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Efficacy of citronella and cinnamon essential oils on *Candida albicans* biofilms

Running Title: Efficacy of essential oils on *Candida* biofilm

Keywords: Anti-Infective Agents; Phytotherapy; *Candida albicans*; Biofilm; Denture stomatitis.

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Efficacy of citronella and cinnamon essential oils on *Candida albicans* biofilms developed on acrylic

Running Title: Efficacy of essential oils on Candida biofilm

Abstract

Introduction: The discovery of new antimicrobials derived from plants could aid the management of biofilm-associated infections, including denture-induced stomatitis (DS). DS is an oral infection caused by *Candida* biofilms on the surface of poorly cleansed dentures. Effective treatment of DS requires the use of an appropriate denture cleanser and preferably one that exhibits antimicrobial properties. **Objective:** This study aimed to evaluate the anti-*Candida* and anti-biofilm efficacy of two essential plant oils from *Cymbopogon winterianus* (citronella) and *Cinnamon cassia* (cinnamon). **Material and methods:** Minimum Inhibitory Concentrations (MICs) and Minimum Fungicidal Concentrations (MFCs) were determined by broth microdilution, whilst anti-biofilm activity was measured against mature (cultured for 72 h) acrylic biofilms. *Candida* cell viability was assessed immediately (0 h) after treatment (T₀) and 48 h after biofilm re-growth (T₄₈). Biofilm structure was determined using Scanning Electron Microscopy (SEM) at T₀ and T₄₈. **Results:** The respective MICs of cinnamon and citronella oils were 65 and 250 µg/ml, and these were also the MFC values. For anti-biofilm efficacy, both oils significantly (p<0.05) reduced the number of viable microorganisms and accumulation of biofilms at T₀. However, at T₄₈, there was no difference between treated and untreated biofilms. **Conclusions:** We conclude that citronella and cinnamon essential oils have potential for daily anti-candidal denture cleansing.

Keywords: Anti-Infective Agents; Phytotherapy; *Candida albicans*; Biofilm; Denture stomatitis.

Introduction

Candida albicans is a frequent opportunistic pathogen of humans that is able to colonise several oral surfaces, including oral epithelia and denture prostheses. Importantly, *Candida* species may also exhibit resistance to antifungal agents and this also manifests when they are components of biofilms [1-3]. Biofilms afford protection to embedded cells of *C. albicans* from removal through the effects of shear forces or the cidal action of antimicrobials and host immune responses [4].

Candida albicans biofilms on denture acrylic are directly associated with the oral infection denture-induced stomatitis (DS) [3,5]. DS is the most prevalent form of oral candidosis, affecting up to 65% of all denture wearers [3]. Typically, DS occurs due to inefficient denture hygiene regimes, which allows persistence of the candidal biofilms, and this in turn drives local inflammatory response in the underlying palatal mucosa [3]. The key factor in the treatment of this chronic infection is efficient denture cleansing, which is problematic for many patients [6-9].

Diverse arrays of chemical denture cleansers are available and these may contain enzymes, sodium hypochlorite, alkaline peroxide, and acidic solutions to promote biofilm removal [6-11]. Whilst these cleansing solutions may exhibit antimicrobial properties [7-10], their ability to remove biofilm and prevent biofilm regrowth is often unclear [8,9].

Although sodium hypochlorite undoubtedly is an effective antimicrobial, it has also been associated with degradation of metal and acrylic components of dentures [12,13]. Alkaline peroxides are alternatives to hypochlorite solutions, although many studies have shown that these are ineffective at inhibiting *Candida* biofilms [8,10]. Besides that, many studies have shown that those commercially available denture cleansers are not completely effective in removing denture biofilms [14-16]. Therefore, new antimicrobial agents should be investigated in order to overlap those limitations.

Throughout history natural antimicrobials derived from plants have been recognised as having therapeutic benefits. Antibiotics and developed synthetic drugs had largely replaced the use of such agents in the treatment of infectious diseases. However, with the global threat of antimicrobial resistance, research into plant essential oils for antimicrobial activity has been revisited in recent decades. Examples of such oils include those present in tea tree oil *e.g.* eugenol, terpinol and terpinen [17]. The advantages of these natural agents is that they have multiple microbial

targets enabling them to exhibit broad spectrum antimicrobial activity with little or no occurrence of antimicrobial resistance [17,20].

Recent clinical studies have revealed anti-biofilm efficacy of agents derived from plants [18-21], and importantly, an absence of detrimental effects on denture base materials [20,21]. Cinnamon oil is an extract from the plant *Cinnamomum cassia*, whilst citronella is derived from *Cymbopogon winterianus*. Recently, these essential oils have been reported as having antimicrobial activity against *C. albicans* [20,22]. As a result of these studies, the aim of this present work was to determine the potential of these essential oils in treating acrylic biofilms formed by *Candida albicans* and therefore highlight their potential as natural treatments for DS.

Tested hypothesis (H1) was that the application of essential oils from cinnamon and citronella affect significantly the viability of *C. albicans* biofilms, immediately and after 48 h regrowth.

Materials and Methods

Experimental design

An *in vitro* study was performed to evaluate the anti-biofilm efficacy of cinnamon (*C. cassia*) and citronella (*C. winterianus*) essential oils. Initially, assays to establish Minimum Inhibitory Concentrations (MICs) and Minimum Fungicidal Concentrations (MFC) were performed to compare relative antimicrobial effects and to inform later studies assessing treatment of mature *C. albicans* acrylic biofilms (72 h).

Citronella and Cinnamon essential oils

Essential oils originating from *C. cassia* (cinnamon) and *C. winterianus* (citronella) were obtained commercially (Ferquima, Vargem Grande Paulista, Brazil) and key components and properties of these oils are presented in Table 1.

Stock solutions of essential oils were prepared at 180 mg/ml mL in water and 2% emulsifier agent (v/v) (Tween 80, Sigma-Aldrich, St. Louis, MO, USA) [23]. Serial dilutions of the stock concentrations were then used to assess antimicrobial effects.

Candida albicans preparation

Candida albicans ATCC 90028 was routinely cultured on Sabouraud dextrose agar (SDA, Difco, Detroit, MI, USA) at 35° C for 24 h. Subculture of a single colony from SDA was then undertaken in yeast nitrogen base (YNB, Difco, Detroit, MI, USA) medium supplemented with 100 mM glucose. Inoculated YNB was incubated at 35° C for 24 h, and the *Candida* cells were harvested by centrifugation (6,000 g). Cell pellets were subsequently washed (×2) in phosphate buffered saline (PBS, pH 7.4) and then re-suspended in fresh YNB containing 100 mM glucose. Optical density of cell suspensions was adjusted to 0.25 at 520 nm, using a spectrophotometer (DU800, Beckman, Rochester, NY). Under these culture conditions, it had previously been established that the prepared cell suspension was equivalent to 1×10^6 colony forming units (CFU)/mL of *C. albicans* [9].

Anti-candidal activity of essential oils

Anti-candidal activity of the essential oils was determined using both microdilution and based upon M27-A3 guidelines [24] to measure MIC and MFC. Briefly, 100 µL of RPMI 1640 culture medium (Sigma-Aldrich, St. Louis, MO, USA) containing two-fold serial dilutions of essential oils were in the wells of 96-well microtitre plates. To each test well 100 µL of *C. albicans* (1×10^3 CFU/mL) inoculum was added. The concentration range of the essential oils ranged between 1000 µg/mL and 7.8 µg/mL. Nystatin and Fluconazole (concentration range 64-0.5 µg/mL) were included as antifungal controls. Positive and negative controls consisted of wells without antimicrobials and without microorganisms, respectively. Plates were incubated at 35° C for 24 to 48 h. The MIC was defined as the lowest concentration that inhibited at least 80% of microbial growth, assessed using a spectrophotometer (600 nm). In order to assess MFC, 20 µL from each well was sub-cultivated on SDA. MFC was defined as lowest concentration that yielded no cultured microorganisms.

Acrylic preparation and biofilm development

Heat-polymerised acrylic resin (QC-20; Dentsply Int, Inc., Weybridge, UK) was fabricated according to the manufacturer instructions and moulded into disc-shaped (2 mm thickness, 10 mm diameter) specimens (n=9/ group) [8,9]. The number of specimens per group was determined in preliminary tests and reproduced by other studies, which reported that the sample size yielded an adequate power ($\beta > 80\%$) for detecting statistically significant differences ($\alpha < 0.05$).

The specimens were finished and softened using progressively finer oxide-aluminum papers (320-, 400- and 600-grit) on a horizontal polisher (APL-4; Arotec, São Paulo, Brazil). The surface roughness was standardized to $0.32 \pm 0.03 \mu\text{m}$. Although the literature has established low impact of surface roughness on biofilm growth [25,26], the standardization of specimen's surface roughness has been reported in the literature to simulate the surface of acrylic dentures [8,27,28]. Prior to use, acrylic specimens were ultra-sonically cleaned with 70% (v/v) alcohol and rinsed with sterile ultra-purified water (20 min) to remove surface debris. Specimens were decontaminated by immersion in 1% sodium hypochlorite solution for 15 min followed by rinsing with sterile water [29]. Absence of microbial growth was confirmed by immersing additional sample of specimens in a nutritive broth for 48 h.

Following local ethical committee research approval (protocol 021/2012) and receipt of informed consent, whole human saliva was collected from two healthy volunteers. Collection of saliva occurred at the same time of day in iced-chilled polypropylene tubes, and over a period of 30 min by chewing on paraffin film (Parafilm M; American Can Co, Neenah, WI, USA). Pooled saliva was clarified by centrifugation (3800 g, 4°C, 10 min) and the supernatant sterilised by filtration (0.22 μm filter membrane, Corning, Horseheads, NY, USA).

Salivary pellicles were subsequently formed on the surface of acrylic resin specimens using pooled fractions of the prepared saliva. Briefly, acrylic specimens were held horizontally in pre-sterilized 24-well plates and incubated (35° C, 75 rpm) with 2 mL of saliva for 90 min to form the salivary pellicle. The acrylic discs were then rinsed with PBS (pH 7.4) and used immediately for experiments [8].

Saliva-coated specimens were inoculated with 2 mL of YNB enriched with 100 mM glucose containing the previously described standardised suspensions of *C. albicans* (1×10^6 CFU/mL). The inoculated acrylic specimens were incubated aerobically (35°C, 75 rev/min) for 1.5 h to allow candidal adherence. Culture medium was removed and the specimens washed ($\times 2$ in PBS) to remove non-adherent cells. Fresh culture medium was added to each well (and renewed every 24 h) and incubation was continued for 72 h at 35°C, under agitation (75 rev/min).

Anti-biofilm activity of essential oils

After 72-h incubation, the acrylic specimens were transferred to new 24-well plates and randomly assigned to one of the following treatments (n=9/group):

cinnamon essential oil (1 mg/mL); citronella essential oil (1 mg/mL) or PBS (control) [8,9]. Cleansing treatment was performed by immersing samples in the treatment solutions (1mL) for 3 min. Specimens were then washed ($\times 2$) in 2 mL of sterilised PBS to remove the cleansing solutions and non-adhered cells. To assess the ability of the test agents to remove biofilms, residual colonising cells were immediately recovered (T_0) from certain acrylic specimens by sonication (7 W for 30 s). For other the remaining acrylic specimens, biofilm regrowth was allowed to proceed for 48 h (T_{48}) prior to collection by sonication or imaging by SEM [8,9].

The number of viable *C. albicans* present at T_0 and T_{48} was calculated from colony counts on SDA (incubated at 37°C under aerobic conditions for 48 h) derived from serial-decimal dilutions of recovered biofilms.

Biofilm formation at T_0 and T_{48} on acrylic was directly assessed using Scanning Electron Microscopy (SEM). Briefly, specimens were fixed with Karnovsky solution (4% (v/v) paraformaldehyde and 2% (v/v) glutaraldehyde in 0.1 M Sorensen buffer, pH 7.3) for at least 24 h. Samples were then washed in purified water, and dehydrated in an ethanol series (70% for 10 min, 95% for 10 min, and 100% for 20 min). Specimens were air-dried in a desiccator and gold sputtered coated before imaging by SEM (JSM 5600LV, JEOL, Tokyo, Japan). Images were taken under high vacuum mode (15 Kv) and visualised at $\times 500$ magnification.

Statistical analysis

Statistical analysis for the quantification of viable microorganisms was performed using the Statistical Package for Social Sciences (SPSS, v. 17, IBM, Chicago, IL, USA). Factors under study consisted of treatments and time of evaluation. Data was analysed regarding its normal distribution using the Kolmogorov-Smirnov test, under 95% confidence level. Due to normal distribution, data was analysed using two-way analysis of variance (ANOVA), using the significance level of 5% ($\alpha < 0.05$). Post-hoc comparison was done for the factor 'treatment', using the Tukey honestly significant difference (HSD) test.

Results

Both of the test essential oils were found to exhibit anti-candidal activity using broth microdilution and the MICs and MFCs of these are presented in Table 2.

Regarding the anti-biofilm activity of the essential oils both were found to significantly ($p < 0.001$) reduce the number of viable microorganisms, immediately after treatment (T_0) (Table 3). Significantly ($p < 0.05$) higher reduction of biofilm accumulation at T_0 , occurred with *C. cassia* (cinnamon). After the regrowth period (T_{48}), there was no significant difference in recovered *C. albicans* between all of the respective test treatments ($p < 0.001$). In addition, the number of viable *C. albicans* of treated groups was significantly ($p < 0.001$) higher than at T_0 . Table 4 shows the two-way analysis of variance in which is observed that interactions between treatment and time are statistically significant ($p < 0,001$).

SEM analysis of biofilms treated with essential oils at T_0 suggest that the essential oils remove a noticeable component of the biofilms (Figure 1), and this appears to include not only adherent cells but also extracellular polymeric substance. At T_{48} biofilms appear to have regrown with to levels greater than those T_0 . However, biofilm accumulation on essential oil treated acrylic does appear to be lower than PBS controls.

Discussion

The results of present study have shown that essential oils from cinnamon and citronella possess anti-*Candida* activity and exhibit anti-biofilm action. This activity can be detected immediately after treatment (T_0). However, there was an incomplete ability of the agents to remove and kill all of the biofilm cells. As a consequence, subsequent biofilm regrowth was encountered. Nevertheless, if extrapolated to a scenario of denture treatment, reducing the level of candidal biofilm present on dentures would be achievable, particularly when undertaken on a frequent (24 h) basis. In DS, reducing candidal load rather than eradication using cleansers would be the aim and this can be achieved with cinnamon and citronella oils based on these current findings.

Previous studies have indeed demonstrated the antimicrobial activity of essential oils from cinnamon and citronella [20,22,30,31]. However, the MIC and MFC values encountered in the present study were lower than those reported by others. Previous studies have used the same methods with standard strains and clinical isolates, having no difference in the microbial resistance [20,22]. Therefore the difference within MIC and MFC may be due to the proportion of chemical constituents present in the composition of the essential oil. In the present study,

cinnamaldehyde and citronellal were primary constituents of cinnamon and citronella oils, respectively.

The antimicrobial effect of cinnamon and citronella likely originates from the natural biological activity of their chemical constituents, such as cinnamaldehyde and citronellal [22,30]. Due to the lipophilic nature of these constituents, essential oils are thought to disrupt cell membranes and degrade other organic structures that may comprise the extracellular polymeric substances (EPS) of biofilms [22,32].

In the present study, SEM analysis did not indicate any obvious cell disruption. However, compared with controls, essential oil treatment appeared to reduce EPS and residual cell presence on acrylic specimens immediately after treatment (T_0). It may follow, that the cleansing with the natural oils primarily influenced superficial biofilm layers, with embedded *C. albicans* being largely protected from the treatments. This effect has been demonstrated by other studies with regards to the use of commercially available denture cleansers [8,9]. Further study, using a repeated exposure of the acrylic soon after T_0 could be of value in confirming this concept.

As confirmed by CFU counts and by imaging biofilms at T_{48} , treatment of biofilms using cinnamon and citronella oils did not prevent biofilm regrowth. This was not surprising based on the residual *C. albicans* observed by SEM at T_0 . Enzymatic and citric acid based denture cleansers also fail to eradicate biofilms after single exposure and as outlined earlier, reducing rather than eradicating biofilms would still have a major impact on managing DS [8-10].

Therefore, daily use of dentures cleansers based on the natural products studied here is suggested to continuous treat biofilms growth upon removable prosthesis. However this condition was not tested in the present study. These treatments would significantly affect the biofilms, reducing its accumulation immediately after treatments, similarly as shown at T_0 . In contrast, a single exposition to the cleansing solutions based on citronella or cinnamon does not have long-term efficacy to avoid, or prevent, biofilm regrowth after 48 h (T_{48}).

Although the authors have not investigated possible deleterious effects of essential oils on soft tissues and on acrylic resin, the literature has demonstrated low cytotoxicity [33,34] and insignificant effect on acrylic surfaces [20,21]. This study has limitations that concern to an *in vitro* design, single-species biofilm and single

exposition to essential oils. Before clinical experimentation, future studies should consider testing a multi-species biofilm and multiple expositions to denture cleansers.

Conclusion

Essential oils from citronella and cinnamon have potential for use as a daily denture cleanser due to their anti-*Candida* and immediate anti-biofilm activity.

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Conflict of interest

Authors declare no conflicts of interest. All the authors declare that contributed significantly to the conception of the study, to the manuscript drafting and critical revision. All authors approved the final version of the manuscript.

Figure caption

Figure 1 – SEM of *Candida* biofilms grown on acrylic specimens and treated (3 min) with PBS (control), essential oil of Citronella (*C. winterianus* – Cw) and essential oil of Cinnamon (*C. cassia* – Cc). Panels on the left show biofilms immediately after treatment (T₀) and panels on the right show biofilms post regrowth (T₄₈). Note the reduced biofilm accumulation and loss of extracellular polymeric substance on samples immediately after treatment with essential oils (Cw–T₀ and Cc–T₀). Also note that biofilm accumulation on samples treated with PBS (control) was consistently higher than for samples treated with essential oils.