

Chamaecyparis obtusa Essential Oil Inhibits Methicillin-Resistant *Staphylococcus aureus* Biofilm Formation and Expression of Virulence Factors

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ABSTRACT The emergence of antibiotic-resistant bacteria has caused difficulty in treating infectious diseases. Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most commonly recognized antibiotic-resistant bacteria. Novel antibiotics are urgently required to treat these bacteria. Raw materials derived from natural sources can be used for the development of novel antibiotics, such as *Chamaecyparis obtusa* (*C. obtusa*), which has been traditionally used in treating asthmatic disease. In this study, the antibacterial activity of the essential oil (EO) extracted from *C. obtusa* leaves against MRSA was investigated. MRSA growth and acid production from glucose metabolism were inhibited at concentrations greater than 0.1 mg/mL *C. obtusa* EO. MRSA biofilm formation was observed using scanning electron microscopy and safranin staining. *C. obtusa* EO inhibited MRSA biofilm formation at concentrations greater than 0.1 mg/mL. Using real-time polymerase chain reaction, mRNA expression of virulence factor genes, *sea*, *agrA*, and *sarA*, was observed. *agrA* expression was inhibited with *C. obtusa* EO concentrations greater than 0.2 mg/mL, whereas inhibition of *sea* and *sarA* expression was also observed at a concentration of 0.3 mg/mL. *C. obtusa* EO was analyzed by gas chromatography (GC) and GC coupled for mass spectrometry, which identified 59 constituents, accounting to 98.99% of the total EO. These findings suggest that *C. obtusa* EO has antibacterial effects against MRSA, which might be associated with the major components of *C. obtusa* EO, such as sabinene (19.06%), α -terpinyl acetate (16.99%), bornyl acetate (10.48%), limonene (8.54%), elemol (7.47%), myrcene (5.86%), γ -terpinene (4.04%), and hibaene (3.01%).

KEY WORDS: *agrA* • antimicrobial activity • cypress • hinoki • *sarA* • *sea*

INTRODUCTION

SOME ESSENTIAL OILS (EOs) are known to have calming effects on stress as well as antibiotic and anticancer effects.^{1–3} Calming EOs contain chemicals such as ketone, terpene, and phenol ether⁴ and have also been used as food flavoring agents^{3,5} with a wide antimicrobial activity owing to the high phenol derivative content.⁶ The rise of antibiotic-resistant bacteria has caused problems in the treatment of infectious diseases. Although some novel antibiotics have been developed, an increasing number of bacteria are becoming drug resistant. Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most well-recognized antibi-

otic-resistant bacteria, and it is not easily eliminated by antibiotics as it forms biofilms on implants, medical supplies, and/or medical devices in patients.^{7–9} Staphylococcal enterotoxin A (*sea*), regulated by accessory gene regulatorA (*agrA*) and staphylococcal accessory regulatorA (*sarA*), is one of the major virulence factors of MRSA. It causes staphylococcal gastroenteritis through the stimulation of T-cell activity and cytokine secretion.^{10–13}

The rise of multidrug-resistant MRSA owing to hospital-acquired infections is becoming an important social issue. Increased MRSA has led to the acquisition and dissemination of resistance factors among various strains as well as virulence factor mutations, creating mutant strains and resulting in the emergence of superbacteria. Historically, various antimicrobial compounds have been found in natural substances through studies on therapeutic agents for resistant and mutant strains, and additional studies are actively underway.^{14,15} There are six members of the genus *Chamaecyparis*, mainly distributed across various regions of

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North America, Japan, and Taiwan.¹⁶ They have been reported to have high levels of monoterpenes, sesquiterpenoids,¹⁷ diterpenes,^{17,18} and lignin,¹⁹ as well as anticancer, antimalarial, and antimicrobial properties. *Chamaecyparis obtusa* (*C. obtusa*) EO has antimicrobial activity against the phytopathogen *Alternaria alternata*, which causes asthma when airborne, has also shown antifungal activity and strong antibacterial activity against Gram-positive bacteria,²⁰ and has been commercially used in soap, toothpaste, and cosmetics as a functional additive due to its potent fragrance.²¹ *C. obtusa* EO also has anti-inflammatory activity, regulates cytokine gene expressions, exerts antibacterial activity against foodborne pathogens, stimulates hair growth, and has antioxidant activity.^{22–26}

However, very few studies have investigated the effects of *C. obtusa* on MRSA. Therefore, the inhibitory effects of *C. obtusa* EO on MRSA growth, acid production and biofilm formation were examined in this study, and the detailed chemical constituents of *C. obtusa* EO were analyzed by gas chromatography (GC) and GC coupled to mass spectrometry (GC-MS).

MATERIALS AND METHODS

Materials

The brain–heart infusion (BHI) broth was purchased from Difco Laboratories (Detroit, MI, USA). Glucose and dimethyl sulfoxide (DMSO) were obtained from Sigma Co. (St. Louis, MO, USA). MRSA ATCC 33591 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA).

Plant material and extraction

The leaves of *C. obtusa* were collected in October 2013 from the Jeollanam-do province, South Korea. The identity was confirmed by Young-Hoi Kim at the College of Environmental & Bioresource Sciences, Chonbuk National University. A voucher specimen (No. 9-12-13) has been deposited at the Herbarium of College of Environmental & Bioresource Sciences, Chonbuk National University. Fresh leaves of *C. obtusa* (1 kg) were ground mechanically and hydrodistilled for 3 h using a Clevenger-type apparatus. The yield of the EO of *C. obtusa* was 1.08% (as fresh weight base) of yellow pale oil. The EO was stored in a deep freezer (–70°C) to minimize the escape of volatile compounds. The EO was dissolved in DMSO to give the desired stock solution. The final concentration of DMSO applied to culture systems was adjusted to 0.1% (v/v), which did not interfere with the testing system. Control groups were treated with media containing 0.1% DMSO.

Bacterial growth and acid production

Bacterial growth was determined using a modification of a previously described method.^{27,28} The growth of MRSA was examined at 37°C in 0.95 mL of BHI broth containing various concentrations of the *C. obtusa*. These tubes were inoculated with 0.05 mL of an overnight culture grown in

the BHI broth (final: 5×10^5 colony-forming units [CFU]/mL) and incubated at 37°C. After 24 h of incubation, the optical density (OD) of cells was measured spectrophotometrically at 550 nm, and the pH of the cultures was determined using a pH meter (Corning, Inc., Corning, NY, USA). Three replicates were measured for each concentration of the test extract. NaF (0.1%) was used as a positive control.

Biofilm assay

The biofilm assay was based on a method described previously.^{29,30} *C. obtusa* extract was added to the BHI broth containing 1% glucose in 35-mm polystyrene dishes or 24-well plates (Nunc, Copenhagen, Denmark). The cultures were then inoculated with a seed culture of MRSA (final: 5×10^5 CFU/mL). After cultivating for 48 h at 37°C, the supernatant was completely removed, and the dishes, wells, or wells containing resin teeth were rinsed with distilled water. The amount of biofilm formed in the wells was measured by staining with 0.1% safranin. The bound safranin was released from the stained cells with 30% acetic acid and the absorbance of the solution was measured at 530 nm. The biofilm formed on the surface of the resin teeth was also stained with 0.1% safranin and photographed.

Scanning electron microscopy

The biofilm on 35-mm polystyrene dishes was also determined by scanning electron microscopy (SEM) using a modification of a previously described method.³¹ The biofilm formed on the dishes was rinsed with distilled H₂O and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 4°C for 24 h. After gradual dehydration with ethyl alcohol (60%, 70%, 80%, 90%, 95%, and 100%), the sample was freeze dried. The specimens were then sputter coated with gold (108A sputter coater; Cressington Scientific Instruments, Inc., Watford, England, United Kingdom). For observation, a JSM-6360 SEM (JEOL, Tokyo, Japan) was used.

Bactericidal effect

The bactericidal effect of *C. obtusa* EO was determined by confocal laser scanning microscopy. The cultured MRSA in BHI was diluted using BHI media to $\sim 1 \times 10^7$ CFU/mL. The bacteria (1×10^7 CFU/mL) were treated with high concentrations (0.2–1.6 mg/mL) of *C. obtusa* EO at 37°C under aerobic conditions. After 30 min of incubation, the bacteria were washed with PBS and stained with the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Eugene, OR, USA), prepared according to the manufacturer's instructions, for 15 min. Stained bacteria were observed with confocal laser scanning microscopy (LSM 510; Zeiss, Oberkochen Germany). This method is based on two nucleic acid stains: green fluorescent SYTO9 stain and red fluorescent propidium iodide stain, which differ in their ability to penetrate healthy bacterial cells. The SYTO9 stain labels live bacteria, in contrast to propidium iodide, which penetrates only bacteria with damaged membranes.

TABLE 1. NUCLEOTIDE SEQUENCES OF PRIMER USED FOR REAL-TIME PCR IN THIS STUDY

Gene ^a	Gene description	Primer sequences (5'-3')	
<i>16srRNA</i>	Normalizing internal standard	Forward	ACTGGGATAACTTCGGGAAA
		Reverse	CGTTGCCTTGGTAAGCC
<i>Sea</i>	Staphylococcal enterotoxin A	Forward	ATGGTGCTTATTATGGTTATC
		Reverse	CGTTTCCAAAGGTACTGTATT
<i>agrA</i>	Accessory gene regulator A	Forward	TGATAATCCTTATGAGGTGCTT
		Reverse	CACTGTGACTCGTAACGAAAA
<i>sarA</i>	Staphylococcal accessory regulator A	Forward	TGTTATCAATGGTCACTTATGCTG
		Reverse	TCTTTGTTTTCGCTGATGTATGTC

^aBased on the NCBI MRSA genome database.

PCR, polymerase chain reaction.

Real-time polymerase chain reaction analyses

To determine the effect of *C. obtusa* EO on gene expression, a real-time PCR assay was performed. The sub-minimal inhibitory concentration (0.1–0.3 mg/mL) of *C. obtusa* EO was used to treat and culture MRSA for 24 h. Total RNA was isolated from MRSA by using the TRIzol reagent (Gibco-BRL, Gaithersburg, MD, USA) according to the manufacturer's instructions. Then, cDNA was synthesized using a reverse transcriptase reaction (Superscript; Gibco-BRL). The DNA amplifications were carried out using an ABI-Prism 7,000 Sequence Detection System with Absolute QPCR SYBRGreen Mixes (Applied Bio systems, Inc., Foster City, CA, USA). The primer pairs that were used in this study were described by previous reports^{8,12,32} and are listed in Table 1. The 16S rRNA was used as an internal control.

GC and GC-MS analysis

GC analysis was performed on a Hewlett-Packard model 6890 series gas chromatograph equipped with a flame ionization detector, and a split ratio of 1:30 using DB-5HT fused silica capillary column (30 m × 0.32 mm, i.d., 0.10 µm film thickness). The column temperature was programmed from 40°C to 230°C, increased 2°C/min, and then kept constant at 230°C for 20 min. The injector and detector temperatures were 230°C and 250°C, respectively. The carrier gas was nitrogen, at a flow rate of 0.80 mL/min. Peak areas were measured by electronic integration and the relative amounts of the individual components are based on the peak areas. The GC-MS analysis was performed on an Agilent Technologies 7890A GC and 5975C mass selective detector operating in the EI mode at 70 eV, fitted with a DB-5MS fused silica capillary column (30 m × 0.25 mm, i.d., 0.25 µm film thickness). The column temperature was programmed from 40°C to 230°C at 2°C/min and then kept constant at 230°C for 20 min. The injector and ion source temperatures were 250°C, respectively. The carrier gas was helium at a flow rate of 1.0 mL/min. The identification of individual components was based on comparisons with Wiley 7n and NIST 05 mass spectra libraries and retention indices with literature data.³³ Linear retention indices were calculated against those of n-paraffin (C₆–C₂₆) series.³⁴

Statistical analysis

All experiments were performed in triplicate. Data were analyzed using SPSS software 12.0 (Chicago, IL, USA). The data are expressed as the mean ± standard deviation values. The differences between the means were analyzed for statistical significance using the Student's *t*-test. The level of significance was set at *P* < .05.

RESULTS

Bacterial growth inhibition by *C. obtusa* EO

After extraction of EO from the leaves of *C. obtusa* by hydrodistillation, the antibacterial activity of the oil was tested against MRSA. *C. obtusa* EO significantly inhibited the growth of MRSA in a concentration-dependent manner at 0.1–0.4 mg/mL. The positive control (NaF) also showed antibacterial activity at 0.1% (Fig. 1).

Inhibitory effect of *C. obtusa* EO on acid production

To investigate whether *C. obtusa* EO can inhibit MRSA organic acid production, changes in pH were measured

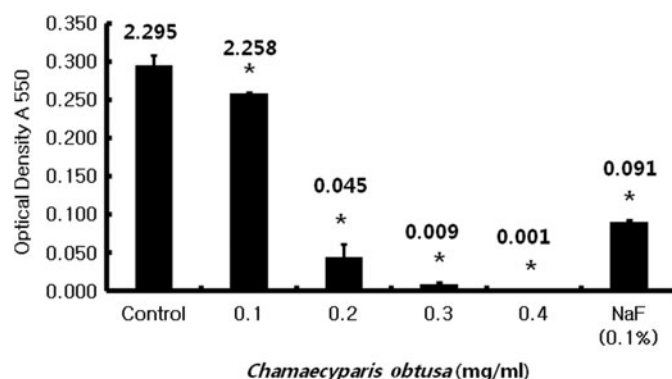


FIG. 1. Bacterial growth inhibition by *Chamaecyparis obtusa* essential oil (EO). Methicillin-resistant *Staphylococcus aureus* (MRSA) was inoculated into brain–heart infusion (BHI) broth with various concentrations of *C. obtusa* and incubated for 24 h at 37°C. The optical density (A550) was read using a spectrophotometer. Data are mean ± standard deviation. **P* < .05 compared to the control group.

TABLE 2. INHIBITORY EFFECT OF *C. obtusa* ESSENTIAL OIL ON ACID PRODUCTION

Concentration (mg/mL)	pH (after incubation)
Control	5.91 ± 0.01 ^a
0.1	5.90 ± 0.01
0.2	7.10 ± 0.01*
0.3	7.26 ± 0.01*
0.4	7.30 ± 0.00*
NaF (0.1%)	7.00 ± 0.01*

^aData (pH) are represented as mean ± standard deviation.

**P* < .05 when compared with the control group after incubation.

after the addition of *C. obtusa* EO to the MRSA culture medium. The pH of the control declined to ~5.91 after bacterial culture, while the initial pH of the media before bacterial culture was 7.3. However, the addition of 0.2, 0.3, and 0.4 mg/mL of *C. obtusa* EO resulted in pH levels of 7.10, 7.26, and 7.30, respectively, indicating inhibition of acid production. NaF (0.1%) used for the positive control also inhibited acid production, resulting in a pH of 7.00 (Table 2).

Inhibitory effect of *C. obtusa* EO on biofilm formation

MRSA can increase antibiotic resistance by forming a biofilm on the implant, medical supplies, or medical device. Biofilm formation was studied using safranin staining, and absorbance was measured at 530 nm. The negative control showed an OD of 2.3, whereas experimental groups at 0.1–0.4 mg/mL *C. obtusa* EO showed ODs of 2.1, 1.2, 0.3, and 0.1, respectively. The positive control of 0.1% NaF resulted in an OD of 0.4 (Fig. 2). Safranin staining results were consistent with those of SEM (Fig. 3).

Bactericidal effect of *C. obtusa* EO

The bactericidal effect of *C. obtusa* EO was also observed using confocal laser microscopy, and a concentration-dependent effect was observed (Fig. 4).

Inhibitory effect of *C. obtusa* EO on virulence factor gene expression

mRNA expression of *sea*, *agrA*, and *sarA*, which are virulence factor genes, was observed by real-time PCR. *agrA* mRNA expression was inhibited at concentrations greater than 0.2 mg/mL of *C. obtusa* EO, whereas the expression of *sea* and *sarA* was inhibited at concentrations of 0.3 mg/mL (Fig. 5).

GC and GC-MS analysis of *C. obtusa* EO

Analysis of *C. obtusa* EO by GC and GC-MS identified 59 constituents, accounting up to 98.99% of the total EO. All unidentified compounds were minor components. The major components included sabinene (19.06%), α -terpinyl acetate (16.99%), bornyl acetate (10.48%), limonene (8.54%), myrcene (5.86%), γ -terpinene (4.04%), and hi-baene (3.01%) (Table 3).

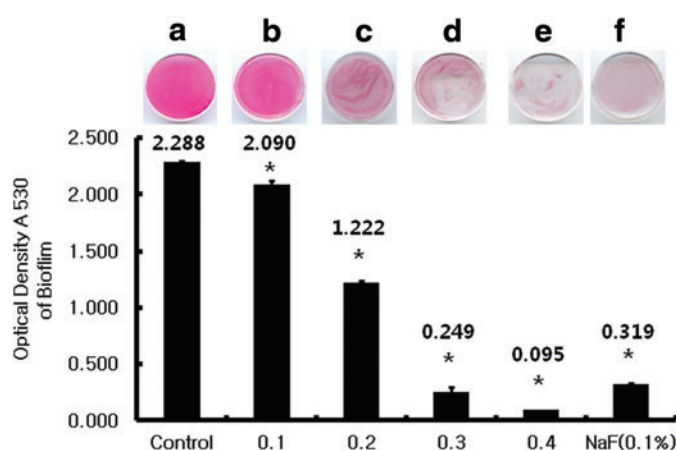


FIG. 2. Inhibitory effect of *C. obtusa* EO on acid production. MRSA was inoculated into BHI broth with various concentrations of *C. obtusa* and incubated for 48 h at 37°C. The biofilms that formed on the dish surface were measured by staining with 0.1% safranin. The bound safranin was released from the stained cells with 30% acetic acid. Data are represented as mean ± standard deviation. **P* < .05 compared to the control group. (a) Control; (b) 0.1 mg/mL; (c) 0.2 mg/mL; (d) 0.3 mg/mL; (e) 0.4 mg/mL; EO of *C. obtusa* (f)-positive control (0.1% NaF). Color images available online at www.liebertpub.com/jmf

DISCUSSION

MRSA is one of the most well-known antibiotic-resistant bacteria. Most of the β -lactam antibiotics, including penicillin, cephalosporin, carbapenem, and monobactam, are ineffective in the treatment of MRSA infection. MRSA also has multidrug resistance to various antibacterial agents, including aminoglycosides, and thus existing drugs effective against MRSA are quite limited.

Fast detection and accurate identification of methicillin-resistant bacteria are essential,^{34,35} as they can cause serious adverse effects, including increased mortality in critically ill patients with conditions such as immunodeficiency. Novel antibiotics are urgently required to treat those bacteria, and natural substances are being studied for antibacterial activity. *C. obtusa* EO has been used in spices, pesticides, and aromatics. It has also been used in Korea as a traditional herbal medicine for treating headache due to cold, leukorrhea, cloudy urine, skin disease, warts, and bleeding,³⁶ and also as an alternative to *Thuja orientalis* L.³⁷ Many studies have examined the antimicrobial and antifungal characteristics of EO as well as their constituents.³⁸ Thus, antimicrobial activities against *Listeria monocytogenes*, *S. aureus*, *Legionella anisa*, and *Candida albicans* were reported.³⁹ Terpinen-4-ol has been shown to be a general antimicrobial constituent, and α -terpineol and α -pinene have shown inhibitory activity against microbial growth.⁴⁰ The main constituents of *C. obtusa* EO analyzed in this study were sabinene (19.06%), a component of the phytoncide. Cypress oils contain about 10% phytoncide and there are also antibacterial agents that prevent asthma caused by *Alternaria alternata*⁴¹; α -terpinyl acetate (16.99%), which is may be important for the typical aroma of the oil; and bornyl acetate,¹⁹ which is found most abundantly in *C. obtusa* EO.

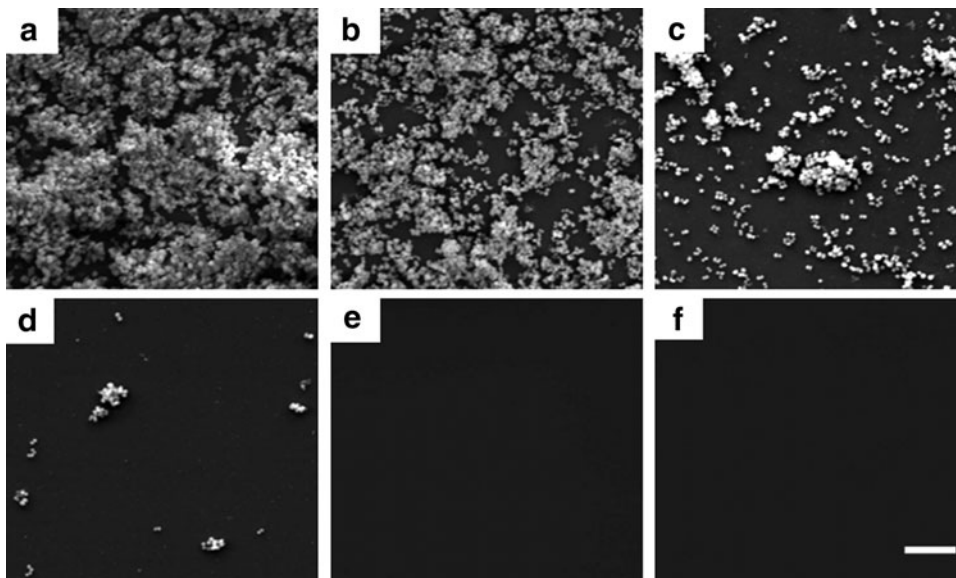


FIG. 3. Inhibitory effect of *C. obtusa* EO on biofilm formation. Scanning electron microscopy of MRSA biofilms grown in *C. obtusa* (a) control; (b) 0.1 mg/mL; (c) 0.2 mg/mL; (d) 0.3 mg/mL; (e) 0.4 mg/mL; (f) positive control (0.1% NaF); scale bar = 10 μ m.

Many natural EOs containing terpenes as major constituents have been found to be active against a variety of microorganisms. The mechanism of antimicrobial action of terpenes is closely associated with their lipophilic character. Monoterpenes preferentially influence membrane structures that increase membrane fluidity and permeability, changing the topology of membrane proteins and inducing disturbances in the respiration chain.⁴² Recently, it was proposed that the antibacterial activity against *S. aureus* depends on the length of the aliphatic chains of terpene alcohols and the presence of double bonds.⁴³

Several studies have suggested that MRSA produces organic acid through carbohydrate metabolism.^{44,45} The main organic acid produced by MRSA is acetic acid, which lowers

the pH in the area of infection (Table 2), and this decreased pH is known to promote biofilm formation.^{6,7,44} Biofilms are a type of bacterial community that is formed on the surface of a living organism or inorganic substance. Biofilms are surrounded by a self-produced extracellular matrix consisting of polysaccharides and proteins. Once formed, it is very difficult to remove the biofilm, and biofilms formed on the surface of implanted medical devices cannot be eliminated using antibiotics alone. The treatment involves long-term administration of antibiotics after removal of the implanted medical device and surgical excision of the tissues surrounding the biofilm.^{6,7} The most commonly known method for measuring biofilm formation is the tissue culture plate assay method.⁸ In this study, biofilm formation was observed by this method

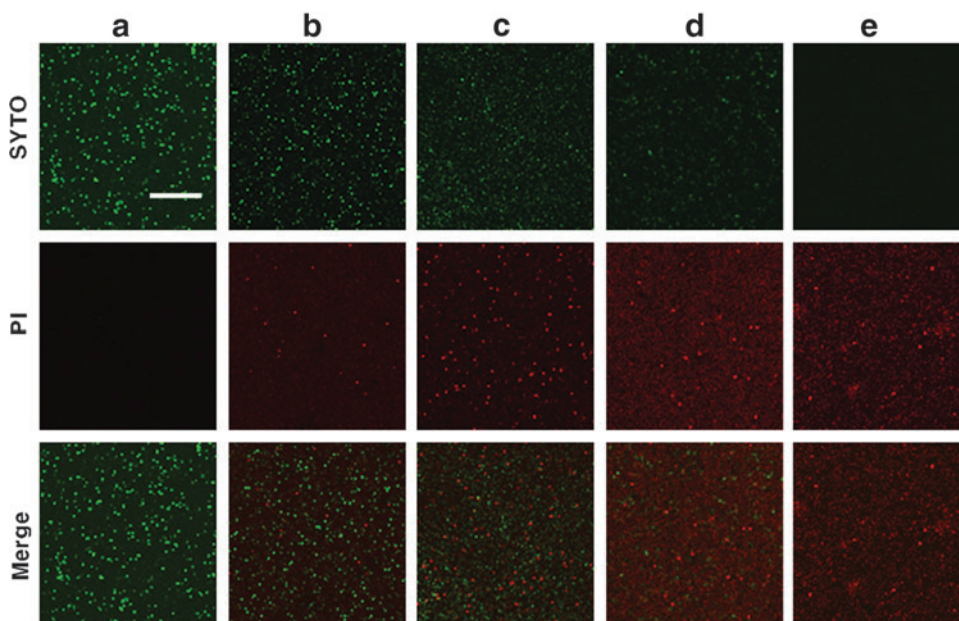


FIG. 4. Bactericidal effect of *C. obtusa* EO. Cultured MRSA was treated with high concentration (0.4–3.2 mg/mL) of *C. obtusa* extract and stained with LIVE/DEAD BacLight Bacterial Viability Kit. The stained bacteria were observed by confocal laser scanning microscopy. Treatment with *C. obtusa* decreased green-labeled living bacteria (SYTO 9 stain) and increased red-labeled dead bacteria (PI stain) in a dose-dependent manner. (a) Control; (b) 0.4 mg/mL; (c) 0.8 mg/mL; (d) 1.6 mg/mL; (e) 3.6 mg/mL; scale bar = 50 μ m.

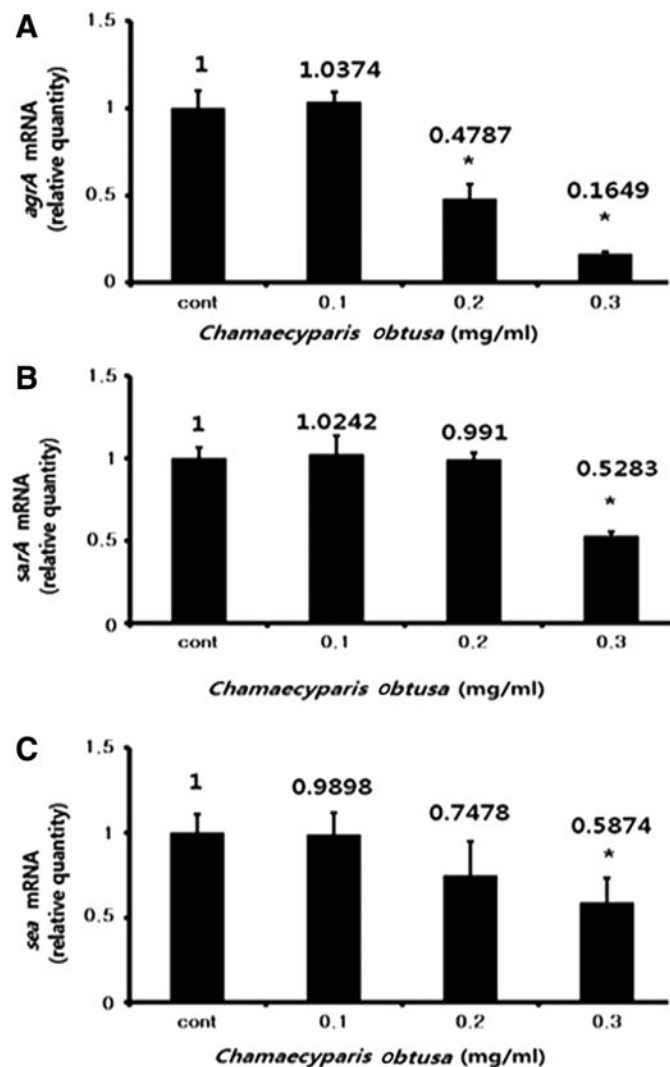


FIG. 5. Inhibitory effect of *C. obtusa* EO on virulence factor gene expression. MRSA was cultured and treated with subminimal inhibitory concentration (0.1–0.3 mg/mL) of *C. obtusa* extract, and real-time polymerase chain reaction (PCR) analysis was then performed as described in the Materials and Methods section. *agrA* expression was significantly inhibited at 0.2 mg/mL of *C. obtusa*, and *sarA* and *sea* expression was significantly inhibited at a concentration higher than 0.3 mg/mL. Each value is expressed as mean \pm standard deviation. *Significance was determined at $P < .05$ when compared with the control.

through safranin staining, and *C. obtusa* inhibited MRSA biofilm formation at concentrations greater than 0.1 mg/mL (Fig. 2). SEM analysis results were also similar to those observed by safranin staining (Fig. 3). According to previous studies, physiological characteristics differ between biofilm culture and planktonic culture. Biofilm formation has been reported to increase the bacterial host immune system and resistance to antibacterial substances.⁴⁶ However, a comparison of biofilm culture and planktonic culture in this study showed no differences in resistance to *C. obtusa* EO, which may be due to the action of the oil before biofilm formation.

It has been shown that, with respect to the mechanism of MRSA resistance, innate chromosomal acquisition of the

TABLE 3. GC AND GC-MS ANALYSIS OF THE ESSENTIAL OIL ISOLATED FROM *CHAMAECYPARIS OBTUSA*

RRT ^a	Compound	Retention index ^b	Peak area (%)
0.77	Tricyclene	917	0.09
0.79	α -Thujene	924	0.45
0.81	α -Pinene	930	1.96
0.85	Camphene	952	0.56
1.00	Sabinene	985	19.06
1.01	β -Pinene	986	0.05
1.06	Myrcene	995	5.86
1.08	α -Phellandrene	1007	0.09
1.14	α -Terpinene	1018	1.03
1.20	Limonene	1031	8.54
1.35	γ -Terpinene	1064	4.04
1.37	<i>cis</i> -Sabinene hydrate	1068	0.19
1.47	α -Terpinolene	1090	1.26
1.51	Dehydro- <i>p</i> -cymene	1098	0.11
1.54	Linalool	1102	0.05
1.61	<i>trans</i> -Sabinene hydrate	1109	0.11
1.63	1-Octen-3-yl acetate	1115	0.22
1.72	<i>trans</i> - <i>p</i> -2-Menthen-1-ol	1137	0.12
1.96	Terpinen-4-ol	1182	2.83
2.02	α -Terpineol	1194	0.24
2.14	Fenchyl acetate	1216	0.06
2.37	Linalyl acetate	1259	0.20
2.57	Bornyl acetate	1297	10.48
2.93	α -Terpinyl acetate	1364	16.99
2.98	Geranyl acetate	1374	0.05
3.08	β -Elemene	1392	0.11
3.16	α -Cedrene	1406	0.06
3.19	β -Cedrene	1414	0.13
3.28	<i>cis</i> -Thujopsene	1429	1.75
3.35	β -Gurjunene	1444	0.70
3.37	α -Humulene	1447	0.06
3.44	<i>cis</i> -Muurolo-4(14),5-diene	1460	1.62
3.49	β -Cadinene	1469	0.21
3.54	Germacrene D	1479	0.17
3.61	β -Hamachalene	1492	0.54
3.62	α -Chamigrene	1494	0.45
3.68	γ -Cadinene	1506	0.13
3.75	δ -Cadinene	1521	0.60
3.77	Cadina-1,4-diene	1526	0.38
3.80	Selina-3,7(11)-diene	1531	0.10
3.96	Elemol	1565	7.47
4.02	Germacrene D-4-ol	1579	0.15
4.04	Caryophyllene oxide	1584	0.12
4.12	Cedrol	1601	0.82
4.18	1,10-Di- <i>epi</i> -cubenol	1612	0.18
4.20	10- <i>epi</i> - γ -Eudesmol	1617	0.10
4.24	1- <i>epi</i> -Cubenol	1624	0.10
4.28	γ -Eudesmol	1633	0.90
4.30	τ -Cadinol	1640	0.20
4.36	α -Eudesmol	1652	1.88
4.44	Bulnesol	1668	0.44
4.51	α -Bisabolol	1682	0.11
5.40	Rimuene	1885	0.57
5.54	Hibaene	1919	3.01
5.64	Pimaradiene	1934	0.19
5.74	13-Isopimaradiene	1972	0.10
5.80	Dolabradene	1987	0.36
5.91	Phyllocladene	2016	0.05
6.94	<i>cis</i> -Totarol	2274	0.05
Total			98.99

^aAnalysis was performed only once, and RRT refers to relative retention time for each compound and a major constituent, sabinene is given a value of 1.00.

^bRetention index on DB-5 HT column.

GC, gas chromatography; GC-MS, GC coupled to mass spectrometry.

mecA (methicillin resistance determinant) gene induces production of PBP 2', a cell wall-synthesizing protein that has a low affinity for drugs. PBP is also involved in bacterial cell wall synthesis and exhibits antibacterial activity through β -lactam binding. MRSA is known to convert PBP into PBP 2' and thus prevents drugs from functioning.⁴⁷ *sea*, one of the major virulence factors of *S. aureus*,¹¹ causes staphylococcal gastroenteritis and also stimulates T-cell activation and cytokine secretion through superantigen immunomodulatory activity. Expression of *sea* was also inhibited by *C. obtusa* EO at concentrations of 0.3 mg/mL (Fig. 5).

The production of virulence factors in *S. aureus* is regulated by global regulators such as *agrA* and *sarA*.¹² *agrA* encodes accessory gene regulator A, and when *agrA* expression is inhibited, the production of virulence factors is inhibited as well. *sarA* regulates the production of several matrix adhesion genes (e.g., *fnbA*), which are virulence factors linked to the adhesion of *S. aureus*, and several exotoxin genes (e.g., *hla*).^{48,49} Expression of *agrA* in this study was significantly inhibited by *C. obtusa* EO at concentrations greater than 0.2 mg/mL, and expression of *sarA* was significantly inhibited by *C. obtusa* EO at concentrations of 0.3 mg/mL, respectively (Fig. 5), which implies antibacterial activity through inhibition of the production of MRSA adhesion and virulence factors.

In conclusion, these results suggest that *C. obtusa* EO may exhibit antibacterial activity against MRSA through inhibition of virulence factor expression, which may be associated with the major components of *C. obtusa* EO, such as sabinene, α -terpinyl acetate, bornyl acetate, limonene, elemol, myrcene, γ -terpinene, and hibaene. Further studies are needed to determine the mechanism of virulence factor gene expression inhibition by *C. obtusa* EO.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

REFERENCES

1. Miladinović DL, Ilić BS, Mihajilov-Krstev TM, *et al.*: Antibacterial activity of the essential oil of *Heracleum sibiricum*. *Nat Prod Commun* 2013;8:1309–1311.
2. Salazar AT, Hoheisel J, Youns M, Wink M: Anti-inflammatory and anti-cancer activities of essential oils and their biological constituents. *Int J Clin Pharmacol Ther* 2011;49:93–95.
3. Ascari J, Sens SL, Nunes DS, *et al.*: Sedative effects of essential oils obtained from *Baccharis uncinella*. *Pharm Biol* 2012;50:113–119.
4. Slamenova D, Horvathova E: Cytotoxic, anti-carcinogenic and antioxidant properties of the most frequent plant volatiles. *Neoplasma* 2013;60:343–354.
5. Lopes CLM, Gonçalves SáC, de Almeida AA, *et al.*: Sedative, anxiolytic and antidepressant activities of *Citrus limon* (Burn) essential oil in mice. *Pharmazie* 2011;66:623–627.
6. Du WX, Olsen CW, Avena-Bustillos RJ, Friedman M, McHughm TH: Physical and antibacterial properties of edible films formulated with apple skin polyphenols. *J Food Sci* 2011;76:149–155.
7. Quave CL, Plano LR, Pantuso T, Bennett BC: Effects of extracts from Italian medicinal plants on planktonic growth, biofilm formation and adherence of methicillin-resistant *Staphylococcus aureus*. *J Ethnopharmacol* 2008;118:418–428.
8. Conrady DG, Brescia CC, Hori K, Weiss AA, Hassett DJ, Herr AB: A zinc-dependent adhesion module is responsible for intercellular adhesion in staphylococcal biofilms. *Proc Natl Acad Sci USA* 2008;105:19456–19461.
9. Jia P, Xue YJ, Duan XJ, Shao SH: Effect of cinnamaldehyde on biofilm formation and *sarA* expression by methicillin-resistant *Staphylococcus aureus*. *Lett Appl Microbiol* 2011;53:409–416.
10. Ortega E, Abriouel H, Lucas R, Gálvez A: Multiple roles of *Staphylococcus aureus* enterotoxins: Pathogenicity, superantigenic activity, and correlation to antibiotic resistance. *Toxins* 2010;2:2117–2131.
11. Becker K, Friedrich AW, Lubritz G, Weilert M, Peters G, VonEiff C: Prevalence of genes encoding pyrogenic toxin superantigens and exfoliative toxins among strains of *Staphylococcus aureus* isolated from blood and nasal specimens. *J Clin Microbiol* 2003;41:1434–1439.
12. Qiu J, Wang D, Xiang H, *et al.*: Subinhibitory concentrations of thymol reduce enterotoxins A and B and alpha-hemolysin production in *Staphylococcus aureus* isolates. *PLoS One* 2010;5:e9736.
13. Qiu J, Zhang X, Luo M, *et al.*: Subinhibitory concentrations of perilla oil affect the expression of secreted virulence factor genes in *Staphylococcus aureus*. *PLoS One* 2011;6:16160.
14. Faizi S, Mughal NR, Khan RA, *et al.*: Evaluation of the antimicrobial property of *Polyalthia iongifolia* var. *Pendula*: Isolation of a lactone as the active antibacterial agent from the ethanol extract of the stem. *Phytother Res* 2003;17:1177–1181.
15. Gibbons S, Leimkugel J, Oluwatuyi M, Heinrich M: Activity of *Zanthoxylum clava-herculis* extracts against multidrug resistant methicillin-resistant *Staphylococcus aureus* (mdr-MRSA). *Phytother Res* 2003;17:274–275.
16. Zhang YM, Xu J, Xiao L, Zeng GZ, Sun ZH, Tan NH: A new phenolic glycoside from *Chamaecyparis obtusa* var. *breviramea* f. *crippsii*. *Molecules* 2013;18:1255–1261.
17. Kuo YH, Chen CH, Wein YS: New dimeric monoterpenes and dimeric diterpenes from the heartwood of *Chamaecyparis obtusa* var. *formosana*. *Helv Chim Acta* 2002;85:2657–2663.
18. Zhang YM, Tan NH, Lu Y, Yang EH, Jia RR: Chamobtusin A, a novel skeleton diterpenoid alkaloid from *Chamaecyparis obtusa* cv. *Tetragon*. *Org Lett* 2007;9:4579–4581.
19. Takaku N, Mikame K, Okunishi T, Suzuki S, Umezawa T, Shimada M: Lignans of *Chamaecyparis obtusa*. *J Wood Sci* 2001;47:493–496.
20. Yang JK, Choi MS, Seo WT, Rinker DL, Han SW, Cheong GW: Chemical composition and antimicrobial activity of *Chamaecyparis obtusa* leaf essential oil. *Fitoterapia* 2007;78:149–152.
21. Kuo YH, Chen CH, Chiang YM: Three novel and one new lignan, chamaecyparones A, bobtulignolide and isootobanone from the heartwood of *Chamaecyparis obtusa* var. *formosana*. *Tetrahedron Lett* 2001;42:6731–6735.

22. Chien TC, Lo SE, Ho CL: Chemical composition and anti-inflammatory activity of *Chamaecyparis obtusa* f. *formosana* wood essential oil from Taiwan. *Nat Prod Commun* 2014;9:723–726.
23. Park HJ, Kim SK, Kang WS, Woo JM, Kim JW: Effects of essential oil from *Chamaecyparis obtusa* on cytokine genes in the hippocampus of maternal separation rats. *Can J Physiol Pharmacol* 2014;92:95–101.
24. Bajpai VK, Sharma A, Baek KH: Antibacterial mode of action of the essential oil obtained from *Chamaecyparis obtusa* sawdust on the membrane integrity of selected foodborne pathogens. *Food Technol Biotechnol* 2014;52:109–118.
25. Park YO, Kim SE, Kim YC: Action mechanism of *Chamaecyparis obtusa* oil on hair growth. *Toxicol Res* 2013;29:241–247.
26. Bajpai VK, Sharma A, Kim SH: Phenolic content and antioxidant capacity of essential oil obtained from sawdust of *Chamaecyparis obtusa* by microwave-assisted hydrodistillation. *Food Technol Biotechnol* 2014;51:360–369.
27. Kim SJ, Gao Zhang C, Lim JT: Mechanism of anti-nociceptive effects of *Asarum sieboldii* Miq. radix: Potential role of bradykinin, histamine and opioid receptor-mediated pathways. *J Ethnopharmacol* 2003;88:5–9.
28. Matsumoto M, Minami T, Sasaki H, Sobue S, Hamada S, Ooshima T: Inhibitory effects of oolong tea extract on caries-inducing properties of mutans streptococci. *Caries Res* 1999;33:441–445.
29. O'Neill E, Humphreys H, O'Gara JP: Carriage of both the *fnbA* and *fnbB* genes and growth at 37 degrees C promote FnBP-mediated biofilm development in methicillin-resistant *Staphylococcus aureus* clinical isolates. *J Med Microbiol* 2009;58:399–402.
30. Petersen FC, Pecharki D, Scheie AA: Biofilm mode of growth of *Streptococcus intermedius* favored by a competence-stimulating signaling peptide. *J Bacteriol* 2004;186:6327–6331.
31. Nakamiya K, Hashimoto S, Ito H, Edmonds JS, Yasuhara A, Morita M: Microbial treatment of bis (2-ethylhexyl) phthalate in polyvinyl chloride with isolated bacteria. *J Biosci Bioeng* 2005;99:118–119.
32. Rosato AE, Craig WA, Archer GL: Quantitation of *mecA* transcription in oxacillin-resistant *Staphylococcus aureus* clinical isolates. *J Bacteriol* 2003;185:3446–3452.
33. Adams RP: *Identification of Essential Oil Components by Gas Chromatography/Mass Spectroscopy*. Allured Publishing Corporation, Carol Stream, IL, 1995, pp. 1–469.
34. Van den Dool H, Kratz PD: A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. *J Chromatogr* 1963;11:463–471.
35. Hartman BJ, Tomasz A: Expression of methicillin resistance in heterogeneous strains of *Staphylococcus aureus*. *Antimicrob Agents Chemother* 1986;29:85–92.
36. Thornsberry C, McDougal LK: Successful use of broth microdilution in susceptibility tests for methicillin-resistant (hetero-resistant) staphylococci. *J Clin Microbiol* 1983;18:1084–1091.
37. Chinese Materia Medica Editorial Committee in State Administration of Traditional Chinese Medicine. *Chinese Materia Medica (Chinese herbology)*, Vol. 2. Shanghai Scientific and Technical Publishers, Shanghai, China, 1999, pp. 319.
38. Ju YS, Kim HJ: *UnGok Comprehensive Bibliography of Origin and Producing Place of Medicinal Herbs*. Korean Studies Information, Paju, Gyeonggi, Korea, 2009, pp. 171.
39. Kalembe D, Kunicka A: Antibacterial and antifungal properties of essential oils. *Curr Med Chem* 2003;10:813–829.
40. Hong EJ, Na KJ, Choi IG, Choi KC, Jeung EB: Antibacterial and antifungal effects of essential oils from coniferous trees. *Biol Pharm Bull* 2004;27:863–866.
41. Raman A, Weir U, Bloomfield SF: Antimicrobial effects of tea-tree oil and its major components on *Staphylococcus aureus*, *Staph. epidermidis* and *Propionibacterium acnes*. *Lett Appl Microbiol* 1995;21:242–245.
42. Paduch R, Kandefer-zerszeń M, Trytek M, Fiedurek J: Terpenes: Substances useful in human healthcare. *Arch Immunol Ther Exp* 2007;55:315–327.
43. Inoue Y, Shiraishi A, Hada T, Hirose K, Hamashima H, Shimada J: The antibacterial effects of terpene alcohols on *Staphylococcus aureus* and their mode of action. *FEMS Microbiol Lett* 2004;237:325–331.
44. Kohzaki K, Gomi K, Kokudo YY, Ozawa R, Takabayashi J, Akimitsu K: Characterization of a sabinene synthase gene from rough lemon (*Citrus jambhiri*). *J Plant Physiol* 2009;166:1700–1704.
45. Manetti AG, Köller T, Becherelli M, et al.: Environmental acidification drives *S. pyogenes* pilus expression and microcolony formation on epithelial cells in a FCT-dependent manner. *PLoS One* 2010;5:e13864.
46. Rode TM, Møretrø T, Langsrud S, Langsrud O, Vogt G, Holck A: Responses of *Staphylococcus aureus* exposed to HCL and organic acid stress. *Can J Microbiol* 2010;56:777–792.
47. Yoshida A, Kuramitsu HK: Multiple *Streptococcus mutans* genes are involved in biofilm formation. *Appl Environ Microbiol* 2002;68:6283–6291.
48. Fuda CC, Fisher JF, Mobashery S: Beta-lactam resistance in *Staphylococcus aureus*: The adaptive resistance of a plastic genome. *Cell Mol Life Sci* 2005;62:2617–2633.
49. Kupferwasser LI, Yeaman MR, Nast CC, et al.: Salicylic acid attenuates virulence in endovascular infections by targeting global regulatory pathways in *Staphylococcus aureus*. *J Clin Invest* 2003;112:222–233.