 mL was taken from the first tube and added to the second tube, with the same procedure performed with the other tubes. Thus, the final volume in each tube was 2.0 mL and the essential oil concentrations in the 5 tubes were 1.0, 0.5, 0.25, 0.125, 0.0625, and 0.03125%, respectively. A 100 μL aliquot of the pre-culture was added to all of the tubes, then each was incubated in a shaker at 37°C for 48 h. The MIC was considered to be the lowest concentration of essential oil to inhibit microorganism growth, which was indicated by clear fluid with no development of turbidity and without visible growth. One hundred milliliters of the first and second lowest concentrations that produced a non-cloudy liquid sample was inoculated into Sabouraud agar at 37°C for 24 h. The MBC was considered to be the lowest concentration without colony formation. MIC and MBC values were used in the adhesion and wear assays.

Effects on removal of biofilm-adhesion assays

A total of 294 specimens were prepared using heat-polymerized acrylic (PMMA) resin for the denture base (Acron, No. 9, GC, Tokyo, Japan), using a conventional method to produce a denture appliance. A wax pattern was formed and buried in dental investment material using a metal dental flask for water-bath polymerization, then the wax was washed out with boiling water. Next, the PMMA resin was packed and polymerized under heating in the metal flask according to the manufacturer's instructions. After polymerization, the resin was trimmed into a disk form (6 mm in diameter, thickness 1.5 mm, weight 0.24 g) and the surface was polished with a silicone point (Big Silicone Points, HP R2, Shofu, Kyoto, Japan), which resulted it what was assumed to be a clinically acceptable denture-fitting surface. The samples were soaked in water overnight. Prior to use in an experiment, each was sterilized with

Table1 Essential oils used in this study

Product	Code	Component
Tea tree oil (*Melaleuca alternifolia*)	TO	Terpene hydrocarbons γ-Terpinen, α-Terpinen, Terpinolene, p-Cymene, α-Pinene, Aromadendrene, Limonene, δ-Cadinene, Ledene, Sabinene Terpene alcohol Terpinen-4-ol, Terpinolene, α-Terpineol, Globulol, Viridiflorol Oxide 1,8-Cineole
Lemongrass oil (*Cymbopogon flexuosus*)	LO	Terpene hydrocarbons β-Karyopherin, Camphene, Limonene Terpene alcohol Geraniol, Linalool Aldehyde Citral Ester Geranyl acetate Ketone Methylheptenone

0.05% sodium hypochlorite for 5 min and immersed in sterilized DW 3 times for 10 min.

C. albicans was inoculated into yeast nitrogen base (YNB) broth (Difco Laboratories, Detroit, MI, USA) supplemented with 50 mM glucose at 37°C with shaking for 18 to 20 h. After incubation, the yeast cells were washed twice with PBS and suspended in YNB supplemented with 100 mM glucose. The concentration of suspensions of *C. albicans* was standardized to approximately 1×10^7 cells/mL. Biofilm formation was determined using the method of Wander *et al.*[21] Briefly, biofilm developed on PMMA surfaces placed into sterilized flat-bottomed 24 well microtiter plates (Iwaki, Tokyo, Japan). An aliquot containing 1.0 mL of a standard yeast cell suspension was transferred into each well containing a single disc and incubated for 90 min at 37°C in an orbital shaker at 75 rpm (adhesion phase). Next, the medium was aspirated and each sample was washed twice with PBS to remove non-adherent cells from the resin surface. Fresh YNB (2.0 mL) supplemented with 100 mM glucose was then added to each well and incubated for 3 days at 37°C in a shaker at 75 rpm, during which each sample was washed with PBS and the medium exchanged for 2.0 mL of freshly every 24 h. After biofilm formation, the samples were separately soaked in different 6 combinations of essential oil and DW, as follows: TO, 1.0 and 0.5%; LO, 1.0, 0.5, 0.25, and 0.125%; based on the MIC and MBC values. The soaking times in each solution were 1, 3, and 5 min. Thus, the samples were divided into 21 groups (n=14). After soaking, each sample was placed into a well of a new 24-well plate with 2.0 mL of RPMI 1,640 medium (Kohjin Bio, Saitama, Japan) supplemented with 10% of a REDOX indicator (Alamar Blue, Trek Diagnostic Systems, Cleveland, OH, USA) and incubation was performed for 24 h at 37°C. Every 6 h, 100 μL of the supernatant solution was gently transferred to a single well of a 96-well plate. Colorimetric changes were determined at 570 and 630 nm using a Microplate Photometer (Multiskan FC, Thermo Scientific, Waltham, MA, USA). Following the instructions of the manufacturer, the AB value of each sample was calculated based on absorbance, then the AB values for the combinations of DW and TO, and DW and LO were compared.

Effects on denture base resin-wear test

Twenty PMMA resin (Acron) samples were polymerized according to the manufacturer's instructions, then trimmed into $25\times15\times2$ mm^3 pieces. For testing, a mirror surface, with one side coming into contact with a glass plate during polymerization. Before and after the wear test, the surface roughness of 5 random areas of each sample was determined using a laser scanning microscope (Super depth surface profile measurement microscope, VK-8550, Keyence, Osaka, Japan). The mean surface roughness of all samples was R_a=0.17±0.01 μm, which was near to the value previously obtained with a conventional denture polishing method[22]. Next, 4 groups of cleaning materials were tested; 1.0%TO, 1.0%LO, 0.5%LO and DW (control). Wear testing was performed with an abrasion testing machine (Rubbing Tester IMC-151B, Imoto Machinery, Kyoto, Japan)[10]. The sample was brushed with a denture brush (Erac Denture Brush, Lion, Tokyo, Japan) at 200 g of loading in the direction of the long axis with a 20 mm trail and up to 10,000 reciprocal strokes, which was considered to simulate denture cleaning for approximately 1 year. Five samples were subjected to each of the cleaning materials. Surface roughness was then measured and surface properties were observed (magnification×1,000) using a digital microscope (VK-5000, Keyence).

Statistical analysis

AB values obtained during the measurement period and surface roughness were analyzed using one-way ANOVA and *post-hoc* Dunnett's analysis. p<0.05 was considered to indicate a significant difference. SPSS Statistics software, version 17.0, was used for all statistical analyses (SPSS, Chicago, IL, USA).

RESULTS

MIC and MBC

In the present study, 2 different essential oil formulations showed antifungal effects towards the ATCC18804 strain, as TO had MIC and MBC values of 0.5%, and LO had an MIC value of 0.0625% and an MBC value of 0.125%. Those values for LO were less as compared to TO. Therefore, for TO and LO, the concentrations used in the adhesion assay were based on the MIC and MBC.

Adhesion assay

In Figs. 1 and 2, the effects of soaking in TO and LO on removal of biofilm attached to the resin samples are shown. The AB value after soaking in TO or LO showed a low tendency at a high concentration and long soaking time. On the other hand, soaking in 1.0% TO for 3 or 5 min resulted in a significantly lower AB value as compared to the control after 12 h (p<0.05) (Fig. 1B). There were no significant differences between any of the groups after 6, 18, and 24 h (Figs. 1A, C, D).

Soaking in 0.5 and 1.0% LO resulted in lower AB values as compared to the control group after 6 and 12 h (Figs. 2A, B), while those after soaking for 5 min group were significantly reduced (p<0.05) (Fig. 2A), with the 1.0% LO group showing a significantly lower value (p<0.05). In addition, 12 h of soaking resulted in significantly lower values for the 0.5 and 1.0% LO groups as compared to the control group after 12 h (p<0.05) (Fig. 2B). There were also significant differences among soaking in 0.5% LO for 5 min, in 1.0% LO for 3 min, in 1.0% LO for 5 min, and the control groups after 6 and 12 h (p<0.05) (Figs. 2C, D).

Wear test

Surface roughness in all groups showed a tendency to increase at the end of the tests (Fig. 3). Surface roughness for the 0.5%LO, 1.0%LO, and DW groups was significantly greater than before the start of the experiment (p<0.05).

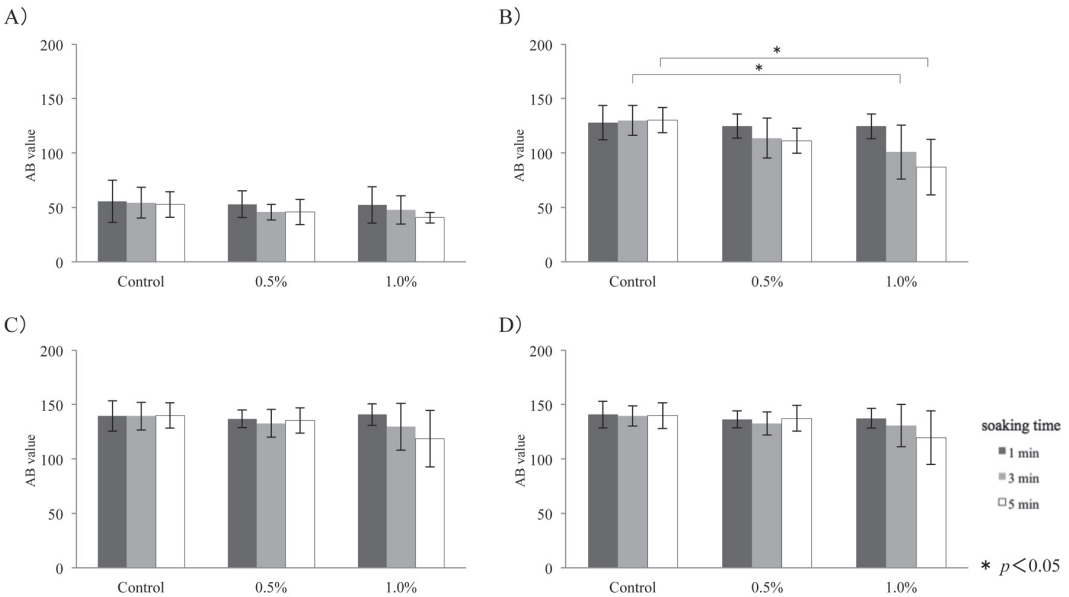

Fig. 1 Effects of soaking in tea tree oil (TO) on removal of biofilm attached to the resins.
After biofilm formation, samples were soaked in TO to remove *Candida* biofilm. Then, samples were cultured for 24 h and the activity of surviving bacteria at 6 h intervals was determined. The AB value of each sample was calculated based on absorbance and the AB value of control and TO were compared. Error bars indicate standard deviation, asterisk (*) indicate statistical differences (Dunnett's test, $p<0.05$). A) 6 h later, B) 12 h later, C) 18 h later, D) 24 h later.

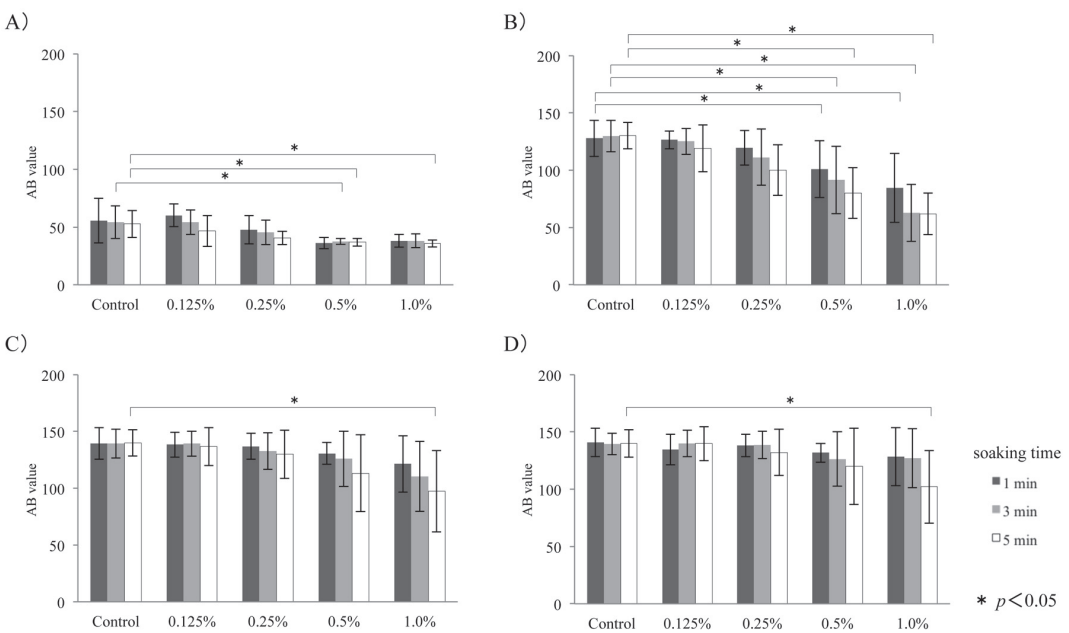

Fig. 2 Effects of soaking in lemongrass oil (LO) on removal of biofilm attached to the resins.
After biofilm formation, samples were soaked in LO to remove *Candida* biofilm. Then, samples were cultured for 24 h and the activity of surviving bacteria at 6 h intervals was determined. The AB value of each sample was calculated based on absorbance and the AB value of control and LO were compared. Error bars indicate standard deviation, asterisk (*) indicate statistical differences (Dunnett's test, $p<0.05$). A) 6 h later, B) 12 h later, C) 18 h later, D) 24 h later.

That value for DW (0.23 μm) was the greatest among all the groups, while surface roughness with 1.0%TO was the lowest. There was no significant difference between before and after exposure to 1.0%TO. Figure 4 presents representative digital microscopic images obtained during the wear tests. With all of the cleaning materials, grooves formed by brushing were observed, though the 1.0%TO, 0.5%LO, and 1.0%LO groups were similar and showed few wear traces, in contrast to the DW group.

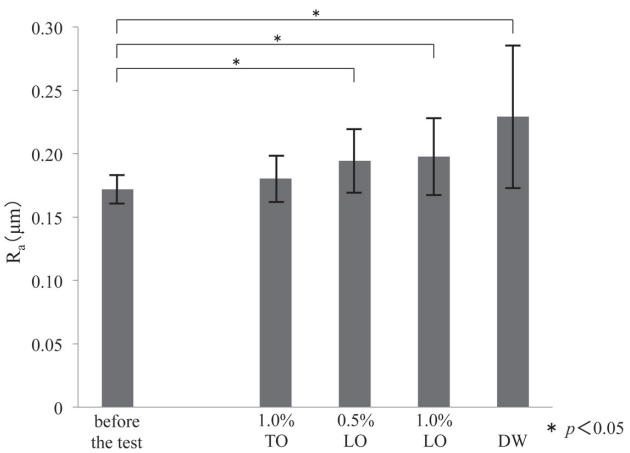

Fig. 3 Results of surface roughness testing.
Error bars indicate standard deviation, asterisk (*) indicate statistical differences (Dunnett's test, $p<0.05$). Surface roughness values (R_a) of resins for the 0.5%LO, 1.0%LO, and DW groups were significantly greater than before the test.

DISCUSSION

The results of the present study show that TO and LO have antifungal effects against *C. albicans*, which has also been reported in other studies[11,14-19]. However, LO were more effective than TO based on the MIC and MBC values, and consider that the differences in antifungal effect were related to the individual components of the essential oils. Those with antibacterial activities include the alcohol terpinen-4-ol and the aldehyde citral[11,12]. We found that the content of terpinen-4-ol in TO was 41.90%, while citral in LO was 67.77%. These components are known to easily combine with proteins, while they promote bacteriolysis by denaturation of cell body proteins, penetration into cytoplasm, and solidification and leakage of cell contents[11,14]. In other studies, these oils showed antibacterial power at a lower density than glutaral, and are anticipated to function well as substitute disinfectants[16,17].

In 2004, the Scientific Committee on Consumer Products in Brussels, Belgium stated that 80–90% of the contents of *M. alternifolia oil* are monoterpenes[23], which is consistent with the TO used in our study [terpinen-4-ol (41.90%), γ-terpinene (20.30%), α-terpinene (9.30%), 1.8-cineole (3.2%), para-cymene (2.80%), α-terpineol (2.90%), α-pinene (2.40%), terpinolene (3.30%), limonene

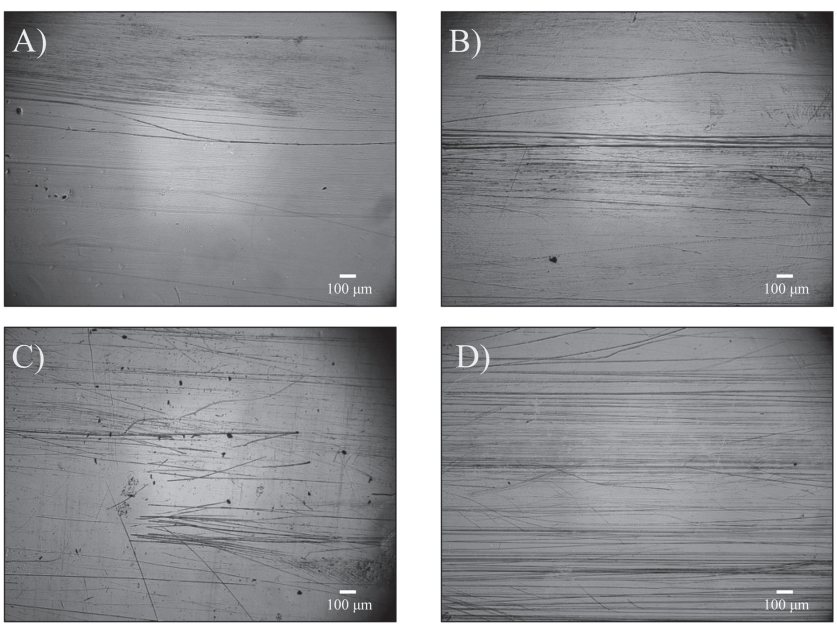

Fig. 4 Representative digital microscopic images of resins obtained during wear test (magnification ×1,000).
With all of the cleaning materials, grooves formed of brush were observed. 1.0% TO, 0.5% LE, 1.0% LE had fewer wear traces than the DW group. A) 1.0% TO, B) 0.5% LO, C) 1.0% LO, D) DW.

(1.00%), sabinene (0.20%); 87.30% of total composition]. Another report also noted that TO showed a MIC of 0.375% towards *C. albicans* strain ATCC10231[18]. We found that the effect of this oil was caused by its higher density due to the difference in content of terpene related to its growth environment or place of origin.

The results of this laboratory study allow only partial acceptance of the research hypotheses. First, the effect of TO on removal of *Candida* biofilm was great, as the number of remaining bacteria at 24 h after immersion in the 1.0% concentration solution for 5 min was the lowest, though compared to other TO group. The numbers of remaining bacteria were also lower with the other experimental groups at each measurement point compared to the control group. In addition, the number of remaining bacteria in the LO groups tended to be reduced when the concentration was higher and immersion time was longer. Particularly, the group with immersion in the 1.0% LO concentration solutions for 5 min showed significantly lower levels of bacteria 24 h later. However, we also found that the number of remaining bacteria tended to increase when the incubation time after immersion was extended. As a result, we considered that sterilization was not completely achieved without the active ingredients being taken in deeply to the biofilm with shorter times of immersion of 1, 3, or 5 min. Therefore, it is expected that biofilm could be removed from resin by TO and LO at a higher concentration and longer immersion period.

Studies have been conducted regarding the antibacterial activities of plant essential oils and their influence on biofilm formation on base resin. Dalwai *et al.* reported that TO was more effective against *Candida* growth inhibition on heat cured resin as compared to fluconazole[17]. Tobouti *et al.* noted that *Candida* have difficulty with surface adhesion following immersion in *Melaleuca alternifolia* oil[18]. There are also other reports describing inhibition of biofilm formation by plant essential oils, while ours is the first to show a removal effect.

We formed biofilm on base resin and examined the removal effects of the tested oils after 3 days. Biofilm is formed by *C. albicans* in 4 sequential steps[21,24]. First, the microorganisms adhere to a surface and the initial colony is organized, which is followed by secretion of extracellular polysaccharide that develops a matrix structure and finally progeny biofilm cells disseminate. In the present experiments, we used AB as a REDOX indicator for determining removal effects. This agent contains tetrazolium salt and shows color change with a reduction in nicotinamide adenine dinucleotide produced during the process of metabolism by living cells. Since the number of living cells can be determined by quantifying colorimetric change, we utilized this indicator to determine *Candida* removal[25].

In general, biofilm formed in the oral cavity environment includes *C. albicans* as well as other bacteria[1,6]. A previous study evaluated the effects of chemical cleansers on the pathogenesis of biofilm composed of a mixture of *C. albicans* and *C. glabrata*, and it was found that the tested denture cleansers were more effective against *C. albicans* than *C. glabrata*[26]. In another study, the predominant oral yeast organisms identified were *C. albicans* (75%) and *C. glabrata* (30%), which were isolated in higher proportions in patients with the highest grades of inflammation (100 and 80%, respectively), as well as in combination from 80% of the examined patients[27]. Additional studies are needed to examine biofilms composed of a mixture of *C. albicans* and *C. glabrata* in regard to antifungal therapy and pathogenicity.

The second research hypothesis of the present study can be accepted. In the present study, the change of roughness value for 1.0%TO was 0.012±0.013 μm, for 0.5%LO was 0.017±0.014 μm, and for 1.0%LO was 0.024±0.027 μm, which were lower as compared to other studies[28], though abrasion traces with use of these agents were observed (Fig. 4). In addition, each was significantly different as compared to DW (0.049±0.035 μm) and the other essential oil formulations. Sorgini *et al.* studied changes in surface roughness of PMMA caused by brushing with a conventional dentifrice and immersion in 0.5% sodium hypochlorite, and reported that the surface roughness values obtained with sorriso (1.617±1.190 μm) and corega (1.634±1.082 μm) were significantly greater as compared to water (−0.063±0.013 μm) [28]. Fernandes *et al.* also investigated surface roughness using denture base acrylic resins and found that it was increased after 30 min of immersion in the examined chemical disinfectants (1% sodium hypochlorite, 2% peracetic acid)[29]. Increased surface roughness can facilitate bacterial accumulation, which then promotes development of denture stomatitis, caries, and periodontal disease[8,9,30]. In addition, it was reported that the morphological transformation between fungal blastospores and hyphae in *C. albicans* biofilm coincides with an increase in surface roughness of denture acrylic resin[31,32], a phenomenon attributed to thigmotropism (contact sensing) by *C. albicans*, in which yeast cells switch to hyphal growth upon contact with a surface[33]. Invasion of hyphae *via* a few cracks in resin induces hyphal growth and formation of biofilm[34], thus increasing pathogenicity and virulence.

On the other hand, different factors are also involved with the increase of adhesion to a resin surface by bacteria. Other studies have highlighted the role of hydrophobicity in *C. albicans* biofilm formation on the surface of denture base materials. For example, Klotz *et al.* observed that a greater number of cells adhered to surfaces with greater hydrophobicity[35], though Minagi *et al.* observed lower adhesion[36]. Camila *et al.* evaluated the effects of surface roughness on the hydrophobicity of denture base acrylic resin and *C. albicans* colonization, and suggested that surface roughness ranging from 0.05 to 3.0 μm significantly increased hydrophobicity but had no significant effect on *C. albicans* adhesion[37]. In addition, Gantait *et al.* examined candidal growth on acrylic resin to elucidate the effects of denture adhesive and denture cleaning methods, and found that denture adhesive increased the adherence of *C. albicans* to a

resin surface and removal of biofilm by cleanser alone was difficult[38].

A limitation of this study is that only a single type of resin was evaluated. Various types of materials with a variety of components, such as artificial tooth resin, metals, and hard and soft denture liners are used clinically. Moreover, there was no simulation of the oral environment or use of denture adhesive. Additional studies are needed to better understand the effects of essential oils with other denture materials.

Several previous investigations regarding denture cleaning methods have found that a combination of mechanical cleaning and chemical washing is desirable for removal of biofilm from denture surfaces[4,5,28]. Based on results of a 21-day clinical trial, Panzeri *et al.* suggested that brushing with denture cleaning paste was more effective for removal of biofilm than brushing with tap water[39]. However, abrasion of the appliance surface by denture cleaning paste and brushing, with subsequent bacterial accumulation in the wear traces are likely to occur[2,10,40]. Our results demonstrated that TO and LO remove biofilm formed by *C. albicans*, and their use during brushing also resulted in a lower level of increased surface roughness of denture base resin. Therefore, addition of an essential oil to a denture cleaner for brushing is rational.

CONCLUSION

Within the limitations of this *in vitro* study, following are our important results. First, TO and LO each had antifungal effects towards *C. albicans*. Also, removal of biofilm attached to resin was accomplished by adjusting the concentration and action time of each tested essential oil. In comparison to DW, TO and LO showed a low level of wear when used for brushing denture base resin. Together, our findings suggest the possibility of using TO and LO for removal of *Candida* as components of denture cleansers for brushing.

ACKNOWLEDGMENTS

This work was supported by JSPS KAKENHI Grant Numbers 16K15806.

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