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Original Article

In vitro method for studying the penetration of cinnamon oil and chlorhexidine through *Candida albicans* biofilm using Franz diffusion apparatus

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Abstract

Biofilm extracellular polymeric substances can act as a barrier to antifungal agents reaching the target site of yeast cells in the basal layer which results in resistance to various antifungal drugs. The aim of this study was to use a Franz diffusion apparatus to investigate the penetration of cinnamon oil and chlorhexidine (CHX) in various combinations through a *Candida albicans* biofilm. This study demonstrated that 0.5% (w/v) CHX and in combination with cinnamon oil solution (8 µL/mL) penetrated the *C. albicans* biofilm. In addition, sessile *C. albicans* biofilms exposed to 0.5% (w/w) CHX and in combination with cinnamon oil (8 µL/mL) for 4 h resulted in log reductions. In conclusion, this *in vitro* method may be used to investigate the penetration of antimicrobial solutions through *C. albicans* biofilm qualitatively and quantitatively at timed intervals.

Keywords: *Candida albicans*, biofilm penetration study, chlorhexidine, cinnamon oil, Franz diffusion apparatus

1. Introduction

Candida biofilms consist of extracellular polymeric substances (EPS) that contain fewer total carbohydrates and proteins than planktonic cells (Baillie & Douglas, 2000). Biofilms appear to be 30–2000 times more resistant than planktonic cells to antifungal agents such as amphotericin B, fluconazole, itraconazole, and ketoconazole (Andes *et al*., 2004; Chandra *et al*., 2001; Hawser & Douglas, 1995). Among the possible resistance mechanisms, EPS play an important role by preventing penetration of drugs through the biofilm matrix so that only the surface of the biofilm is exposed to lethal concentrations of an antifungal agent. The penetration of drugs through the biofilm barrier depends on the physico-chemical properties of both the drug and the biofilm matrix (Al-Fattani & Douglas, 2004; Donlan & Costerton, 2002; Mathé & Dijck, 2013; Samaranayake, Ye, Yau, Cheung, & Samaranayake, 2005; Tobudic, Kratzer, Lassnigg, Graninger, & Presterl, 2010). For example, fluoroquinolone antibiotics can readily penetrate *Pseudomonas aeruginosa* biofilm, but tobramycin cannot because it binds to the matrix polymers (Gordon, Hodges, & Marriott, 1988; Nichols, Dorrington, Slack, & Walmsley, 1988). Similarly, fluconazole and flucytosine penetrate *Candida* biofilms faster than amphotericin B (Samaranayake *et al*., 2005). Furthermore, the resistance of *Candida albicans* to antifungal agents such as azoles and polyenes is growing. In our previous study, we found that a combination of chlorhexidine (CHX) and cinnamon oil had a synergistic effect against *C. albicans* in both planktonic and biofilm forms. The most effective combination against *C. albicans* biofilm was 0.2% (w/v) CHX in combination with 8 µL/mL cinnamon oil (Satthanakul, Taweechaisupapong, Luengpailin, & Khunkitti, 2019).

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In our preliminary study, we found that an inhibition zone was not observed when Bacto agar containing antiseptic was used as the drug reservoir. This might be because CHX and cinnamon oil were not released from the agar to the paper disk. CHX was reported to be incompatible with starch which is a polymeric carbohydrate (Rowe, Sheskey, & Quinn, 2009; Zimbro, Power, Miller, Wilson, & Johnson, 2009). To avoid this problem, the penetration of CHX and cinnamon oil solution either alone or in combination through the *C. albicans* biofilm was investigated using a Franz diffusion cell apparatus. The advantages of using this apparatus are that it is simple to use and avoids interactions between the sample and vehicle. Moreover, this method also enables the investigation of cinnamon oil and CHX penetration through *C. albicans* biofilms both qualitatively and quantitatively at timed intervals and the oral cavity conditions are simulated by controlling the temperature with continuous mixing.

2. Materials and Methods

2.1 Materials

Cinnamon oil was purchased from Thai China Flavours & Fragrances Industry Co. (Bangkok, Thailand). Chlorhexidine digluconate (CHX), 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2*H*-tetrazolium hydroxide (XTT) and 2,3-dimethyl-5-methyl-1,4-benzoquinone (Coenzyme Q) were purchased from Sigma-Aldrich (USA).

2.2 Preparation of CHX and cinnamon oil solutions

CHX stock solution at a concentration of 2% (w/v) was diluted with deionized water to concentrations in the range of 0.2%–0.7% (w/v). Cinnamon oil solution in 5% Tween 80 and 5% ethanol at a concentration of 32 µL/mL was prepared and diluted in deionized water to concentration ranges of 6–12 µL/mL (0.6%–1.2% [v/v]).

2.3 Preparation of *C. albicans* inoculum

C. albicans ATCC 10231 was obtained from the culture collection of the Faculty of Dentistry, Khon Kaen University. *C. albicans* was reconstituted from the lyophilized stock and cultured on Sabouraud dextrose agar (Becton, Dickinson and Company, Sparks, MD, USA) at 37 ºC. A single colony of *C. albicans* ATCC 10231 was re-suspended in Sabouraud dextrose broth (Becton, Dickinson and Company, Sparks, MD, USA) and incubated overnight at 37 °C for 24 h. The density of *C. albicans* was adjusted to 10^6 CFU/mL at optical density = 0.1 (600 nm) with Sabouraud dextrose broth (Taweechaisupapong, Aieamsaard, Chitropas, & Khunkitti, 2012).

2.4 Generating the calibration curves of CHX and cinnamon oil concentration versus *C. albicans* inhibition zone using the agar disk diffusion assay

A 200 µL aliquot of the *C. albicans* inoculums was spread evenly on a Sabouraud dextrose agar plate. Paper disks (8 mm; Advantec, Japan) were placed on the surface and 45 µL of sample (either 0.2%–0.7% [w/v] CHX, 6–12 µL/mL cinnamon oil, or the combination that was 0.07%–0.2% [w/v] CHX: 3–8 µL/mL cinnamon oil) was pipetted onto the paper disks and incubated at 37 °C for 48 h. Inhibition zones were measured with Vernier calipers to two decimal points. The experiments were conducted in triplicate. Calibration curves were plotted between the sample concentrations and the inhibition zones. Regression equations and coefficients of determination (r^2) of the test samples were determined.

2.5 *C. albicans* biofilm preparation

Biofilms of *C. albicans* were grown on filter membranes (cellulose acetate membrane filters: diameter 25 mm; pore size 0.2 µm; Sartorius, Germany). Briefly, filter membranes were sterilized by UV exposure for 15 min on both sides and placed onto the surfaces of Sabouraud dextrose agar plates. A 50 µL aliquot of the *C. albicans* inoculum was dropped onto the filter membrane and incubated at 37 °C for 24 h before transfer to a fresh Sabouraud dextrose agar plate and incubated at 37 °C for a further 24 h to obtain a *C. albicans* mature biofilm (Al-Fattani & Douglas, 2004; Samaranayake *et al*., 2005).

2.6 *In vitro* study of test sample penetration through *C. albicans* biofilm

The penetration of sample solutions of CHX in a cinnamon oil solution in various combinations through the *C. albicans* biofilm was studied using the Franz diffusion cell apparatus. This method is a modification of the filter disk method (Al-Fattani & Douglas, 2004; Samaranayake *et al*., 2005). The schematic model is shown in Figure 1. The Franz diffusion cell (sample holder) was filled with 5.3 mL of sample solution (0.5% [w/v] CHX or 8 µL/mL cinnamon oil or the combination of 0.2% [w/v] CHX and 8 µL/mL cinnamon oil). The volume was maintained at all times by adding sample solution at the sample port. Homogeneity of the sample solutions was maintained by stirring at 50 rpm and the solutions were kept at 37 °C to simulate oral cavity conditions. *C. albicans* biofilms cultured on 25 mm membrane filters were then placed onto the sample holder (Figure 2a). Membrane filters of a smaller size (cellulose acetate membrane filters, diameter, 13 mm; pore size 0.2 µm; Sartorius, Germany) were placed on top of the mounted biofilm (Figure 2b). Then, paper disks (8 mm; Advantec, Japan), moistened with 10 µL distilled water to prevent capillary effects were placed on the membrane filter (Figure 2c). The sample holder was covered with the cell cap, fixed with a clamp (Figure 2d and Figure 2e), and covered with a sterile plastic lid to maintain aseptic conditions. The Franz diffusion cell was assembled with a multistation Franz diffusion apparatus (Keshary-Chien Diffusion Cell, Crown Glass Company, USA) (Figure 2f). The temperature was set at 37 °C with a temperature controller (Model 9010, Poly Science, IL, USA). The paper disks were collected at different time intervals (15, 30, 45, 60, 120, and 240 min) and placed on Sabouraud dextrose agar plates seeded with *C. albicans*. Inhibition zones were measured after incubation at 37 °C for 48 h and compared with zones from control cellulose acetate membranes filters without biofilms.

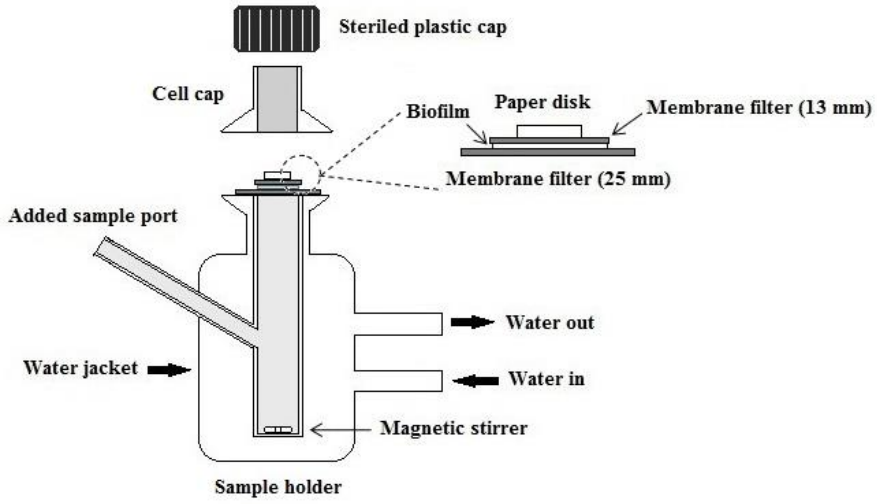

Figure 1. Schematic of the Franz diffusion model.

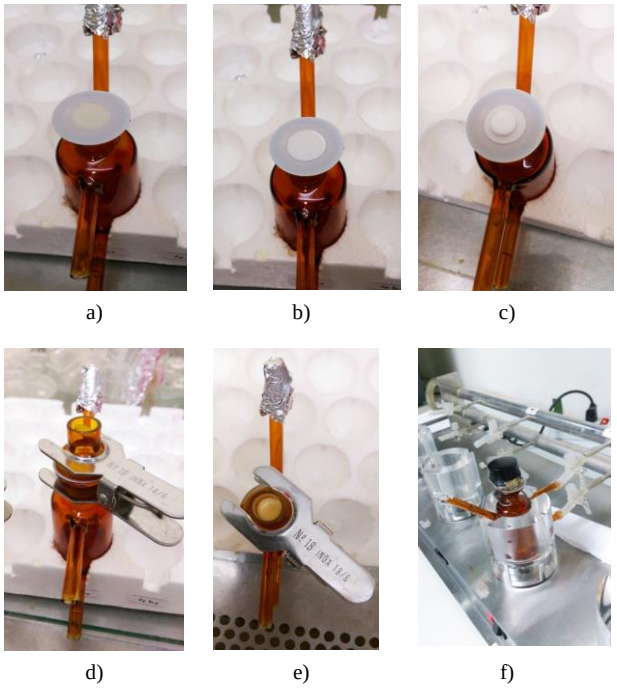

Figure 2. Biofilm penetration study model on the Franz diffusion apparatus

2.7 Viability of sessile *C. albicans* in biofilms

After the penetration study, the viability of sessile *C. albicans* in the biofilms on the cellulose acetate membrane filters was investigated. Briefly, the biofilms on the membranes were transferred into centrifuge tubes containing 3 mL of Sabouraud dextrose broth supplemented with 0.75% (w/v) azolectin and 5% (w/v) Tween 80 to inactivate the CHX carry over effect (i.e. prevent biocidal activity of any remaining CHX that would underestimate the viability in the biofilm (Fitzgerald, Davies, & Russell, 1989). The membrane-cell suspensions were mixed for 30 s using a vortex mixer (Model S0100-220, Labnet, NJ, USA) and sonicated for 10 min (Model 6210HP, Kudos, Shanghai, China). Survival of sessile *C. albicans* in the biofilms was determined by the plate count method. Cell suspensions were serially 10-fold diluted in Sabouraud dextrose broth dispensed onto Sabouraud dextrose agar plates and incubated at 37 ºC for 24 h. Colonies of *C. albicans* were counted to determine the number of viable cells.

2.8 Statistical analysis

All experiments were performed in triplicate as three independent experiments. The results are expressed as mean±SD. The statistical analyses were performed using the Statistical Package for Social Sciences (SPSS version 17.0, SPSS Inc., Chicago, IL, USA). Kolmogorov tests were applied to examine the normality of the distributions. Differences in the sizes of the inhibition zones at different time intervals were analyzed with repeated measures ANOVA. The Bonferroni *post hoc* test was used for pairwise comparisons within the groups. Differences in sample penetrations between the biofilms and membranes were analyzed using the independent t-test. A P-value <0.05 was considered statistically significant.

3. Results and Discussion

The main chemical components of cinnamon oil in this study were 68.1% cinnamaldehyde and 18.5% eugenol. Neither sub-chronic oral toxicity of cinnamaldehyde was found in mice at 2,620 mg/kg/day for 28 days nor dermal toxicity in rats at 940 mg/kg/day for 90 days using repeated doses in both cases (Hébert, Yuan, & Dieter, 1994). Acute oral toxicities of cinnamaldehyde were reported in guinea pig at 2,200 mg/kg and in rat at 2,250–3,350 mg/kg, and cinnamon caused oral toxicity at 2,800 mg/kg (Baker & Grant, 2018; Jenner *et al*., 1964; U.S. National Library of Medicine,

2014). Acute dermal toxicity of cinnamaldehyde was reported to be higher than 1,200 mg/kg in rat and higher than 1,000 mg/kg in rabbit (U.S. Environmental Protection Agency, 20 15; Baker & Grant, 2018). Therefore, 8 µL/mL cinnamon oil appears to be safe as a mouthrinse. However, a clinical study on the use of this mouthrinse must be conducted.

Figure 3 shows the calibration curves of cinnamon oil (Figure 3a) and CHX (Figure 3b). The coefficients of determination (r^2) of CHX and cinnamon oil were greater than 0.99 which indicated that these calibration equations could be used to quantify the test agent concentrations according to the size of their inhibition zones. The inhibition zones of the 0.5% (w/v) CHX, 8 µL/mL cinnamon oil, and the combination solution which penetrated through *C. albicans* biofilms and the uninoculated control cellulose membranes in the Franz diffusion cell system were compared.

The concentrations of CHX and cinnamon oil at each time interval were calculated from the calibration curve for each agent (Figure 3). Figure 4 shows that 0.5% (w/v) CHX was able to penetrate through the uninoculated control membrane in the amount of 0.53±0.05% (w/v) at 15 min, whereas the 0.5% (w/v) CHX took at least 30 min to penetrate through the *C. albicans* biofilm at a concentration 0.30±0.02% (w/v) which was significantly less than that of the uninoculated control membrane at a concentration of 0.57±0.09% (w/v) ($P<0.05$). The penetration of 0.5% (w/v) CHX through the biofilm during the periods of 45 to 120 min were not significantly different from the corresponding penetrations of the uninoculated control membrane. Thus, 0.5% (w/v) CHX was retarded by the biofilm layer for at least 30 min prior to its penetration at 45 min to 120 min. Penetration of CHX through the biofilm was greater than the uninoculated control membrane at 120 min and higher than the 0.5% (w/v) of stock solution in the sample holder which could be due to the capillary action of the thick biofilm structure (Sinko, 2011) which increased the volume of CHX absorbed by the paper disk. Therefore, it might increase the inhibition zone and cause the concentration of the CHX to be higher than the 0.5% (w/v) stock solution in the sample holder.

Our preliminary study showed from the inhibition zone of CHX that at a concentration of 0.2% (w/v) the biofilm was not penetrated, whereas the penetration of 0.5% (w/v) CHX increased as time increased. CHX, which is a cationic compound, might interact with the negatively charged EPS in the biofilm, resulting in slower penetration through the biofilm. After this interaction is complete, CHX could penetrate through the biofilm (Al-Fattani & Douglas, 2004; Rowe *et al.*, 2009).

The comparison of cinnamon oil penetration through uninoculated control membrane with *C. albicans* biofilm is shown in Figure 5. The mean inhibition zone size for 8 µL/mL cinnamon oil solution through the uninoculated control membrane was 7.23±0.02 mm at 15 min which increased to 10.89±0.56 mm at 30 min and the penetration increased

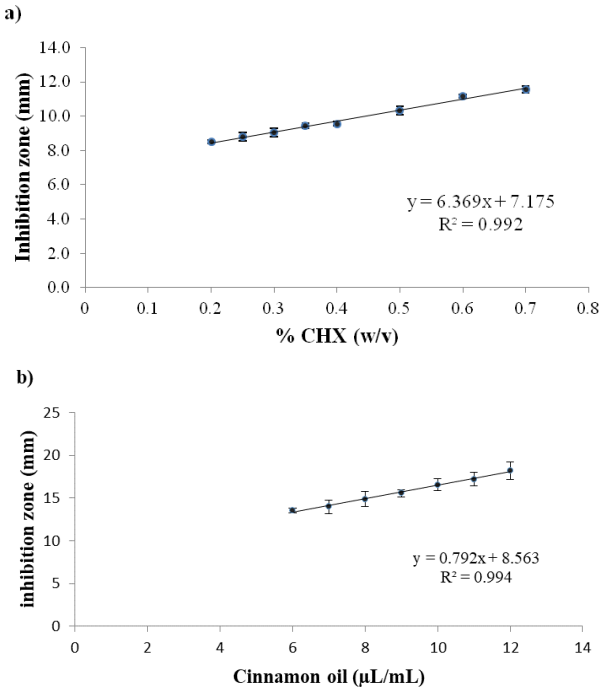

Figure 3. Calibration curve of CHX (a) and cinnamon oil (b) showing the relationship between the drug concentration and inhibition zone

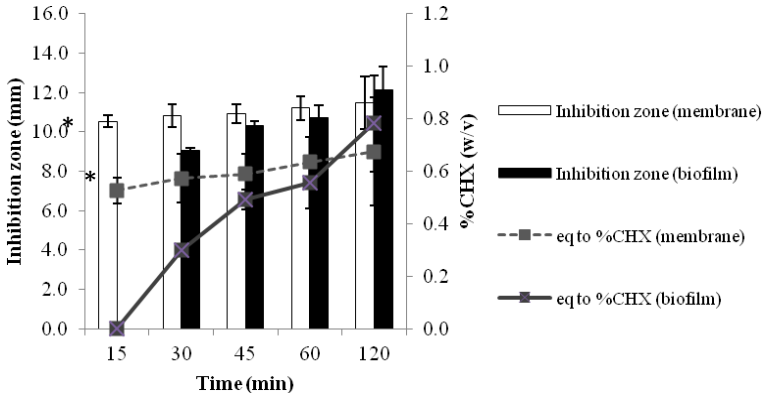

Figure 4. Penetration of 0.5% (w/v) CHX through uninoculated control membrane and *C. albicans* biofilm.
* $P<0.05$, Independent t-test of inhibition zones between the membrane and *C. albicans* biofilm.

further during the period of 45–240 min. However, the cinnamon oil solution was retarded by the biofilm from 120 min. At 240 min, the penetration of the solution through the *Candida* bioflim was not significantly different from the uninoculated control membrane. Figure 5 shows that the concentration of cinnamon oil solution that passed through the uninoculated control membrane at 30 min was 2.94±0.70 µL/mL before leveling off at a concentration in the range of 4.72±0.28 to 4.86±0.57 µL/mL from 60 to 120 min and increased to 5.69±1.69 µL/mL at 240 min. In contrast, penetration of cinnamon oil solution was completely retarded by the biofilm layer at 60 min, reached a concentration of 1.05±0.04 µL/mL at 120 min before penetration sharply increased to 6.59±0.73 µL/mL at 240 min.

These results showed that cinnamon oil obviously took longer to penetrate through the biofilm than the CHX. This effect might have occurred because the biofilm is composed of a hydrophilic extracellular matrix and cinnamon oil, which is a hydrophobic substance which requires more time for the excess oil solution to penetrate through the biofilm at an equivalent amount that penetrated the uninoculated control membrane at 240 min.

The combined cinnamon oil-CHX solution (8 µL/mL cinnamon oil: 0.2% [w/v] CHX) appeared to penetrate through both the uninoculated control membrane and the biofilm at 15 min (Figure 6) with mean inhibition zones of 11.11±0.22 mm and 7.90±0.18 mm, respectively. This finding was different from the penetration study of the agents alone. CHX alone took at least 30 min to penetrate through the biofilm with the inhibition zone of 9.08±0.12 mm and CHX could penetrate through the biofilm at 120 min with an inhibition zone of 12.16±1.17 mm (Figure 4). Cinnamon oil alone took at least 120 min with an inhibition zone of 9.39±0.04 mm (Figure 5), whereas the combination solution that penetrated

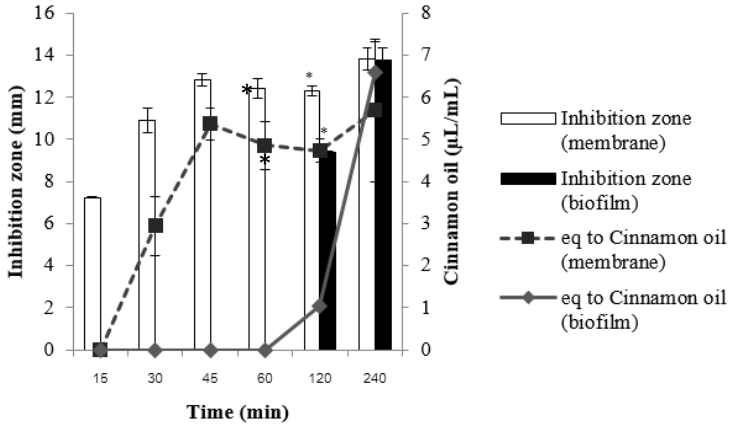

Figure 5. Penetration of 8 µL/mL cinnamon oil solution through uninoculated control membrane and *C. albicans* biofilm.
* P<0.05, Independent t-test of inhibition zones between the membrane and *C. albicans* biofilm.

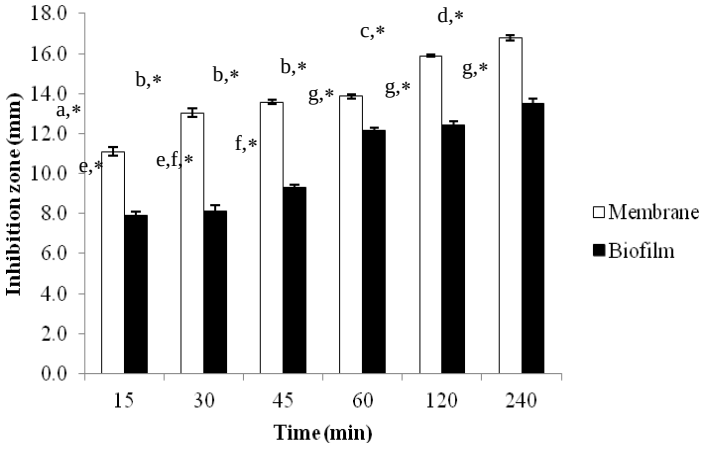

Figure 6. Penetration of the combination solution (8 µL/mL cinnamon oil: 0.2% (w/v) CHX) through uninoculated control membrane and *C. albicans* biofilm.

* Significant difference of inhibition zone between membrane and biofilm (Independent t-test at P-value <0.05).

[a - g] Significant difference of inhibition zone among tested time intervals of either membrane (a-d) or biofilm (e-g), respectively (Repeated measure at P-value <0.05) (a different alphabet indicates significant difference among the times).

through uninoculated control membrane and the biofilm at 120 min showed inhibition zones of 15.89±0.05 mm and 12. 40±0.21 mm, respectively, which was higher than each agent alone. Therefore, this finding demonstrated that the combination of CHX and cinnamon oil solution improved penetration through the biofilm compared to the agents alone. When the CHX and cinnamon oil were used individually, penetration was retarded by the EPS in the biofilm. The possible mechanism of the improved performance of the combination might be because CHX interacts with the negatively charged EPS to change the chemical microenvironment in the biofilm which allows cinnamon oil to penetrate through the biofilm and thereby improves the penetration of both agents (Al-Fattani & Douglas, 2004; Rowe *et al*., 2009)

The viability of sessile *C. albicans* in the biofilms was determined following the penetration studies (Table 1 and Table 2). At 120 min, the viability of the *C. albicans* that recovered from the biofilms exposed to 8 µL/mL cinnamon oil was 0.80±0.01 log reduction (84.16±0.52% kill), whereas 0.2% (w/v) CHX and 0.5% (w/v) CHX caused 1.37±0.15 log reduction (95.57±1.52% kill) and 3.46±0.42 log reduction (99.96±0.04% kill), respectively. When the viable *C. albicans* recovered from the biofilms exposed to the combination solution (8 µL/mL cinnamon oil: 0.2% CHX), it caused a 1.27± 0.24 log reduction (95.18±2.57% kill) which indicated that *C. albicans* was not completely killed. However, within 4 h, the viable *C. albicans* did not recover from the biofilms exposed to either 0.5% (w/v) CHX or the combination solution which indicated complete killing of the sessile *C. albicans* by exposure to these two solutions.

Table 1. Viability of *C. albicans* biofilm after penetration study.

Sample	Time (min)	CFU/mL	Log	Log reduction
Control	120	7.72×10^7	7.89±0.04	-
	240	7.06×10^7	7.85±0.03	-
Cinnamon oil	120	1.22×10^7	7.08±0.01	0.80±0.01
(8 µL/mL)	240	3.79×10^5	5.55±0.21	2.30±0.21
0.2% CHX	120	3.13×10^6	6.48±0.15	1.37±0.15
	240	9.11×10^5	5.96±0.03	1.93±0.03
0.5% CHX	120	3.27×10^4	4.42±0.42	3.46±0.42
	240	0	0	7.85±0.00
Combination	120	4.62×10^6	6.62±0.24	1.27±0.24
(8 µL/mL Cin: 0.2% CHX)	240	0	0	7.85±0.00

Table 2. Percent reduction of *C. albicans* biofilm after penetration study

Sample	Time (min)	% Kill
Cinnamon oil (8 µL/mL)	120	84.16±0.52
	240	99.46±0.23
0.2% CHX	120	95.57±1.52
	240	98.82±0.08
0.5% CHX	120	99.96±0.04
	240	100.00±0.00
Combination	120	95.18±2.57
(8 µL/mL Cin: 0.2% CHX)	240	100.00±0.00

These findings demonstrated that penetration of the 0.5% (w/v) CHX and the combination of cinnamon oil and CHX through the biofilm was higher than each agent alone and their fungicidal activity against sessile *C. albicans* in the biofilm was both concentration- and time-dependent. 48-hour old biofilms are heterogeneous by nature, which consist of blastospores, pseudohyphae, and hyphae surrounded by matrix material (i.e. EPS). The distribution of water channels among the cellular components facilitate both the diffusion of nutrients from the environment to the bottom layers and the disposal of waste products (Seneviratne, Jin, & Samaranayake, 2008). In the filamentous structural form of biofilms the yeast cells are located in the basal layer (Douglas, 2003). Suci and Tyler (2002) found that CHX was able to penetrate the filamentous forms at significantly higher levels than the yeast forms. Moreover, there might be capillary action within the biofilm structure that can increase the penetration of the CHX solution (Sinko, 2011). This might explain why CHX was detected penetrating biofilms at high concentrations at 2 h but the sessile cells were not completely killed, whereas at 4 h viable sessile cells were not detected. Cinnamon oil, which is lipophilic in nature, did not eradicate the sessile cells within 4 h. This study suggests that the biofilm penetration ability depends on the nature of the antifungal agent and the binding capacity between the EPS and the tested agent. It might be possible that the EPS might act as a barrier to the diffusion of the tested agents and limit the access of the agents to the yeast cells (Baillie & Douglas, 2000; Donlan & Costerton, 2002). Susceptibility of the sessile cells might depend on the concentration reached and the exposure time of the antifungal agents.

4. Conclusions

The Franz diffusion apparatus was relatively simple to use to investigate the penetration of antifungal agents through biofilms and this method can be used for qualitative and quantitative investigations. Moreover, this method can be used to simulate the conditions of oral cavities and it was able to demonstrate that the active compound could directly penetrate the biofilm using the Franz cell. Therefore, this method of study may be used to investigate the resistance to penetration of other microorganism biofilms by other antimicrobial agents.

Acknowledgements

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