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Inhibitory effect of farnesol on biofilm formation by *Candida tropicalis*

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ABSTRACT

Background: Candidiasis associated with indwelling medical devices is especially problematic since they can act as substrates for biofilm growth which are highly resistant to antifungal drugs. Farnesol is a quorum-sensing molecule that inhibits filamentation and biofilm formation in *Candida albicans.* Since in recent years *Candida tropicalis* have been reported as an important and common *non-albicans Candida* species with high drug resistance pattern, the inhibitory effect of farnesol on biofilm formation by *Candida tropicalis* was evaluated.

Methods: Five *Candida tropicalis* strains were treated with different concentration of farnesol (0, 30 and 300 µM) after 0, 1 and 4 hrs of adherence and then they were maintained under biofilm formation condition in polystyrene, 96-well microtiter plates at 37°C for 48 hrs. Biofilm formation was measured by a semiquantitative colorimetric technique based on reduction assay of 2,3- bis -2H-tetrazolium- 5- carboxanilide (XTT).

Results: The results indicated that the initial adherence time had no effect on biofilm formation and low concentration of farnesol (30 µM) could not inhibit biofilm formation. However the presence of non-adherent cells increased biofilm formation significantly and the high concentration of farnesol (300 µM) could inhibit biofilm formation.

Conclusion: Results of this study showed that the high concentration of farnesol could inhibit biofilm formation and may be used as an adjuvant in prevention and in therapeutic strategies with antifungal drugs.

Keywords: Farnesol, Biofilm, *Candida tropicalis,* Azole

INTRODUCTION

Biofilms are colonies of microbial cells wrapped in a self-product organic polymeric matrix which are formed in response to attachment to a surface and are highly resistant to antimicrobial drugs (1, 2). They are often performed in indwelling medical devices (IMD) (1). Although majority of implant infections are caused by gram-positive bacteria, such as *staphylococci*, infections due to gram-negative bacteria and fungi are more serious (3). Transplantation procedures, immuno-suppression, chronic use of indwelling devices, and prolonged staying in intensive care unit have increased the prevalence of fungal disease. The most commonly fungi associated with such diseases are *Candida* species, notably *Candida albicans*, which causes both superficial and systemic diseases. Even with antifungal therapy, mortality of patients with invasive candidiasis may be up to 40% (4).

Farnesol one of the extracellular compounds released by *C. albicans* is a quorum-sensing molecule that inhibits conversion of yeast to *Candida* mycellium which is essential for initiation of infection and biofilm formation on catheter surface (5).

Non-albicans Candida species such as *C. tropicalis, C. parapsilosis, C. krusei, C.lipolytica, C. dubliniensis* also form biofilm on IMD surfaces (1). These species cause infections of higher complication and death rates compared to those resulting from *Candida albicans* (6).

Among *non-albicans Candida* species, the prevalence of *C. tropicalis* is about 30% and it is an important colonizing fungus causing hematogenous disseminated Candidiasis (7, 8). *Candida tropicalis* is the second or third agent of candidemia especially in patients with neutropenia, leukemia, lymphoma and diabetes (9).

To our knowledge there are only few studies about inhibitory effects of farnesol on biofilm formation by *C. albicans*, *C. parapsilosis* and *C. dubliniensis* (10-12). Since farnesol production differs significantly between *Candida* spp. (13) and because *C. tropicalis* have been noted as an important and common *non-albicans Candida* species (7) with the low susceptibility to azole

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isolates in recent years (14), in these study inhibitory effects of farnesol on biofilm formation by *Candida tropicalis* was evaluated. To our knowledge there has been no documented study in Iran in this area of investigation.

MATERIAL AND METHODS

Organisms

C. tropicalis strain ATCC 750 and four other strains which obtained from patients referred to Medical Mycology Laboratory, School of Public Health, Tehran University of Medical Sciences were used in this study.

Biofilm Formation

According to Ramage et al experience (10), *C. tropicalis* strains were maintained on Sabouraud dextrose agar (Difco) plates and after 48 hrs subcultured onto yeast peptone dextrose (YPD) agar plates (1% [W/V], yeast extract (Oxoid); 2% [W/V], peptone (Biolife); 2% [W/V], dextrose (Merck); 2% [W/V], agar agar (BBL)). After 48 hrs one loop of colonies were transferred to 20 ml YPD broth in 250-ml Erlenmeyer flasks and incubated overnight in an orbital shaker (100 rpm) at 30°C under aerobic condition. All *C. tropicalis* strains were grown in the budding-yeast phase under this condition. Yeast cells were harvested and washed twice in sterile phosphate-buffered saline (PBS) ([0.8% [W/V], sodium chloride (Merck); 0.02% [W/V], KH_2PO_4 (Merck); 0.31% [W/V], $Na_2HPO_4+12H_2O$ (Merck); 0.02% [W/V], potassium chloride (Panreac); pH 7.4). Then they were resuspended in RPMI 1640 supplemented with L-glutamine (Gibco) and buffered with morpholinopropanesulfonic acid (MOPS) (Merck) and adjusted to 1.0×10^6 cells/ml after counting with a hematocytometer.

Farnesol (Trans-Trans Sigma Chemical Co.) was as a 3 M stock solution and then diluted to obtain a 30 mM working stock solution in 100% (V/V) methanol. Working stock solution was used for the preparation of 300 µM and 30 µM concentrations of farnesol in RPMI 1640. All experiments were performed in presterilized, polystyrene, flat-bottom, 96-well microtiter plates (Nunc).

In this study for each biofilm formation, five different groups were considered. The first, as control group consisted of three wells containing 0, 30 and 300 µM of farnesol. The other groups named as H0A, H0B, H1 and H4 and consisted of three wells containing 100 µl of 1.0×10^6 cells/ml and 100 µl of farnesol solution (0, 30 and 300 µM) which were added after 0, 0, 1, and 4 hrs respectively.

For H0A and H0B groups, standardized cell suspensions were seeded into sterile 0.5 ml microtubes (TreffClear, Switzerland) and double strength of different concentration of farnesol also were added into these sterile tubes at the same time to obtain 0, 30, 300 µM as final concentration of farnesol and both of them were transferred into wells of microtiter plates. After one (in H0A group) and four hrs (in H0B group) the medium was aspirated, and non adherent cells were removed by washing of the preparations thoroughly three times in sterile PBS. Then farnesol solution was added at different concentrations (0, 30, and 300 µM) to the adherent cells. However in H1 and H4 groups since farnesol was added to the aspirated and pre-washed wells, single strength of farnesol solutions were used (0, 30, and 300 µM).

Biofilm formation assay

A semiquantitative measure of biofilm formation was assayed by using a 2,3-bis(2–methoxy–4–nitro–5–sulfo-phenyl)-2H-tetrazolium-5-carbox-anilide (XTT) reduction assay. XTT (Sigma Chemical Co.) was prepared as a saturated solution at a concentration of 0.5 mg/ml in Ringer's lactate. This solution was filter sterilized through a 0.22-µm-pore-size filter, divided into aliquots, and then stored at -70°C. Prior to each assay, an aliquot of the XTT stock solution was thawed, and treated with menadione sodium bisulfite (10 mM prepared in Distilled Water; Sigma Chemical Co.) to obtain a final concentration of 1 µM of menadione. A 100-µl aliquot of XTT-menadione was then added to each pre-washed biofilm and wells to measure background XTT levels. The plates were then incubated in dark for 2 hrs at 37°C, and the colorimetric change at 490 nm (a reflection of the metabolic activity of the biofilm) was measured with a microtiter plate reader (Titertekplus-MS_2 reader, UK) (10).

Statistical analyses

Overall in this study 25 independent biofilms were performed for 5 strains and the optical density (OD) of control group was considered as baseline which was exerted to other groups. After standardization of the biofilm formation test, normality distribution of data were analyzed with Kolmogorov-Smirnov test and it was shown that all of the data had normal distributions in significant level $\alpha=0.05$. Therefore for comparison of the means, One-Way Anova test and post Hoc (LSD test) were used. SPSS version 11.5 was used as statistical software and significant level was $\alpha=0.05$.

RESULTS

In this study the mean OD in H0A group increased from 0.25 ± 0.08 in the absence of farnesol to 0.27 ± 0.12 when the farnesol concentration was 30 µM and then it was decreased to 0.12 ± 0.07 in 300 µM farnesol solution (Table 1) H0B group Study showed that the mean OD increased from 0.26 ± 0.09 in the absence of farnesol to 0.27 ± 0.11 in 30 µM farnesol solution and then it decreased to 0.14 ± 0.08 in 300 µM farnesol solution (Table 1), In H1 group it was found that the mean (OD) decreased from 0.27 ± 0.10 in the absence of farnesol to 0.24 ± 0.14 in 30 µM of farnesol and then it decreased to 0.12 ± 0.08 in 300 µM farnesol solution (Table 1), One-Way Anova test showed this change in biofilm formation was significant for different concentration of farnesol (P<0.001) and Post Hoc (LSD Test) showed that this significant difference was related to 300 µM farnesol solution (Fig. 1).

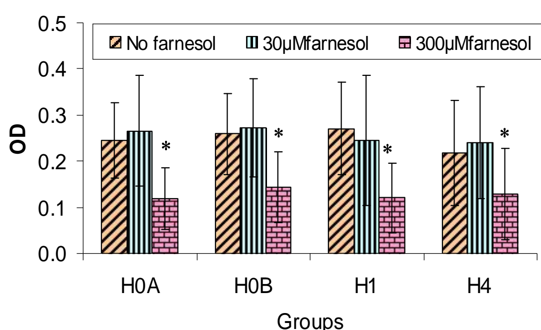

Figure 1. Effect of time and Farnesol concentration on biofilm formation. Different concentration of Farnesol (0, 30, and 300 µM) were added to *C.tropicalis* cells after different time (0, 1 and 4 hrs) and then the yeasts were incubated under biofilm formation conditions. Biofilm formation was measured by the reduction of tetrasolium salt (XTT) and Optical Density (OD) was measured at 490 nm. H0A: Farnesol was added to *C.tropicalis* cells at zero time and wells were washed 1 h later. H0B: Farnesol was added to *C.tropicalis* cells at zero time and wells were washed 4 hrs later. H1: Farnesol was added to *C.tropicalis* cells after 1h. H4: Farnesol was added to *C.tropicalis* cells after 4h. One-Way Anova, Post Hoc (LSD Test). *: P < 0.05. (Values are the mean OD ± standard deviation).

H4 group showed that the mean (OD) increased from 0.22 ± 0.11 in the absence of farnesol to 0.24 ± 0.12 in 30 µM farnesol and then it decreased to 0.13 ± 0.10 in 300 µM farnesol solution (Table 1), One-Way Anova test showed this change in biofilm formation was significant for different concentration of farnesol (P<0.01) and Post Hoc (LSD Test) showed that this significant difference was related to 300 µM farnesol solution (Fig. 1).

A comparison of H0A and H0B groups showed that the mean OD was 0.21 ± 0.11 in H0A and 0.23 ± 0.11 in H0B (Table 1) and the difference with Paired Sample T-Test was significant (Fig. 2) but with splitting data due to different concentration of farnesol (0, 30, 300 µM farnesol) in these two groups it was shown that the difference was significant only in 300 µM farnesol concentration (Fig. 3).

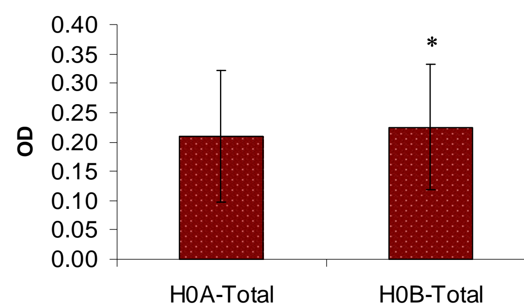

Figure 2. The comparison of biofilm formation in H0A and H0B groups. H0A: Farnesol was added to *C.tropicalis* cells at zero time and wells were washed 1 h later. H0B: Farnesol was added to C.tropicalis cells at zero time and wells were washed 4 hrs later. Paired Sample T-Test. *: P < 0.05. (Values are the mean OD ± standard deviation).

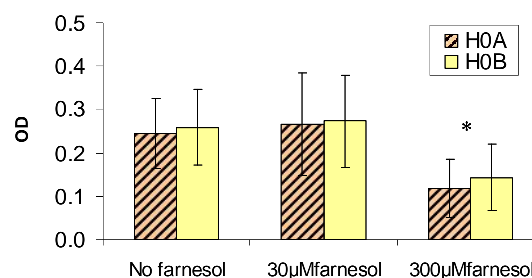

Figure 3. The comparison of biofilm formation in H0A and H0B groups in different concentrations of Farnesol. H0A: Farnesol was added to *C.tropicalis* cells at zero time and wells were washed 1 h later.H0B: Farnesol was added to *C.tropicalis* cells at zero time and wells were washed 4 hrs later. Paired Sample T-Test. *: P < 0.05. (Values are the mean OD ± standard deviation).

DISCUSSION

In Recent years, there has been an increase in frequency of invasive candidiasis as an opportunistic infection in human being (15) and high morbidity and mortality of disseminated candidiasis have been one of the most important problems (4). A survey of nosocomial blood stream infections of more than 24,000 cases in 49 US hospitals from 1995 to 2002 showed that 9.5% of these infections were caused by *Candida* species (1). Even with antifungal therapy, high mortality of patients with invasive candidiasis is notable (40%) (4). Although *Candida albicans* is the most frequently isolated species, *non- albicans*

Table 1. Standardized mean of optical density (OD) according to different concentrations of Farnesol in study groups.

Group	Farnesol Concentration (μM)	N	Mean OD	Standard Deviation
H0A	No Farnesol	25	0.24508	0.081340
	30	25	0.26592	0.119244
	300	25	0.11908	0.067272
	Total	75	0.21003	0.111759
H0B	No Farnesol	25	0.25896	0.087247
	30	25	0.27288	0.106609
	300	25	0.14380	0.077653
	Total	75	0.22521	0.107248
H1	No Farnesol	25	0.27044	0.099674
	30	25	0.24400	0.141228
	300	25	0.12052	0.075666
	Total	75	0.21165	0.125994
H4	No Farnesol	25	0.21740	0.113837
	30	25	0.24032	0.121359
	300	25	0.12840	0.098629
	Total	75	0.19537	0.120400

Candida species with less susceptibility to antifungal drugs have been increased drastically (9, 16).
Overall biofilm is an organized community of cells under the control cell signaling and these cells of microbial biofilms release chemical compounds such as farnesol to regulate the number of microbial population. This phenomenon is similar to communication of the cells of a tissue by autocrine and paracrine stimulation. So it is essential for the whole population of biofilm to prevent overgrowth and spreading yeast cells far from initial biofilms (10). Biofilm formation by *Candida* species is a phenomenon which helps survival, dissemination and resistance of infection to drug (4). Around 2-24% of all candidemia are caused by *Candida tropicalis* resistance pattern of this species to drug is notable (15).
The number of strains and independent biofilm which were formed in this study were almost similar to those of previous studies (10-12). Five *Candida tropicalis* strains used in this study and 5 independent biofilm were formed for each strain. In the previous study data were tested with Student's t test compared to control group (10) but in this study the mean OD of control group was considered as baseline (blank) and exerted to other groups to standardize the test and then these adjusted data were analyzed. Therefore, this study consisted of 4 groups: H0A, H0B, H1 and H4. Since in H0A and H0B groups, cell suspension and farnesol were added at the same time, farnesol was used in double strength, after one hrs (in H0A) and 4 hrs (in H0B), medium was aspirated and farnesol solutions were added again in single strength to pre-washed wells to obtain similar condition to the other groups. It was hypothesized that non adherent cells can bias. In H0B a three-hrs delay in washing the wells, increased biofilm formation drastically in this study, whereas it was not noted in previous studies (10-12).
In the previous study the decrease of OD from 0 to 30 μM of farnesol concentration at 0, 1 and 24 hrs was significant and the initial adherence time, prior to the addition of farnesol (0, 1, 2, 4 and 24 h), was important in the ability of farnesol to inhibit biofilm formation except for 24-h biofilms in which treated biofilms with 30 and 300 μM farnesol were noticeably lower than those of the untreated controls. This was explained by the presence of high concentration of farnesol which could inhibit newly budded yeast cells which were formed in mature biofilms (10). In another report, the initial adherence time (0 and 2 h) was effective only in 3 of 4 phenotypes of *C.parapsilosis* (11) and In another study for both *C.albicans* and *C. dubliniensis,* as the initial adherence time increased (from 0 to 24) the effect of farnesol on biofilm development diminished and minimal visible defect was noted on 24-h

performed biofilms (12) but in this study in H0A and H0B and H4 groups the mean OD (biofilm formation) increased from 0 to 30 µM concentration of farnesol. Although it was not statistically significant but it can be considered in future studies and it may be due to difference between *Candida* strains that low concentration of farnesol is unable to inhibit biofilm formation in these strains. Differences in biofilm formation and inhibitory effect of farnesol are shown among different phenotypes of *Candida parapsilosis* (11). In this study the initial adherence time (0, 1 and 4 hrs) was not effective for inhibition of biofilm formation.

While potential ability of farnesol as an adjuvant in prevention and therapeutic strategies with antimicrobial drugs has been reported (12, 17) the doses of farnesol as an adjuvant with antifungal drugs to inhibit biofilm formation by *Candida* species should be determined.

Overall in this study the inhibitory effect of farnesol was related to its concentration and similar to result of other studies, farnesol at concentration of 300 µM reduced biofilm formation decisively (10-12).

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