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ORIGINAL ARTICLE

The inhibitory effect of the methanolic extract and the essence of *Anvillea garcinii* on expression of the genes related to *Staphylococcus aureus* biofilm formation

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ABSTRACT

Background and Objectives: *Staphylococcus aureus (S. aureus)* is a pathogenic bacterium whose virulence is attributed to its extracellular compounds and biofilm-forming ability. This study aimed to evaluate the inhibitory effects of the methanolic extract (AGME) and the essential oil (AGEO) of *Anvillea garcinii* on the growth and the biofilm formation of *S. aureus.*
Materials and Methods: The antibacterial and antibiofilm activities of AGME and AGEO against *S. aureus* ATCC 6538 were assessed using the microbroth dilution method and the Crystal Violet Staining Assay, respectively. The expression levels of *sarA*, *spa,* and *icaA*, genes involved in biofilm formation, were analyzed using real-time PCR.
Results: AGME and AGEO inhibited *S. aureus* growth at minimum inhibitory concentrations (MIC) of 1 mg/ml and 0.6 mg/ml, respectively. AGME exhibited a 72% inhibition of biofilm formation at ¼ MIC, whereas AGEO showed no significant antibiofilm activity. AGME downregulated the expression of *sarA*, a key regulator of biofilm formation, as well as *spa*, and *icaA* genes.
Conclusion: This study demonstrated that *A. garcinii* essential oil (AGEO) exhibits significant antimicrobial activity, while its methanolic extract (AGME) effectively inhibits biofilm formation in *S. aureus*. These findings suggest the potential application of AGEO and AGME as antimicrobial and antibiofilm agents. Further investigations on their efficacy against other bacterial pathogens are recommended.

Keywords: *Staphylococcus aureus*; Biofilm; Plant extract; Antibacterial agents; Gene expression

INTRODUCTION

Staphylococcus aureus is a Gram-positive coccus commonly colonizing the skin and the upper respiratory tract (1). It is responsible for various infections, including skin abscesses, respiratory infections, soft tissue infections, bacteremia, and endocarditis. Antibiotic resistance in *S. aureus* poses a significant global challenge in clinical medicine, particularly for patients with open wounds, as the bacterium can disseminate through the bloodstream, leading to severe conditions such as endocarditis, osteomyelitis, pneumonia, and sepsis (2).

The ability of *S. aureus* to form biofilms is a key factor for its virulence and antibiotic resistance. These biofilms frequently develop on medical im-

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plants, human tissues, and catheters, where bacteria exhibit high resistance to antimicrobial treatments.

Biofilm formation of *S. aureus* is primarily mediated by the polysaccharide intercellular adhesin (PIA) protein, which is synthesized under the regulation of the intercellular adhesion (*ica*) locus (3). Another crucial regulator of biofilm formation is the staphylococcal accessory regulator (SarA), encoded by the *sarA* gene (4). Additionally, protein A, a surface protein encoded by the *spa* gene, binds to the peptidoglycan of the bacterial cell wall and facilitates biofilm formation by mediating protein interactions with the cell wall (5). Given their essential roles in biofilm development, the *sarA*, *spa*, and *icaA* genes represent potential targets for compounds with antibiofilm activity (5).

Numerous studies have been conducted to identify novel compounds with antibacterial and antibiofilm activities, including silver nanoparticles, bacteriophages, and plant-derived antimicrobial agents (5). *Anvillea garcinii*, a plant from the Asteraceae family, is distributed from North Africa to Iran (6, 7). Phytochemical studies on this plant have identified various secondary metabolites with potential biological activities (8, 9). Traditionally, *A. garcinii* has been used in Arab regions to treat intestinal, pulmonary, hepatic, and digestive disorders, as well as diabetes (10).

In 2009, the antibacterial activity of the methanolic extract of *A. garcinii* against *S. aureus* was evaluated using the minimal inhibitory concentration (MIC) and disk diffusion methods (11). In a study in 2020, the bioactive compounds of *A. garcinii* were isolated, and the MIC of each compound against *S. aureus* was determined (6). Previous studies have shown that *A. garcinii* contains secondary metabolites, including flavonoids and sesquiterpene lactones (12-14). These compounds are capable of disrupting bacterial biofilm formation (15, 16). However, no studies have investigated the antibiofilm effects of *A. garcinii* at both the phenotypic and molecular levels. This study aimed to evaluate the inhibitory effects of the methanolic extract and the essential oil of *A. garcinii* native to Iran on growth and biofilm formation of *S. aureus*, as well as the expression of the *sarA*, *spa*, and *icaA* genes.

MATERIALS AND METHODS

Preparation of the extract and the essential oil from *A. garcinii*. *A. garcinii* was obtained from the Research and Education Center for Agriculture and Natural Resources of Bushehr Province. The plant leaves were dried at room temperature in the shade and subsequently used for extraction (17).

To prepare the methanolic extract, 60 grams of dried leaf powder was subjected to extraction in a Soxhlet apparatus using 300 milliliters of methanol for 8 hours. The resulting extract was then concentrated and dried using a rotary evaporator at 40°C (17).

For essential oil extraction, 800 grams of dried leaf powder was hydro-distilled with one liter of distilled water using a Clevenger apparatus. The collected essential oil was dehydrated with sodium sulfate. Subsequently, the sealed containers were stored at ambient temperature, protected from light (17).

***Staphylococcus aureus* strain.** *S. aureus* strain ATCC 6538 was obtained in lyophilized form from the Pasteur Research Center, Tehran. Confirmatory tests for *S. aureus* included culture on blood agar, Gram staining, culture on mannitol salt agar, coagulase, catalase tests, and sensitivity to novobiocin (18).

Determination of the antibacterial activity of AGME and AGEO. A stock solution of *A. garcinii* extract was prepared at a concentration of 16 mg/ml in dimethyl sulfoxide (DMSO). Serial dilutions of the methanolic extract were prepared in a 96-well microplate by adding 100 µl of bacterial suspension with a turbidity equivalent to 1 × 10^6 cfu/ml to each well. Concentrations of 4, 2, 1, 0.5, 0.25, 0.125, and 0.062 mg/ml were obtained. Wells containing the extract and physiological serum, as well as the bacterial suspension and Mueller-Hinton broth, were used as negative and positive growth controls, respectively. After incubation at 37°C for 24 hrs, the optical absorbance of the wells was measured at 570 nm using an ELISA reader. The lowest concentration at which no bacterial growth was observed compared to the control group was considered the minimum inhibitory concentration (MIC) (19). Each test was performed in triplicate.

The inhibitory percentage of AGME against S. *aureus* strain ATCC 6538 was calculated using the following formula (20):

Bacteria growth inhibitory percentage = [1 − ((treated OD570nm – negative control OD570nm) / positive control OD570nm)] × 100

To prepare the initial stock of *A. garcinii* essential oil, the volume and the mass of the essential oil were first determined. Then, 15 microliters of plant essential oil (equivalent to 15 mg) was dissolved in 285 mi-

croliters of DMSO solution (95% DMSO) to achieve a concentration of 50 mg/ml. This stock solution was subsequently used to prepare serial concentrations of 5, 2.5, 1.25, 0.6, 0.3, and 0.15 mg/ml in the wells. To each well, 200 microliters of Mueller-Hinton broth was added. A bacterial suspension of *S. aureus* was prepared by cultivating the bacteria in Mueller-Hinton agar (MHA) medium, and a 0.5 McFarland standard was prepared in sterile physiological saline. The bacterial suspension was diluted to a final concentration of 1.5 × 10^6 cfu/ml, and 5 microliters of this suspension was added to each well. After 24 hours of incubation at 37°C, the optical absorbance at 570 nm was measured using an ELISA reader. The lowest concentration at which no bacterial growth was observed compared to the control group was considered as the MIC.

Ten microliters of the culture medium from wells with concentrations higher than the MIC were inoculated onto MHA plates. After 24 hrs of incubation at 37°C, the lowest concentration at which no colony growth was observed was considered the minimum bactericidal concentration (MBC) (17).

Determination of the antibiofilm activity of AGME and AGEO. A suspension of *S. aureus* in the logarithmic phase was prepared in Tryptic Soy Broth (TSB) medium containing 1% glucose, with a turbidity equivalent to 1 × 10^6 cfu/ml. Serial concentrations of AGME and AGEO, including ½ MIC (0.5 mg/ml), ¼ MIC (0.25 mg/ml), and ⅛ MIC (0.125 mg/ml), were prepared in sterile microplate wells by adding 100 microliters of each concentration to the wells containing 100 microliters of TSB medium with 1% glucose. Subsequently, 100 microliters of the bacterial suspension was added to each well. A well containing 100 microliters of bacterial suspension and 100 microliters of TSB medium was used as a positive control, while a well containing 200 microliters of TSB medium alone served as a negative control (20).

All of the above steps were repeated for AGEO, with the exception that the ½ MIC, ¼ MIC, and ⅛ MIC concentrations for the essential oil were 0.3 mg/ml, 0.15 mg/ml, and 0.070 mg/ml, respectively.

To assess biofilm formation, the microplates were incubated for 24 hours at 37°C. After incubation, the planktonic bacteria were removed by washing the wells twice with Phosphate-buffered saline (PBS) solution. The biofilm was then stained with 200 microliters of 0.4% crystal violet solution for 5 minutes and washed twice with distilled water to remove the excess dye. Subsequently, 200 microliters of cold 20% acetic acid was added to each well, and after 30 minutes, the optical absorbance was measured at 570 nm (21). The percentage of biofilm inhibition following exposure of *S. aureus* to AGME and AGEO was calculated using the following formula (19).

% Biofilm formation inhibition = [1 − (treated OD570 nm/ positive control OD570 nm)] × 100

Evaluation of the effect of AGME and AGEO on gene expression. To assess the impact of the methanolic extract and essential oil on gene expression, *S. aureus* ATCC6538 was incubated for 24 hrs in Brain Heart Infusion (BHI) medium. One milliliter of BHI medium containing bacteria, with an optical density of 0.5-0.6 at 600 nm, was exposed to the extract and the essential oil of *A. garcinii* for 4, 16, and 48 hrs. Following incubation, the bacterial suspension was centrifuged at 3000 rpm for 5 minutes to collect the bacterial pellet. RNA was extracted from the bacteria to evaluate the expression of the *sarA*, *spa*, and *icaA* genes. The sediment was transferred to RNase-free microtubes, and the treatments were stored at -80°C for further analysis (17). Three repetitions were performed for each test.

Assessment of the expression of *sarA*, *spa*, and *icaA* genes by Real-time PCR. The nucleotide sequences of the *sarA*, *spa*, and *icaA* genes were determined and sent to Metabion Company (Germany) for the synthesis of specific primers (Table 1). Primers were prepared at a concentration of 10 µM and stored at -20°C until use.

RNA extraction and cDNA synthesis. RNA extraction was performed using the Total RNA Prep Kit (BioFACT, Korea). Genomic DNA was subsequently removed using DNase enzyme (Ekta Tajhiz Azma, Iran). Following the manufacturer's instructions (Pars Toos, Iran), cDNA was synthesized using the temperature program outlined in Table 2.

Synthesized cDNAs were prepared according to the instructions of the RealQ Plus 2x Master Mix Green High ROX™ kit (AMPLIQON, Denmark). The temperature program for Real-time PCR included an initial denaturation step at 95°C for 15 minutes, followed by 40 amplification cycles: denaturation at 95°C for 20 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 30 seconds. A final denatur-

Table 1. The specific sequence of the gene primers (*sarA, spa, icaA*)

Gene	Primer sequence (5′–3′)	Length of fragment	References
spa	F: ATAAGAAGCAACCAGCAAAC	113	(22)
	R: CGCTAATGATAATCCACCAA		
sarA	F: TTCTTTCTCTTTGTTTTCGCTG	115	(23)
	R: GTTATCAATGGTCACTTATGCT		
icaA	F: CTGGCGCAGTCAATACTATTTCGGGTGTCT	195	(24)
	R: GACCTCCCAATGTTTCTGGAACCAACATCC		
16SrRNA	F: ACTCCTACGGGAGGCAGCAG	197	(25)
	R: ATTACCGCGGCTGCTGG		

Table 2. cDNA synthesis temperature program

Level	First	Second	Third
Temperature	25°C	47°C	85°C
Time	10 minutes	60 minutes	5 minutes

ation step was performed at 95°C for 15 seconds, followed by annealing at 60°C for 1 minute and elongation at 72°C for 15 seconds.

Statistical analysis. Statistical analysis was performed using two-way analysis of variance (ANOVA). Tukey's post-hoc test was conducted for pairwise comparisons between gene expression levels and different concentrations at the same time point. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Antibacterial activity of the methanolic extract and the essential oil of *A. garcinii.* The results indicated that the methanolic extract of *A. garcinii* completely inhibited the growth of *S. aureus* at concentrations of 1 mg/ml and higher. At lower concentrations, growth inhibition was observed at 33%, 22%, and 18% for concentrations of 0.5 mg/ml, 0.25 mg/ml, and 0.125 mg/ml, respectively.

On the other hand, the MIC value of AGEO for *S. aureus* ATCC6538 was found to be 0.6 mg/ml, demonstrating greater antibacterial activity of the essential oil compared to the methanolic extract of *A. garcinii* (Fig. 1).

Anti-biofilm activity of AGME and AGEO. The inhibition percentage of biofilm formation by AGME was 35% at 0.5 mg/ml (½MIC), 72% at 0.25 mg/ml (¼MIC), and 26% at 0.125 mg/ml (⅛MIC) concentration (17). The paired sample test revealed that the ¼ MIC concentration of AGME significantly inhibited *S. aureus* biofilm formation ($p<0.05$) (Fig. 2).

The inhibitory effect of AGEO on *S. aureus* biofilm formation was observed at various concentrations: 8% inhibition at 0.3 mg/ml (½MIC), 6% at 0.25 mg/ml (¼MIC), and 3% at 0.125 mg/ml (⅛MIC) (Fig. 3).

***spa* gene expression.** Tukey's post hoc test results showed that *spa* gene expression was significantly downregulated when exposed to AGEO at three time intervals (4, 16, and 48 hours) compared to the control, with the exception of the ¼ MIC concentration at 48 hours. The most substantial decrease in expression was observed at 4 hrs across all concentrations (Fig. 4A).

For AGME, the greatest decrease in *spa* gene expression occurred after 16 hours at ½ and ¼ MIC concentrations ($p<0.001$). However, AGME treatment increased *spa* gene expression at 48 hrs at both ½ and ¼ MIC concentrations ($p<0.001$) (Fig. 5C).

***sarA* gene expression.** Tukey's post-hoc test results indicated that AGEO did not significantly downregulate *sarA* gene expression at any concentration or exposure time. The highest increase in expression was observed at ½ MIC after 16 hrs (Fig. 4B).

For AGME, *sarA* gene expression was decreased at all concentrations after 48 hrs of treatment. However, after 16 hrs, expression increased at all concentrations, while a decrease was observed at the ¼ MIC concentration after 4 hrs of treatment (Fig. 5).

***icaA* gene expression.** The greatest decrease in *icaA* gene expression was observed after 16-hour of expo-

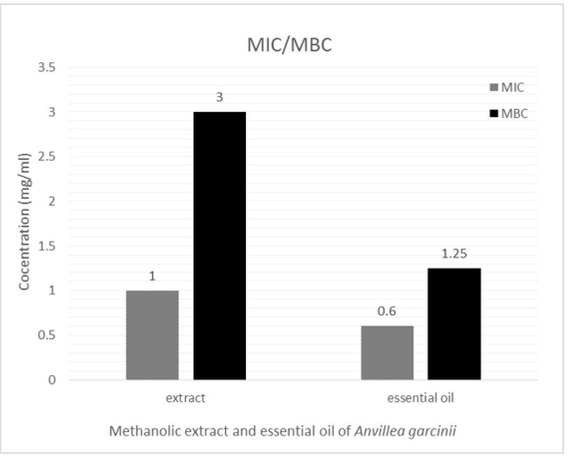

Fig. 1. MIC and MBC values of the methanolic extract and the essential oil of the *A. garcinii* for *S. aureus* ATCC6538.

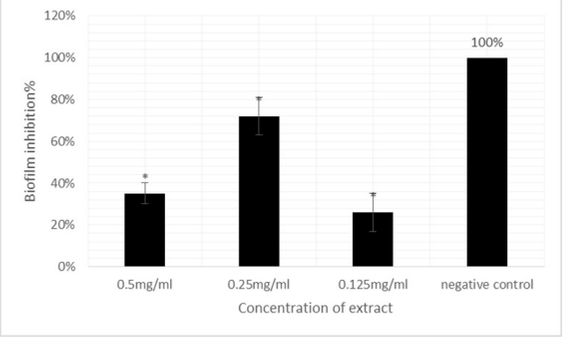

Fig. 2. The inhibitory effect of sub-MIC concentrations of methanol extract of *A. garcinii* on *S. aureus* ATCC6538 biofilm formation. ns: non-significant (p>0.05), *: p<0.05

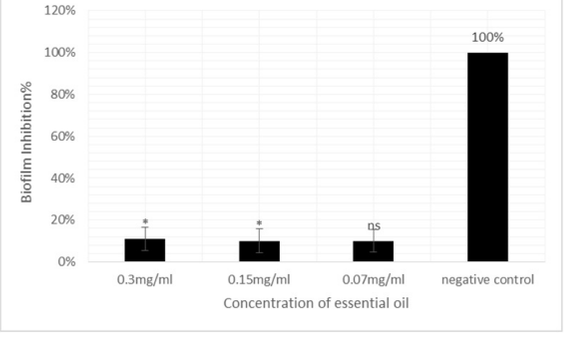

Fig. 3. The inhibitory effect of sub- MIC concentrations of essential oil of *A. garcinii* on *S. aureus* ATCC6538 biofilm formation. ns: non-significant (p>0.05), *: p<0.05

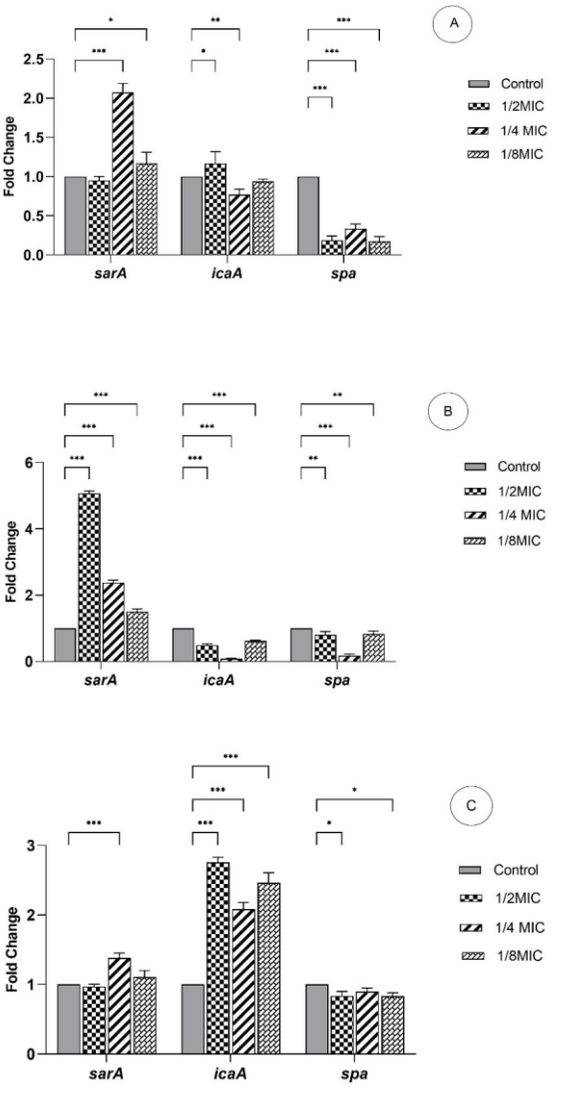

Fig. 4. Comparison of fold changes in *sarA, icaA* and *spa* genes expression of *S. aureus* ATCC6538 in sub-MIC concentrations of essential oil of *A. garcinii* between different treatments (A: 4 h, B: 16 h and C: 48 h). ns: non-significant (p>0.05), ***: p<0.001, **: p<0.01,*: p<0.05

sure to AGEO, particularly at the ¼ MIC concentration (p<0.001) (Fig. 4B).

For AGME, significant reduction in *icaA* gene expression occurred at the ¼ MIC concentration after 4 hours of treatment. No reduction was observed at other concentrations or exposure times (Fig. 5).

Chemical composition of AGEO. The bioactive compounds in AGEO were identified using GC-MS. The major compounds identified were Nerolidyl acetate (31.32%), alpha-Himachalene (7.13%), Caryo-

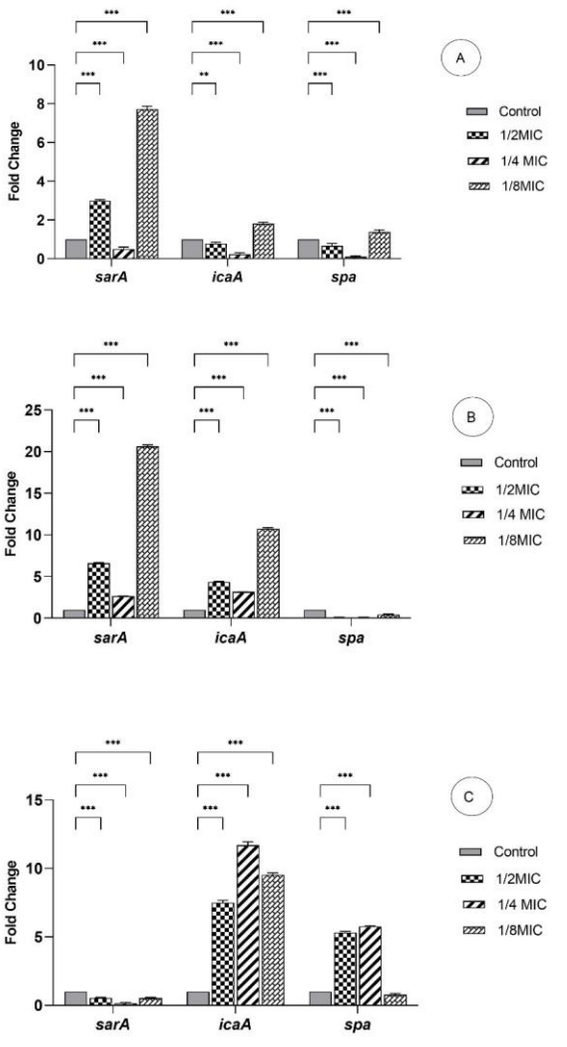

Fig. 5. Comparison of fold changes in *sarA*, *icaA* and *spa* genes expression of *S. aureus* ATCC6538 in sub-MIC concentrations of methanolic extract of *A. garcinii* between different treatments (A: 4 h, B: 16 h and C: 48 h). ns: non-significant ($p>0.05$), ***: $p<0.001$, **: $p<0.01$,*: $p<0.05$

phyllene oxide (5.45%), Propanoic acid, 2-methyl-, 3,7-dimethyl-2,6-octadienyl ester (3.77%), and pichtosin (3.34%) (Table 3).

DISCUSSION

In this study, the anti-biofilm activity of the methanolic extract (AGME) and the antibacterial activity of *A. garcinii* essential oil (AGEO) against *S. aureus* strain ATCC6538 were significant. Similarly, Javidnia et al. (2009) examined the antimicrobial activity of AGME on *S. aureus* using two methods, MIC and disk diffusion, with an MIC of 2 mg/ml (11). Perveen et al. (2020) identified effective compounds from the methanolic extract of *A. garcinii* and determined the MIC for each compound against *S. aureus.* MIC values ranged from 2.3 to 9.4 µg/ml for some compounds, while others exceeded 25 µg/ml (6). In our study, the MIC of AGME against *S. aureus* ATCC6538 was 1 mg/ml, determined using the microtiter plate method. The variations in antimicrobial activities between studies may be attributed to differences in growing conditions, harvest times, plant growth stages, and extraction methods (26-28). For instance, in a study on AGME from *A. garcinii* native to Saudi Arabia, two compounds—4α,9α,10α-trihydroxyguaia-11 (13) en-12,6α-olide and germacrane, 9β-hydroxyparthenolide-9-O-β-D-glucopyranoside—exhibited antifungal properties, while chlorogenic acid, a non-sesquiterpenoid, showed antibacterial activity against certain Gram-negative bacteria (6).

In this study, the essential oil of *A. garcinii* demonstrated significant antimicrobial activity. GC-MS analysis of the plant essential oil revealed that the highest percentage of compounds were attributed to Nerolidyl acetate and alpha-Himachalene. Acidic compounds like Nerolidyl acetate are known for their strong antibacterial and anti-sediment properties. Additionally, Chaudhary's study highlighted the antibacterial activity of alpha-Himachalene, particularly against Gram-positive bacteria, including *S. aureus.* This supports the observed low MIC of the plant's essential oil (29-31).

To the best of our knowledge, no previous studies have investigated the anti-biofilm effect of *A. garcinii* methanolic extract (AGME) and the essential oil. Our findings demonstrated that AGME inhibited biofilm formation at a concentration of ¼MIC by 75%, although this effect was not dose-dependent. Previous research indicated that the sub-MIC concentrations of antimicrobial agents can trigger stress responses in bacteria, affecting their physiological and biochemical functions and, consequently, inducing biofilm production. The dose-dependent relationship of anti-biofilm agents varies depending on their nature and the bacterial strain (32). Therefore, the observed reduction in biofilm inhibition at ½MIC compared to ¼MIC may reflect a biofilm production signal triggered by AGME at this concentration. This observation aligns with the genetic studies in this work, as all three studied genes were significantly downreg-

Table 3. Compounds identified in the essential oil of *A. garcinii* in GC-MS

Compound		Formula	Molecular Weight (g/mol)	RT	Area Sum%
Nerolidyl acetate	fragrance component	$C_{17}H_{28}O_2$	264/4	19/751	31/32
alpha-Himachalene	sesquiterpene	$C_{15}H_{24}$	204/35	18/801	7/13
Caryophyllene oxide	Sesquiterpenes	$C_{15}H_{24}O$	220/35	22/202	5/45
Propanoic acid, 2-methyl-, 3,7-dimethyl-2,6-octadienyl ester, (Z)-	carboxylic ester	$C_{14}H_{24}O_2$	208/3398	20/297	3/77
Pichtosin	Camphanes	$C_{12}H_{20}O_2$	196/29	14/442	3/34
Oxandrolone	Hormones	$C_{19}H_{30}O_3$	306/4	20/929	2/64
Geranyl isopentanoate	Fatty Acyls	$C_{15}H_{26}O_2$	238/37	22/662	2/61
Methyl 5,8,11,14-eicosatetraenoate	Fatty acids	$C_{21}H_{34}O_2$	318/5	19/031	2/57
hexahydrofarnesylacetone	Terpenes	$C_{18}H_{36}O$	268/5	28/67	2/41
1,2-Longidione	Terpenoids	$C_{15}H_{22}O_2$	234/33	22/867	2/37
nerolidol acetate	fragrance component	$C_{17}H_{28}O_2$	264/4	22/069	2/25
Neointermedeol	Terpenoids	$C_{15}H_{26}O$	222/37	23/909	1/95
Estragole	Phenols	$C_{10}H_{12}O$	148/2	14/535	1/91
cis-Damascenone	terpenoid	$C_{13}H_{18}O$	190/28	17/034	1/73
alpha-Longipinene	Sesquiterpenes	$C_{15}H_{24}$	204/35	20/103	1/48
2-dodec-7-ynoxyoxane		$C_{22}H_{40}O_2$	266/4	17/233	1/38
Neryl (S)-2-methylbutanoate		$C_{15}H_{26}O_2$	238/37	21/857	1/18
Caryophyllene	Sesquiterpenes	$C_{15}H_{24}$	204/35	17/994	1/13
3,7-Nonadien-2-one, 4,8-dimethyl-		$C_{11}H_{18}O$	166/26	14/086	1/05
5-Isopropyl-2-methylbicyclo[3.1.0]hexan-2-ol	carbonyl compound	$C_{10}H_{18}O$	138/21	11/522	1/03
Aromandendrene	Sesquiterpenes	$C_{15}H_{24}$	204/35	19/968	1/03
Glutaric acid, tridec-2-yn-1-yl isopropyl ester		$C_{28}H_{46}O_4$	352/5	13/608	1/02
Isopulegol	Terpenes	$C_{10}H_{18}O$	154/25	12/881	0/94
Cembrene		$C_{20}H_{32}$	272/5	26/548	0/91
(-)-Carvone	terpenoid	$C_{10}H_{14}O$	150/22	13/313	0/86
2-((2R,4aR,8aS)-4a-Methyl-8-methylenedecahydronaphthalen-2-yl)acrylaldehyde	terpenoid	$C_{15}H_{22}O$	250/33	24/3	0/84
Fitone	Terpenes	$C_{18}H_{36}O$	268/5	17/519	0/84
(Z)-3,7Dimethylocta-2,6-dienyl pentanoate		$C_{22}H_{40}O_2$	238/37	21/621	0/82
Lilac aldehyde C	carbonyl compound	$C_{10}H_{16}O_2$	168/23	10/698	0/8
Methyl 10,12-pentacosadiynoate		$C_{26}H_{44}O_2$	388/6	21/385	0/78
Oxirane, hexadecyl-		$C_{18}H_{36}O$	597	25/18	0/72
5-Methylene-6-isopropenyl-3-cyclohexen-1-ol acetate		$C_{10}H_{14}O$	192/25	11/772	0/63
octadecane-1-sulfonyl chloride		$C_{18}H_{37}ClO_2S$	353	22/381	0/62
Methyl 5,8,11,14-eicosatetraenoate	Fatty acids	$C_{21}H_{34}O_2$	318/5	24/802	0/59
Pinocarveol	Terpenes	$C_{10}H_{16}O$	152/23	10/448	0/52
methyl eicosapentaenoate	fatty acid ester	$C_{20}H_{30}O_2$	316/5	12/046	0/51
3-Eicosene, (E)-		$C_{20}H_{40}$	280/5	15/66	0/44
9-Heptadecene-4,6-diyn-8-ol, (Z)-		$C_{17}H_{26}O$	244/37	17/864	0/42
		$C_{25}H_{42}O_2$		15/759	0/4
3,7-Nonadien-2-one, 4,8-dimethyl-		$C_{11}H_{18}O$	166/26	13/021	0/4
.alpha.-Terpineol	Terpenes	$C_{10}H_{18}O$	154/25	11/907	0/34
p-Mentha-1,4-dien-7-ol	Terpenoids	$C_{10}H_{16}O$	152/23	14/855	0/33
Ylangene	Terpenoids	$C_{15}H_{24}$	204/35	16/17	0/32
octadecane-1-sulfonyl chloride		$C_{18}H_{37}ClO_2S$	353	16/729	0/32
Longiborneol	Sesquiterpenes	$C_{15}H_{26}O$	222/37	24/183	0/31

Table 3. Continuing ...

1-Heptatriacontanol	Fatty acids	$C_{37}H_{76}O$	537	23/425	0/31
Chloroicosane		$C_{20}H_{41}C_{1}$	317	27/253	0/28
gamma.-Dehydro-Ar-himachalene		$C_{15}H_{20}$	200/32	20/763	0/21
(2,4-dimethylcyclohex-3-en-1-yl)methanol		$C_{10}H_{18}O$	140/22	14/776	0/11
6,9-Octadecadienoic acid, methyl ester		$C_{19}H_{30}O_{2}$	294/5	19/386	0/08

ulated at ¼MIC, irrespective of treatment duration.

On the other hand, previous studies have shown that exposure of *S. aureus* to sub-MIC concentrations of methicillin results in a significant increase in biofilm production. This increase is likely attributed to a genetic mechanism that induces bacterial lysis, releasing extracellular DNA (eDNA) and promoting biofilm formation (32). In our study, the effect of sub-MIC concentrations on biofilm formation did not exhibit a proportional decrease with increasing concentrations, supporting the possibility of a mechanism similar to the one observed in the aforementioned research. However, further studies are needed to confirm this hypothesis.

The anti-biofilm effect of the essential oil on *S. aureus* was minimal (10%). This finding aligns with the results of GC-MS analysis, which revealed that the bioactive compounds in the essential oil do not exhibit significant anti-biofilm activity. Additionally, several studies have reported varying results regarding the anti-biofilm activity of essential oils. These findings confirm that, despite containing more potent compounds than extracts, not all essential oils necessarily possess strong anti-biofilm properties (28, 29).

The results of the effect of AGME and AGEO on the expression of biofilm-related genes (*sarA, icaA, spa*) were significantly different, mirroring the phenotypic findings that showed low anti-biofilm activity of AGEO. The reduction in gene expression was also less pronounced in AGEO compared to AGME. This difference can be attributed to the variation in the active compounds present in these two preparations.

The *SarA* protein is a major regulator that influences the expression of various genes involved in the pathogenesis of *S. aureus* and plays a crucial role in biofilm formation (33-35). It functions both as an activator and a repressor of the transcription process. One of the key systems regulated by *SarA* is the *agr* locus, which controls the transition from surface protein synthesis in the logarithmic phase to toxin and destructive protein synthesis in the stationary phase (36). The primary role of *SarA* is to suppress the expression of extracellular proteases. Mutants lacking *SarA* exhibit reduced biofilm-forming capacity due to the inability to prevent biofilm destruction by proteases and nucleases. It seems that the significant downregulation of the *sarA* gene at 48 hrs with the ¼ MIC of the methanolic extract is linked to the function and the expression timing of this gene (36, 37). However, the expression of the other two genes, *icaA* and *spa*, increased after 48 hrs of AGME treatment. This diminished effect on *icaA* and *spa* expression could be due to the specific roles of these genes and their expression timing (30).

The *icaA* and *icaD* gene products are integral to the synthesis of exopolysaccharides, with *IcaA* being a transmembrane protein that synthesizes N-acetyl-glucosamine oligomers through transferase activity (34). The *spa* gene encodes surface protein A, which induces cell aggregation and biofilm formation (38). Protein A attaches to the peptidoglycan in the cell wall, facilitating the binding of other proteins and promoting biofilm formation. Therefore, the significant reduction in the expression of these genes early in growth may be attributed to their high expression during this phase in the normal state (36). Additionally, Merino et al. reported that *spa* gene deletion significantly impaired *S. aureus* colonization. The observed reduction in biofilm formation in this study may be due to the downregulation of the *spa* gene (38-41). Furthermore, other minor antimicrobial agents in AGME, which were not investigated in this study, may also influence the expression of regulatory genes by interacting with the translation process (32-44).

In this study, the effects of AGME on additional genes involved in *S. aureus* biofilm production and virulence factors were not investigated. Future research should address these aspects to more precisely determine the antimicrobial properties of this compound.

CONCLUSION

This study demonstrated that *A. garcinii* essential oil (AGEO) exhibits promising antimicrobial activity, while the methanolic extract (AGME) not only possesses antimicrobial properties but also significantly downregulates key genes involved in *S. aureus* biofilm formation. Therefore, AGME may serve as an effective anti-biofilm agent against *S. aureus*, and further investigations into its anti-biofilm potential against other pathogens are warranted.

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