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ORIGINAL ARTICLE

Impact of oleuropein on *Candida albicans* and *Staphylococcus aureus* adhesion and its mediated toxicity in Zebrafish (*Danio rerio*) embryos

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ABSTRACT

Background and Objectives: The rising prevalence of antibiotic resistance and biofilm-associated infections poses significant challenges in clinical settings. This study investigates the antimicrobial and anti-adhesive properties of oleuropein, a compound derived from olive leaves, against *Candida albicans* and *Staphylococcus aureus.*
Materials and Methods: This study was conducted on *Candida albicans* (fluconazole-resistant/susceptible) and *Staphylococcus aureus* (methicillin-resistant/susceptible). The antifungal, antibacterial, anti-adhesion, and cell surface hydrophobicity (CSH) effects of oleuropein were evaluated. The impact of oleuropein on germ tube formation (GTF) in *C. albicans* was assessed. Finally, the toxicity of oleuropein was evaluated in zebrafish embryos.
Results: Oleuropein exhibited MIC values of 10 mg/ml for *C. albicans* and 5 mg/ml for *S. aureus*. It significantly ($P< 0.05$) reduced the adhesion of both microorganisms in a dose-dependent manner, with inhibition percentages of 78.43% and 75.91% for *C. albicans* and *S. aureus*, respectively. Additionally, oleuropein reduced the CSH of *C. albicans*, indicating its potential to interfere with adhesion mechanisms. In addition, oleuropein exhibited inhibition of GTF in *C. albicans.*
Conclusion: Oleuropein demonstrates significant antimicrobial and anti-adhesive properties against *C. albicans* and *S. aureus*, indicating its potential as a therapeutic agent for preventing biofilm-related infections. However, careful dosage management is crucial due to its observed toxicity at higher concentrations.

Keywords: Oleuropein; *Candida albicans*; *Staphylococcus aureus*; Adhesion; Biofilm

INTRODUCTION

Candida albicans is a common commensal fungus found in various body regions, including the oral cavity, pharynx, gastrointestinal tract, vagina, and skin of healthy individuals. It forms part of the natural microbiota in approximately 50% of the population (1).

Both *Staphylococcus aureus* and *C. albicans* are key components of the human microbiota. However, they can cause mixed infections on the skin, mucous membranes, and in the bloodstream. Notably, *S. au-*

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reus is the third most common pathogen in hospital-acquired bloodstream infections associated with *C. albicans* (2). Co-infection with *C. albicans* and *S. aureus* demonstrates a "lethal synergy," where concurrent infections lead to increased mortality. These two pathogens are frequently co-detected in biofilm-associated infections (3).

Biofilm formation begins with microbial adhesion to a surface, followed by the development of a protective matrix that shields the microorganisms from environmental pressures and immune system attacks. Approximately 65% of clinical infections are associated with biofilms formed on tissues, organs, or medical devices (4, 5).

The mechanism of antimicrobial resistance related to biofilm formation involves several key factors. In biofilms, microorganisms exhibit altered gene expression, reduced growth rates, and increased production of extracellular matrix components that act as a physical barrier to antimicrobial agents. The matrix also limits the penetration of antibiotics and enables microbial cells to enter a dormant or less metabolically active state, making them less susceptible to antimicrobial action (6). Additionally, the close proximity of microorganisms within a biofilm promotes horizontal gene transfer, potentially spreading resistance genes between different species or strains (5-7).

C. albicans and *S. aureus* are frequently co-isolated from biofilm-associated diseases such as periodontitis, cystic fibrosis, denture stomatitis, urinary tract infections; burn wound infections, and infections involving medical devices such as central venous catheters. The complexity of these polymicrobial infections presents a significant clinical challenge in developing effective treatment strategies (7). *C. albicans* can cause systemic infections by transitioning from yeast to invasive hyphal forms. *S. aureus* specifically adheres to the hyphae of *C. albicans*, with *C. albicans* hyphae serving as a scaffold for the attachment of *S. aureus* cells (8).

These two microorganisms were specifically chosen for this study because of their prevalence in co-infections and their ability to form biofilms that contribute to treatment resistance. Additionally, their dual role as a fungal-bacterial interaction system provides a unique model to study polymicrobial biofilm formation and the subsequent challenges in overcoming antimicrobial resistance (7, 8).

Oleuropein, a phenolic secoiridoid compound found in olive leaves and unripe olives, exhibits significant antimicrobial properties through its interaction with membrane lipids (9). Structurally, oleuropein consists of three subunits: elenolic acid (a secoiridoid), hydroxytyrosol (a polyphenol), and a glucose molecule. Upon hydrolysis, oleuropein breaks down into hydroxytyrosol and tyrosol, both of which are potent antioxidants. By integrating into the lipid bilayers of microbial cell membranes, oleuropein disrupts membrane structure, leading to compromised membrane integrity, leakage of cellular contents, and ultimately, cell death. Research has shown that oleuropein affects the ordering and fluidity of phospholipid membranes by inserting itself into the lipid bilayer and altering its physical properties, thereby reducing membrane stability (9, 10).

Additionally, oleuropein's antioxidant properties enable it to scavenge free radicals within the membrane, protecting it from oxidative damage and preventing lipid peroxidation (11). Studies indicate that oleuropein has a stronger interaction with specific lipids, such as phosphatidylglycerol (PG) monolayers, which are abundant in Gram-positive bacteria like *S. aureus* (12). This targeted interaction enhances oleuropein's antibacterial efficacy against these bacteria. Given the growing concern over antimicrobial resistance, plant-derived antimicrobial agents like oleuropein are considered promising alternatives for developing new treatments (13).

The numerous properties of oleuropein-including its antioxidant, anti-inflammatory, anti-atherogenic, anticancer, antimicrobial, and antiviral effects-have been well-documented (9, 14). This study aims to investigate the effect of oleuropein in preventing the adhesion of *C. albicans* and *S. aureus* cells, which is the initial stage of biofilm formation. Additionally, to determine the safe dosage range of oleuropein, its toxicity was examined using a zebrafish larva model.

MATERIALS AND METHODS

Ethical approval. This study was conducted in accordance with ethical guidelines and was approved by the relevant institutional ethics committee. The ethical approval code for this research is 212506/76.

Microorganisms and materials. In this study, we employed clinical isolates: fluconazole-resistant *C. albicans* (FRCA), fluconazole-susceptible *C. al-*

bicans (FSCA), and a standard strain, *Candida albicans* ATCC 10231. The clinical fungi were obtained from our previous study (15). In addition, the clinical bacterial isolates included methicillin-resistant *S. aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) from the study (16), along with *S. aureus* ATCC 6538 as a standard strain. The standard fungi and bacterial strains were purchased from the Persian Type Culture Collection (PTCC, Tehran, Iran).

C. albicans was cultured on Sabouraud's Dextrose (SAB) agar, while *S. aureus* grew on Luria Bertani (LB) agar. For long-term preservation, we maintained them in tryptone soy broth (TSB) and yeast–peptone–dextrose (YPD) broth with 20% v/v sterile glycerol at -80°C. Overnight cultures were prepared by incubating *C. albicans* in YPD medium at 30°C and *S. aureus* in LB broth at 37°C. *S. aureus* cells were diluted to an optical density (OD_{600}) of 0.6 (approximately 1×10^8 cells/mL), while *C. albicans* concentrations were adjusted to 1×10^6 cells/mL using a Neubauer hemocytometer. The oleuropein (purity 98%) employed in this research was previously extracted, purified, and verified from Iranian olive leaves (17). All additional chemicals and reagents were sourced from Sigma (St. Louis, MO, USA).

Assessing the minimum inhibitory concentration of oleuropein. The minimum inhibitory concentration (MIC) of oleuropein against *S. aureus* and *C. albicans* was determined using the M07-A8 and M27-A3 serial broth microdilution methods, as outlined by the Clinical and Laboratory Standards Institute (CLSI) (18). Oleuropein was diluted in yeast-YPD broth for *C. albicans* and in TSB for *S. aureus.* The concentration range was adjusted from 0.625 to 80 mg/mL concentrations. The experiments were performed in sterile, flat-bottom 96-well microtiter plates, with each well inoculated separately with 100 µl of *S. aureus* or *C. albicans* suspension. After aerobic incubation at 37°C for 24 hours, the lowest concentrations without visible growth were defined as the MICs, representing the concentrations that completely inhibited bacterial and/or fungal growth (MIC_{90}).

Adhesion assays. To assess the impact of oleuropein on the adhesion of *C. albicans* ATCC 10231 and *S. aureus* ATCC 6538, a 96-well polystyrene plate pre-coated with 50% inactivated fetal bovine serum (FBS) was utilized, following the protocols described previously (19). The fungal and bacterial suspensions were standardized to a final concentration of 1×10^6 CFU/mL by adjusting the OD at 600 nm (OD_{600}) to 0.08-0.1 for *S. aureus* and OD at 530 nm (OD_{530}) to 0.5 for *C. albicans.* Colonies were suspended in the appropriate broth medium (TSB for *S. aureus* and YPD for *C. albicans*) and adjusted to the desired OD using a spectrophotometer before further use in adhesion assays. Subsequently, 200 µL of these suspensions, containing various concentrations of oleuropein (MIC, MIC/2, MIC/4, and MIC/8) were added to the wells and incubated at 37°C for 1.5 hrs. Post-incubation, the supernatants were carefully removed, and each well was gently rinsed with sterilized phosphate-buffered saline (PBS) to remove non-adherent cells. The adhered cells were then disaggregated by vigorous pipetting. Following serial dilution, the adhered cells were plated on TSB plates. The total viable count was determined after 24 hours of incubation at 37°C. Finally, the inhibition percentage of bacterial and fungal cell adhesion treated with different concentrations of oleuropein was obtained according to the following equation

Inhibition percentages = % I = 100 - (CFU sample/ CFU control) × 100

Assessing cell surface hydrophobicity in *C. albicans* with oleuropein treatment. To evaluate the hydrophobicity of planktonic *C. albicans* ATCC 10231 cells, both untreated and treated with oleuropein at concentrations of MIC, MIC/2, MIC/4, and MIC/8, the biphasic hydrocarbon/aqueous method as described previously (20). Initially, a 5 mL yeast suspension (at 1×10^6 CFU/mL) was prepared. For each yeast sample, two sterile glass test tubes containing 2.5 mL of the suspension were prepared as test and control samples. Additionally, one test and one control tube were used as spectrophotometer blanks, containing only the medium. Each test suspension received different doses of oleuropein (MIC, MIC/2, MIC/4, and MIC/8).

The samples were incubated at 37°C for 10 minutes to equilibrate, followed by 30 seconds of vortexing and additional 30-minute incubation. The aqueous phase at the bottom was carefully extracted using a pipette and transferred to another test tube. To remove any xylene contamination, air was bubbled through the suspension at a rate of 180 mL per minute for two minutes. After a 5-second vortex to break up and re-suspend any aggregates, the OD was measured

again at 520 nm.

The cell CSH was determined by comparing the decrease in OD values of the test suspension to the control. A greater difference in OD values indicated a higher displacement of yeast cells from the bulk medium to the interface, signifying increased hydrophobicity of the yeast strain. Negative controls consisted of suspensions without oleuropein. All tests were conducted in triplicate.

Effect of oleuropein on *C. albicans* germ tube formation in liquid media. A standard suspension of *C. albicans* ATCC 10231 cells was prepared and diluted in RPMI 1640 medium with 10% FBS (21). This medium was then supplemented with different concentrations of oleuropein: MIC, MIC/2, MIC/4, and MIC/8. The cells were incubated at 37°C with shaking at 250 rpm for 3 hrs. Germ tube formation (GTF) was evaluated by examining a sample of the culture under a light microscope (ZEISS Primo Star, Germany) at 400x magnification. The results were reported as the number of germ tube-forming cells per 100 cells counted (GTF/100 cells). The germination reduction percentage (GRP) for each oleuropein concentration was calculated using the following formula:

$$GRP=(GTF\%\ control-GTF\%\ test)\times 100$$

Zebrafish embryo toxicity test. The toxicity of oleuropein was assessed using the zebrafish embryo model, following the standard guidelines outlined in OECD236 (22). Briefly, 2 female and 4 wild-type male zebrafish, approximately 7 months old and in optimal health, were obtained from the zebrafish laboratory of the Toxicology Department at the Faculty of Veterinary Medicine, University of Tehran. The fish were kept under a 14-hour light and 10-hour dark cycle, and fed three times daily with two meals of commercial food and one meal of Artemia. On the morning of the experiment, eggs were collected using a sampler 60 minutes post-fertilization. The eggs were then washed twice with E3 solution (composed of 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2{\cdot}2H_2O$, 0.33 mM $MgCl_2{\cdot}7H_2O$) to eliminate any contaminants that might interfere with the test.

After confirming fertilization, 20 eggs were placed in each well of a 96-well plate for each concentration of oleuropein, and 24 eggs were placed in wells containing the control solution (E3 solution). The eggs were then exposed to oleuropein concentrations of 100, 1000, 2000, 3000, and 4000 ppm. Over a 96-hour period, the zebrafish embryos were observed under an inverted microscope (Zeiss, Germany) to monitor for any signs of toxicity. To maintain the accuracy of the results, the solutions in the wells were refreshed every 24 hours to prevent changes in concentration due to evaporation or degradation.

The concentrations of oleuropein that resulted in the death of 50% (LC_{50}) and 90% (LC_{90}) of the zebrafish larvae were determined using the probit method (23).

Statistical analysis. Statistical analyses were performed to identify significant differences between treated and untreated samples using one-way analysis of variance (ANOVA). Mean comparisons were performed using the Dunnett's multiple comparisons test to examine if differences were significant at $P < 0.05$. These analyses were conducted with GraphPad Prism version 5 (GraphPad Software, CA, USA). All experiments were performed as triplicates at three independent occasions.

RESULTS

Table 1 presents the in vitro antifungal and antibacterial activity of oleuropein against *C. albicans* and *S. aureus* isolates. The MIC of oleuropein was determined to be 10 mg/ml for *C. albicans* and 5 mg/ml for *S. aureus*. Additionally, the MFC of oleuropein ranged from 20 to 40 mg/ml for *C. albicans* and from 10 to 20 mg/ml for *S. aureus.*

In the next step of the study, the impact of MIC

Table 1. Antifungal and antibacterial activity of oleuropein against *C. albicans* and *S. aureus* isolates

Microorganisms	MIC (mg/ml)	MFC-MBC (mg/ml)
Candida albicans ATCC 10231	10	20
Candida albicans FRCA[A]	10	40
Candida albicans FSCA[B]	10	40
Staphylococcus aureus ATCC 6538	5	20
Staphylococcus aureus MRSA[C]	5	20
Staphylococcus aureus MSSA[D]	5	10

A; Fluconazole-resistant *C. albicans*, B; Fluconazole- susceptible *C. albicans*, C; Methicillin-resistant *S. aureus*, D; Methicillin-susceptible *S. aureus*

and sub-MIC concentrations of oleuropein was assessed on the adhesion of *C. albicans* and *S. aureus* cells to polystyrene surfaces. The findings revealed that oleuropein, in a dose-dependent manner, significantly reduced the attachment of both *C. albicans* and *S. aureus* cells to the bottoms of wells in polystyrene cell culture plates compared to the control group. As illustrated in Figs. 1 and 2, the inhibition percentages for *C. albicans* and *S. aureus* cells at the MIC_{50} of oleuropein were 78.43 ± 1.7% and 75.91 ± 1.8%, respectively, indicating a significant reduction in cell attachment relative to the control group (P<0.05). Even at a concentration of MIC/8 of oleuropein, a significant decrease in binding was observed. The binding inhibition percentages for *C. albicans* and *S. aureus* cells treated with MIC/8 of oleuropein were calculated to be 20.85% and 18.16%, respectively (P<0.05).

The current study's findings on the CSH properties of *C. albicans* are illustrated in Fig. 3. The hydrophobicity test results demonstrated that oleuropein significantly altered CSH values. In the control group, the mean CSH of *C. albicans* was 55.17 ± 3.6%. However, after exposing planktonic cells to various concentrations of oleuropein (MIC, MIC/2, MIC/4, and MIC/8), a significant reduction in CSH was observed in the treated groups compared to the control group (P<0.05). Specifically, the CSH percentages for the groups treated with MIC and ½ MIC of oleuropein were 2.17 ± 0.9% and 10.11 ± 1.19%, respectively. For the groups treated with ¼ MIC and 1/8 MIC, the CSH percentages were 25.74 ± 3.45% and 34.64 ± 2%, respectively.

We also examined the germination patterns of *C. albicans* isolates treated with oleuropein in liquid media. Sub-inhibitory concentrations of oleuropein significantly (P<0.05) inhibited germ tube formation in *C. albicans* at all tested concentrations compared to the control group (Fig. 4). The average germ tube formation percentages for *C. albicans* treated with MIC, MIC/2, MIC/4, and MIC/8 of oleuropein were 69.67 ± 13.3%, 61 ± 11.5%, 52.3 ± 2.9%, and 35.3 ± 6.4% after 3 hours of incubation, respectively.

In this study, the toxicity of oleuropein was also examined using the zebrafish larvae model, and the statistical analysis was performed using SPSS software and the probit method (Fig. 5). The results

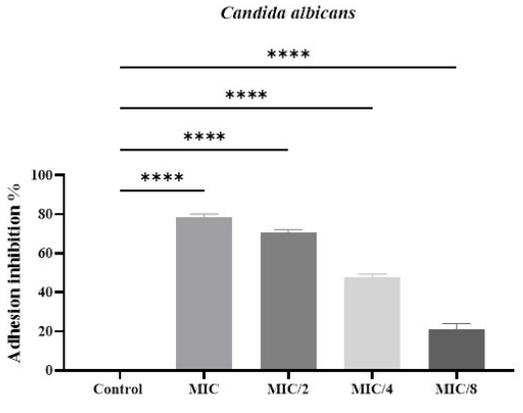

Fig. 1. Percentage inhibition of *C. albican*s adhesion following treatment with various concentrations of oleuropein (****P < 0.0001, Dunnett's multiple comparisons HSD test).

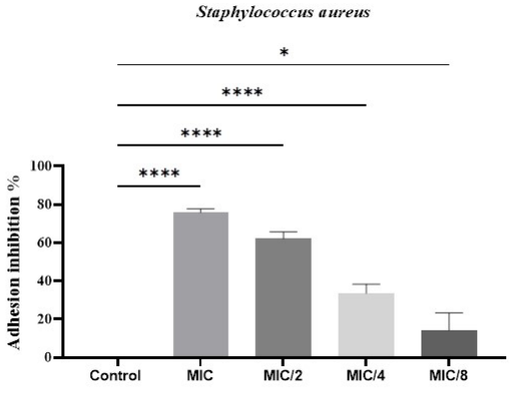

Fig. 2. Percentage inhibition of *S. aureus* adhesion following treatment with various concentrations of oleuropein (****P < 0.0001,*P =0.016, Dunnett's multiple comparisons HSD test).

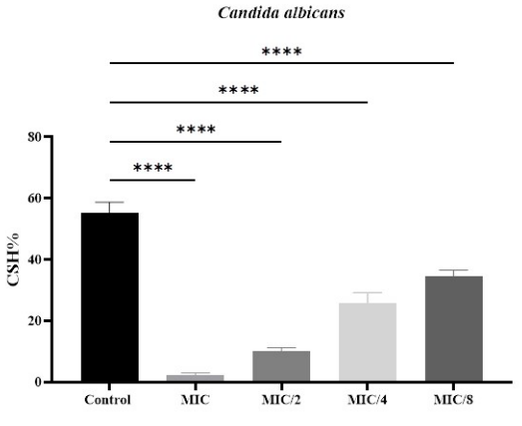

Fig. 3. Cell Surface Hydrophobicity (CSH) in *C. albicans* with oleuropein treatment (****P < 0.0001, Dunnett's multiple comparisons HSD test).

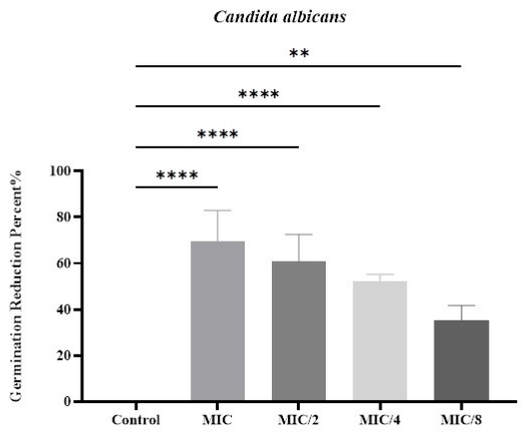

Fig. 4. Effect of oleuropein on germ tube formation of *C. albicans* ATCC 10231 after 3 h. (****P < 0.0001, **P =0.0016) Dunnett's multiple comparisons HSD test).

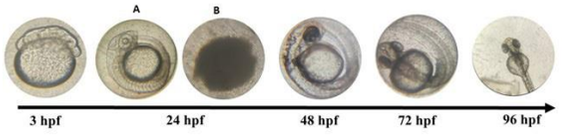

Fig. 5. Timeline of Zebrafish Larvae Development: The figure displays the life cycle of zebrafish larvae from 3 hours post-fertilization to 96 hours post-fertilization. At 24 hours post-fertilization, 'A' indicates a healthy egg observed at concentrations of 100, 1000, and in the control group. 'B' indicates a coagulated egg observed at concentrations of 4000, and some eggs at concentrations of 3000 and 2000.

showed that at a concentration of 4000 ppm, all 20 zebrafish eggs coagulated, resulting in 100% mortality. At a concentration of 3000 ppm, 13 zebrafish eggs coagulated, but no signs of subacute toxicity were observed in the 7 remaining healthy larvae. The toxicity at this concentration was limited to egg coagulation, as all 7 larvae remained healthy and normal until the end of the test, after which they were euthanized at 96 hours according to the OECD protocol. At a concentration of 2000 ppm, 6 eggs coagulated on the first day post-fertilization, but no signs of subacute toxicity were observed in the remaining 14 eggs. These larvae remained healthy and normal until the end of the test, and they were euthanized according to protocol. At concentrations of 1000 and 100 ppm, no mortality or toxicity was observed, and all 20 eggs remained completely normal and comparable to the control group until the end of the test. After 96 hours of observation, all 24 zebrafish eggs in the control group were found to be completely normal and healthy. According to the results, the LC_{50} value for oleuropein was determined to be 2547 ppm, and the LC_{90} value was measured at 3465 ppm.

DISCUSSION

C. albicans is a common commensal fungus present in various body regions, and along with *S. aureus*, can cause severe biofilm-associated infections. These pathogens exhibit "lethal synergy" in co-infections, leading to increased mortality, particularly in hospital settings (3). Oleuropein, a compound from olive leaves, disrupts microbial membranes, offering potential as an alternative antimicrobial agent due to its ability to reduce biofilm formation by preventing cell adhesion (10).

In this study, oleuropein demonstrated in vitro antimicrobial activity against *C. albicans* (with an MIC of 10 mg/ml and an MFC of 20 to 40 mg/ml) and against *S. aureus* (with an MIC of 5 mg/ml and an MBC of 10 to 20 mg/ml) isolates. Our findings align with previous studies that have investigated the antimicrobial properties of oleuropein. For instance, it was reported that oleuropein effectively disrupts bacterial membranes by interacting with phospholipid bilayers, particularly in Gram-positive bacteria such as *S. aureus* (13). Similarly, Omar (2010) highlighted the broad-spectrum antimicrobial and antioxidant effects of oleuropein, which enhance its potential as a therapeutic agent (9). Studies on plant-derived compounds like oleuropein have shown promising results in reducing biofilm formation by various pathogens (9, 10). Baxter et al. (2024) emphasized the ability of oleuropein to hinder the initial adhesion of biofilm-forming bacteria, which is consistent with our results showing significant inhibition of adhesion by *C. albicans* and *S. aureus* (8). Furthermore, It was demonstrated that the combination of *C. albicans* and *S. aureus* in biofilms contributes to increased resistance to antibiotics (24), underscoring the need for agents like oleuropein that can target both pathogens simultaneously.

The potential a mechanism by which oleuropein exerts its antimicrobial and biofilm-inhibiting effects may be attributed to its interaction with microbial cell membranes (25). Structurally, oleuropein consists of elenolic acid, hydroxytyrosol, and a glucose mole-

cule. Upon hydrolysis, hydroxytyrosol and tyrosol, both potent antioxidants, are released. Oleuropein integrates into the lipid bilayers of microbial cells, disrupting membrane fluidity and stability. This disruption leads to leakage of intracellular contents and eventual cell death (26). In particular, oleuropein has been shown to target PG lipids, which are prevalent in Gram-positive bacteria like *S. aureus.* This selective interaction enhances oleuropein's antibacterial efficacy (9).

This study evaluated the effects of oleuropein at MIC and sub-MIC levels on the adhesion of *C. albicans* and *S. aureus* to polystyrene surfaces. Results showed that oleuropein significantly reduced cell attachment in a dose-dependent manner, with both *C. albicans* and *S. aureus* exhibiting reduced adherence to polystyrene plates compared to the control. Even at lower concentrations, oleuropein led to a notable decrease in cell binding, highlighting its potential to inhibit early stages of biofilm formation. The anti-adhesive activity of oleuropein observed in this study is consistent with other research findings on its antimicrobial and anti-biofilm properties. For example, a previous study evaluated the antimicrobial effects of olive leaf extracts, rich in oleuropein, and reported that it not only inhibited microbial growth but also interfered with bacterial adhesion to surfaces (27). This ability to reduce adhesion is critical in the context of biofilm formation, as microbial adhesion to surfaces is the first step in biofilm development. Disruption of this process can significantly limit the establishment and maturation of biofilms, which are notoriously resistant to antimicrobial treatments (28).

The molecular mechanism behind oleuropein's anti-adhesive properties is thought to involve its ability to disrupt the surface hydrophobicity and cellular surface proteins of microorganisms, which play critical roles in the adhesion process (29).

Moreover, the reduction in adhesion observed even at MIC/8 concentrations suggests that oleuropein's anti-adhesive effects can be achieved at sub-inhibitory levels, making it a promising candidate for use in medical and industrial applications where preventing biofilm formation is crucial. This finding aligns with the previous studies, which demonstrated that plant-derived compounds, including oleuropein, are effective at sub-lethal concentrations in inhibiting the attachment of bacterial and fungal pathogens (30).

In summary, the anti-adhesive properties of oleuropein, as demonstrated in this study and supported by previous research, indicate its potential for preventing biofilm-associated infections. Its ability to inhibit adhesion at low concentrations makes it a valuable agent in the fight against biofilm-related infections, particularly in the context of increasing antimicrobial resistance (31).

The study also investigated germination patterns of *C. albicans* treated with oleuropein in liquid media. Sub-MIC concentrations of oleuropein significantly inhibited germ tube formation at all concentrations compared to the control. *C. albicans* showed progressively reduced germ tube formation with increasing doses of oleuropein, indicating a strong inhibitory effect on germination. The inhibition of *C. albicans* germ tube formation by oleuropein is clinically significant, as germ tube formation is a key step in the transition of *C. albicans* from harmless yeast to an invasive pathogen. Germ tubes enable the fungus to adhere to host tissues and form biofilms, which are associated with increased resistance to both the immune system and antifungal treatment. By preventing germ tube formation, oleuropein effectively disrupts the initial stages of *C. albicans* pathogenicity, reducing its ability to invade and establish infections (31).

The toxicity of oleuropein was assessed using a zebrafish larvae model. At high concentrations, oleuropein caused coagulation of zebrafish eggs and resulted in complete mortality. Lower concentrations showed no signs of subacute toxicity, with the larvae remaining healthy throughout the test. At concentrations of 1000 ppm and below, no toxicity or mortality was observed, and the larvae developed normally. The LC_{50} and LC_{90} values for oleuropein were determined to be 2547 ppm and 3465 ppm, respectively, indicating that oleuropein has a relatively safe profile at lower concentrations.

CONCLUSION

This study demonstrates the strong antimicrobial and anti-adhesive effects of oleuropein against *C. albicans* and *S. aureus.* Oleuropein effectively inhibits microbial growth and significantly reduces cell attachment and germ tube formation, suggesting its potential in preventing biofilm-related infections. While toxicity at higher concentrations was observed, it emphasizes the importance of careful dosage in maximizing its therapeutic benefits.

Oleuropein shows promise as a novel approach for combating antimicrobial resistance and addressing biofilm-associated infections. Future studies should explore its mechanism of action in greater detail, evaluate its effectiveness in in vivo models, and investigate potential formulations to enhance its clinical application and safety profile.

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