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Original Article

Inhibitory Activity of *Oliveria decumbens* Essential Oil on *Staphylococcus aureus* Biofilm and Planktonic Cells

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Abstract

Background: *Staphylococcus aureus* is a pathogenic bacterium that forms biofilms on biotic and abiotic surfaces. Biofilm formation is important for researchers because it increases the risk of food contamination in the food industry, increases the pathogenicity of bacteria, and damages the equipment. The main purpose of this study was to find out the antimicrobial and antibiofilm properties of *Oliveria decumbens* essential oil (*Od*-EO) against *Staphylococcus aureus*.

Methods: In this study, the antibacterial and anti-biofilm properties of *Od*-EO were tested against four strong biofilm producers. *S. aureus* isolates were obtained from food and humans. The antibacterial properties of *Od*-EO on planktonic *S. aureus* were investigated using the disk diffusion method; further, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined. The microtiter plate (MTP) method and slime production evaluation were used to assess the inhibitory effect of *Od*-EO on *S. aureus* biofilm formation.

Results: *Od*-EO indicated strong antimicrobial activity against planktonic *S. aureus*. After performing tests related to the anti-biofilm activity of *Od*-EO, it was found that *Od*-EO significantly reduced slime production and thus inhibited biofilm formation.

Conclusions: *Od*-EO and its components can be used as a new anti-biofilm agent in medical, dental, and food industry equipment.

Keywords: Biofilm, Essential oil, *Oliveria decumbens, Staphylococcus aureus*

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Introduction

Staphylococcus aureus is a pathogenic and harmful bacterium that can cause various diseases from simple skin infections to fatal systemic diseases (1,2). In addition, *S. aureus* is a foodborne pathogen, inducing staphylococcal food poisoning (3). *S. aureus* can attach, grow, and form biofilms on different biotic and abiotic surfaces and can resist in clinical and food-processing environments (4). These surfaces include foods and food processing equipment or indwelling medical devices such as joint prosthetics, artificial heart valves, catheters, and dental implants (5). Biofilms increase bacterial resistance against the actions of typical antimicrobial agents, harsh environmental conditions, and host immunity compared to planktonic cells (3).

Biofilm formation is the result of the interaction of three main factors: the microbial cell, attachment surface, and surrounding medium (6). During biofilm formation, bacteria produce a self-produced extracellular matrix that consists of materials such as proteins, carbohydrates, and extracellular DNA, which encloses the cells in an adhesive matrix (7). Accumulated data indicate that biofilm formation is facilitated by the production of various microbial surface components that recognize adhesive matrix molecules and polysaccharide intercellular adhesion during the development of an actual biofilm (1,8). Since biofilms are of great concern, many studies have been conducted to gain more information about their development, spread, and control strategies (9).

In recent years, researchers have been interested in the investigation of natural antimicrobial compounds. Most of the natural compounds originated from plants and investigated in different studies have medicinal and antimicrobial properties, including antibiofilm activity. Antibiofilm activity of plant extracts and essential oils has been reported against *Listeria monocytogenes* (10), *Escherichia coli* (11), *S. aureus* (12,13), and other pathogens.

Oliveria decumbens belongs to Umbelliferae family and is endemic to Iran. Most plants belonging to this family produce volatile compounds such as terpenes. Several studies have been conducted on the antimicrobial and antioxidant properties of Umbelliferae family EOs (14), but no study has been conducted on the antibiofilm effects of *Oliveria decumbens* so far.

The purpose of this study was to determine the chemical composition of *Oliveria decumbens* and assess its antibacterial and antibiofilm activity against *S. aureus* isolates from foodstuff, humans, and standard strains that form strong biofilms.

Materials and Methods

Bacterial Strains

This study investigated four strong biofilm producers of *S. aureus* from 20 isolates of respiratory patients and food samples. All isolates were confirmed in a previous epidemiology study conducted by Sharifi et al (15). The Congo red method was used to investigate biofilm formation by the isolates. *S. aureus* ATCC 29213 was used as a standard strong biofilm constituent strain, and *Staphylococcus epidermidis* ATCC 12228 was used as a negative control in this study based on previous studies (16). Bacterial stocks were kept at −80˚C in tryptic soy broth (TSB) containing 25% glycerol (v/v), and the frozen strains were subcultured in tryptic soy agar after thawing.

Preparation of Collected Plants

Oliveria decumbens plants were collected from Khuzestanin province during the full flowering stage of the plant. The collected plants were identified and confirmed as *Oliveria decumbens* using the Faculty of Agriculture at the Shahid Chamran University of Ahvaz. They were then washed (with distilled water) and dried in the dark at 25˚C. Subsequently, the *Od*-EO was extracted using a Clevenger device for three hours.

GC-MS Analysis of the Od-EO

Od-EO main compounds were analyzed using gas chromatography/mass spectrometry (GC-MS, Agilent 5977B, USA) by an HP-5MS capillary column (5% phenyl methyl silicone and 95% dimethylpolysiloxane) with a column length of 30 meters, the internal diameter of 0.25 mm, and film thickness of 0.25 µm. Further, helium was used as carrier gas (99.99%) with 1.1 mL/min, and 0.2 µL of *Od*-EO was injected for GC-MS analysis which was repeated three times (17).

Anti-microbial Activity of Od-EO on S. aureus

Agar Disc Diffusion Assay

One of the tests performed to determine the antimicrobial properties of *Od*-EO was agar disk diffusion according to CLSI (18). *S. aureus* suspensions (~1.5×10^8 CFU/mL) were cultured on Mueller–Hinton agar plates using sterile swabs. Sterilized paper discs containing 20 µL of *Od*-EO were prepared in advance, and after drying, they were placed on Mueller Hinton agar. The diameter of the inhibition zone was measured at the end of the incubation period (24 hours at 37˚C). Negative control (sterile discs with dimethyl sulfoxide [DMSO]) and positive controls were also used. Negative contained sterile discs with DMSO, and positive controls included ampicillin (10 µg/disk), vancomycin (30 µg/disk), amoxicillin (25 µg/disk), trimethoprim/sulfamethoxazole (23.75 µg/disk), and oxytetracycline (30 µg/disk) (Padtan Teb, Iran).

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration Determination

minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using double broth dilution of *Od*-EO (0.015–4 µL/mL). At first, *Od*-EO was dissolved in a TSB medium supplemented with 0.1% DMSO. Then, 180 µL of the broth containing various concentrations of *Od*-EO was distributed into each well of a 96-well polystyrene microtiter plate (MTP), and 20 µL of the bacterial culture (~10^6 CFU/mL) was added to each well. After incubating the plates (37˚C for 24 hours), the lowest concentration of *Od*-EO that inhibited the visible growth of *S. aureus* was determined as the MIC. The wells with the lowest concentration of *Od*-EO that showed no growth on tryptic soy agar were recognized as MBC. Sterile TSB without bacterial culture was used as the negative control (8).

Phenotypic Identification of Slime Production

Slime production by the *S. aureus* isolates was investigated using the Congo red method. All strains were separately cultured in Congo red agar (CRA) medium. ATCC29213 and ATCC22812 were used as positive and negative controls, respectively, and the color of the *S. aureus* isolate colonies was compared to that of the controls (15). Internal reference six-color scales were used to accurately assess all possible colors of cultured colonies. The color scale varied from strong black to red. Bacteria that produce strong black and black colonies were considered slime producers, whereas those that form dark (almost black) colonies were considered weak slime producers. In contrast, red-colored colonies were considered strains unable to produce slime (19).

Evaluation of Anti-biofilm Activity of Od-EO

The effect of *Od*-EO on the *S. aureus* biofilm formation was studied using an MTP test. An aliquot (180 µL) of TSB containing 1/2 × MIC (0.25 µL/mL), 1/4 × MIC (0.125 µL/mL), 1/8 × MIC (0.0625 µL/mL), and 1/16 × MIC (0.0312 µL/mL) concentrations of the *Od*-EO (5 wells for each concentration) was added to each well of a sterile 96-well polystyrene plate. Then, 20 µL of bacterial culture (~10^6 CFU/mL) was added to each well (final volume of 200 µL in each well). A positive control contained TSB-DMSO with *S. aureus*, and negative controls included TSB-DMSO plus *S. aureus* and 0.1 mg/mL vancomycin. The plates were incubated at 37˚C for 48 hours without shaking to allow the cells to attach to the surface and form biofilms.

After incubation, the contents of the wells were drained and washed three times with sterile phosphate-buffered saline to remove unattached and poorly-attached cells. The plates were then air-dried. Afterward, the wells were stained with 200 µL of 1% crystal violet at room temperature for 15 minutes to validate the cell attachment to the surface. Then, plates were rinsed with phosphate-buffered saline to remove unabsorbed crystal violet, and the wells were distained with 100 µL of ethanol-acetic acid. Finally, optical density was measured using a microplate reader (BOX998, BioTek, Winooski, VT, USA) at 570 nm (20).

Effect of Od-EO on Slime Production

To investigate the effect of *Od*-EO on the rate of exopolysaccharide production, a culture medium containing TSB supplemented with Congo red, saccharose, and DMSO was used. When a bacterium was inoculated into the medium and incubated for 24 hours, the bacteria that were able to produce slime changed the color of the environment to black. The darker color of the environment indicates a higher production of slime, and the red color of the environment denotes a lack of slime production. To perform this test, a single colony of bacteria was inoculated into a microtube containing 1 mL of the above-mentioned TSB supplemented with 1/2×MIC, 1/4×MIC, and 1/8×MIC concentrations of *Od*-EO. After incubation for 24 hours at 37°C, the color of each tube was compared to that of the controls without EO, and the production of slime was checked (21,22).

Statistical Analysis

IBM SPSS Statistics 23 software was used to perform the statistical analysis of data. One-way analysis of variance (ANOVA) was used to evaluate the differences and check the significance of the differences, the *P* value was considered to be less than 0.05. All assays were performed in triplicates.

Results

GC-MS Analysis

GC-MS analysis revealed 15 compounds in the *Od*-EO, representing 99.56% of the total oil. Table 1 presents the concentrations of the *Od*-EO compounds. Thymol has the highest concentration of the constituent compounds (53.4%), and then γ-terpinene (20.48%), p-cymene (18.02%), and myristicin (2.7%) were the dominant compounds of *Od*-EO with the highest concentration.

Table 1. Chemical Compositions of the *Oliveria decumbens* Essential Oil

Compounds	%	RI	RT
Thymol	53.4	1289	18.988
γ-Terpinene	20.48	1054	9.604
Cymene	18.02	1020	8.368
Myristicin	2.7	1517	28.458
Limonene	1.5	1024	8.488
β-Pinene	1.16	974	6.823
Carvacrol	0.66	1298	19.337
Terpinene-4-ol	0.6	1174	14.228
β-Myrcene	0.4	988	7.23
α-Thujene	0.24	924	5.45
α-Pinene	0.15	932	5.633
α-Terpinene	0.12	1014	8.071
Terpinolene	0.05	1086	10.669
1,8-Cineole	0.04	1026	8.569
α-Terpineol	0.04	1186	14.806
Total	99.56		

Note. RI: Retention index; RT: Retention time.

Anti-microbial Activity of Od-EO on Staphylococcus aureus

Table 2 illustrates the results of the *Od*-EO antimicrobial activity tests using the agar disk diffusion method. The MIC and MBC of *Od*-EO against planktonic *S. aureus* were 0.5 µL/mL and 1 µL/mL, respectively.

Phenotypic Identification of Slime-Producing Strains

The CRA plate test is a classic method for the phenotypic detection of slime-producing bacteria of this species (21). By direct analysis of the colonies grown on CRA, slime-producing strains were identified. On CRA, the slime-producing strains formed strong black to almost black colonies, whereas the non-producing slime strains developed strong red colonies (Table 3). Therefore, CRA is not a quantitative test and depends entirely on the subjective color assessment. Furthermore, the color change of colonies from black to red is progressive and sometimes difficult for researchers to classify the results (19). The standard strain ATCC29213 produced slime and formed completely black colonies, while ATCC12228 was a non-slime producer as illustrated in Figure 1. As Table 3 depicts, four *S. aureus* isolates that formed strong black colonies on the CRA were selected for further investigation.

Effect of Od-EO on Staphylococcus aureus Biofilm Inhibition

Staphylococcus aureus biofilm formation in the presence of 1/2×MIC, 1/4×MIC, 1/8×MIC, and 1/16×MIC concentrations of *Od*-EO was measured using a crystal violet assay. As shown in Figure 2, the inhibitory effect of *Od*-EO on *S. aureus* biofilm formation reached a significant level at 0.0312 µL/mL (MIC/16) compared to that of the control ($P<0.001$). The data demonstrated that biofilm formation decreased with increasing *Od*-EO concentration.

Effect of Od-EO on Slime Production

As illustrated in Table 4 and Figure 3, by comparing the treatments with positive and negative controls, *Od*-EO at concentrations of 1/2×MIC (0.25 µL/mL), 1/4×MIC (0.125 µL/mL), and 1/8×MIC (0.0625 µL/mL) significantly inhibited slime production ($P<0.05$).

Table 2. Agar Disc Diffusion Assay

Microorganism	Diameter of the Inhibitory Zone				
	Od-EO	Vancomycin	SXT	Ampicillin	DMSO
Standard strain (ATCC 29213) and 4 biofilm-producer isolates of *Staphylococcus aureus*	39 ± 2.18	22 ± 1.5**	27 ± 1.26*	24 ± 4.9*	0.00 ± 0.00***

Note. *Od*-EO: *Oliveria decumbens* essential oil; SXT: Trimethoprim/sulfamethoxazole; DMSO: Dimethyl sulfoxide.
Mean values of three replicates ± the standard deviation of the mean (*$P<0.05$, **$P<0.01$, and ***$P<0.001$).

Table 3. Phenotypic Identification of Biofilm Formation Production on CRA

Isolate Number	Source of *Staphylococcus aureus*	Colony Color on CRA
1	ATCC29213	++
2	ATCC12228	-
3	Human isolate	++
4	Human isolate	++
5	Food isolate	++
6	Food isolate	+

Note. CRA: Congo red agar; Strong Black++, Almost black+, Red –.

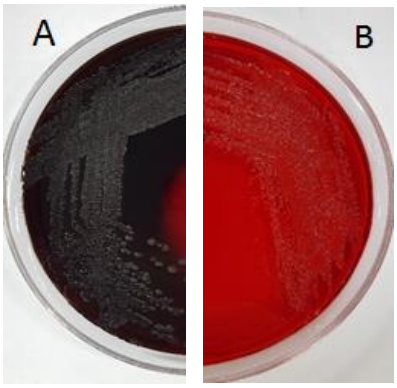

Figure 1. (A) *Staphylococcus aureus* ATCC29213 Cultured on CRA (positive control); (B) *Staphylococcus aureus* ATCC12228 cultured on Congo Red Agar Medium (negative control)

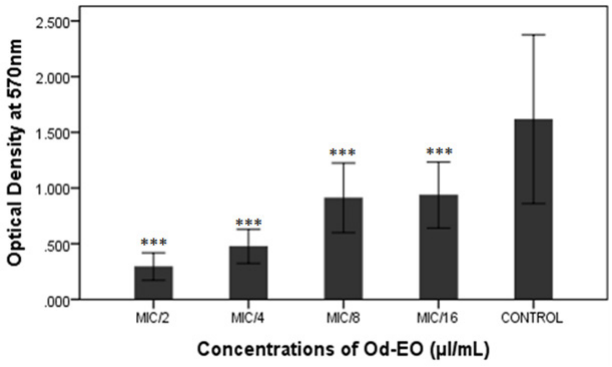

Figure 2. Anti-biofilm Effect of Different Concentrations of *Od*-EO on the *Staphylococcus aureus* Biofilm. *Note*. *Od*-EO: *Oliveria decumbens* essential oil; The error bars represent the standard deviation of three replicates (***$P<0.001$)

Discussion

The formation of bacterial biofilms on biotic and abiotic surfaces has become important in the food industry, public health, and medicine. Biofilms are more resistant to chemical agents and antibiotics compared to planktonic cells (3). Biofilm resistance is related to various factors, including physical barriers for penetrating the chemical agents into the biofilm such as extracellular polysaccharides and changes in gene expression in cells embedded in the biofilm (23). Attachment and stabilization of bacteria into surfaces are the first and most important steps in biofilm formation and development. The production of exopolysaccharides termed slime plays an important role in the mechanisms involved in microbial attachment to surfaces (19).

In recent years, EOs and their constituent compounds have been used for their antimicrobial properties, but there is not enough data regarding the effect of EOs on biofilm formation. The present study explored the preliminary biofilm-forming abilities of the four *S. aureus* isolates. In addition, this study investigated the antibacterial and anti-biofilm effects of *Od*-EO against standard strains and four

Table 4. Effect of *Od*-EO on the Amount of Slime Production in *Staphylococcus aureus*

Isolates Number	Source of *Staphylococcus aureus*	Concentrations of Od-EO (µL/ML)			
		MIC/2	MIC/4	MIC/8	Without Od-EO
1	ATCC29213	-	+	+	++
2	ATCC12228	-	-	-	-
3	Human isolate	-	-	+	++
4	Human isolate	-	-	+	++
5	Food isolate	-	-	-	+
6	Food isolate	-	-	+	++

Note. *Od*-EO: *Oliveria decumbens* essential oil; MIC: Minimum inhibitory concentration. Strong Black++, Almost Black+, Red –.

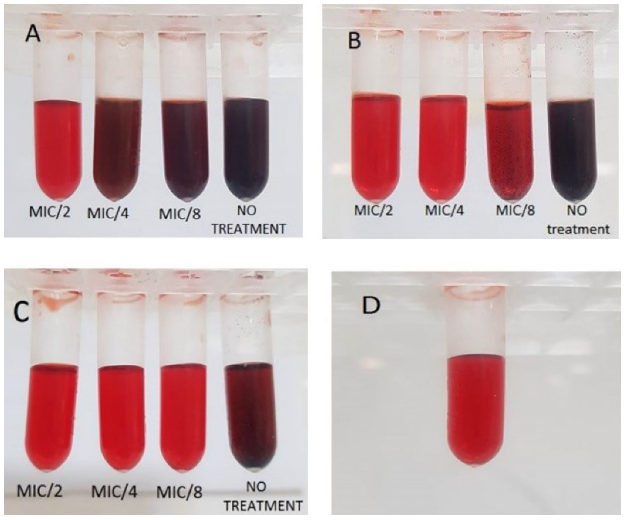

Figure 3. Inhibitory Effect of *Od*-EO on the Presence of Exopolysaccharide (Slime Production)
Note. *Od*-EO: *Oliveria decumbens* essential oil; A: ATCC29213 (Positive control); B: Human isolate; C: Food isolate, and D: Negative control without bacteria.

S. aureus isolates obtained from food and humans.

The antimicrobial properties of *Od*-EO have been previously studied (14,24,25), but their anti-biofilm properties have not been explored. The antimicrobial properties of *Od*-EO appear to be related to oxygenated monoterpenes such as thymol and carvacrol (24). According to the results obtained from GC-MS analysis, thymol (53.4%) was the major compound.

Amin et al (24) reported thymol (47.06%) as the major compound in *Od*-EO. In another study, 10 components in the *Od*-EO were reported, among which γ-terpinene, myristicin, thymol, ρ-cymene, and carvacrol were predominant (24). To some extent, the results of the current study are analogous to the results of the above-mentioned studies. The chemical composition of a plant EO depends on various factors such as season, climate, and growth stage.

In parallel with similar studies, the current data indicated that *Od*-EO had potent antimicrobial activity against *S. aureus*. An average diameter of 39 mm inhibition zone was observed in the disc diffusion test, which was significantly ($P<0.05$) larger than that in the positive controls. The MIC and MBC of *Od*-EO for four isolates and standard strains were determined to be 0.5 and 1 µL/mL, respectively. These results are consistent with previous studies (24,25)

The MTP order was used to determine the anti-biofilm properties of *Od*-EO. This assay indicated that the effect of *Od*-EO on initial bacterial cell attachment and prevention of biofilm formation was dosage-dependent ($P<0.001$). The effect of *Od*-EO on slime production also confirmed the potent anti-biofilm properties of this EO against *S. aureus* (Figure 3). The use of Congo red in this experiment is due to the fact that this color indicates the presence of exopolysaccharide (22). In all tested isolates, *Od*-EO at MIC/2 and MIC/4 prevented slime production. Many studies have investigated the anti-biofilm effects of different EOs on bacteria such as yarrow EO against *Listeria monocytogenes* (23), cardamom EO against methicillin-resistant *S. aureus* biofilm (26), and peppermint EO against *S. aureus* (5). In all these studies, researchers concluded that plant EOs are effective in preventing biofilm formation. Because the use of natural compounds prevents microorganism attachment or biofilm formation, it seems to be a useful method for the sanitization of biotic and abiotic surfaces (10).

Conclusion

Od-EO at low concentrations has significant anti-bacterial and anti-biofilm effects on *S. aureus* (standards, humans, and foodstuff isolates). *Od*-EO or its compounds can be used as anti-biofilm and anti-bacterial sanitizers in various fields, including medicine, dentistry, and the food industry. In addition, the synergistic effects of the combination of *Od*-EO with other chemical and natural compounds to produce more powerful sanitizers need to be investigated. These sanitizers may apply on different surfaces in medical and food surfaces to remove or prevent bacterial populations.

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Conflict of Interests

The authors declare that they have no conflict of interests.

Ethical Approval

The experiments were conducted according to the protocol approved by the Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz.

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