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Effect of Farnesol on Responsive Gene Expressions in Hyphal Morphogenesis Transformation of *Candida albicans*

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ABSTRACT

Aims *Candida albicans* a polymorphic fungus can grow as yeast, pseudohyphae and true hyphae forms. The hyphal form has a key role in infection process during invasion to mucosal membrane. A cluster of genes contribute in controlling of hyphae formation in *C. albicans*, include *SAP6*, *HWP1* and *RIM101*. Farnesol is a quorum sensing molecule which inhibits switching of yeast-to-hyphae form. The aim of this study was to investigate the effect of farnesol on yeast-to-hyphae morphogenesis and its related gene expressions in *C. albicans*.

Materials & Methods In this laboratory trial study, *C .albicans* was exposed to various concentration (5, 10, 20, 50, 100, 150 and 300µM) of farnesol and the rate of yeast cell proliferations and germ tube formation was evaluated by different methods and microscopic examination. Real time-PCR was performed to assess the expression levels of the hyphae-specific genes *SAP6*, *HWP1* and *RIM101*. The results were analyzed by IBM SPSS 23 software using Student's t-test and one-way ANOVA.

Findings The yeast growth reduced 5% in 300µM of farnesol approximately ($p<0.05$). Germ tube formation strongly suppressed. Moreover, Real time-PCR analysis showed that 300µM farnesol decreased *HWP1* and *SAP6* gene expressions significantly in comparison to control group ($p<0.05$), whereas, there was no difference in the expression of *RIM101* gene.

Conclusion Farnesol in 300µM concentration can inhibits growth and proliferation of *C. albicans* yeast cells and also inhibits hyphal formation. Farnesol can affect the expression of virulent genes including pathogenic genes that are associated with hyphae morphogenesis such as *SAP6* and *HWP1*.

Keywords Candida albicans; Farnesol; RIM101; SAP6; HWP1

CITATION LINKS

[1] Virulence and pathogenicity of fungual pathogens with special reference to Candida albicans [2] Growth of Candida albicans hyphae [3] Genetic control of Candida albicans biofilm development [4] Adhesion in Candida spp [5] Hwp1 and related adhesins contribute to both mating and biofilm formation in Candida albicans [6] Candida albicans transcription factor Rim101 mediates pathogenic interactions through cell wall functions [7] Environmental sensing and signal transduction pathways regulating morphopathogenic determinants of Candida albicans [8] Farnesol, a fungal quorum-sensing molecule triggers Candida albicans morphological changes by downregulating the expression of different secreted aspartyl proteinase genes [9] Farnesol biosynthesis in Candida albicans: Cellular response to sterol inhibition by Zaragozic acid B [10] Antimicrobial effect of farnesol, a Candida albicans quorum sensing molecule, on Paracoccidioides brasiliensis growth and morphogenesis [11] Susceptibility evaluation of aspergillus fumigatus to silver nanoparticles compared with voriconazole [12] Investigation of bcr1 gene expression in Candida albicans isolates by RT-PCR technique and its impact on biofilm formation [13] Identification of Candida species isolated form Iranian women eith vaginal candidasis by PCR-RFLP method [14] Candida albicans dimorphism as a therapeutic target [15] From attachment to damage: Defined genes of Candida albicans mediate adhesion, invasion and damage during interaction with oral epithelial cells [16] Expression of transglutaminase substrate activity on Candida albicans germ tubes through a coiled, disulfide-bonded N-terminal domin of Hwp1 requires C-terminal glycosylphosphatidylinositol modification [17] Morphogenesis in Candida albicans [18] Quorum sensing controls hyphal initiation in Candida albicans through Ubr1-mediated protein degradation [19] Inhibition of filamentation can be used to treat disseminated candidiasis [20] Farnesol-induced apoptosis in Aspergillus nidulans reveals a possible mechanism for antagonistic interactions between fungi [21] Inhibition of Candida albicans biofilm formation by farnesol, a quorum-sensing molecule [22] Biodegradation of 1,2-dichloroethane (1,2-DCA) by cometabolism ... by ... [34] Optimizing aerobic biodegradation of dichloromethane ...

Introduction

Candida albicans is a normal flora of mucosal membranes which cause a wide spectrum of infections including mucosal as well as systemic diseases that lead to life-threatening diseases [1]. It is well known that transition from yeast to hyphae in Candida has a critical role in invasion and pathogenesis [2, 3]. Morphological transformation is undergoing to micro-environmental conditions such as pH, temperature and others. In different stage of infection, transition of the morphology plays a major role. Generally, colonization mainly is composed with yeast cells only while hyphal cells are more invasive to host cells. In addition, in *C. albicans* biofilm formation, adhesion of yeast cells to the surfaces stimulates hyphal formation [2, 3]. A network of genes contributes in controlling of hyphae formation in Candida that includes *SAP6* (Aspartyl Proteinase 6), *HWP1* (Hyphal Wall Protein 1) and *RIM101* (alkaline-responsive transcriptional regulator).

The *SAP6* gene as a member of Aspartyl Proteinase family is considered as one of the significant pathogenesis factors of *C. albicans* [4]. The *HWP1* gene encoded cell wall protein1 has a special role in hyphal morphogenesis which is more important in biofilm formation [5]. As colonization of *C. albicans* to infection induction and hyphal morphogenesis needs to environmental condition adaptation; transcription factor *RIM101* (Cys2 His2 Zinc finger transcription factor- alkaline-responsive transcriptional regulator) regulates such gene expressions that are responsible to extracellular pH regulation [6]. Farnesol is one of the produced quorum sensing molecule that is as an intermediate product of sterol biosynthesis pathway-farnesyl pyrophosphate [7]. Studies show that farnesol in addition to its effect on biofilm formation, can also influence the expression of virulent genes. According to farnesol property, it inhibits hyphae formation by blocking of yeast to hyphae morphogenesis transformation [8, 9].

In the exploration of new antifungal agents, several studies have been conducted with the focus on unique biological characteristics of natural components with high efficiency and limited side effects. There is limited information about the farnasol effect on hyphae formation. In this study farnesol was considered as a new target for a developing of antifungal agents.

The aim of this study was to determinate the *in vitro* inhibiting effect of such farnesol concentrations on hyphae formation and to evaluate the blocking of hyphae formation through assessing of gene expressions.

Materials and Methods

This laboratory trial study was carried out at Tarbiat Modares University in Tehran, Iran from 2015 to 2017. *C. albicans* (ATCC 10231) cultured on Sabouraud dextrose agar (Sigma-Aldrich; USA) for 24h and incubated at 37^0C. Farnesol (Sigma-Aldrich; USA) prepared in methanol with 300, 150, 100, 50, 20, 10, 5µM concentrations.

Effects of farnesol on the cell proliferation of *C. albicans*: The activity of farnesol on the *C. albicans* cell proliferation was evaluated as reported previously with a minor modification [10]. For the *in vitro* proliferation assay, 10^3 yeast cells per milliliter (ml) were inoculated in yeast nitrogen base (YNB; Merck; Germany) medium supplemented with farnesol at different final concentrations (5, 10, 20, 50, 100, 150 and 300µM). The 96 well-plates were used and farnesol-free wells were as positive control and Itraconazole containing wells were as negative control. The wells were allowed to incubate at 32^0C for 24hours. After this period, growth rate was determined by measuring of wells optical density (OD) at 630nm by using ELISA reader Stat Fax 2000 (Stat Fax; Germany). Minimum inhibitory concentration (MIC) of farnesol on *C. albicans* growth was defined in comparison to farnesol-free control well [10, 11].

Effect of farnesol on the *C. albicans* cell viability: The effect of farnesol on cell viability was also estimated by colony counting. Yeast cell of *C. albicans* were suspended in YNB medium at a density of 10^3cells/ml and farnesol added at final concentration of 5, 10, 20, 50, 100, 150 and 300µM. Farnesol-free wells were as positive control and Itraconazole containing wells were as negative control. After incubation at 32^0C for 24hours, each farnesol-treated and farnesol-untreated wells were cultured on the Sabouraud dextrose agare plates and incubated for 24h at 30^0C. Percentage of inhibitory growth was determined using of following formula: 1-N1/N2×100, (N1 is the mean of farnesol-treated colonies and N2 is the mean of farnesol-untreated colonies) [10].

Effect of farnesol on yeast- to- hyphae transition: Determination of farnesol effect on yeast- to- hyphal transition and germ tube formation in *C. albicans* was carried out as reported previously with a few modifications [10]. After obtaining of farnesol MIC on *C. albicans* growth (300µM), the 10^3cells/ml *C. albicans* inoculated into 1ml of YNB medium containing 300µM farnesol and incubated overnight at 32^0C. Harvested budding yeast cells then inoculated in serum and incubated at 37^0C for 2h [10]. Finally, hyphal formation and germination were examined by optical microscope and microscopic photographs were taken from treated and untreated samples as negative control.

Real-time PCR (RT-PCR): After obtaining of farnesol MIC on *C. albicans* growth, fresh culture colonies with 10^3cells/ml of *C. albicans* were

prepared and treated with a 300µM concentration of farnesol for 24h in 30^{0}C. Farnesol-free wells were as positive control and Itraconazole containing wells were as negative control. After collecting of yeast cells and washing with phosphate buffer solution (PBS), total ribonucleotide acid (RNA) was extracted by glass bead and lysis buffer method according to the previously described method [12, 13]. Then, cDNA was synthesized using kit (vivantis; Malaysia) as recommended by the manufacturer's protocol. Real-time PCR was accomplished using AMPLIQON (Real Q plus 2 x master mixes Green High Rox). In order to PCR performance, the mixture were prepared containing 12.5µl of master mix (Green High Rox), 0.5µl of each specific primers, 2µl of each cDNA templates were adjusted to final volume of 25µl using Diethyl pyrocarbonate (DEPC) water. Thermo cycling condition was optimized for incubation at 95^{0}C for 5minutes (min.), followed by 40 cycles at 95^{0}C for 15seconds (sec.), 58^{0}C for 30sec., 72^{0}C for 30sec. and finally 72^{0}C for 5min. All steps were done in the ABI one step (Applied Biosystems; Rotkreuz; Switzerland). The specific primers for *HWP1*, *SAP6* and *Rim101* were designed using all-ID design software version 7.5 (Table 1).

The *ACT1* (Actin1gene) was used for normalization of the RT-PCR data. All reactions were performed as duplicate form and the analysis was done using REST 2009 Ver. 2.0.13 software.

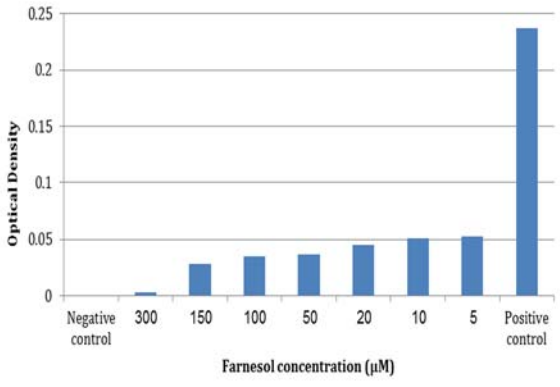
Statistical analysis: The experiments in this survey were performed in duplicate and results were analyzed by IBM SPSS Statistics version 23 software. We used Student's t-test and one-way ANOVA for analysis of data.

Table 1) Primers sequences used for Real-time PCR

Gene	Sequence (5'to 3')	Amplicon size (bp)	Accession no.
ACT1	5'-GCGGTAGAGAGACTTGACCAACC-3' 5'-GACAATTTCTCTTTCAGCACTAGTAGTG-3'	200	XM019475182.1
SAP6	5'-TTACGCAAAAGGTAACTTGTATCAAGA-3' 5'-CCTTTATGAGCACTAGTTAGACCAAAC-3'	101	XM714012.1
HWP1	5'-ACTGCTCAACTTATTGCTATCGC-3' 5'-ACCGTCTACCTGTGGGACAG-3'	69	XM704869.2
RIM101	5'-ATTACAACATTCATCCCGATAA-3' 5'-CCAACATGGTCGTCACAC-3'	81	XM709954.1

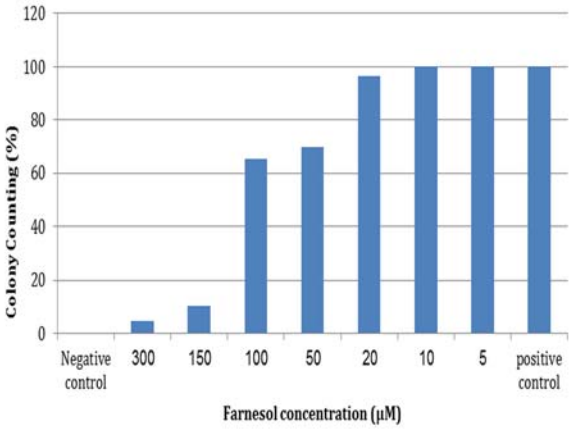
Findings

It was observed that reducing of farnesol concentrations in term of cultures cause to increasing of microorganism density in medium and led to increasing of OD. It was verified that 300µM concentration of farnesol, strongly inhibited yeast cells growth ($p<0.05$; Diagram 1).



Digram 1) Results of different farnesol concentrations effect on *C. albicans* proliferation, the cell mass at each concentration was assessed by measuring of OD based on farnesol concentrations at 630nm. Positive control is farnesol-free wells and negative control is Itraconazole containing wells.

Colony counting of the treated-cells in 300µM concentration of farnesol showed the least growth of *C. albicans* cells in comparison to other concentrations and negative control ($p<0.05$; Diagram 2).



Digram 2) Result of the different farnesol concentrations effect on the *C. albicans* cells viability by colony counting. Positive control is farnesol-free wells and negative control is Itraconazole containing wells.

The results of hyphal growth and germ tube formation indicated that 300µM farnesol is able to inhibit hyphae and germ tube formation and only blastospores were observed. This phenomenon confirmed the inhibitory effect of farnesol on hyphal growth (Figure 1).

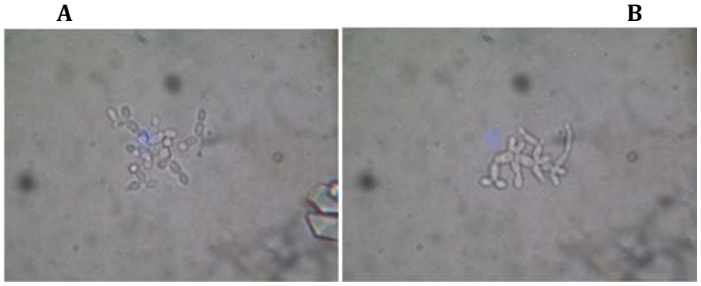
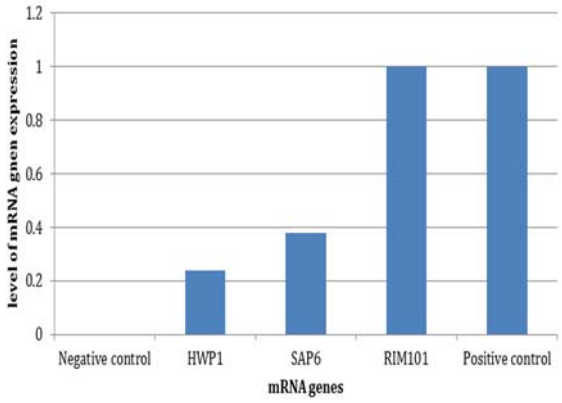


Figure 1) Filamentation and germ tube formation of *C. albicans*. A: 300µM farnesol treated *C. albicans* after 24h shows only blastospores in slide filed. B: Untreatment farnesol *C. albicans* that yeast cells have started to germination and filamentation

Effect of farnesol on expression *SAP6*, *HWP1* and *RIM101* genes using Real-time PCR showed significant decreasing in expression of *HWP1* gene in 300µM farnesol-treated *C. albicans*, followed decreasing in expression of *SAP6* gene in comparison to untreated control group ($p<0.05$), Whereas, no effect was observed on expression of *RIM101* genes in comparison to untreated sample (Diagram 3).



Digram 3) Effect of 300µM farnesol concentration on *SAP6*, *HWP1* and *RIM101* gene expressions of *C. albicans*. *ACT1* was used as internal control (housekeeping gene). Negative control is Itraconazole containing wells that expression of even 4 mentioned genes was negative. In contrast, farnesol free wells used as positive control (untreated group). By normalizing and fold changing evaluation, expression was 1 in positive control.

Discussion

C. albicans is a commensal fungus, which can able to invade to host epithelial cells in certain conditions [14, 15]. *C. albicans* can cause diseases of mucosal membranes due to morphogenesis to the hyphal form [14]. This morphology has key role in the infection process. During the infection, the hyphal form invades to epithelial and endothelial cells via releasing of hydrolytic enzymes such as Aspartyl Proteases family (SAP4-6) and cause damages in mucosal membranes [8]. Also, hyphal cell wall protein encoded by *HWP1* gene attaches to the host cells through transglutaminase with covalent bond and thereby penetrates to host mucosal membrane epithelial cells [16]. The *RIM101* gene of *C. albicans* is responsible to regulate the environmental pH. [17].

Farnesol as quorum sensing molecule is secreted by Candida cells into the environment and inhibit hyphal formation during biofilm formation [18, 19]. Previous studies indicated that farnesol can prevent hyphal morphogenesis and influences the invasion of fungus to host tissue and its expansion and pathogenesis [20]. In a previous study conducted by Ramage *et al*. in 2002 showed using 300µM farnesol has inhibition effect on biofilm formation of Candida at 24h exposing [21]. Mosel *et al.* in 2005 in other study showed that 250µM farnesol prevents the germ tube formation and suppresses filamentation of Candida in biofilm formation [22]. In the present study, the results showed that growth and proliferation of *C. albicans* at concentrations of 300µM farnesol has been significantly inhibited in comparison to other concentrations ($p<0.05$). Also, our finding showed that farnesol in 300µM inhibits the hyphae formation and blocks germ tube formation in comparison to other concentrations.

In the present study effect of farnesol on the expression of *HWP1*, *SAP6* and *RIM101* genes of *C. albicans* evaluated and results showed significant decreasing in expression of *HWP1* and *SAP6* genes in comparison to control groups ($p<0.05$), whereas, in the *RIM101* gene expression no difference was observed. According to a study conducted by Decanis *et al.* in 2011, 300µM farnesol reduces expression of *SAP4-6* genes with a significant difference [8]. Also, other study verifies this inhibitory effect on gene expressions [20].

In the current study, for the first time farnesol were used for evaluation of its effect on the expression *HWP1* and *Rim101* genes, just analysis did on standard isolate ATCC 10231 *C. albicans*. However, in order to obtain more accurate and comprehensive results, further studies should be carried out on the causative agents of fungal diseases and clinical isolate at *in vivo* condition. Future studies at *in vitro* and *in vivo* conditions can evaluate the molecular mechanisms of farnesol effects. Also, farnesol inhibitory effects can be used

on new targets and for designing natural-based antifungal agents.

Conclusion

Farnesol in 300µM concentration can inhibits growth and proliferation of *C. albicans* yeast cells and also inhibits hyphal formation. Farnesol can affect the expression of virulent genes including pathogenic genes that are associated with hyphae morphogenesis such as *SAP6* and *HWP1*. This action can reduce the pathogenicity and invasion of *C. albicans*.

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