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*Research Article*

# INVESTIGATION OF THE EFFECTIVENESS OF TYROSOL ON CANDİDA TROPICALIS HYPHAL WALL PROTEIN (HWP1)

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**ABSTRACT.** Microorganisms of the genus *Candida* are fungal pathogens that cause mucosal and invasive infections in immunocompromised individuals. In particular, *C. tropicalis* is one of the most frequently isolated species from candidemia and candiduria after *C. albicans*. Biofilm formation is a very important virulence factor regulating *Candida* pathogenesis, and combating biofilm-related infections is very difficult. Tyrosol (2-[4-hydroxyphenyl] ethanol), a fungal quorum sensing molecule, induces germ tube formation and hyphal development in the early and intermediate stages of biofilm formation, and when added exogenously to the medium, it reduces the biofilm in a concentration-dependent manner. However, the effectiveness of tyrosol on fungal pathogens has not yet been fully elucidated. Hyphal wall protein (HWP1) is an important adhesin protein found in some *Candida* species and is required for biofilm formation *in vivo*. In this study, the antimicrobial activity of tyrosol on a clinical *Candida* isolate (*C. tropicalis* 1660) was investigated microbiologically, and its activity on the fungal cell wall was examined under a transmission electron microscope (TEM) using the immunold labeling technique. The minimum inhibitory concentration (MIC) value of tyrosol was determined as 18.75 µg/mL by broth microdilution test. Our TEM results revealed that the HWP1 protein, which was localized on the cell wall in the control group, dispersed along with the damage in the cell after tyrosol application. The damaging effect of exogenous tyrosol on cell ultrastructure and the reducing effect on HWP1 protein should also be taken into consideration in antibiofilm activity studies.

**Keywords:** *Candida tropicalis, tyrosol, immuno-electronmicroscopy*

## INTRODUCTION

The incidence of fungal infections caused by *Candida* is increasing and shows high morbidity and mortality rates. *Candida* species, which exist as commensals in the human body, cause opportunistic infections in cases where immunity is suppressed. The fact that *Candida* adheres to mucosal surfaces or abiotic surfaces such as indwelling medical devices and forms biofilms makes treatment very difficult. Although the most frequently isolated species is *C. albicans*, in recent years species such as *C. tropicalis*, *C. glabrata*, and *C. krusei* have also been frequently reported to cause biofilm-related and treatment-resistant infections.

Biofilm formation is a growth form observed in the majority of microorganisms and facilitates survival in the presence of adverse environmental conditions and stress factors and shows resistance to drugs. Microorganisms that settle by adhering to the host tissue or indwelling devices form a biofilm structure by being embedded in an extracellular polymeric substance (EPS) they produce. This structure is an important virulence factor

of *Candida* and new and effective treatment strategies continue to be investigated in the fight against biofilm.

The species *C. tropicalis* shows yeast and filamentous forms, like *C. albicans*. Yeast form is seen as single-celled and oval shaped cells that develop by budding. Pseudohypha and hyphae structures contain filamentous form. While the pseudohyphal structure is ellipsoidal and constrictions are observed at the detection points, there are long parallel-sided cells in the hyphae structure and they do not show constriction. When non-budding yeast cells are induced into hyphal form, the germ tube structure is formed through induction from the parent cell. It is easier for cells in hyphal form to invade host tissues, and this form is necessary for biofilm formation and virulence [20] , [22].

The outer side of the cell wall of *Candida* species is very rich in glycoprotein. HWP1, a gene encoding an important adhesion protein and it is frequently expressed on the hyphal surface and germ tube of *Candida* species [1]. It is reported that this gene is expressed at a high level, especially in *C. albicans*, *C. dubliniensis* and *C. africana* species, after the transition from the blastoconidia form to the germ tube or hyphae [18].In a previous study, it was stated that HWP1 adhesin retained *Candida* biofilm in an *in vivo* model [14]. Like *C. albicans*, germ tube transformation, hyphal switching and HWP1 expression occur in also *C. tropicalis*. However, studies on the role of adhesion proteins in this species are quite limited [24], [21].

In recent years, new and effective treatment options to combat biofilm have been intensively researched. The use of exogenously applied signaling molecules, biosynthetic enzymes in the cell wall, wall cross-linking enzymes and adhesins, or proteins involved in biofilm formation offer various and potential targets in this regard [23]. Farnesol, an autoregulatory molecule, prevents germ tube formation, but cannot prevent the existing germ tube from elongating. It is also reported that farnesol inhibits biofilm formation, blocks yeast-hyphal morphological transformation, and changes fungal gene expression [16]. Tyrosol, another quorum sensing molecule produced by *C. albicans*, stimulates germ tube formation and hyphal development in the early and middle stages of biofilm development [7]. In other words, the morphogenesis of *C. albicans* is under complex control with the effects of tyrosol and farnesol [5]. When added exogenously to the medium, tyrosol can show biofilm-reducing effects, but the molecular mechanisms involved in this phenomenon are still not fully elucidated.

In this study aimed to microbiologically investigate the antimicrobial activity of tyrosol on a clinical *Candida* isolate (*C. tropicalis* 1660) and to examine its activity on the fungal cell wall HWP1 protein under a transmission electron microscope (TEM) using the immunogold labeling technique.

## MATERIALS AND METHODS

### *Isolates and chemicals*

Clinical isolate used in this study was collected from Eskişehir Osmangazi University Medical Faculty. It was obtained from the Microbiology Department of the hospital. Tyrosol was purchased from Sigma-Aldrich Chemie GmbH, Germany. Yeast isolate containing 20 % glycerol in Yeast Peptone Dextrose (YPD) was stored in liquid medium as a stock at -80 °C. It was cultured in RPMI 1640 broth at 37 °C. *C. tropicalis* 1660 isolate was used as the study microorganism.

### *Antifungal susceptibility tests*

In determining the Minimum Inhibition Concentration (MIC) value of tyrosol, the Laboratory Standards Institute (CLSI) Microdilution (CLSI [M27-A3] reference method was used [6]. Tyrosol, 0.0017 %-0.9 % (vol/vol) prepared in RPMI 1640 medium at different concentrations and minimum inhibitory concentration (MIC) values has been determined. Amphotericin B was used as a standard antifungal. All experiments were performed three times and the results were averaged.

### *Transmission electron microscopic examination (TEM)*

In our study, we evaluated the morphological changes caused by tyrosol with TEM. For this purpose, 10 mL cell suspensions were exposed to tyrosol at MIC concentration and further incubated at 37 °C for 24 hours. A control group without tyrosol was also included in the study. Cell suspensions were centrifuged at 5000 g for 15 minutes in sterile plastic centrifuge tubes and added to PBS three times (10 minutes), consecutively. The supernatant was then discarded and the pellet was harvested. Then, the cells were fixed with 2.5% glutaraldehyde prepared in PBS at 4°C overnight. The samples were washed in PBS buffer and fixed in 1% osmium tetroxide for 2 hours. Later, they were washed in PBS (Three times, 15 minutes each). For dehydration, samples were treated with 40%, 60%, 75%, 80% and 95% in ethanol dilution series, respectively. The final dehydration step was in 100% ethanol for 1 hour. After the samples were embedded in epoxy resin for 48 hours, they were left in polymerization at 60°C. Ultrathin sections of the blocks of the samples were cut using an ultramicrotome (Leica Ultracut). 60 nm thick ultrathin sections were taken from the obtained blocks with the help of an ultramicrotome and mounted on copper grids. After staining with uranyl acetate and lead citrate, grids were imaged with a Hitachi HT7800 TEM.

### *Immuno-electron microscopic examination*

In conventional TEM studies, double fixation application (glutaraldehyde and osmium tetroxide) suppresses immune labeling. However, to overcome this problem, the use of strong oxidants such as periodic acid can reverse the effect of osmium tetroxide. So, previously recommended protocol was used in our immunolabeling studies. For this purpose, ultrathin sections (60 nm) of the embedded samples were treated with 5% periodic acid aqueous solution for 10 minutes at room temperature. Thin sections were washed thoroughly with ultra pure water. Then they were labelled with polyclonal anti-hwp1 (MyBioSource) primary antibody (1/1000 concentration) in a moisture trap overnight at 4°C. After incubation, immunogold labeling was performed with protein A-gold complexes (1/100 Protein A Gold Conjugate 20nm Abcam) for 2 hours at room temperature. The prepared samples were analyzed with Hitachi HT 7800TEM [2], [25], [9].

## RESULTS AND DISCUSSION

According to the antifungal susceptibility tests result obtained by the broth microdilution method, the MIC value of tyrosol for *C. tropicalis* 1660 isolate was determined as 18.75 µg/mL and the MIC value of amphotericin B used as the control antifungal drug was 0.5 µg/mL. Amphotericin B is more effective than tyrosol against *C. tropicalis*.

In TEM examinations prepared with the immunogold labeling technique, a healthy-appearance normal ultrastructure was observed in the control group cells that were not treated with tyrosol. Cell membrane and wall structures are regular, the general appearance of the cell is oval and regular, the cytoplasm is homogeneous, the nucleus is centrally located and the mitochondria are regular (Figure 1 A). Gold nanoparticles indicating HWP1 protein were observed along the cell wall (Figure 1B). In tyrosol-treated cells, damage such as membrane-wall damage in yeast cells, ruptures in the cytoplasm, fusions and involutions in the cells are evident. In cells damaged by the effect of tyrosol, gold probes showed a random distribution in the cytoplasm (Figure 1 C, D, E, F).

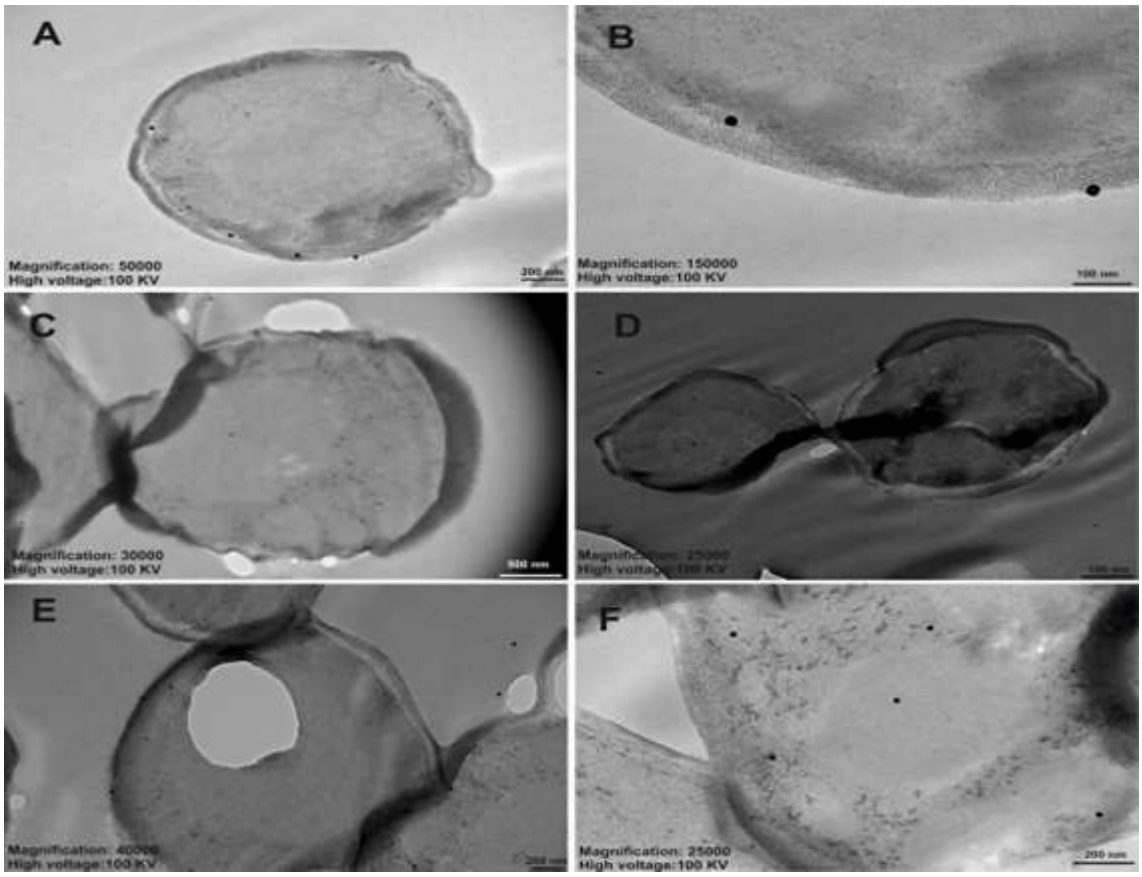


***Fig. 1.*** *TEM micrographs of immunogold labeled cells using anti-hwp antigen and protein A secondary antibody. A and B Control group; C, D, E and F are at MIC concentration shows tyrosol-treated C. tropicalis 1660 cells (A, E, F = 200 nm, B, D = 100 nm, C = 500 nm).*

## DISCUSSION

The immuno electronmicroscopy method is a valuable method that can combine the structural details of cells or tissues with the localization of a selected protein. Labeling can be done with pre-embedding and post-embedding protocols, and samples can be examined with TEM or SEM. The gold particles used in the method can be bound to specific binding molecules, such as antibodies, which can provide direct labeling of proteins of interest [11].

Characterization of cell surface proteins of *Candida* is important in understanding both adhesive interactions with host cells and interactions with microbial cells in the biofilm structure [19]. This study aims to reveal the change in cell wall protein HWP1

localization of a clinical *C. tropicalis* isolate in the presence of tyrosol at MIC concentration.

MIC values obtained from studies examining the antifungal activity of quorum sensing molecules in the literature show significant differences depending on concentration and time. In the study of Cordeiro et al., the effects of exogenous tyrosol on planktonic and biofilm cells of *C. albicans* and *C. tropicalis* isolates were examined by broth microdilution test, and the synergistic effects of tyrosol with different antifungal drugs were investigated. The MIC values of tyrosol obtained for planktonic cells were found to be between 2.5-5 mM for both species. In the same study, exogenous tyrosol added to the medium at the zero hour significantly reduced biofilm formation, and a biofilm reducing effect was observed when used in combination with antifungals. [7]. Kovacs and colleagues also investigated the *in vitro* effectiveness of caspofungin and micafungin against *C. parapsilosis* biofilm in the presence of tyrosol. The authors stated that echinocandins tested in the presence of tyrosol had a higher lethal effect [13]. In the study of Monteiro et al., the effect of tyrosol on the adhesion of *C. albicans* and *C. glabrata* on acrylic surfaces was examined. The authors reported that *C. albicans* (MIC = 50 mM) was much more sensitive to tyrosol than *C. glabrata* (MIC = 90 mM), [15]. The MIC value obtained for tyrosol in our study was 18.75 µg/ml and was found to be lower than the literature data.

In addition to being a fungal quorum sensing molecule, tyrosol is a phenolic component with antioxidant properties and is also found in olive oil, which has an important place in human nutrition [7]. It is reported in the literature that tyrosol accumulated in the culture medium increases fungal cell density [5]. Information about the mechanism of action of tyrosol on the fungal cell wall is very limited, but it is thought that tyrosol specifically affects fungal cell membrane permeability. On the other hand, it is suggested that tyrosol affects both the cell and nuclear membranes by causing protein and nucleic acid loss in the cell. Studies have shown that there is a decrease in the amount of ergosterol extracted from fungal strains after adding a high concentration of tyrosol to the medium, and it has been suggested that tyrosol can inhibit ergosterol synthesis [3]. TEM data obtained in our study also supports that tyrosol exerts its most important effect on the cell wall and membrane.

The biofilm structure shows an extremely heterogeneous structure and its molecular mechanism is still being investigated. During *C. albicans* biofilm formation *in vitro*, the early phase lasts for approximately 11 hours and microcolonies are formed following adhesion. The intermediate phase occurs during the 12-30 hour period of biofilm formation, and at this stage, EPS production, a community consisting of yeast, germ tubes and young hyphae are observed. 38-72 hours, called the mature phase, thick EPS layer, yeast cells and a dense network of hyphae are formed. In the dispersion phase, cells separated from the mature biofilm leave the environment to form new colonizations [4].

Cell wall adhesins are also thought to be important in biofilm formation and maintenance. Konduri and colleagues also stated that the HWP1 gene, which is the main hyphal cell wall protein, is required for biofilm formation in *C. albicans* isolated from a patient with keratitis [12]. In a previous study, it was reported that HWP1 was required for *C. albicans* to form a catheter biofilm in a rat model, and reported that HWP1 interacted with other cell surface adhesins from the agglutinin-like sequence (Als) family [17]. According to immunogold labeling findings in our study, HWP1 protein, which was localized along the cell wall in control cells, showed a random distribution in the cytoplasm due to cell damage as a result of tyrosol application.

In conclusion, the pathogenesis of *Candida* species depends on many specific factors. These factors include the presence of genes that enable the transfer from yeast cells to filamentous form and surrounding the host tissue, the expression of adhesions, the production of hydrolytic enzymes or the continuation of biofilm formation. HWP1 protein, located in the *Candida* cell wall, is responsible for the formation of biofilms structure [10]. Due to the damaging effect of tyrosol on the yeast cell, the regular localization of the HWP1 protein is also disrupted. This supports that tyrosol may also affect the synthesis of cell wall proteins. It is important to evaluate of detail studies on different *Candida* species at different tyrosol concentrations and times in the future. Investigating the changes in the structure and localization of the HWP1 protein, which is important in biofilm formation, such as Als (Agglutinin-Like Sequence), may provide important clues for the development of new antibiofilm strategies.

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