Research Article

Liang Peng, Yunhao Xiong, Mei Wang, Manman Han, Weilan Cai, Zhimin Li*

Chemical composition of essential oil in *Mosla chinensis* Maxim cv. Jiangxiangru and its inhibitory effect on *Staphylococcus aureus* biofilm formation

Abstract: The essential oil of *Mosla chinensis* Maxim cv. Jiangxiangru is known for its antibacterial ability. This study aimed to investigate the chemical composition of Jiangxiangru essential oil and its inhibitory effect on *Staphylococcus aureus* biofilm formation. Gas chromatography/mass spectrometry (GC–MS) was used to determine the chemical composition of Jiangxiangru essential oil. Subsequently, the eight major chemical components were quantitatively analyzed using GC–MS, and their minimum inhibitory concentration (MIC) values against *S. aureus* were tested. Biofilm formation was detected by crystal violet semi-quantitative method and silver staining. Of the 59 peaks detected, 29 were identified by GC–MS. Of these peaks, thymol, carvacrol, *p*-cymene, γ-terpinene, thymol acetate, α-caryophyllene, 3-carene, and carvacryl acetate were present at a relatively higher concentration. The results of the quantitative test showed that thymol, carvacrol, *p*-cymene, and γ-terpinene were the major components of the essential oil. Among the eight reference substances, only thymol, carvacrol, and thymol acetate had lower MICs compared with the essential oil. Essential oil, carvacrol, carvacryl acetate, α-caryophyllene, and 3-carene showed the better inhibition of *S. aureus* biofilm formation. When one fourth of the MIC concentrations were used for these substances (0.0625 mg/mL for essential oil, 0.0305 mg/mL for carvacrol, 1.458 mg/mL for carvacryl acetate, 0.1268 mg/mL for α-caryophyllene, and 2.5975 mg/mL for 3-carene), the inhibition rates were over 80%. However, thymol, γ-terpinene, thymol acetate, and *p*-cymene showed a relatively poor inhibition of *S. aureus* biofilm formation. When 1× MIC concentrations of these substances were used, the inhibition rates were less than 50%. In conclusion, Jiangxiangru essential oil and its major components, carvacrol, carvacryl acetate, α-caryophyllene, and 3-carene, strongly inhibited biofilm formation in *S. aureus*.

Keywords: *Mosla chinensis* Maxim cv. Jiangxiangru; essential oil; GC–MS analysis; *Staphylococcus aureus*; biofilms; antibacterial ability

1 Introduction

Staphylococcus aureus is an important foodborne pathogen and one of the most common species responsible for nosocomial infection [1-3]. The incidence of nosocomial infections caused by *S. aureus* has increased in recent years. Multiple *S. aureus* mutants obtained as clinical isolates have been found to be resistant to a majority of antibacterial agents, including macrolides, quinolones, and β-lactams [4-7]. Also, the resistance to methicillin and β-lactam has shown an annual increasing trend [8, 9]. *S. aureus* employs various mechanisms to resist the killing and inhibitory effects of antibiotics, such as enzymatic inactivation of the antibiotic (penicillinase and aminoglycoside-modifying enzymes), altering the target by reducing its decreased affinity to the antibiotic (e.g., penicillin-binding protein 2a of methicillin-resistant *S. aureus*, and d-Ala-d-Lac of peptidoglycan precursors of vancomycin-resistant strains), trapping antibiotics within the cell (vancomycin) [10, 11], and using bacterial biofilms (BBFs).

BBFs are defined as an aggregation of bacteria adherent to an inert contact surface embedded within a self-produced slimy extracellular matrix composed of

*Corresponding author: Zhimin Li, Jiangxi Provincial Key Laboratory of Drug Design and Evaluation, School of Pharmacy, Jiangxi Science & Technology Normal University, Nanchang 330013,P.R. China, E-mail: lzmin3026@163.com
Liang Peng, Yunhao Xiong, Mei Wang, Manman Han, Weilan Cai, Jiangxi Provincial Key Laboratory of Drug Design and Evaluation, School of Pharmacy, Jiangxi Science & Technology Normal University, Nanchang 330013,P.R. China

polysaccharides, proteins, and other polymers. Biofilms are involved in multiple infections and often contribute significantly to the difficulties encountered in treatment [3]. Staphylococci are commensals on the skin, therefore mucous surfaces are most likely to infect medical devices that penetrate the skin or these surfaces. The presence of *S. aureus* biofilms pose difficulties in eradicating *S. aureus* infection in patients because the biofilms protect bacteria from the effects of both the host immune system and antibacterial drugs [12]. A previous study demonstrated that staphylococci residing in biofilms exhibit increased mutability, accelerating the emergence of heritable antibiotic resistance through spontaneous mutations [13]. Another study demonstrated that *S. aureus* biofilms dramatically increased the frequency of plasmid transfer events by both conjugation and mobilization, thereby promoting horizontal spread of antibiotic resistance [14]. Therefore, identifying drugs against BBFs has become an important focus of current pharmaceutical drug development research.

Effective alternative modalities against biofilm-producing microorganisms have gained interest. Of note, natural substances offer a wide repository of compounds with potential efficacy against bacterial pathogens, including antibiotic-resistant strains. A number of studies have explored the therapeutic and antimicrobial use of plant materials. The antibiotic effects of many plants are already known and applied in many forms of traditional medicine [15]. *Mosla chinensis* Maxim cv. Jiangxiangru is an annual herb belonging to the Lamiaceae family. It is recorded in Chinese Pharmacopoeia [16] and distributed mainly in Fenyi and Xinyu, Jiangxi province, China. As a medicinal and edible herb, it exerts a good therapeutic effect [17]. It can remove dampness. It is also used to treat stomach ailments and relieve external symptoms by diaphoresis. Moreover, it can alleviate symptoms such as summer heat and dampness, chills, and fever, no sweat and chest tightness, abdominal pain, vomiting, and diarrhea [18]. *M. chinensis* polysaccharide extract has also been shown to have a preventive action against oxidative damage in the immune system of mice [19]. Apart from polysaccharides, the essential oil is one of the major components in *M. chinensis* Maxim cv. Jiangxiangru having antibacterial, immunity-improving, and antioxidant effects [18]. Several studies have shown the antimicrobial and antioxidant effects of *M. chinensis* essential oil extract against a variety of pathogens, including *S. aureus* [20]. However, whether the *M. chinensis* Maxim cv. Jiangxiangru essential oil or its major chemical components can inhibit the growth and development of *S. aureus* biofilm has not been reported.

2 Materials and Methods

2.1 Preparation of the essential oil in *M. chinensis* Maxim cv. Jiangxiangru

The traditional Chinese medicine of Jiangxiangru prepared in ready-to-use forms was provided by Jiangxi Zhangshu Tianqitang Chinese Medicine Co. Ltd., China (lot number 1508005) and identified by the Department of Biological Medicine in Jiangxi Science and Technology Normal University School of Pharmacology as the dried leaves of *M. chinensis* Maxim cv. Jiangxiangru. The essential oil was prepared according to the Appendix XD of Essential Oil Determination Method A in the "Chinese Pharmacopoeia" 2015 edition. Briefly, 100 g of Jiangxiangru Chinese medicine was placed in a round-bottomed flask and subjected to steam distillation for 5 h using an essential oil extractor. The resulting faint yellow oily liquid was collected and dried using anhydrous sodium sulfate, transferred to a brown bottle, and stored at 2–8°C for future use.

2.2 GC–MS analysis of essential oil in *M. chinensis Maxim* cv. Jiangxiangru

An Agilent 6890N+5975i GC–MS system (Agilent Technologies, USA) was used. An HP-5MS (Agilent Technologies, USA) fused silica capillary column (30m × 0.25mm × 0.25μm) was used along with helium (He) with 99.999% purity as the carrier gas. The following chromatographic conditions were used: injector temperature, 250°C; carrier gas flow rate, 1.0 mL/min; inlet pressure, 7.69 psi; sample volume, 1.0 μL; and injected in the split mode with a split ratio of 50:1. The initial inlet temperature was programmed to increase from 50°C (held for 2 min) to 100°C at 3°C/min, and then held for 10 min at 100°C. The temperature was further increased to 120°C at the rate of 1°C/min (held for 5 min), and then to 150°C at the rate of 2°C/min (held for 3 min). Finally, the temperature was increased to 250°C at the rate of 4°C/min and held for 5 min.

The MS conditions used were as follows: the ionization mode was set to EI, with an ion source temperature of 230°C, quadrupole temperature of 150°C, ionization energy of 69.9 eV, interface temperature of 280°C, solvent delay of 3 min, and scanning mass range of m/z 35–550 amu.

The essential oil extracted from *M. chinensis* Maxim cv. Jiangxiangru was examined to obtain total ion

chromatograms using GC–MS under the aforementioned conditions. A mass spectrogram was obtained after scanning the mass spectra of the peaks in total ion chromatograms and matching them with the spectra in the NIST11 mass spectrum database. The chemical composition of essential oil in *M. chinensis* Maxim cv. Jiangxiangru was identified according to the retention index of each peak and comparison of mass spectrogram of each peak with the mass spectra in the NIST11 mass spectrum database in combination with manual spectrum interpretation and verification of the mass spectrograms of each peak with the mass spectra in the literature [20]. The relative percentages of each component were determined by the peak area normalization method.

2.3 Determination of the content of each main component in the *M. chinensis* Maxim cv. Jiangxiangru essential oil

The thymol standard (99.9%) content was obtained from the National Institutes for Food and Drug Control. The carvacrol, 3-carene, γ-terpinene, and *p*-cymene standards (>95% content) were procured from Dr Ehrenstorfer Company. The thymol acetate standard (≥98% content) was purchased from Shanghai Yihe Biological Technology Co., Ltd., China. The carvacrol acetate standard (RG) was procured from ChromaDex Inc., USA. The α-caryophyllene standard (≥93.0% content) was produced by Tokyo Kasei Kogyo Co. Further, 1616.02, 197.00, 221.70, 172.00, 148.95, 52.81, 378.00, and 1604.80 mg of carvacrol, carvacryl acetate, thymol acetate, *p*-cymene, 3-carene, γ-terpinene, α-caryophyllene, and thymol standards, respectively, were transferred to 10-mL volumetric flasks and dissolved in ethanol to a constant volume to determine the essential oil components. Hence, 161.60, 19.70, 22.17, 17.20, 14.90, 5.28, 37.80, and 160.480 mg/mL of carvacrol, carvacryl acetate, thymol acetate, *p*-cymene, 3-carene, γ-terpinene, α-caryophyllene, and thymol standard stock solutions, respectively, were prepared. Each standard stock solution was measured to obtain solutions of five different concentrations, and 1.5 mL of the standard solutions were placed in 2-mL centrifuge tubes. The five different concentrations of the standard solutions were 1/2, 1/4, 1/8, 1/16, and 1/32 of the standard stock solution. Standard solutions with different concentrations were analyzed and examined by GC–MS. The standard curve was drawn with the peak area as the ordinate and the concentration as the abscissa.

The *M. chinensis* Maxim cv. Jiangxiangru essential oil was examined by the GC–MS analysis. The contents of eight chemical components in the essential oil, namely, carvacrol, carvacrol acetate, thymol acetate, *p*-cymene, 3-carene, γ-terpinene, α-caryophyllene, and thymol, were calculated.

2.4 GC–MS analysis and verification

Precision test: Standard solutions of carvacrol, carvacrol acetate, thymol acetate, *p*-cymene, 3-carene, γ-terpinene, α-caryophyllene, and thymol with 1/2 stock concentration were obtained for six replicate sample experiments. The peak area and retention time were measured and recorded.

Stability test: Standard solutions with the same concentration were taken for injection at 0, 2, 4, 6, 8, and 10h. The peak area and retention time were measured and recorded.

Reproducibility test: For each standard, five standard solutions with the same concentration were prepared under the same conditions six times. The peak area and retention time were measured and recorded.

Recovery test: For the recovery test, the same amount of *M. chinensis* Maxim cv. Jiangxiangru essential oil was injected with the addition of 60%, 80%, 100%, 120%, and 140% standards. The recovery rate was calculated.

2.5 Pretreatment of strains

Lyophilized powder of the *S. aureus* standard strain ATCC 25923 (National Institutes for Food and Drug Control) was cultured at 37°C for 24 h in tryptic soy broth (TSB) containing 2.5% glucose. An appropriate amount of bacteria was spread on Luria-Bertani (LB) plates and cultured at 37°C for 24 h. Single colonies were selected from the LB plates and inoculated in Congo red medium [21]. After incubation at 37°C for 48 h, positive strains that turned black and grew well were screened for subsequent experiments.

2.6 MIC determination

For the determination of MIC, reagent solutions containing 10.00, 10.00, 19.52, 207.80, 204.00, 233.28, 7.80, 205.68, and 20.28 mg thymol, carvacrol, 3-carene, γ-terpinene, carvacryl acetate, thymol acetate, *p*-cymene, and α-caryophyllene, respectively, were precisely measured. The reagents to be tested were first fully dissolved in an appropriate amount of dimethyl sulfoxide (final content ≤2%, volume fraction) and then diluted to 0.5 mL using

MH broth. After mixing, a 0.22-μm sterile filter was used to filter the reagents for future use.

Briefly, 10 strains of *S. aureus* were inoculated on LB agar plates using a sterile inoculation loop and incubated in a 37°C incubator for 24 h. Then, the colonies were picked up, dispersed in MH broth, and diluted to A_{600} of 0.10, which was measured in a UV-visible light spectrophotometer (UV-2550; Shimadzu, Japan). The bacterial count in the suspension was approximately 1×10^7 CFU/mL. The MIC of *M. chinensis* Maxim cv. Jiangxiangru against *S. aureus* was measured using the broth macrodilution antimicrobial susceptibility test [22]. The MIC value obtained by this method was used to determine the concentration of drug solution in the *S. aureus* biofilm test.

2.7 Inhibition of *S. aureus* biofilm formation by reference substances using the crystal violet semi-quantitative method

The crystal violet semi-quantitative method was performed as described previously [23]. An *S. aureus* bacterial suspension was prepared in TSB containing 2.5% glucose at an absorbance (A_{600}) of 0.05. The bacterial suspension was then used to prepare drug solutions of 2, 1, 0.5, 0.25, 0.125, and 0.0625× MIC. Briefly, 200-μL aliquots of bacterial suspension containing each concentration of drug were transferred into the wells of a 96-well plate. Six wells were used for each drug concentration, in addition to six blank wells (medium only) and six negative control wells (biofilm without drugs). After incubation at 37°C for 48 h, the supernatant was discarded, and the cells were washed thrice with phosphate-buffered saline (PBS; pH 7.4). The cells were then stained with 0.1% crystal violet for 30 min. The crystal violet dye was discarded, and the cells were washed with distilled water until the water ran clear. Finally, 200 μL of anhydrous ethanol was added for dissolution, and each well was measured at an absorbance of 570 nm using a microplate reader (MK3; Thermo Fisher Scientific Co., Ltd., Shanghai, China).

The inhibition rate of drugs on *S. aureus* biofilm (A_{570}) was calculated as follows:

$$\text{Inhibition rate (\%)} = \frac{A_2 - A_1}{A_2 - A_0} \times 100$$

where A_0 is the average value of A_{570} in the control group, A_1 is the average value of A_{570} in the drug effect group, and A_2 is the average value of A_{570} in the negative control group.

2.8 Morphological observation of *S. aureus* biofilm using silver staining

An *S. aureus* bacterial suspension was prepared in TSB containing 2.5% glucose at an absorbance (A_{600}) of 0.05, as described previously [24]. The bacterial suspension was then used to prepare drug solutions of 2, 1, 0.5, and 0.25× MIC. Briefly, 10 mL of bacterial suspension containing different concentrations of drugs was measured and transferred into Petri dishes. A separate negative control with 10 mL of bacterial suspension without drugs was prepared. Subsequently, four coverslips soaked in anhydrous ethanol for 30 min were placed in each dish. After incubation at 37°C for 48 h, nonadherent bacteria were removed from the coverslips by rinsing them thoroughly with saline. The coverslip with a biofilm was soaked in 2.5% glutaraldehyde in PBS for 1 h, washed with distilled water for 1 min, soaked in saturated calcium chloride solution for 15 min and washed with distilled water for 1 min, soaked in 5% silver nitrate solution for 15 min, stained with 1% hydroquinone for 2 min, and washed with distilled water for 1 min. Finally, the cells were fixed using 5% sodium thiosulfate and rinsed with distilled water for 1 min. After air-drying, *S. aureus* BF morphology was observed under an optical biological microscope (BH300-OPTEC; Chongqing Ott Optical Instruments Co., Ltd).

3 Results

3.1 Reliability of GC–MS analysis of essential oil in *M. chinensis* Maxim cv. Jiangxiangru

Precision test: The relative standard deviation (RSD) values were all less than 2.0% for the area of GC chromatographic peaks measured for each standard. The RSD values of retention time were all less than 1.0%, indicating that the instrument had good precision.

Stability test: The RSD values of peak areas for injection at 0, 2, 4, 6, 8, and 10 h for all the eight standards were <1.5%, and the RSD values of retention time were all less than 1.5%, indicating that the reference standard solution was stable within 10 h.

Reproducibility test: The RSD values of peak areas of the prepared solutions were all less than 1.6%, and the RSD values of retention time were all less than 1.1%, indicating that the reaction conditions had good reproducibility and repeatability.

Recovery test: The experimentally measured recovery rates ranged from 99.0% to 101.5%. The average recovery

rates of carvacrol, carvacryl acetate, thymol acetate, *p*-cymene, 3-carene, γ-terpinene, α-caryophyllene, and thymol were 99.97%, 99.50%, 100.11%, 100.32%, 99.83%, 99.45%, 101.21%, and 99.87%, respectively, indicating that the standard curve had good accuracy.

3.2 GC–MS analysis of the chemical composition of *M. chinensis* Maxim cv. Jiangxiangru essential oil

The GC–MS analysis of the essential oil extracted from *M. chinensis* Maxim cv. Jiangxiangru was performed. Figure 1 shows the total ion chromatography of the essential oil.

The results of GC–MS revealed a total of 59 peaks in the essential oil. A total of 29 compounds were identified through a search of the NIST11 MS library. The components accounted for 99.316% of the total essential oil according to the MS ion peak area normalization method. Table 1 presents the results of the GC–MS analysis. As shown in Table 1, the *M. chinensis* Maxim cv. Jiangxiangru essential oil was composed primarily of phenols, esters, olefins, terpenes, aromatics, and alcohols. Thymol accounted for 58.514% of the essential oil, followed by carvacrol, which accounted for 22.195%. Additionally, 3-carene accounted for 0.937% of the essential oil; *p*-cymene, 5.601%; γ-terpinene, 3.545%; thymol acetate, 2.223%; carvacrol acetate, 0.611%; and α-caryophyllene, 1.848%. Together, these eight components accounted for 95.774% of the total essential oil.

3.3 Content of each major component in the *M. chinensis* Maxim cv. Jiangxiangru essential oil

A standard solution was prepared by double dilution. The measured peak area was plotted against the concentration of the standard solution. The resulting linear relationship, using concentration as the abscissa and peak area as the ordinate, is shown in Supplementary Table 1. The GC peak of every standard was integrated using three different integration methods. Different R^2 values and linear equations were calculated using different integration methods. Therefore, the linear equation with the best linear relationship, which had the maximum R^2 value, was chosen to calculate the percentages of the eight essential oil components.

As shown in Table 2, the thymol, carvacrol, thymol acetate, and carvacryl acetate contents calculated via the standard curve (Supplementary Table 1) were all greater than their corresponding relative contents measured by the peak area normalization method. However, the calculated contents of 3-carene, *p*-cymene, γ-terpinene, and α-caryophyllene were all lower than their corresponding contents measured by the peak area normalization method. On the whole, the contents of carvacrol and thymol were higher than the content of the remaining six compounds among the relative contents and the contents measured according to the standard curve.
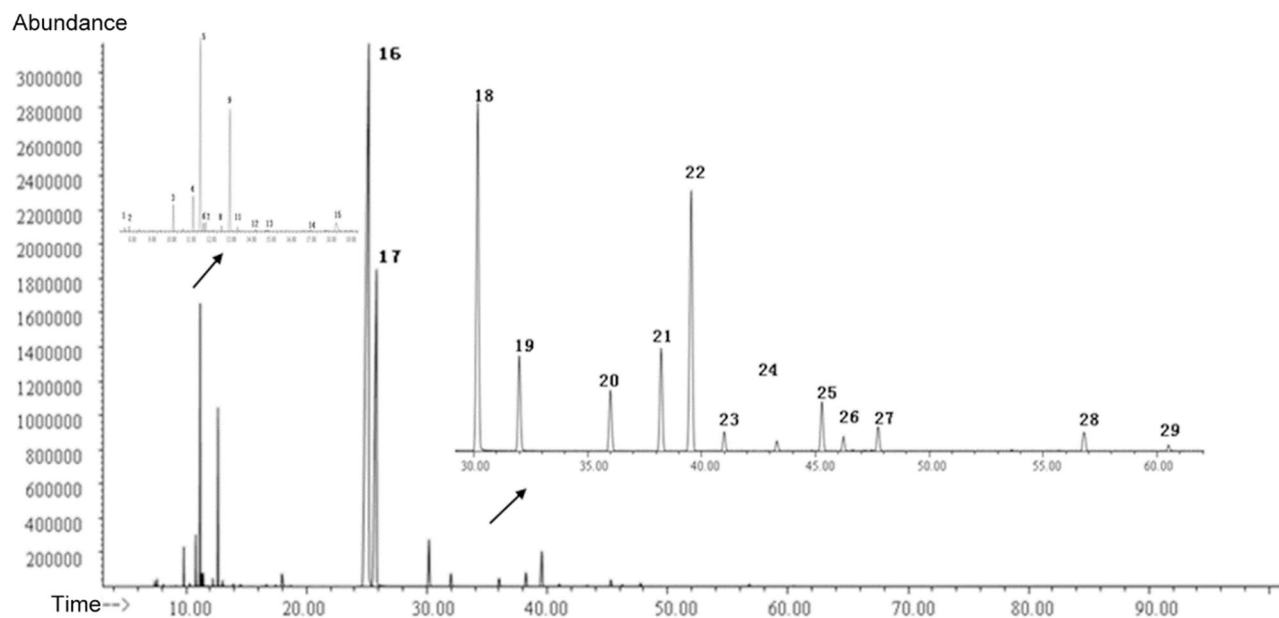

Figure 1. Total ion chromatography of the essential oil from *Mosla chinensis* Maxim cv. Jiangxiangru.

Table 1. Gas chromatography–mass spectrometry analysis of the *M. chinensis* Maxim cv. Jiangxiangru essential oil

Peak number	Retention time (min)	Compound name	Molecular formula	Relative molecular mass	Retention Index	Relative content (%)
1	7.375	β-Thujene	$C_{10}H_{16}$	136	1068	0.078
2	7.592	α-Pinene	$C_{10}H_{16}$	136	1031	0.111
3	9.810	β-Myrcene	$C_{10}H_{16}$	136	979	0.674
4	10.286	α-Phellandrene	$C_{10}H_{16}$	136	997	0.064
5	10.804	3-Carene	$C_{10}H_{16}$	136	1078	0.937
6	11.163	*p*-Cimene	$C_{10}H_{16}$	136	1025.4	5.601
7	11.310	Sylvestrene	$C_{10}H_{16}$	136	1016	0.217
8	11.421	Eucalyptol	$C_{10}H_{18}O$	154	1023	0.254
9	12.216	*cis*-β-Ocimene	$C_{10}H_{16}$	136	1024	0.147
10	12.645	γ-Terpinene	$C_{10}H_{16}$	136	1047	3.545
11	13.021	*trans*-4-Thujanol	$C_{10}H_{18}O$	154	1051	0.151
12	13.921	Terpinolene	$C_{10}H_{16}$	136	1078	0.062
13	14.533	*cis*-Sabinene hydrate	$C_{10}H_{18}O$	154	1059	0.061
14	16.668	Sabinol	$C_{10}H_{16}O$	152	1107	0.060
15	17.962	Terpinen-4-ol	$C_{10}H_{18}O$	154	1161	0.610
16	25.179	Thymol	$C_{10}H_{14}O$	150	1266	58.476
17	25.803	Carvacrol	$C_{10}H_{14}O$	150	1278	22.239
18	30.179	Thymyl acetate	$C_{12}H_{16}O_2$	192	1338	2.235
19	31.997	Carvacryl acetate	$C_{12}H_{16}O_2$	192	1354	0.611
20	36.008	β-Caryophyllene	$C_{15}H_{24}$	204	1424	0.418
21	38.220	α-Bergamotene	$C_{15}H_{24}$	204	1407	0.691
22	39.555	α-Caryophyllene	$C_{15}H_{24}$	204	1456	1.848
23	40.996	(*Z*)-β-Farnesene	$C_{15}H_{24}$	204	1449	0.114
24	43.302	cis-β-Farnesene	$C_{15}H_{24}$	204	1449	0.066
25	45.278	(*Z,E*)-α-Farnesene	$C_{15}H_{24}$	204	1486	0.321
26	46.237	γ-Muurolene	$C_{15}H_{24}$	204	1480	0.085
27	47.754	β-Sesquiphellandrene	$C_{15}H_{24}$	204	1516	0.160
28	56.795	Humulene-1,2-epoxide	$C_{15}H_{24}O$	220	1601	0.137
29	60.510	Eudesma-3,7(11)-diene	$C_{15}H_{24}$	204	1440	0.015

Table 2. Content of the eight main components of the *M. chinensis* Maxim cv. Jiangxiangru essential oil

Sample	3-Carene	*p*-Cymene	γ-Terpinene	Thymol	Carvacrol	Thymyl acetate	Carvacryl acetate	α-Caryophyllene
Relative content (%)	0.937	5.601	3.545	58.476	22.239	2.235	0.611	1.848
Content (%)	0.118	1.025	1.303	63.370	25.258	3.575	0.791	0.694

3.4 Antibacterial effect of the major essential oil components and essential oil on *S. aureus*

Table 3 shows the MICs of essential oil and its eight major components against *S. aureus*. As shown in Table 3, only thymol, carvacrol, and thymol acetate had smaller MICs compared with the essential oil among the eight reference substances. The MICs of the other reference substances were all higher compared with the essential oil; in particular, the MICs of 3-carene, γ-terpinene, and *p*-cymene were all >10 mg/mL. This suggested that thymol, carvacrol, and thymol acetate were the major components of the *M. chinensis* Maxim cv. Jiangxiangru essential oil contributing to its excellent antibacterial effect.

3.5 Morphological observation of the inhibition of *S. aureus* biofilm formation by the major essential oil components and essential oil

Figure 2 illustrates the morphological analysis of the *S. aureus* biofilm using silver staining. The microscopic observation of the stained biofilm revealed an evenly distributed biofilm in the negative control group. In comparison, biofilm formation in the group containing less than 1/4× MIC of the test substance was found to be significantly reduced and sparsely distributed.

3.6 Inhibition of *S. aureus* biofilm formation by the major essential oil components and essential oil using the crystal violet semi-quantitative method

Figure 3 illustrates the morphological analysis of the *S. aureus* biofilm using crystal violet staining. Figure 4 illustrates the rate of inhibition of the essential oil and its eight components on the formation of *S. aureus* biofilm, and half maximal inhibitory concentration (IC$_{50}$) is shown in Table 4. Of all the tested substances, *M. chinensis* Maxim cv. Jiangxiangru essential oil, thymol, carvacrol, carvacryl

acetate, α-caryophyllene, and 3-carene showed better inhibition, and IC$_{50}$ values were 0.018 ± 0.005, 0.007 ± 0.005, 0.008 ± 0.007, 0.343 ± 0.012, 0.019 ± 0.025 and 0.356 ± 0.019 mg/mL, respectively (Table 4). When concentrations of 1/4× MIC were used for these substances, the inhibition
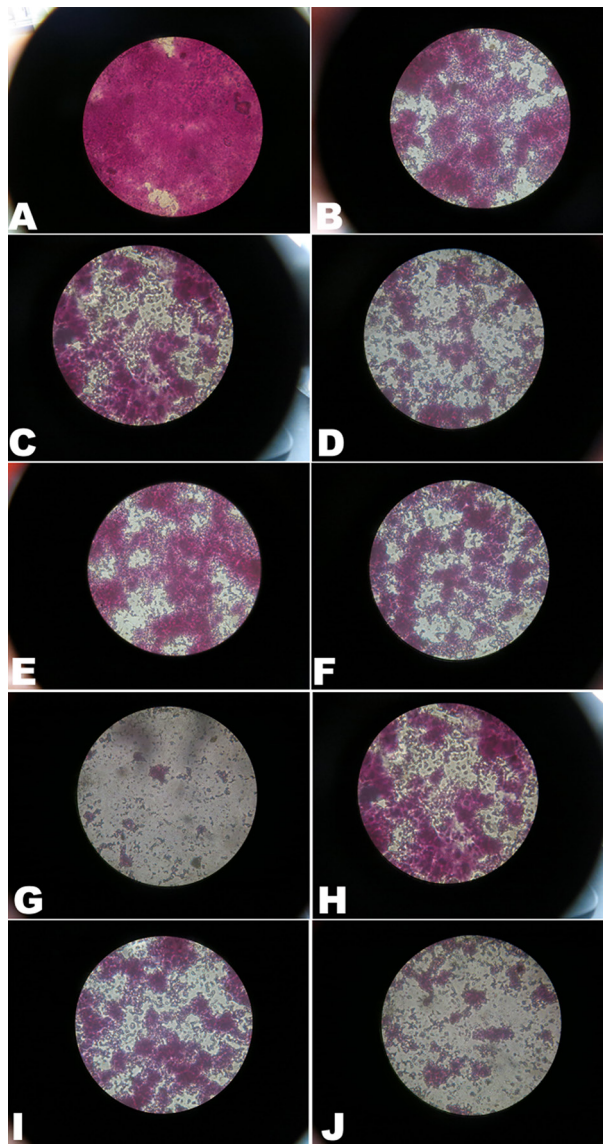

Figure 2. Effect of essential oil from *M. chinensis* Maxim cv. Jiangxiangru on *S. aureus* biofilm morphology using silver staining. (A) Negative control; (B) thymol 1/16 MIC; (C) thymol 1/4 MIC; (D) thymol 2 MIC; (E) carvacrol 1/16 MIC; (F) carvacrol 1/4 MIC; (G) carvacrol 2 MIC; (H) carvacrol acetate 1/16 MIC; (I) carvacrol acetate 1/4 MIC; (J) carvacrol acetate 2 MIC. Magnification: ×1000.

Table 3. MIC of the *M. chinensis* Maxim cv. Jiangxiangru essential oil and its main components against *S. aureus*

Sample	Essential oil	Thymol	Carvacrol	3-Carene	γ-Terpinene	Carvacryl acetate	*p*-Cymene	Thymyl acetate	α-Caryophyllene
MIC (mg/mL)	0.25	0.245	0.122	10.39	10.2	5.832	10.284	0.195	0.507

rates were >80% (Fig. 2). However, thymol, γ-terpinene, thymol acetate, and *p*-cymene showed a relatively poor inhibition of *S. aureus* biofilm formation. When 1× MIC concentrations of these substances were used, the inhibition rates were less than 50% (Fig. 2), indicating that carvacrol, carvacryl acetate, α-caryophyllene, and 3-carene were the main components in the *M. chinensis* Maxim cv. Jiangxiangru essential oil that inhibited *S. aureus* biofilm formation.

4 Discussion

Plants have a long evolutionary history with respect to developing resistance against viruses and are increasingly drawing attention as potential sources for development of antiviral drugs [25]. *M. chinensis* Maxim cv. Jiangxiangru has a good therapeutic effect as a medicinal and edible herb, especially its essential oil and polysaccharides [19, 20]. In this study, the chemical composition of essential oil in *M. chinensis* Maxim cv. Jiangxiangru was first analyzed using GC–MS. Of the 59 peaks detected, 29 were identified, accounting for 99.316% of the total essential oil. The thymol, carvacrol, *p*-cymene, carvacryl acetate, thymol acetate, and α-caryophyllene contents in the essential oil were relatively high, consistent with that previously reported [21]. Additionally, nine of the essential oil components, namely, 3-carene, sylvestrene, eucalyptol, *trans*-4-thujanol, (*Z*)-β-farnesene, *cis*-β-farnesene, (*Z,E*)-α-farnesene, γ-muurolene, and eudesma-3,7(11)-diene, were newly identified in the present study. Another 30 compounds were not identified. This might be because fresh *M. chinensis* Maxim cv. Jiangxiangru specimens were used in previous studies, but in the present study, the ready-to-use preparation of this plant according to traditional Chinese medicine was used. The *M. chinensis* Maxim cv. Jiangxiangru essential oil was altered during the preparation and storage of herb drugs. The quantitative analysis of the essential oil components showed a good linear relationship within the measured concentration range, indicating a highly significant linear relationship between the concentration of each component and the corresponding chromatographic peak area. An established regression equation could be used to

calculate the concentration of tested compounds in the essential oil. The concentration results of each component showed higher concentrations of thymol and carvacrol
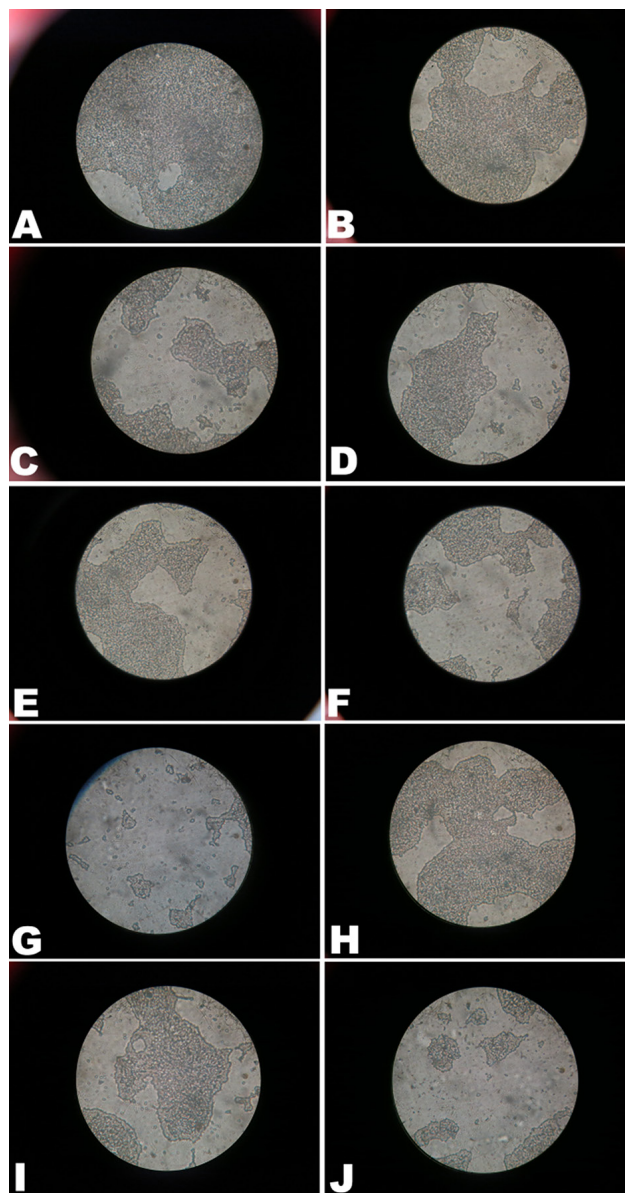

Figure 3. Effect of essential oil from *M. chinensis* Maxim cv. Jiangxiangru on *S. aureus* biofilm morphology using crystal violet staining. (A) Negative control; (B) thymol 1/16 MIC; (C) thymol 1/4 MIC; (D) thymol 2 MIC; (E) carvacrol 1/16 MIC; (F) carvacrol 1/4 MIC; (G) carvacrol 2 MIC; (H) carvacrol acetate 1/16 MIC; (I) carvacrol acetate 1/4 MIC; (J) carvacrol acetate 2 MIC. Magnification: ×1000.

Table 4. Inhibition of *S. aureus* biofilm formation by essential oil and its eight main components (IC50 values)

Sample	Essential oil	Thymyl acetate	Carvacrol	Carvacrol acetate	α-Caryophyllene	3-Carene	Thymol	γ-Terpinene	*p*-Cymene
IC$_{50}$ (mg/mL)	0.018 ± 0.005	0.278 ± 0.025	0.008 ± 0.007	0.343 ± 0.012	0.019 ± 0.025	0.356 ± 0.019	0.259 ± 0.149	22.55 ± 0.044	237.6 ± 0.183

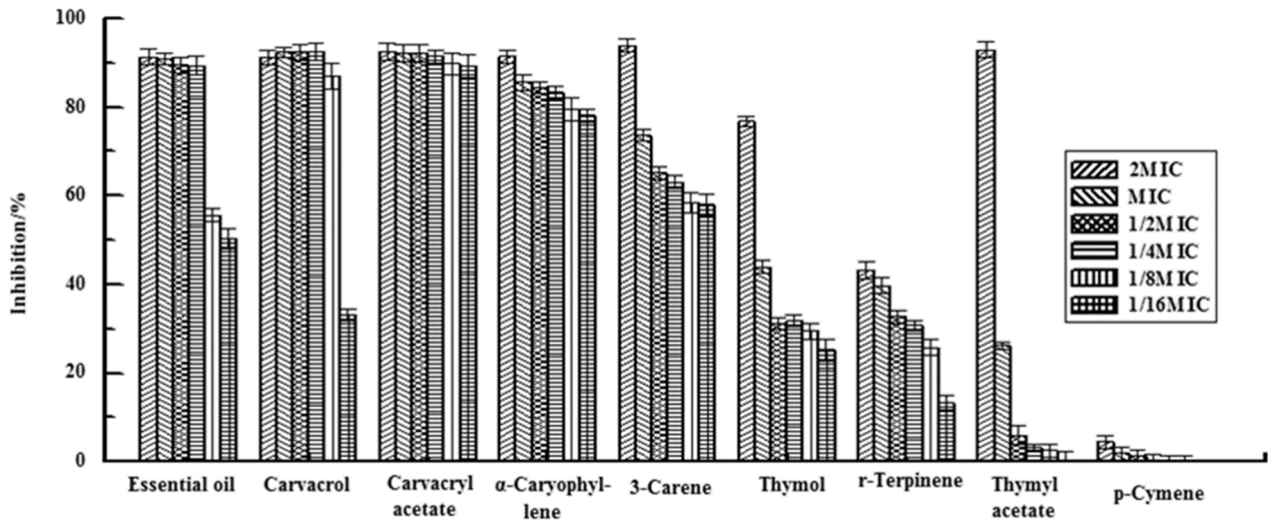Data are shown as means ± standard deviation from six independent experiments.

Figure 4. Inhibition of *S. aureus* biofilm formation by essential oil and eight reference substances. Data are shown as means ± standard deviation from six independent experiments.

in the essential oil; these results were consistent with the quantification results from the area normalization method.

The results of the GC–MS analysis showed that the main essential oil components were thymol, carvacrol, 3-carene, *p*-cymene, γ-terpinene, thymol acetate, carvacryl acetate, and α-caryophyllene. These eight chemical components accounted for 95.774% of the total essential oil. Then antibacterial effect was further studied. Among the eight reference substances, only thymol, carvacrol, and thymol acetate had smaller MICs compared with the essential oil. In a previous study on the effects of carvacrol and thymol on *S. aureus* and *S. aureus* biofilms, the biofilm inhibitory concentration ranged from 0.031% to 0.125% v/v for carvacrol and thymol, which were two to four times greater than the concentration required to inhibit the planktonic growth [26]. These data supported the antibacterial effect of the essential oil components found in the present study. This study also indicated that a greater concentration of the antimicrobial agent was required to have the same inhibitory effect on sessile bacteria compared with planktonic bacteria, suggesting the protective effect of BBFs. Many previous studies have reported that essential oils extracted from plants exhibit antibiotic and antimicrobial activities in *vitro* against many bacteria, including antibiotic-resistant strains [27, 28]. For instance, Adukwu et al. tested the MICs of lemongrass, grapefruit, bergamot, and lime against *S. aureus* strains. They found that although all strains were susceptible to these essential oils, lemongrass oil exhibited the most effective antimicrobial and anti-biofilm activities against the *S. aureus* strains [29].

Therefore, as a follow-up, this study then examined the effects of *M. chinensis* Maxim cv. Jiangxiangru essential oil and its eight components on *S. aureus* biofilms at concentrations of 2, 1, 0.5, 0.25, 0.125, and 0.0625× MIC using the semi-quantitative method. After co-incubation of test substances at a concentration of 2× MIC and *S. aureus* for 48 h, the essential oil, carvacrol, 3-carene, carvacryl acetate, thymol acetate, and α-caryophyllene all significantly inhibited *S. aureus* biofilm formation, with inhibition rates of >90%. This result indicated that the aforementioned tested substances were capable of killing a large number of floating *S. aureus* at 2× MIC, thereby inhibiting biofilm formation. However, at a concentration of 0.25× MIC, only carvacrol, carvacryl acetate, α-caryophyllene, and 3-carene showed an inhibition rate of >80%. The morphological observation in the biofilm formation experiment revealed that at 0.25× MIC, the biofilm distribution significantly decreased in the test drug group than in the negative control and showed a sparse distribution. This indicated that 0.25× MIC could significantly inhibit the formation of *S. aureus* biofilm. Meanwhile, thymol, γ-terpinene, thymol acetate, and *p*-cymene had a relatively weak inhibitory effect on *S. aureus* biofilm formation at all concentrations. Moreover, No biofilm formation occurred when these compounds were used at 2× MIC. All these results showed that carvacrol, carvacryl acetate, α-caryophyllene, and 3-carene were the major components inhibiting biofilm formation in the essential oil. They might exert their effect by reducing the secretion of extracellular DNA, inhibiting the expression of polysaccharide intercellular adhesion,

or regulating genes associated with biofilm formation, but not by killing planktonic bacteria. Further studies are required to determine the specific mechanism by which the essential oil and its components can inhibit biofilm formation.

5 Conclusions

In summary, the essential oil in *M. chinensis* Maxim cv. Jiangxiangru is a potential drug against *S. aureus* biofilm. Low doses of this essential oil and its major components, carvacrol, carvacryl acetate, α-caryophyllene, and 3-carene, can significantly inhibit the formation of *S. aureus* biofilm.

Conflicts of interest: Authors state no conflict of interest.

References

[1] Archer G.L., Staphylococcus aureus: a well-armed pathogen, Clin Infect Dis, 1998, 26, 1179-1181

[2] Lowy F.D., Staphylococcus aureus infections, N Engl J Med, 1998, 339, 520-532

[3] Otto M., Staphylococcal biofilms, Curr Top Microbiol Immunol, 2008, 322, 207-228

[4] Alekshun M.N., Levy S.B., Molecular mechanisms of antibacterial multidrug resistance, Cell, 2007, 128, 1037-1050

[5] Lowy F.D., Antimicrobial resistance: the example of Staphylococcus aureus, J Clin Invest, 2003, 111, 1265-1273

[6] Shalit I., Berger S.A., Gorea A., Frimerman H., Widespread quinolone resistance among methicillin-resistant Staphylococcus aureus isolates in a general hospital, Antimicrob Agents Chemother, 1989, 33, 593-594

[7] Ito H., Yoshida H., Bogaki-Shonai M., Niga T., Hattori H., Nakamura S., Quinolone resistance mutations in the DNA gyrase gyrA and gyrB genes of Staphylococcus aureus, Antimicrob Agents Chemother, 1994, 38, 2014-2023

[8] Seal J.B., Moreira B., Bethel C.D., Daum R.S., Antimicrobial resistance in Staphylococcus aureus at the University of Chicago Hospitals: a 15-year longitudinal assessment in a large university-based hospital, Infect Control Hosp Epidemiol, 2003, 24, 403-408

[9] Nimmo G.R., Bell J.M., Mitchell D., Gosbell I.B., Pearman J.W., Turnidge J.D., Agar, Antimicrobial resistance in Staphylococcus aureus in Australian teaching hospitals, 1989-1999, Microb Drug Resist, 2003, 9, 155-160

[10] Hiramatsu K., Vancomycin-resistant Staphylococcus aureus: a new model of antibiotic resistance, Lancet Infect Dis, 2001, 1, 147-155

[11] Pantosti A., Sanchini A., Monaco M., Mechanisms of antibiotic resistance in Staphylococcus aureus, Future Microbiology, 1974, 12, 323-334

[12] Davies D., Understanding biofilm resistance to antibacterial agents, Nat Rev Drug Discov, 2003, 2, 114-122

[13] Ryder V.J., Chopra I., O'Neill A.J., Increased mutability of Staphylococci in biofilms as a consequence of oxidative stress, PLoS One, 2012, 7, e47695

[14] Savage V.J., Chopra I., O'Neill A.J., Staphylococcus aureus biofilms promote horizontal transfer of antibiotic resistance, Antimicrob Agents Chemother, 2013, 57, 1968-1970

[15] Aumeeruddy-Elalfi Z., Gurib-Fakim A., Mahomoodally M.F., Chemical composition, antimicrobial and antibiotic potentiating activity of essential oils from 10 tropical medicinal plants from Mauritius, Journal of Herbal Medicine, 2016

[16] Stewart P.S., A review of experimental measurements of effective diffusive permeabilities and effective diffusion coefficients in biofilms, Biotechnol Bioeng, 1998, 59, 261-272

[17] Brown M.R., Allison D.G., Gilbert P., Resistance of bacterial biofilms to antibiotics: a growth-rate related effect?, J Antimicrob Chemother, 1988, 22, 777-780

[18] Cao L., Si J.Y., Liu Y., Sun H., Jin W., Li Z., Zhao X.H., Le Pan R., Essential oil composition, antimicrobial and antioxidant properties of Mosla chinensis Maxim, Food Chemistry, 2009, 115, 801-805

[19] Li J.E., Nie S.P., Xie M.Y., Huang D.F., Wang Y.T., Li C., Chemical composition and antioxidant activities in immunosuppressed mice of polysaccharides isolated from Mosla chinensis Maxim cv. jiangxiangru, Int Immunopharmacol, 2013, 17, 267-274

[20] Li J.E., Nie S.P., Qiu Z.H., Che M.J., Li C., Xie M.Y., Antimicrobial and antioxidant activities of the essential oil from Herba Moslae, J Sci Food Agric, 2010, 90, 1347-1352

[21] Yang S., Ouyang H., Su X., Gao H., Kong W., Wang M., Shu Q., Fu Z., Dual-recognition detection of Staphylococcus aureus using vancomycin-functionalized magnetic beads as concentration carriers, Biosens Bioelectron, 2016, 78, 174-180

[22] CLS I., Performance standards for antimicrobial susceptibility testing; eighteenth informational supplement M100-S18, Wayne, PA: Clinical and Laboratory Standards Institute, 2008

[23] Karaolis D.K., Rashid M.H., Chythanya R., Luo W., Hyodo M., Hayakawa Y., c-di-GMP (3'-5'-cyclic diguanylic acid) inhibits Staphylococcus aureus cell-cell interactions and biofilm formation, Antimicrob Agents Chemother, 2005, 49, 1029-1038

[24] Wang Y., Wang T., Hu J., Ren C., Lei H., Hou Y., Brantner A.H., Anti-biofilm activity of TanReQing, a Traditional Chinese Medicine used for the treatment of acute pneumonia, J Ethnopharmacol, 2011, 134, 165-170

[25] Sawai R., Kuroda K., Shibata T., Gomyou R., Osawa K., Shimizu K., Anti-influenza virus activity of Chaenomeles sinensis, J Ethnopharmacol, 2008, 118, 108-112

[26] Nostro A., Sudano Roccaro A., Bisignano G., Marino A., Cannatelli M.A., Pizzimenti F.C., et al., Effects of oregano, carvacrol and thymol on Staphylococcus aureus and Staphylococcus epidermidis biofilms, J Med Microbiol, 2007, 56, 519-523

[27] Fisher K., Phillips C.A., The effect of lemon, orange and bergamot essential oils and their components on the survival of Campylobacter jejuni, Escherichia coli O157, Listeria monocytogenes, Bacillus cereus and Staphylococcus aureus in vitro and in food systems, J Appl Microbiol, 2006, 101, 1232-1240

[28] Warnke P.H., Becker S.T., Podschun R., Sivananthan S., Springer I.N., Russo P.A., et al., The battle against multi-resistant strains: Renaissance of antimicrobial essential oils as a promising force to fight hospital-acquired infections, J Craniomaxillofac Surg, 2009, 37, 392-397

[29] Adukwu E.C., Allen S.C., Phillips C.A., The anti-biofilm activity of lemongrass (Cymbopogon flexuosus) and grapefruit (Citrus paradisi) essential oils against five strains of Staphylococcus aureus, J Appl Microbiol, 2012, 113, 1217-1227