In vitro antibiofilm efficacy of farnesol against *Candida* species

Mihrinur Yapıcı[1] · **Bükay Yenice Gürsu**[2] · **İlknur Dağ**[2,3]

Abstract

Candida species are opportunistic fungi that can cause mucosal or invasive infections. Especially in biofilm-related infections, resistance is very high to anifungals; therefore more effective treatment strategies are needed. Farnesol(3,7,11-trimethyl-2,6,10-dodecatriene-1-ol) is the quorum sensing (QS) signal molecule and can interact with *Candida* species both as a QS molecule and as an exogenous agent. The aim of this study was to investigate the effects of farnesol on both the planktonic and biofilm forms of *Candida* species by colorimetric, microbiological, and electron microscopic methods. Obtained results demonstrated the inhibitory effect of farnesol on the planktonic and biofilm forms of *Candida*. Farnesol showed a biofilm-enhancing effect at lower concentrations. TEM findings showed the membrane and wall damage, vacuolization, or granulation in cells. SEM images confirmed biofilm reduction in pre-/post-biofilm applications as a result of farnesol treatment. In conclusion, farnesol can be used as an alternative agent to reduce the *Candida* biofilms, with future studies.

Keywords Farnesol · Biofilm · *Candida* · Electron microscopy

Introduction

Candida species are found as normal microbiota members in healthy individuals; however they can lead to life-threatening opportunistic infections in immunocompromised individuals (Ferreira et al. 2013). While these infections can be seen superficially or systemically, they are also among the main causes of nosocomial blood stream infections in hospitals (Yapar 2014). Although *C. albicans* is the most common pathogen species, infections caused by *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. krusei*, and *C. guillermondii* species have also been frequently reported in recent years (Spampinato and Leonardi 2013). The development of *Candida* associated multidrug resistance (MDR) is also one of the most important clinical problems (Prasad and Kapoor

2005). More recently, *C. auris* is also identified as MDR species and have a high mortality (Kunyeit et al. 2019; Lone and Ahmad 2019). Therefore, treatment is very difficult due to the narrow spectrum of drugs used in treatment and toxicity problems (Zhao et al. 2013).

Biofilm is a community formed by microorganisms that are irreversibly attached to biotic or abietic surfaces and microrganisms were embedded in the extracellular matrix they produce (Cavalheiro and Teixeira 2018). This complex structure is one of the most important virulence factors contributing to the *Candida* pathogenicity, and some *Candida* species have high biofilm formation capacity (Öztürk 2019). In *Candida* biofilms, matrix enclosed yeast microcolonies, hyphae, or pseudohyphae are arranged as a complex structure, and this structure may differ between species. Biofilm is very difficult to eradicate and is the primary source of many persistent infections. Besides, *Candida* colonization and biofilm formation are observed on many biomaterials used in clinical applications. Especially, as a result of influence of implanted devices such as permanent catheters or prosthetic heart valves, the implant should be removed (Douglas 2003; Hasan et al. 2009; Ramage et al. 2006). For all these reasons, there is an urgent need to develop new antifungal agents or alternative therapeutics against *Candida* biofilms (Öztürk et al. 2020).

Biofilms are specific and organized communities controlled by the quorum sensing (QS) signal molecules, and this

✉ Bükay Yenice Gürsu
bgursu@ogu.edu.tr

1 Department of Biotechology and Biosafety, Institute of Life Science, Eskisehir Osmangazi University, Eskisehir, Turkey

2 Central Research Laboratory Application and Research Center, Eskisehir Osmangazi University, Eskisehir, Turkey

3 Vocational Health Services High School, Eskisehir Osmangazi University, Eskisehir, Turkey

system is the cell-cell communication mechanism formed by a synchronized gene expression in response to the cell density population (Solano et al. 2014; Sharma et al. 2019). Farnesol is also the first described QS molecule in *Candida* and is released as a by-product of the sterol synthetic pathway by dephosphorylation of farnesol pyrophosphate in *C. albicans*. (Leonhardt et al. 2015). It can interfere with *Candida* species both as a QS molecule and as an exogenous agent. The exogenous addition of QS molecules such as farnesol to the growth environment may have a negative effect on the QS mechanism, limit yeast adhesion on the surface, or create a toxic effect on the metabolic pathway involved in biofilm formation. In this regard, a number of physiological responses are particularly emphasized. Among these, the most studied issue is reported as farnesol inhibiting the development of biofilms by inhibiting hyphae formation (Rodrigues and Cernakova 2020). On the other hand, farnesol is thought to reduce cell surface hydrophobicity and may also induce oxidative stress and apoptosis (Krom et al. 2016). So, farnesol may also induce the general stress response through a toxic component. In some studies, it is also reported that farnesol reduces azole resistance, and this may be explained by the modulation of Cdr1 efflux pumps, the generation of reactive oxygen species, or the changes in glutathione homeostasis. Farnesol also has effects on genes linked to ergosterol synthesis (Kovács and Majoros 2020). On the other hand, it has been reported that the amphiphilic property of farnesol affects fungal membrane fluidity and permeability.

So far, intense researches on *C. albicans* and farnesol attract attention in the literature. However, comparative studies on the effects of exogenous farnesol on non-albicans *Candida* species are quite limited (Sebaa et al. 2019; Ramage et al. 2002). In a study just published, Bezerra et al. studied the antifungal activity of farnesol-containing liposomes associated with fluconazole against *C. albicans* and non-albicans *Candida* isolates. The authors stated that farnesol-containing liposomes may have an important potential in antifungal drug development studies (Bezerra et al. 2020). Rossignol et al. also reported that *C. parapsilosis* cells were arrested after exogenous farnesol was added, but that this was not at a certain stage of the cell cycle. Therefore, cells were not found sensitive to farnesol and the cells eventually recover from arrest. So, the authors investigated the transcriptional response of *C. parapsilosis* to the presence of exogenous farnesol in their study. When both *C. albicans and C. parapsilosis* cultures are exposed to farnesol, the expression of the oxidoreductases GRP2 and ADH7 is increased, and the expression of genes involved in sterol metabolism is altered. However, genes involved in hyphae growth in *C. albicans* had no effect on the expression of *C. parapsilosis* orthologs. Thus, farnesol showed significant differences on *C. parapsilosis* and *C. albicans* (Rossignol et al. 2007).

Detection of microbial biofilms can be done by different analyses such as crystal violet and XTT method. While the CV method can be evaluated visually, the XTT method is performed with multi-well plate readers, and the absorbances obtained are evaluated. On the other hand, while the CV method determines biofilm biomass, the XTT method measures the metabolic activity. (Corte et al. 2019). So these two measurements are not fully correlated, but both methods are used in biofilm studies. However, *Candida* biofilm measurement with XTT method was recommended as more effective, more reliable and less costs (Chavez-Dozal et al. 2016; Taff et al. 2012).

This paper aims to examine the effects of exogenous farnesol addition on planktonic growth and biofilm formation in different *Candida* species. For this purpose, two different *C. albicans* strains as well as 5 non-albicans *Candida* species were included to study by adding 10 different farnesol concentrations in microtiter plate assays before and after initiation of biofilm formation. In addition, the pre- and post biofilm activities of farnesol on the biofilm formation of standard and clinical strains of *C. albicans* were compared with SEM on two different biomaterials. In addition, ultrastructural effects of farnesol on planktonic *C. albicans* isolate were investigated by TEM.

Materials and methods

Microorganisms

The clinical isolates used in this study were obtained from Eskişehir Osmangazi University, Faculty of Medicine, Department of Microbiology. These isolates belong to 6 different species and were isolated from blood and peritoneal fluid samples (*C. albicans* 1766, *C. krusei* 1561, *C. tropicalis* 1678, *C. glabrata* 1744, *C. kefyr* 1798 and *C. parapsilosis* 1799). API 20C (bioMerieux, Marcy l'Etoile-France) commercial assimilation test and conventional methods including germ tube test, microscopic morphology in Cornmeal Tween 80 agar, and CO_2 fermentation tests were carried out for identification procedures. *C. albicans* ATCC 14053 isolate was used as standard microorganism. The isolates were stored at −80 °C in Yeast Extract Peptone Dextrose (YPD) containing 20% glycerol. To fresh culture preparation, isolates from stock were incubated in RPMI 1640 medium and at 37 °C for 24 h (CLSI M27-A2).

Following in vitro growth in *Candida* isolates by farnesol treatment

In the study, yeast isolates incubated in RPMI 1640 medium and 37 °C for 24 h were taken in 5 ml 0.85% normal saline. Cell suspensions were then adjusted with a densitometer (Biosan McFarland densitometer, Latvia) to be 0.5 McFarland turbidity ($1\text{-}5 \times 10^6$ cells / ml) (CLSI M27-A2).

Yeast suspensions prepared as 0.5 McFarland for in vitro growth follow-up test were inoculated into RPMI 1640 medium containing farnesol diluted in concentration ranges of 600–1.171 µM. In the study of 96-well U-based microplates, the final volume for each well was adjusted to be 200 µl. Control group without farnesol was also included in the study. After the microplates were incubated at 37 °C for 24 h, the cell densities obtained were determined by measuring optical density (OD) absorption at 490–600 nm. The percentage growth rates corresponding to the OD values shown by farnesol (Sigma-Aldrich, ABD) on *Candida* cells were prepared as column charts. All the tests were performed in duplicate in three independent experiments (Derengowski et al. 2009; Pammi et al. 2011; Cordeiro et al. 2019; Zhang et al. 2011).

Determination of the effect of farnesol on biofilm formation by XTT reduction test

After *Candida* cell suspensions were prepared, they were added to 96-well sterile flat bottom polystyrene microplates containing RPMI 1640 medium and and farnesol diluted at a concentration ranges of 600–1.171 µM. Control group without farnesol was also included in the study. Thus, it was aimed to investigate the effect of farnesol on biofilm formation before incubation (0 h). After 72 h of incubation at 37 °C, the medium was aspirated and the wells were washed with PBS in a microplate washer (Combiwash Human Diagnostic Worldwide, Germany) to remove non-adherent cells.

Semi-quantitative measurement of biofilm formation was performed with the XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl) -2H-tetrazolium-5-carboxanilide) reduction test (Ramage et al. 2002). For this purpose, XTT (Sigma-Aldrich, USA) was completely dissolved in Ringer's Lactate solution at a concentration of 1 mg/ml. The solution was then passed through a 0.22 µm filter. Menadione (Sigma-Aldrich, USA) was dissolved in acetone to a concentration of 10 mM and diluted with 1:10 PBS. Menadione was added so that the final concentration was 1 µM in the XTT-Menadione solution containing 5 mg/ml XTT.

XTT-menadione of 50 µl was added to the previously washed and dried wells. Plates were incubated for 2 h at 37 °C in the dark. Colorimetric changes at 490–630 nm as a reflection of the metabolic activity of the biofilm were measured with a microplate reader (Chromate Microplate Reader Model 4300, Awareness Technology Inc. Doc) (Ramage et al. 2002).

Determination of the effect of farnesol on the formed biofilm by XTT reduction test

In this study, *Candida* biofilms were developed in flat bottom microplates at 37 °C for 24 h using the procedure using for prebiofilm study. Following biofilm formation, the medium was aspirated, and non-adherent cells were washed away with PBS. Diluted farnesol in the concentration range of 600–1.171 µM prepared in RPMI 1640 was added. The microplates were incubated at 37 °C for 72 h. The effects of farnesol on the previously formed biofilm were determined by XTT reduction test.

Investigation of the effect of farnesol on planktonic *Candida* cells with transmission electron microscopy (TEM)

To study the morphological effects of farnesol on planktonic *Candida* cells by TEM, *C. albicans* ATCC 14053 isolate was used and sterile 6-well plates were used. Cells were suspended to be 0.5 McFarland ($1\text{-}5 \times 10^6$ cells / ml). Four mililiters RPMI 1640 broth was added into the per wells. Farnesol was prepared diluted in concentration ranges of 600–37.5 µM and added to wells of 4 ml each. Concentration ranges were determined based on the effective concentration we obtained for the *C. albicans* ATCC 14053 isolate. Finally, microorganism suspensions were inoculated into wells of 200 µl. Control group without farnesol was also included in the study. It was left after 24 h incubation at 37 °C and absorbance measurements were made at 490-600 nm for each concentration. At the end of the analysis, control group and suspension at the lowest concentration were included in the study.

Then, *C. albicans* ATCC 14053 cell suspensions prepared for the control and farnesol group were taken into sterile plastic centrifuge tubes and centrifuged for 15 min and the cell pellet was washed 3 times with PBS for 10 min. After the supernatant was removed, the obtained pellet was kept at +4 °C for 24 h in 2.5% glutaraldehyde in PBS. The cells were washed 3 times with PBS the next day, and the they were removed from the primary fixative, and then secondary fixed with osmium tetraoxide solution for 1 h in dark with a rotator. After washing three times again with PBS, the cells are embedded in 5% agar and then block stained with 1% uranyl acetate. Then these blocks were dehydrated at 40, 60, 75%, and 95% ethyl alcohol series at +4 °C. The 100% final ethanol dehydration was carried out at room temperature. After clarification and embedding, the blocks were left to polymerize for 48 h at 60 °C. Ultrathin sections which were taken with an ultramicrotome (Leica UltraCutR) were obtained at 60 nm thickness and taken on copper grids. They were stained with uranyl acetate for 15 min and then with lead nitrate for 1 min. The samples were examined with the Hitachi HT7800 TEM.

Investigation of the effects of farnesol on *Candida* biofilm on different biomaterials by scanning electron microscope (SEM)

In this study, the effects of farnesol on the formation and development of *Candida* biofilms were investigated with

SEM. Sterile silicon (Chilecom Medical Devices Co. Ltd.) and polyvinyl chloride (Bıçakcılar Medical Devices Industry and Trade Inc.) biomaterial surfaces were chosen and 24 well plates were used. On these surfaces, RPMI 1640 broth was added as 1 ml for per well. *Candida* isolates were suspended to be 0.5 McFarland ($1-5 \times 10^6$ cells / ml) and added to each well as 50 μl.

In order to examine the effects of adding farnesol to the medium at different times, 1 ml 300 μM farnesol was added to the medium at at 0, 6, and 24 h. In order to examine the effect of addition of farnesol at 0 h, incubation was stopped at 6 and 24 h and kept at +4 °C for 24 h in the primary fixative. The materials were then washed 3 times for 15 min with PBS. In order to investigate the effect of addition of farnesol at 6 and 24 h after the addition of farnesol, the incubation was stopped and the materials were left in the primary fixative at +4 °C for 24 h. The materials were washed 3 times with PBS for 15 min and removed from the fixative. In addition, for the control group without the farnesol, the 0, 6, and 24 h samples were kept in the primary fixative, and washing with buffer solution was performed in the same way. After all these processes, the samples were dehydrated with increasing concentration of ethyl alcohol (50, 70, 90, 95, and 100%). The samples were then gold-palladium plated with the SEM sample coating device (Polaron SC7620 Sputter Coater). Samples for analysis were analyzed with HITACHI Regulus 8230 SEM.

Results

Results of in vitro growth in *Candida* species by farnesol treatment

Figure 1 shows in vitro growth conditions of planktonic *Candida* isolates exposed to different concentrations of farnesol. As also seen in Figure 1, for *C. albicans* ATCC 14053 and *C. albicans* 1766 isolates at 37.5–300 μM concentrations, up to 10% minimal inhibition was observed. At concentrations of 300–600 μM, approximately 20–30% growth inhibition was determined. For the other five different isolates in the study, this inhibition value varies considerably depends to isolate. While minimal inhibition was observed for *C. kefyr* 1798 at a concentration of 75–300 μM, a marked inhibition of *C. tropicalis* 1678, *C. krusei* 1561, *C. parapsilosis* 1799, and *C. glabrata* 1744 at a concentration of 37.5–600 μM was observed. In the concentration ranges at 18.75–1.17 μM, generally, it increased the growth compared with the control group. Considering all the results obtained, farnesol did not observe any fungicidal or fungistatic effects, but decreased or slowed growth in comparison with the control group at 37.5–600 μM concentration ranges.

Results of the effect of farnesol on biofilm formation by XTT reduction test

The effects of farnesol on *Candida* biofilm formation at different concentrations are presented in Figure 2. Farnesol at between 600–37.5 μM concentrations inhibited the biofilm formation of the *C. albicans* ATCC 14053 isolate at a range of 30-40%; this rate is 15-30% for C. tropicalis 1678, respectively. For the concentration ranges at 600–150 μM, percent inhibition values are 30-40% for *C. albicans* 1766 and 10-70% for *C. krusei* 1561. Farnesol at between 600–4.687 μM concentrations inhibited the biofilm formation of *C. kefyr* 1798 isolate at a range of 5-60%. At 600–9.375 μM concentrations, it inhibited the biofilm formation of *C. glabrata* 1744 isolate by 5-60%. Considering all these data, farnesol generally showed a biofilm reduction effect when treated on *Candida* cells before the formation of biofilm (at 0 h). Interestingly, this effect is much lower than the concentrations of antimicrobial effects farnesol exerts on planktonic cells.
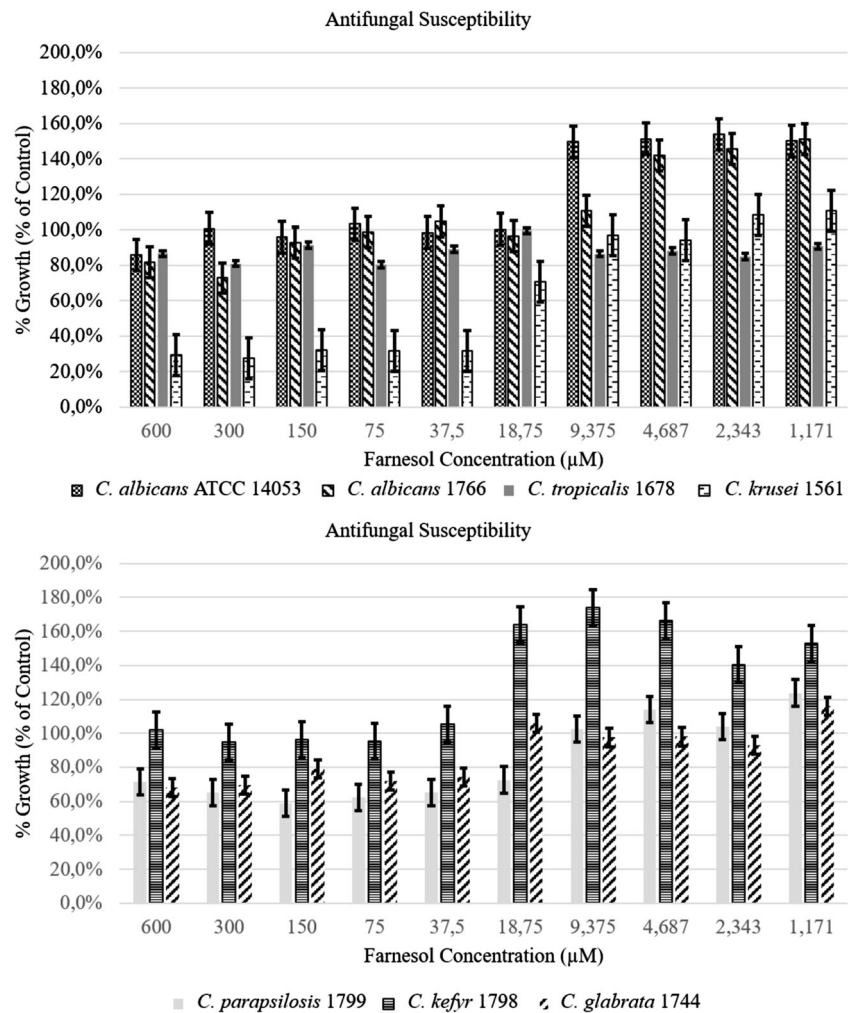
Determination of the effect of farnesol on the formed biofilm by XTT reduction test

The results obtained by XTT reduction test of the effect of farnesol on the formed *Candida* biofilm are presented in Figure 3. At this stage of study, limited concentration range was used (600–37.5 μM), taking into account the data obtained in microdilution and prebiofilm (at 0 h) applications. Farnesol was applied on preformed biofilm (at 24 h), and the XTT results obtained at 72 h were evaluated. Considering the inhibition percentages on the formed biofilm in certain concentration ranges of farnesol, a 7-30% inhibition was observed in the *C. albicans* ATCC 14053 isolate for 600–300 μM concentrations. Considering the concentration ranges from 600 to 37.5 μM, inhibition percentages were determined as 30–85% for *C. albicans* 1766 isolate; 15–75% for *C. tropicalis* 1678 isolate; 40–95% for *C. krusei* 1561 isolate; 50–85% for *C. parapsilosis* 1799 isolate; 85–96% for *C. kefyr* 1798; and 10–75% for *C. glabrata* 1744 isolate (Fig. 3). The graphs express the effect of farnesol on the development and biofilm formation on the basis of the species, and the graphical data created separately for each species are collected for comparison, so SD values were calculated on the basis of species. In addition, these graphs indicate that the effect of farnesol on *Candida* spp is concentration dependent.

Results of the effect of farnesol on planktonic *Candida* cells with TEM

In order to examine the ultrastructural effects of farnesol on the planktonic *C. albicans* ATCC 14053 isolate, at least 10 areas of the control and experimental group were scanned in detail, and common findings were based. Demonstrative TEM

Fig. 1 In vitro growth in *Candida* species following exposure to farnesol at different concentrations (600–1.171 μM). The growth of the control group without farnesol is considered as 100% (OD: 490–600 nm)

Antifungal Susceptibility

⊠ *C. albicans* ATCC 14053   ◨ *C. albicans* 1766   ▪ *C. tropicalis* 1678   ▤ *C. krusei* 1561

Antifungal Susceptibility

▪ *C. parapsilosis* 1799   ▤ *C. kefyr* 1798   ⸜ *C. glabrata* 1744

images were presented in Figs. 4a–f. Control group *Candida* morphology was observed as normal, oval, and round appearance, and cell wall and membrane structures were observed as a whole (Figs. 4a–b). When the *Candida* cells treated with a concentration of 37.5 μM of farnesol were examined, it was determined that some *Candida* cells continued to maintain their normal healthy appearance, but some cells showed wall-membrane separations, vacuol formations, cytoplasm damage, electron dense appearance, and completely lysis. Therefore few ghost cells and some granule formations in cytoplasm were also observed (Figs. 4c–f).

Results of the effects of farnesol on *Candida* biofilm on different biomaterials by SEM

For SEM examinations, reference *C. albicans* ATCC 14053 and clinical *C. albicans* 1766 isolates were used. The results of the farnesol-free control group were compared with the pre- and post-biofilm group results on polyvinyl chloride and silicone materials. The addition of farnesol at 0 time point zero (starting point of incubation) was defined as the prebiofilm,

and the addition at the 6th and 24th h of the incubation was defined as the postbiofilm.

SEM images obtained after 6 h of incubation in the control group without farnesol and experimental groups are presented in Figs. 5 and 6. Growth of standard isolate on polyvinyl chloride material in the control group is much more intense than silicone material. For the clinical isolate, there was no obvious difference between the two materials. According to the prebiofilm data obtained after the application of farnesol at 0 time point zero and incubation for 6 h, the biofilm growth of the standard isolate on polyvinyl chloride is more intense than both the silicone material and the clinical material. In the post-biofilm data obtained by adding farnesol after 6 h of incubation and stopping the incubation after 24 h, no biofilm was observed on polyvinyl chloride and silicone materials for both isolates. Only locally yeast cells were detected (Figs. 5 and 6).

Similarly, 24-h application results are presented in Fig. 7 and 8. Accordingly, in the farnesol-free control group, the biofilm growth of the standard isolate on both materials was much more intense than the clinical isolate; EPS (Exopolysaccharides) and and filamentous elements are marked. According to the

Fig. 2 The effects of farnesol (600–1.171 μM) on biofilm formation by XTT reduction test. The growth of the control group without farnesol is considered as 100% (OD: 490–600 nm)
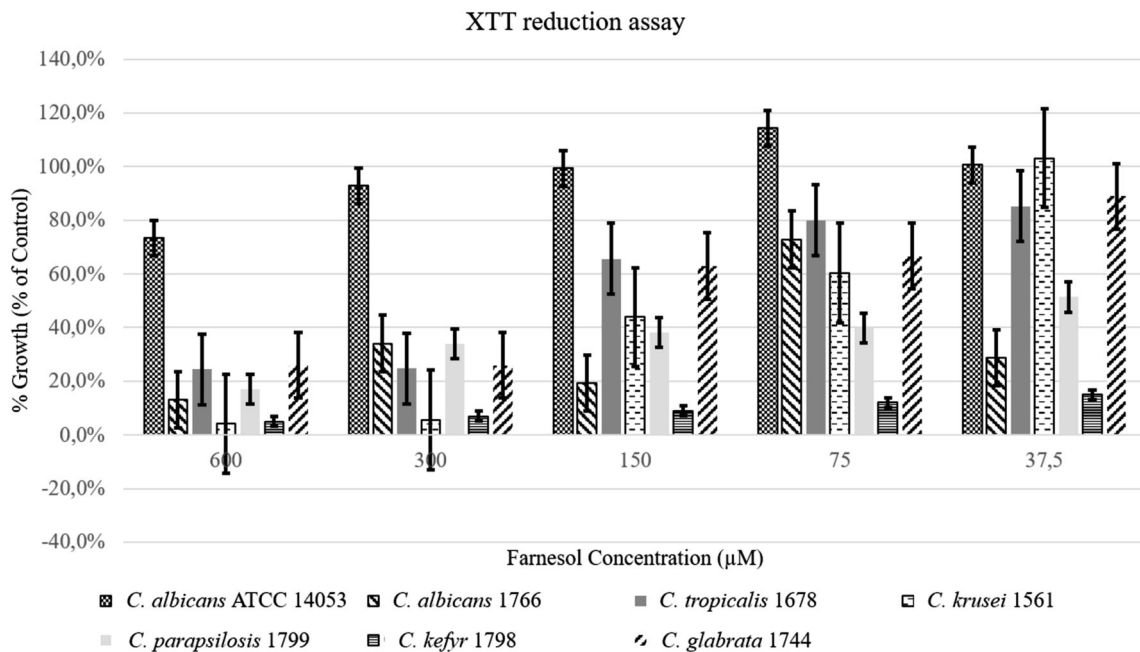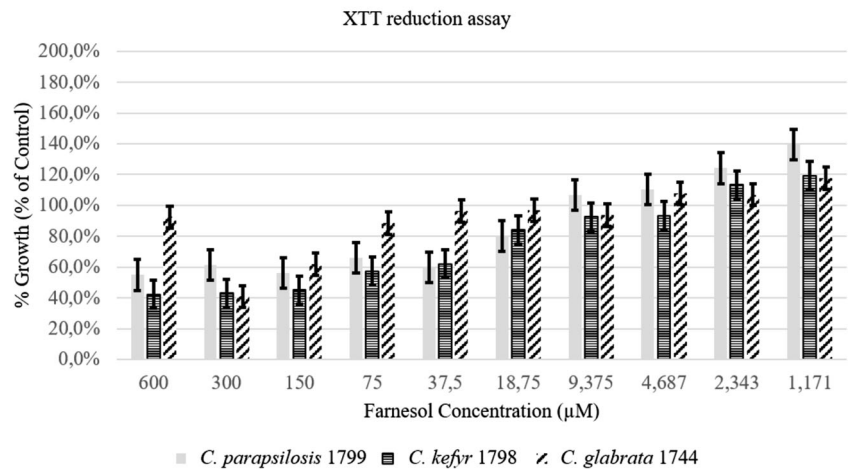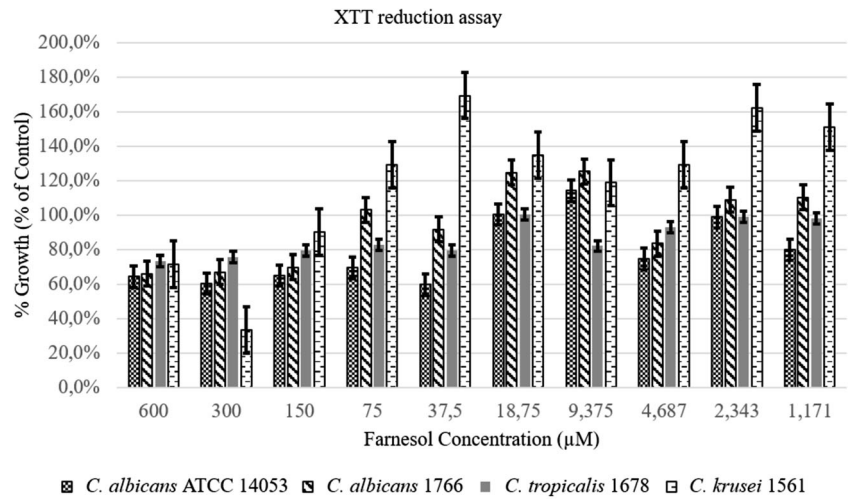

Fig. 3 The effects of farnesol on the formed biofilm by XTT reduction test . The growth of the control group without farnesol is considered as 100% (OD: 490–600 nm)

Fig. 4 TEM images of *C. albicans* ATCC 14053 cells before and and after exposure to 37.5 μM farnesol, showing ultrastructural changes. (A and B): Untreated control cells revealed characteristic ovoid and regular morphology, dense cytoplasm and intact cell wall and membrane structure; (C, D, E and F): Cells treated with 37.5 μM farnesol showed cell wall-membran separations, ghost cell formations, cytoplasm damage, vacuol formation, some completely lyzed cells. Scale bar is 1 μm for A image; 2 μm for B, C, D and E images; 500 nm for F image. Abbreviations: cms cytoplasm-membrane separations; gc ghost cell formation; cd cytoplasm damage; V vacuol formation and lc lyzed cells.
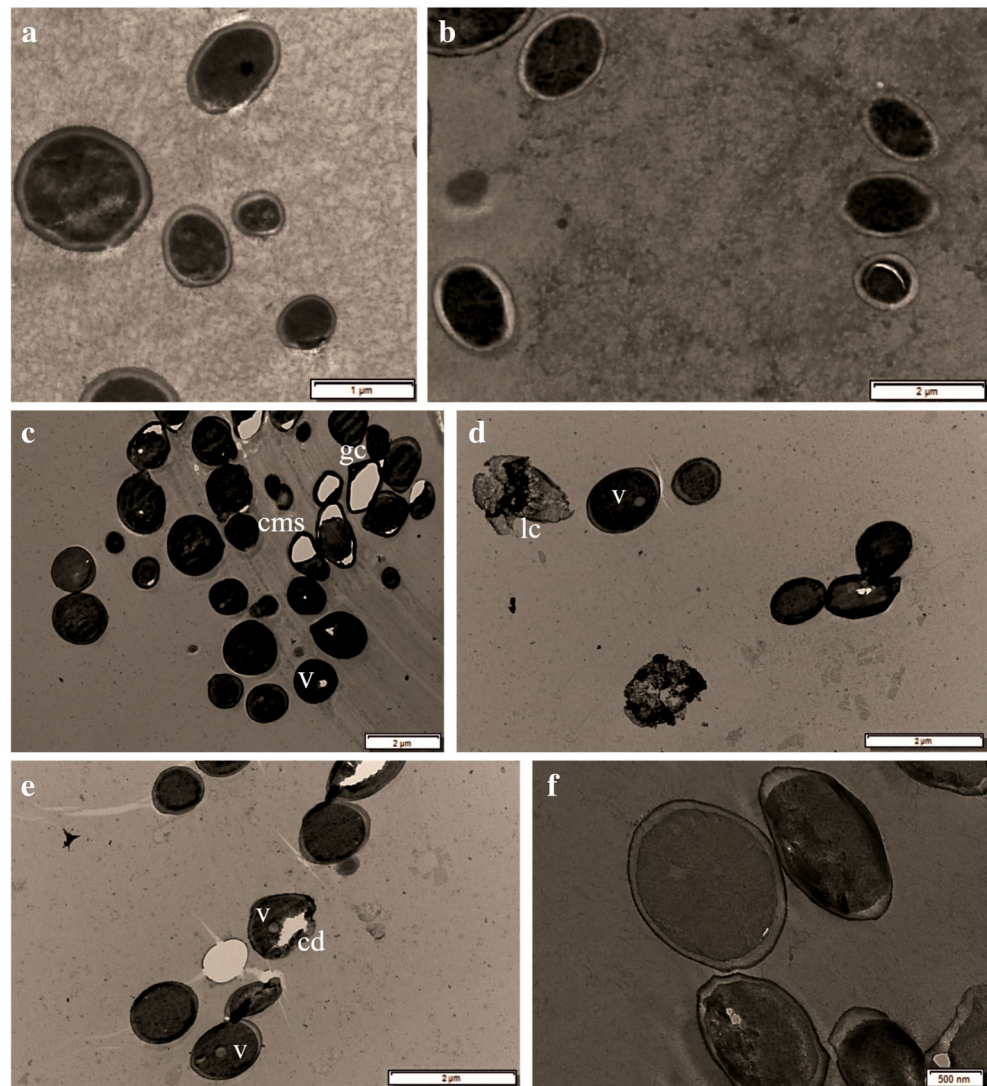
prebiofilm results obtained after adding farnesol at 0 time point zero and stopping the incubation at the 24th h, the EPS layer of the clinical isolate on the polyvinyl chloride material is denser. However, no dense biofilm formation was observed for both isolates. Yeast communities rather than EPS were observed in both samples. Postbiofilm data obtained by adding farnesol after 24 h of incubation and after an additional 24 h of incubation was stopped, showing that there was no biofilm formation in both groups. Only yeast fragments were observed, and these results supported the strong biofilm-reducing effect of farnesol (Fig. 7 and 8).

Discussion

Biofilm-associated *Candida* infections show resistance to current treatment methods, cause prolonged intensive care unit stay and economic problems (Katragkou et al. 2015; Donlan 2001; Wu and Yan 2019). In the formation of biofilm regulated by the QS

mechanism, various signal molecules are secreted into the environment, and microbial communication is provided (Paluch et al. 2020; Fazli et al. 2014). Farnesol is a QS molecule synthesized by *C. albicans* and an autoinducer that blocks yeast hyphae transition. It is thought to have an important role in fungal pathogenicity, and its antimicrobial and anticancer properties are also prominent (Monteiro et al. 2017; Jung et al. 2018). Exogenous application of QS molecules may lead to concentration and time-dependent biofilm inhibition. However, there are limited studies especially on non-albicans *Candida* species. In the present study, the effects of different farnesol concentrations on planktonic and biofilm forms of different *Candida* species were investigated by microbiological and electron microscopic methods.

According to the XTT data, farnesol showed the growth inhibition on *Candida* isolates at approximately 37.5–600 μM concentration rates, and the inhibition percentages showed concentration-dependent differences dependent on the studied isolates. For *C. albicans* isolates, the minimal inhibition rate of farnesol at concentrations of 37.5–300 μM is up to 10%. At 300
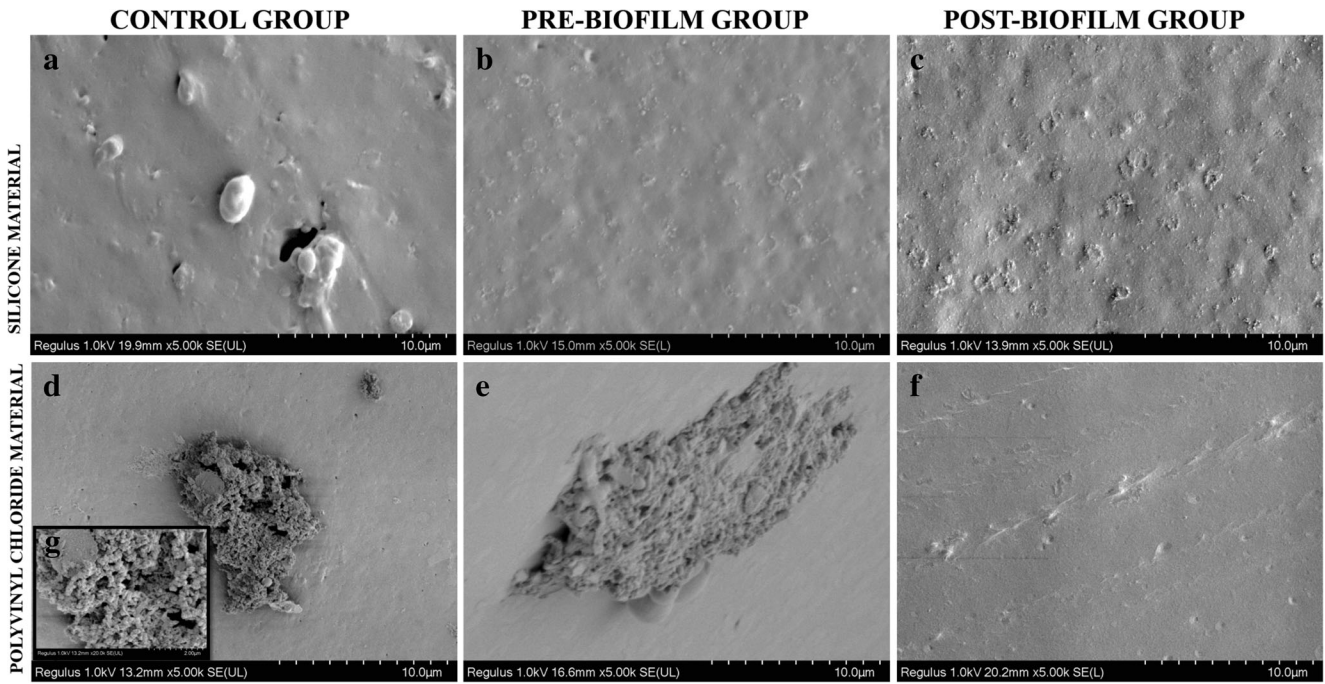
Fig. 5 SEM images of *C. albicans* ATCC 14053 biofilm. Control groups not treated with farnesol after 6-h incubation (A) silicone material, (D) polyvinyl chloride material and (G) larger magnification of same image; Images of prebiofilm condition: Addition of 300 μM farnesol at 0 time point zero (starting point of incubation) and stopping incubation after 6-h (B) silicone material, (E) polyvinyl chloride material; Images of postbiofilm condition: Addition of 300 μM farnesol after 6-h incubation and stopping incubation after 24-h (C) silicone material, (F) polyvinyl chloride material. Scale bar is 10 μm

−600 μM concentrations, this rate is approximately 20-30%. For *C. tropicalis* 1678, *C. krusei* 1561, *C. parapsilosis* 1799, and *C. glabrata* 1744, there was a marked inhibition at 37.5–600 μM farnesol concentrations. For *C. kefyr* 1798, at 75–300
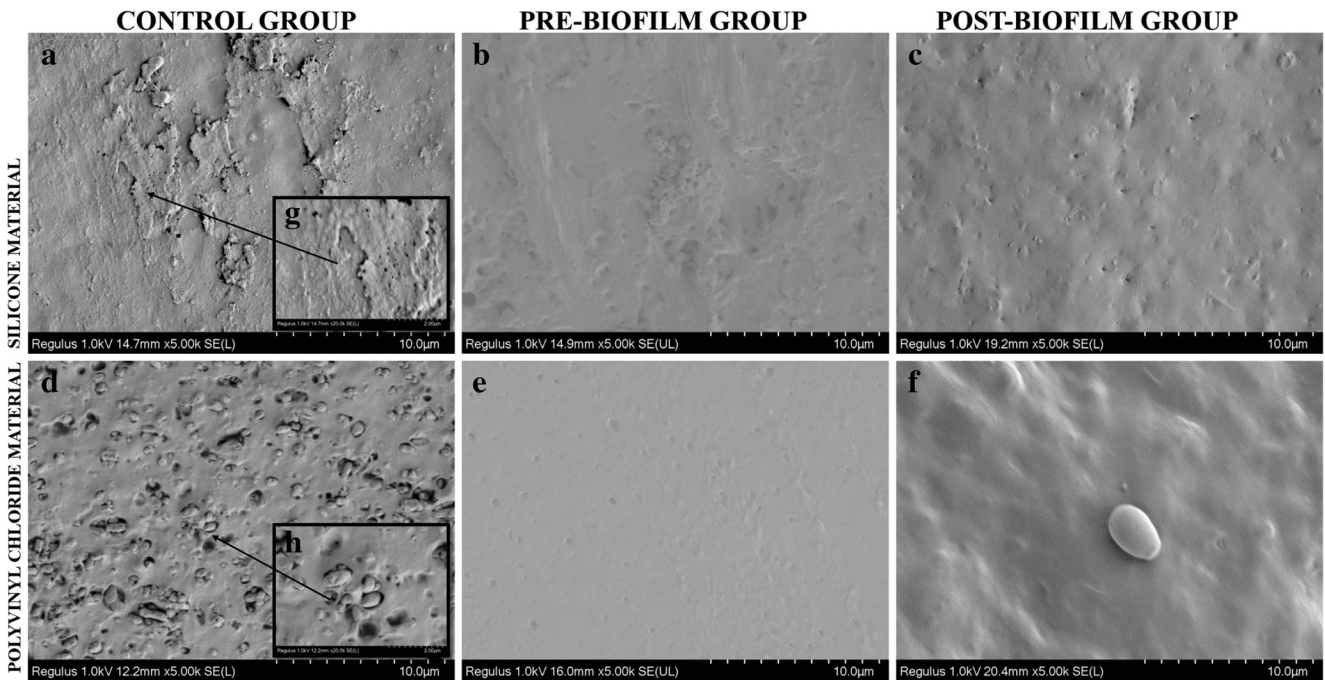

Fig. 6 SEM images of *C. albicans* 1766 biofilm. Control groups not treated with farnesol after 6-h incubation (A) silicone material and (G) larger magnification of same image; (D) polyvinyl chloride material and (H) larger magnification of same image; Images of prebiofilm condition: Addition of 300 μM farnesol at 0 time point zero (starting point of incubation) and stopping incubation after 6-h (B) silicone material, (E) polyvinyl chloride material; Images of postbiofilm condition: Addition of 300 μM farnesol after 6-h incubation and stopping incubation after 24-h (C) silicone material, (F) polyvinyl chloride material. Scale bar is 10 μm

CONTROL GROUP

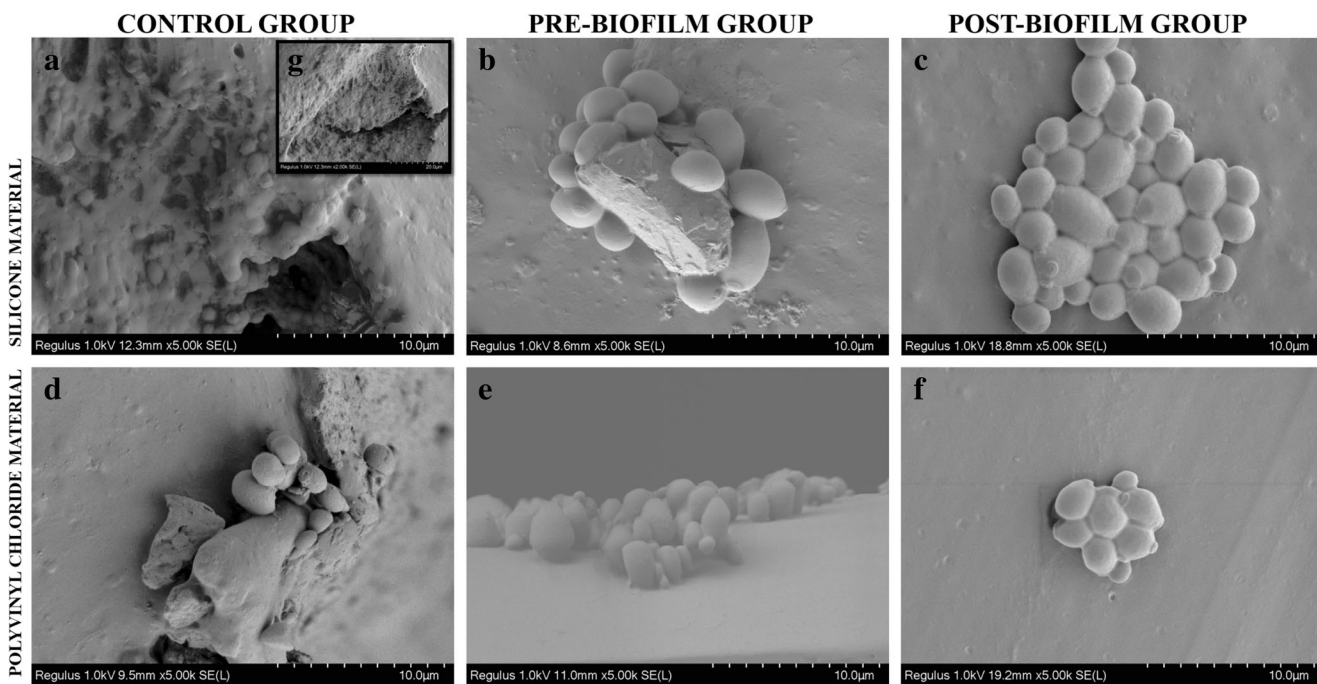## PRE-BIOFILM GROUP

POST-BIOFILM GROUP

Fig. 7 SEM images of *C. albicans* ATCC 14053 biofilm. Control groups not treated with farnesol after 24-h incubation (A) silicone material and (G) larger magnification of same image; (D) polyvinyl chloride material; Images of prebiofilm condition: Addition of 300 μM farnesol at 0 time point zero (starting point of incubation) and stopping incubation after 24-h (B) silicone material, (E) polyvinyl chloride material; Images of postbiofilm condition: Treatment with 300 μM farnesol after 24-h incubation and stopping incubation after additional 24-h (C) silicone material, (F) polyvinyl chloride material. Scale bar is 10 μm.

CONTROL GROUP

PRE-BIOFILM GROUP

POST-BIOFILM GROUP

Fig. 8 SEM images of *C. albicans* 1766 biofilm. Control groups not treated with farnesol after 24-h incubation (A) silicone material, (D) polyvinyl chloride material; Images of prebiofilm condition: Addition of 300 μM farnesol at 0 time point zero (starting point of incubation) and stopping incubation after 24-h (B) silicone material, (E) polyvinyl chloride material; Images of postbiofilm condition: Treatment with 300 μM farnesol after 24-h incubation and stopping incubation after additional 24-h (C) silicone material, (F) polyvinyl chloride material. Scale bar is 10 μm
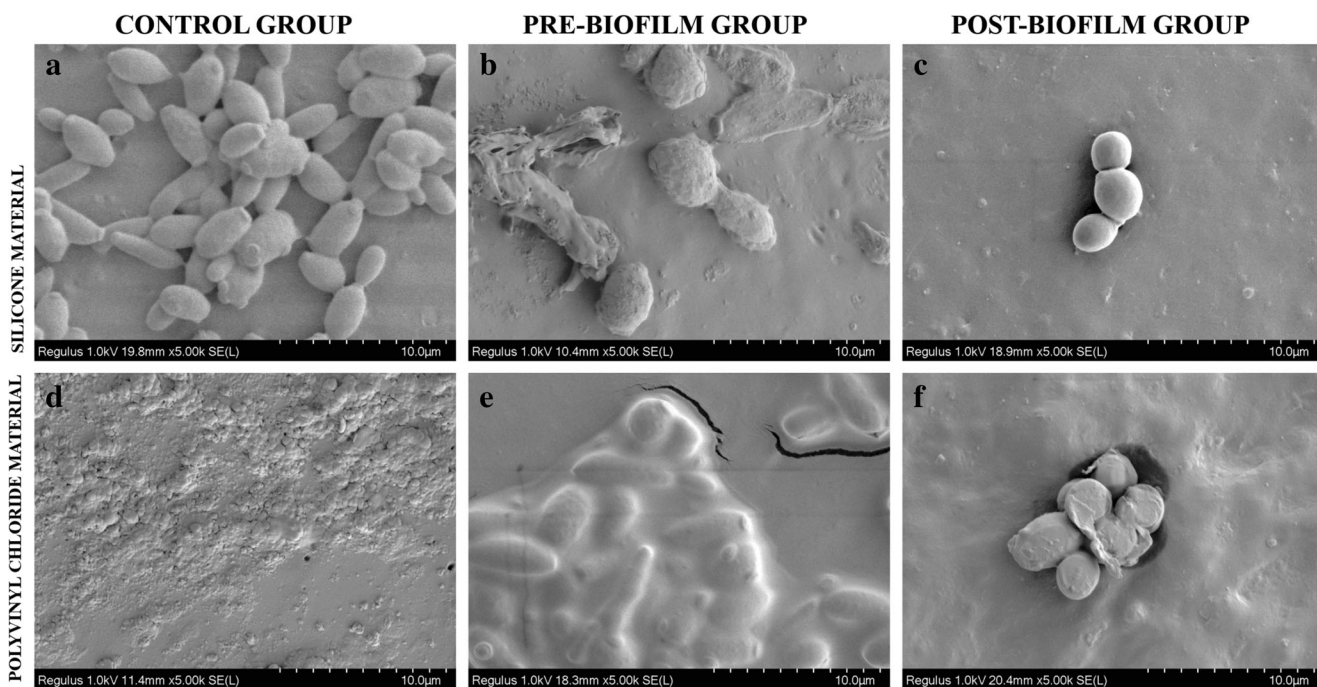
μM farnesol concentrations showed only a minimal inhibition. Interestingly, farnesol increased the growth at 18.75–1.17 μM concentration range compared with the control group. Thus, although no any fungicidal or fungistatic effects of farnesol are observed, it has been found to reduce the growth compared with the control group at certain concentration ranges. Similar results are seen in the literature when the effects of farnesol on planktonic cells are examined. Nagy et al. investigated the efficacy of farnesol on the of *C. auris* biofilm development, and they reported that farnesol showed significant growth inhibition against *C. auris* planktonic cells at concentrations of 100–300 μM (Nagy et al. 2020). Cordeiro et al. also investigated the effect of farnesol on the planktonic and biofilm forms of the clinical isolates of *T. asahii* and *T. inkin*. When using farnesol at concentrations of 75 to 150 μM, *T. asahii* reduced the development of strains by up to 50%. At 600 μM concentration, 80% inhibition was observed. For *T. inkin* strains, farnesol reduced the growth up to 50% at 37.5–150 μM concentrations and up to 80% at 75 to 600 μM concentrations (Cordeiro et al. 2019).

Many experiments indicated important inhibition rates at efficient farnesol concentrations, but data at lower concentrations of without inhibition are very limited. Langford et al. (2010) investigated farnesol concentrations that can be lethal for *C. albicans* based on different phases of the cell. In this study, minimal cell death was observed at farnesol concentrations up to 300 μM, and growth inhibition was detected only at concentrations of ≥ 300 μM. Similarly to our results, Dizova and Bujdakova stated that the effect of farnesol on *C. albicans* is concentration-dependent, and they reported that a concentration of 200–300 μM creates stress for yeast, while a concentration of 40 μM protects it from stress (Dizova and Budjakova 2017). Ramage et al. also investigated the effects of different concentrations (0, 3, 30, and 300 μM) of farnesol on 0, 1, 2, 4, and 24 h *C. albicans* biofilms and compared the obtained XTT data. Authors reported that the effect of farnesol was dependent on both concentration and initial adhesion time and pre-incubation with 300 μM farnesol completely inhibited biofilm formation. (Ramage et al. 2002). Also, in another study of Ramage et al. (2005), the effect of farnesol on biofilm development was reported to be time dependent. In this study, biofilm development was inhibited by adding 30–300 μM farnesol at initial time. Researchers stated that when farnesol was added after 1–2 h, biofilm development continued. Based on this, it has been reported that the addition of farnesol after the hyphae formation has not been able to inhibit biofilm development. Interestingly, at the 24th h, the mature biofilm became sensitive to farnesol again (Ramage et al. 2005). Also in our study, considering the effect of farnesol on *Candida* species, biofilm reduction at a concentration of approximately 37.5–600 μM was generally seen clearly in all isolates. On the other hand, farnesol has increased biofilm formation at concentrations below about 37.5 μM. Here, it is not known exactly by which mechanism the growth-

enhancing effect of farnesol at lower concentrations, or whether it is related to the lack of growth inhibition at the lower concentrations correlate with lack of biofilm formation inhibition, but this situation needs to be clarified with detailed studies.

According to TEM data obtained with farnesol treatment at 37.5 μM concentration on planktonic *C. albicans* cells, membrane, and wall damages, vacuolization or granulations are seen in the cells. In a previous study, Decanis et al. observed large and irregular vacuols in cells treated with farnesol, and these vacuols are particularly dense in the periphery. In the same study, granulation increase in cytoplasm, decrease in cell wall integrity and weakness in the membrane were detected (Decanis et al. 2011). Although our TEM data is also similar to this study, some cells treated with farnesol have a severe wall-membrane separation and there are completely emptied or lysed cells. In addition, an electron-dense appearance was observed in the cells compared with the control group.

According to SEM results, the growth on the polyvinyl chloride surface at the 6th h for the farnesol-free control group of the standard isolate is more intense than the silicone surface. For the clinical isolate, there was no significant difference between both surfaces. While a decrease in biofilm development is observed in prebiofilm group applications compared with control, biofilm development on standard isolate and polyvinyl chloride surface is higher than silicon surface. In postbiofilm applications, there was a strong biofilm reduction on both isolates and both surfaces, and more effective results were obtained. Especially in the control and prebiofilm experiments, the positive effects of the silicone surface compared with the polyvinyl chloride surface were observed because the silicone surface both delayed the biofilm formation and reduced the prebiofilm effects of farnesol. This effect may be due to the surface properties of the silicone. Similarly, it is thought that the differences between standard and clinical isolates observed in control and prebiofilm groups may be due to factors such as adhesion bonds and hydrophobicity. However, these data should be clarified with further studies.

In a study of Xia et al., the effects of farnesol and some antifungals on biofilm formation of standard and resistant *C. albicans* isolates were compared with XTT, SEM, and confocal laser scanning microscope. The authors stated that the biofilm formed by the standard isolate was more sensitive than the resistant isolate. In the same study, farnesol was found more effective on *C. albicans* biofilm formation when applied at an early stage (Xia et al. 2017). Also in our study, farnesol showed both pre- and post-biofilm reducing effects, but the postbiofilm effects were much stronger. Similarly, Fernandes et al. investigated the effect of farnesol on single and mixed species biofilms of *C. albicans* ATCC 10231 and *Streptococcus mutans* ATCC 25175. Farnesol significantly reduced both single and mixed biofilms especially at 12.5 mM concentration, and their SEM results also supported

these findings and reduced the amount of cells in the biofilm (Fernandes et al. 2016). According to these findings and literature information, it is thought that different phase-specific mechanisms, surface properties, farnesol concentration, and strain differences play a role in biofilm formation.

Farnesol is a signal molecule with very broad effects, but the most important of these is its ability to change *Candida* cell morphology without affecting the growth rate. For example, farnesol suppresses hyphae formation at high concentrations but cannot prevent the elongation of the previously formed hyphae. On the other hand, it is also reported that farnesol can induce gene expression of interest in a variety of phenotypic conditions such as drug resistance, pathogenicity, cell wall protection, surface hydrophobicity, or phagocytic response. Perhaps farnesol also exerted an effect by inhibiting the surface adhesion required to inhibit biofilm formation in non-albicans *Candida* species. Considering these different effects of farnesol on cell biology, various studies have shown that it can affect more than one signaling pathway. However, the molecular mechanisms of farnesol on this subject need to be elucidated with further studies.

In conclusion, farnesol showed concentration-, time-, and species-dependent inhibitory effects on *Candida* biofilms at concentrations of 37.5–600 μM, but it increased biofilm formation at concentrations less than 37.5 μM interestingly. Farnesol may protect cells against stress at lower concentrations, but our data need to be confirmed by detailed studies. However, the promising antibiofilm properties of farnesol could potentially be an adjunct or alternative to traditional antifungal treatment strategies. On the other hand, with regard to surface and medical device disinfection in hospital settings, its protective biofilm reducing effects in inhibiting biofilm formation should be explored in detail. The in vivo toxic effects of farnesol have not yet been fully elucidated, but the effects of its use as a surface coating material in body biomaterials may also be among the research topics that may shed light on treatment strategies.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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