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The Effects of Ellagic Acid on Growth and Biofilm Formation of *Candida albicans*

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ABSTRACT

Introduction: Biofilm formation is one of the specific features of *Candida albicans* that protects it from antifungal agents and the host immune system. Also, Biofilm formation by *C. albicans* on the mucosal surfaces and medical devices are responsible for causing *Candida* nosocomial infection. Here, we investigated the effects of ellagic acid on *C. albicans* growth and biofilm formation regarding the expression of two essential genes that are involved in adhesion and yeast-hypha transition. **Methods:** The yeasts were treated with serial two-fold concentrations of ellagic acid (3.125-100 µg/ml) for 48 h at 35°C. The weights of the cultured yeasts were measured as an indicator of the fungal growth, and the biofilm formation was assessed by a tetrazolium salt (XTT) reduction assay. The expression of *HWP1* and *ALS3* genes was assayed by real-time PCR. **Results:** Ellagic acid inhibited *C. albicans* growth 0.68%-82.44%, dose-dependently. The biofilm formation also reduced 2.61%-68.318%. Also, The expression of *HWP1* and *ALS3* genes was notably suppressed by ellagic acid at different concentrations. **Conclusion:** Our results showed that ellagic acid is a potential candidate to eliminate *C. albicans*-generated biofilm by suppressing the expression of the involved genes.

INTRODUCTION

Candida albicans is an essential normal flora in the human body and one of the critical opportunistic organisms that cause infection in certain conditions such as immunodeficiency. Several factors, such as adhesion, phenotypic switching, yeast-hyphal transition, biofilm formation, and proteolytic enzyme secretions, contribute to *C. albicans* virulence [1, 2]. Biofilm formation is one of the essential factors that contribute to the pathogenicity of *C. albicans*. A biofilm consists of a population of microorganisms that are attached firmly to the mucosal cells or the medical devices, which may lead to nosocomial infections. *C. albicans* is the most common species associated with the biofilm formation on the medical devices [3, 4].

Moreover, biofilm is one of the common causes of opportunistic blood infections, with a mortality rate of ≈50% [5, 6]. Biofilm made by *Candida* species can also cause superficial and systemic infections in immunocompromised patients [7].

C. albicans in the biofilm structure exhibits higher resistance to antifungal agents such as fluconazole (FLU) and amphotericin B (AMB), compared to the planktonic cells [6, 7]. Adhesion is the first step of biofilm formation in *C. albicans*. Adhesins and cell wall glycosyl-phosphatidylinositol proteins are required for the attachment of the yeasts to other cells or surfaces. In *C. albicans*, one of the notable families of the adhesions is the hyphal wall protein (Hwp), including Hwp1, Hwp2, Rbt1, Eap1, and Ywp1. The Hwp1, a mannoprotein, exists in the cell wall of germ-tubes and hyphal cells and plays a vital role in biofilm formation [8, 9]. Another important family of the adhesions is the agglutinin-like sequence (Als), among which Als3 is the most important of the eight members of the family with a critical role in the biofilm production [8].

Various studies have attempted to find effective strategies for limiting, preventing, and controlling biofilm production by *C. albicans* [9]. Besides, many studies have aimed to find natural compounds or products with antifungal or anti-biofilm properties. For instance, Raut *et al.* (2013) studied 28 terpenoids with plant origins and analyzed their antifungal and anti-biofilm activities against *C. albicans* [10]. Molales *et al.* (2013) demonstrated that phenazines produced by *P. aeruginosa* regulated *C. albicans* metabolism, hyphal transition, and biofilm formation [11]. Also, Wong *et al.* (2014) have introduced useful compounds with inhibitory activity on growth and yeast- to- hypha transition of *C. albicans* [12].

Ellagic acid, a phenolic compound found in various plants and fruits, has shown antioxidant, antimicrobial, and anti-inflammatory activities [13]. In the present study, the effects of ellagic acid on growth and biofilm formation of *C. albicans* were evaluated by monitoring the expression of

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HWP1 and *ALS3* genes, which are involved in adhesion and yeast-hyphal transition of *C. albicans*, respectively.

MATERIAL AND METHODS

Chemicals. Sabouraud dextrose agar (SDA) was obtained from Merk (E. Merck, Germany). Sabouraud dextrose broth (SDB) was purchased from Scharlau Chemie (S. A., Barcelona, Spain). RPMI 1640 was obtained from Biosera (France). 3-bis (2-methoxy-4-nitro- 5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) and ellagic acid (CAS. 476-66-4) were purchased from Sigma (St. Louis, MO, USA). GITC (Guanidium isothiocyanate) reagent, RNase-free DNase, random hexamer primers, Revert Aid M-MuLV reverse transcriptase, and SYBR Green master mix were obtained from Thermo Fisher Scientific (USA). The stock solution of ellagic acid was prepared in dimethyl sulfoxide, purchased from Sigma Chemical.

Strain and culture condition. *C. albicans* ATCC10231, from pathogenic fungi culture collection (PFCC) of the Pasteur Institute of Iran, was cultured on Sabouraud dextrose agar and incubated at 28°C for 48 h. The fungal suspensions were prepared in final inoculum sizes of $0.5\text{-}2.5\times10^3$ cells/ml for the biofilm formation and, 10^5 cells/ml for the growth inhibition analyses. Sabouraud dextrose broth and RPMI 1640 were used for liquid fungal cultures in quantitative antifungal and biofilm assays.

Determination of the minimum inhibitory concentration. The antifungal activity of ellagic acid was carried out by the broth microdilution method according to CLSI standard M27-A3 [14]. Two-fold serial concentrations of ellagic acid (3.125 to 100 µg/ml) were prepared, and RPMI 1640 with MOPS (pH 7.0) was used to carry out the test with final inoculum concentrations of $0.5\text{-}2.5\times10^3$ cells/ml in 96-well U-bottomed microtitration plate, incubated at 37°C for 48 h. After incubation time, the optical density was measured at 600 nm for the analysis of minimum inhibitory concentrations (MICs) with a plate reader (BioTek, USA). The inhibitory concentration of 50% (MIC_{50}) was defined by comparing the growth of *C. albicans* in treated samples compared with the controls [15]. The experiments were repeated in triplicate.

Biofilm formation. *C. albicans* 10231 was cultured on Sabouraud dextrose agar plates for 24 h at 37°C. The fungal suspension at 10^6 cells/ml concentrations in RPMI-1640 was transferred into flat-bottom 96-well microtiter plates, treated with different concentrations of ellagic acid (3.125 to 100 µg/ml) to a final volume of 200 µl per each well. The biofilm formation was determined after 24 h at 37°C using 2, 3-bis (2-methoxy-4-nitro- 5-sulphophenyl)-2H-tetrazolium-5-carboxanilide. The metabolic activity was measured spectrophotometrically at 490 nm using a microplate reader [16]. The experiments were done in triplicate.

Gene expression by real-time PCR. *C. albicans* 10231 was treated with different concentrations (0 to 100 µg/ml) of ellagic acid in RPMI 1640 and incubated at 37°C for 24 h. The fungal cells were homogenized by glass beads; the total RNA was extracted using GITC (Guanidium isothiocyanate) reagent, and treated with RNase-free DNase [17]. The cDNA was prepared using 1000 ng RNA (normalized with an equal amount of RNA weight in all reactions) with random hexamer primers and Revert Aid M-MuLV reverse transcriptase. A Real-Time quantitative RT-PCR determined the expression of *HWP1* and *ALS1* genes. The amplification was performed using the SYBR Green master mix in 25 µl reactions containing 20 ng cDNA and 0.2 μM of each primer by a Rotor gene 6000 (Corbett, Australia) sequence detection system. The specific primer sequences are shown in Table 1 [18]. Real-time RT-PCR program included an initial incubation at 95°C for 10 min, followed by 35 cycles of 95°C for 60 s, 60°C for 60 s and 72°C for 45 s. The reactions were repeated in triplicate. The *β-actin* gene was used as a reference, and the folding changes were determined using the relative threshold method ($2^{-\Delta\Delta CT}$) [19].

Statistical analysis. The data of the biofilm formation and gene expression were analyzed by One way ANOVA using GRAPHPAD PRISM 6 (GraphPad Prism Software Inc, San Diego, CA, USA). The differences with $P < 0.05$ were considered significant.

Table 1. Primers used for Real-time PCR

Gene	Forward (5'-3')	Reverse (5'-3')
HWP1	CTCCAGCCACTGAAACACCA	GGTGGAATGGAAGCTTCTGGA
ALS3	ACCTGACTAAAACTGCACCAA	GCAGTGGAACTTGCACAACG
ACT1	CGTTGTTCCAATTTACGCTGGT	TGTTCGAAATCCAAAGCAACG

RESULTS

Effects of ellagic acid on the fungal growth. Ellagic acid at different concentrations inhibited the growth of *C. albicans* ATCC10231 (Table 1). The results revealed that the fungal growth was inhibited dose-dependently with increasing concentrations of the acid. Ellagic acid was a potent growth inhibitor with an MIC_{50} of approximately 12.5 µg/ml concentration at 48 h (Table 2).

Effects of ellagic acid on Candida biofilm formation. Biofilm formation of *C. albicans* ATCC10231 was determined after 24 h. Yeast cells were incubated in the presence of various concentrations of ellagic acid. Inhibition of the biofilm formation was dose-dependent at all ellagic acid concentrations as it caused a 50% reduction at a concentration of about 25 µg/ ml (Fig. 1).

Effect of ellagic acid on expression of *HWP1* and *ALS3* genes. Following the treatment of *C. albicans* with different concentrations of ellagic acid, the total RNA was extracted, and the expressions of two critical genes connected to biofilm formation, namely *HWP1* and *ALS* 3, were evaluated by a real-time PCR. The result of the real-time PCR reaction was confirmed by displaying a single

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peak in the melt curves and the correct size of the amplicons, verified by agarose gel electrophoresis. The results indicated that the genes expressions in *C. albicans* were suppressed significantly ($P < 0.05$) after treatment with ellagic acid, and there were reductions at the transcriptional level of these genes in a dose-dependent manner. The expression of *ALS* 3 was more suppressed compared to *HWP1*, at all concentrations of ellagic acid. At the highest concentration of ellagic acid, the expression was 9.1 % and 20.77% for *ALS3* and *HWP1*, respectively (Fig. 2).

Table 2. Inhibitory effect of various concentrations of ellagic acid on *C. albicans* growth

Ellagic acid (µg/ml)	Fungal growth (%)	Growth inhibition (%)
0	100	0.00
100	17.56	82.44
50	40.57	59.43
25	46.71	53.29
12.5	52.71	47.29
6.25	65.85	34.15
3.125	99.32	0.68

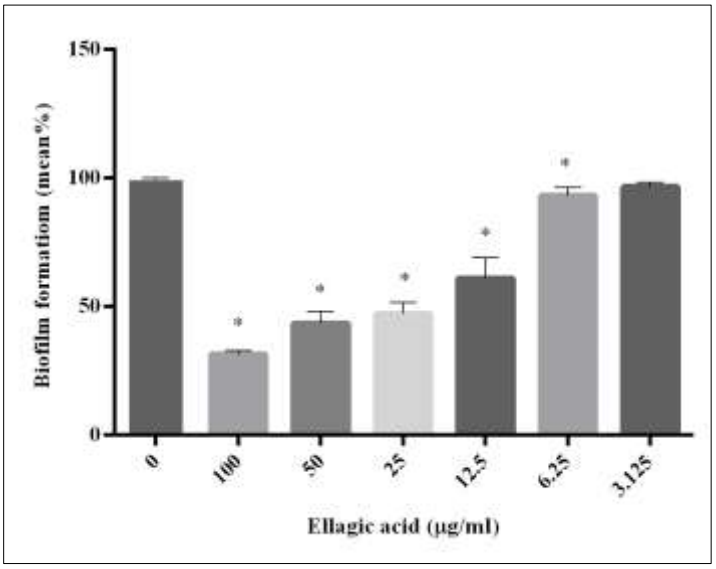


Fig. 1. The mean percent of biofilm formation of *C. albicans* ATCC10231 after treatment with different concentrations of ellagic acid, compared with the non-treated (control) sample.

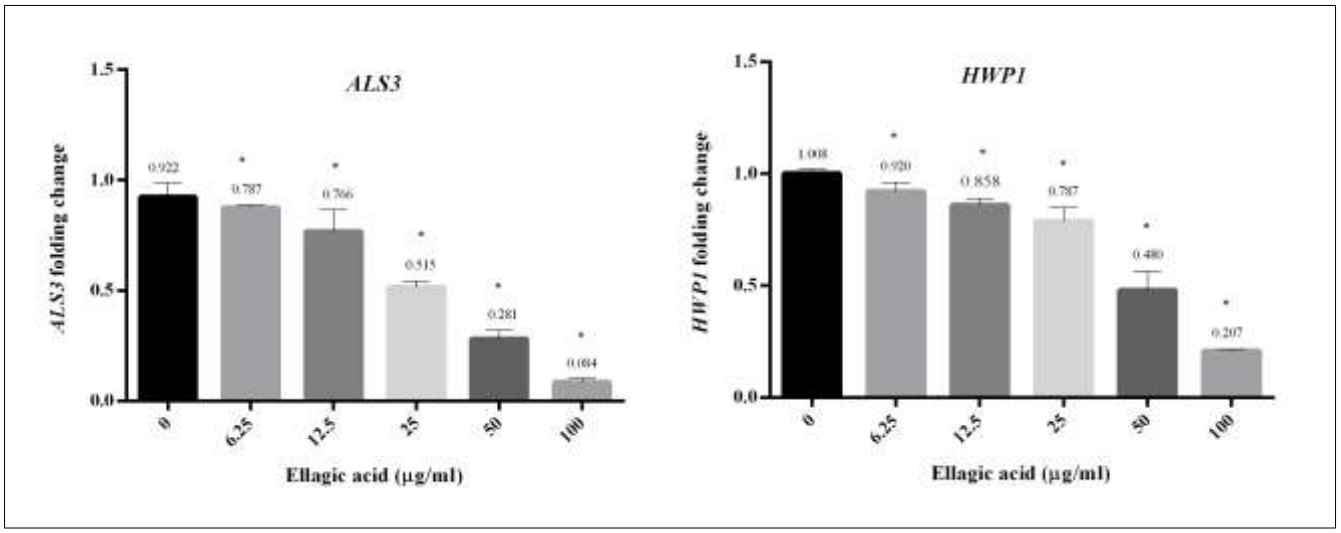


Fig. 2. The level of *ALS3 and HWP1* mRNA expression in ellagic acid-treated (100 to 6.25 µg/ml) and non-treated (control) *C. albicans* ATCC10231. Each sample was normalized for the amount of the template to the levels of *β- actin*. The treated and non-treated samples showed significant differences in the mRNA level in both genes. Asterisks show statistically significant differences with the controls ($P < 0.05$).

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DISCUSSION

Plants are essential sources of compounds with different biological and antimicrobial activities. There have been many studies aiming to find bioactive molecules of plants with inhibitory activity on fungal growth and biofilm formation [20]. Studies showed that a high percentage of human infections are related to biofilm formations [21], and most antifungal drugs have no effect on the biofilm formation or only affect at high concentrations [22]. Here, the anti-biofilm activities of ellagic acid on *C. albicans* were studied, and the expression of dominant genes responsible for yeast adhesion and hyphae formation was investigated. Ellagic acid, a natural compound with antioxidant, antimicrobial, and antimutagenic activities, has been used in traditional medicine for a long time [20].

Many studies have evaluated the antifungal effects of this natural compound. For instance, Brighenti *et al.* (2016) reported that among phenols identified in bioactive extracts of *Buchenavia tomentosa*, ellagic acid had a noticeable anti-*C. albicans* activity with a MIC of 3.2 µg/ml [23]. Studies also demonstrated a wide range of antifungal activities for ellagic acid with MICs ranging from 18.75 to 58.33 µg/ml and 25.0 to 75.0 µg/ml for *dermatophyte* and *Candida* strains, respectively. These studies suggested that ellagic acid has a strong potential to be used for the development of new natural antifungal agents [24]. Pani *et al.* (2016) also demonstrated the antifungal activity of ellagic acid on trichothecene producer *Fusarium culmorum* [25]. In another study, ellagic acid showed inhibitory effects on biofilm formation in *Escherichia coli, Burkholderia cepacia, Staphylococcus aureus*, and *C. albicans* biofilms at 15 to 40 µg/ml concentrations [15].

In the present study, the growth and biofilm formation of *C. albicans* was inhibited ≈50% at 12.5 µg/ml and 25 µg/ml concentrations of ellagic acid (Table 2, Fig. 1). The abilities of adhesion and filamentation are the critical virulence factors of *Candida* spp. The most significant gene involved in biofilm formation is *ALS3*. Suppression or deletion of *ALS3* leads to intense defects in the biofilm formation process [26]. *HWP1*, encoding a cell wall mannoprotein, also has a significant role in biofilm formation [27].

Many studies have exhibited the effects of different substances on the expression of genes responsible for biofilm formation. Khodavandi *et al.* (2011) showed that allicin had a potent effect on *HWP1* gene expression, suggested allicin as a molecular-targeted anti-biofilm compound [28]. Purpurin, with an MIC_{50} of 3 µg/ml, showed a strong inhibitory effect on biofilm formation of *C. albicans* compared to amphotericin B [29]. Purpurin also could down-regulate *ALS3, ECE1, HWP1, HYR1,* and *RAS1*, the crucial genes related to filamentation of *C. albicans*. Another study showed that ellagic acid and gallic acid extracted from longan seed had an antifungal effect on *Candida* species and *Cryptococcus neoformans* with a MIC of 500-4000 µg/ml [30].

Here we showed for the first time that ellagic acid could actively suppress the expression of *HWP1* and *ALS3* genes under the *in vitro* test conditions. Our data showed an increase in ellagic acid concentration decreased expression of *ALS3* and *HWP1* in *C. albicans* proportionally, up to ≈90% and ≈80%, respectively. Meanwhile, the reduction of *ALS3* at the transcriptional level was higher than *HWP1* under different concentrations of ellagic acid, indicating the effectiveness of this compound on the gene responsible for adhesion at the early steps of the biofilm formation. The suppression in such genes expression revealed the underlying molecular mechanism for biofilm formation by *C. albicans*. Finally, due to the considerable effect of ellagic acid in preventing the adhesion phase of biofilm formation, we suggest it as a potential natural compound for developing medicinal drugs against *C. albicans*.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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