



RESEARCH ARTICLE

Antibacterial activity of plant essential oils against indigenously characterized methicillin-resistant *Staphylococcus aureus* (MRSA)

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ABSTRACT

Plant essential oils were evaluated for antimicrobial activity against Methicillin-resistant *Staphylococcus aureus* (MRSA). The isolates (n=03) were procured from Institute of Microbiology, UVAS Lahore, Pakistan. After biochemical and 16S rRNA gene-based PCR characterization, accession numbers were retrieved from NCBI i.e. MW344063.1, MW344064.1 and MW344065.1. These isolates exhibited molecular positivity by multiplex PCR for *mecA*, *coa* and *eta* toxin genes. Moreover, these isolates exhibited resistance to cefoxitin, ampicillin, amoxicillin, penicillin, amoxicillin clavulanate, ciprofloxacin, erythromycin and gentamicin. The antibiotic resistant isolates were evaluated for antimicrobial activity of plant essential oils. The highest zone of inhibition (mean ZOI±S.D.) was measured for *Cinnamomum verum* (22.67±1.52 mm) followed by *Eucalyptus globulus* (18.67±2.51 mm) and *Syzygium aromaticum* (12.67±2.51 mm). Lowest mean MIC value (0.33±0.11 mg/mL) was recorded for *E. globulus*. *Eucalyptus globulus* was processed for fractionation by column chromatography and n-hexane, chloroform, n-hexane + chloroform and ethyl-acetate fractions were evaluated for antibacterial activity. Lowest mean MIC (10.04±5.80 mg/mL) was recorded for *E. globulus* n-hexane fraction. Cell survival percentage of BHK21 cell line was 51.7% at 54.87mg/mL concentration of *E. globulus* n-hexane fraction. Through gas chromatography-mass spectrometry (GC-MS) analysis of n-hexane fraction, benzene was found abundant (29.9%) as active compound. It was concluded that *E. globulus* n-hexane fraction exhibited significantly promising results against MRSA.

Keywords: Essential oil; *Eucalyptus globules*; methicillin resistance; n-hexane; *Staphylococcus aureus*.

INTRODUCTION

Staphylococcus aureus is a gram positive bacterium having the diameter of 0.5-1.5µm, non-motile and non-spore forming facultative anaerobe. The biochemical profile of *S. aureus* exhibits catalase-positive, oxidase-negative, coagulase-positive and it can tolerate high salt concentration and resistant to heat treatment (Vrbovska et al., 2020). Being natural inhabitant of skin microbiota, it usually causes nosocomial staphylococcal infections especially in immunocompromised individuals: septicemia, pyaemia, localized skin and other body infections. This ubiquitous bacterium is a significant pathogen due to combination patterns of toxin-mediated virulence, antibiotic resistance and invasiveness (Kadariya et al., 2014).

Several strains of *S. aureus* produce enterotoxins A, B, C, D and E which can lead to food poisoning and also resistant to methicillin and β-lactams because of *mecA* gene that encodes penicillin binding protein PBP2a which are the

specific characters of MRSA strains (Batista et al., 2013). Coagulase (*coa*) protein is a clotting factor secreted by MRSA that acts as virulence factor as well phenotypic determinant (Effendi et al., 2019). Multiplex PCR assay can be used for the detection of *mecA*, *coa* and staphylococcus exfoliative toxin A (*eta*) genes (Bhowmik et al., 2021). It is a significant pathogen of public health concern because of increasing antibiotic resistance pattern. It shows a wide pattern of resistance against traditional antibiotics like macrolides, lincosamides, streptogramins (MLS), amino-glycosides, quinolones, β-lactams and glycopeptide groups and leads to emergence of multi-drug resistance (MDR) (Gurung et al., 2020).

Plant essential oils are being used as alternative therapeutics for the treatment of staphylococcal infections. Essentials oils (EOs) are the bioactive secondary metabolites of plants and also known as volatile oils. The EOs are extracted from vegetative parts (herbs, bark, leaves, wood and roots) and reproductive parts (flowers and fruits) of

plants by different processes such as fermentation, extraction and steam distillation (Dorman & Deans, 2000). The inhibitory effects of plant essential oils can be monitored by performing agar disc diffusion and broth microdilution methods such as minimum inhibitory concentration (MIC) against pathogenic *S. aureus* (Lopez-Romero et al., 2015).

Essential oil from the leaves of *E. globulus* is known as antioxidant and natural tonic. The chemical components of *E. globulus* oil can be analyzed by GC/MS which are oxygenated monoterpenes, 1, 8-cineole, spathulenol and α -terpineol (Harkat-Madouri et al., 2015). The *C. verum* leaf EO shows antimicrobial activity against *S. aureus* due to major components such as cinnamaldehyde, eugenol, cinnamyl acetate, linalool, cinnamyl alcohol and p-cymene. The *S. aromaticum* EO is commonly known as clove oil and rich in phenolic compounds like eugenol, eugenol acetate and gallic acid as an effective antibacterial agent (Moemenbellah Fard et al., 2020). The active antimicrobial components of *Ferula assa-foetida* EO (hing oil) are propenyl sec-butyl disulfide, β -pinene, α -pinene, thiophene and thiourea (Karimian et al., 2020).

This study was designed to identify the alternative therapeutic potential of various plant essential oils along with chemical component analysis by GC/MS of effective plant essential oil fractions against MRSA.

MATERIALS AND METHODS

Revival of *S. aureus* isolates

The *S. aureus* (n=10) isolates were procured from Institute of Microbiology, University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan which were characterized on the basis of morphology and biochemical profile. The isolates stored on microbeads were revived on mannitol salt agar (MSA) at 37°C temperature for 24-48 hours.

Molecular Confirmation

The DNA of *S. aureus* isolates was extracted through GeneAll® Exgene™ DNA extraction kit by following the manufacturer recommendations. The 16S rRNA gene based PCR was performed by using n=1 pair of forward and reverse primers (Table 1). The PCR amplification was carried out in 25 μ L reaction mixture and PCR reaction conditions were maintained as initial denaturation at 94°C for 10 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 10 min.

Phylogenetic analysis

The DNA amplicons were sequenced by using Sanger di-deoxy sequencing and retrieved in FASTA format. Sequences were

cleaned by JUSTbio program then found the regions of high similarity using Basic Local Alignment Search Tool (BLAST-n) resulted in 97% to 98% homology between the sequences. These sequences also submitted to GenBank NCBI and accession numbers were obtained. A phylogenetic tree was constructed by picking the random sequences of *S. aureus* from GenBank NCBI along with sequenced data of 10 isolates of *S. aureus* using MEGA-X software.

Antibiotic resistant, coagulase and toxin genes based Multiplex PCR

Multiplex PCR amplification was based on *mecA*, *coa* and *eta* toxin using gene specific designed primers. A total of 25 μ L reaction mixture was used for each PCR reaction containing 12.5 μ L master mix, 8.5 μ L nuclease free water, 2 μ L of genomic DNA of each isolate and 1 μ L of 10pmol/ μ L each *mecA*, *coa* and *eta* forward and reverse primers (Table 1). Amplification was done as initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 2 min, annealing at 57°C for 2 min, extension at 72°C for 1 min and final extension at 72°C for 7 min. A DNA ladder of 100 to 1500 bp was used and amplified products were separated on 1.8% agarose gel at voltage 5V/cm of gel length using 1X TAE tank buffer then observed under UV light for DNA bands visualization (Green & Sambrook, 2021).

Antibiotic sensitivity testing

The *S. aureus* isolates (n=3) confirmed by using *mecA*, *coa* and *eta* gene specific primers were analyzed for antibiotic susceptibility testing. By following clinical and laboratory standards institute (CLSI) 2020 manual, culture sensitivity testing was performed using Kirby-Bauer method for antibiotics such as amoxicillin, clavulanate, ampicillin, gentamicin, penicillin, ciprofloxacin, erythromycin, amoxicillin and cefoxitin. The inoculum of *S. aureus* cultures was prepared to 0.5 McFarland by adjusting the optical density to 0.1 at 630nm. The prepared inoculum cultures were swabbed on respective nutrient agar plates and dispensed antibiotic discs by disc dispenser. Plates were incubated at 37°C for 24 hours and after incubation, clear zone of inhibition was measured in millimeter by ruler. Zones of inhibition were marked as sensitive, resistant or intermediate by comparing with CLSI-2020 manual (Shahid et al., 2021).

Antimicrobial activity of plant essential oils against *S. aureus* resistant isolates

Multiple antibiotic resistant isolates were further processed for antibacterial activity of commercially available plant essential oils (n=4) including *S. aromaticum*, *C. verum*, *E. globulus* and *F. assa-foetida*. To check antimicrobial activity, well-diffusion method was used and 6mm wells were cut using sterile well-borer. Nutrient agar plates were swabbed

Table 1. Primer sequences used for molecular confirmation

Gene	Primer name	Primers sequence	Amplicon bp	References
16S rRNA	8FLP-F XB4-R	5'-AGTTTGATCCTGGCTCAG-3' 5'-GTGTGTACAAGGCCCGGGAAC-3'	1500	(Asghar et al., 2016)
Methicillin (<i>mecA</i>)	MECA1-F1 MECA2-R	5'-ACTGCTATCCACCCTCAAAC-3' 5'-CTGGTGAAGTTGTAATCTGG-3'	163	(Shahsavan et al., 2012)
Coagulase (<i>coa</i>)	COA1-F COA1-R	5'-CGAGACCAAGATTCAACAAG-3' 5'-AAAGAAAACCACTCACATCA-3'	970	(Goh et al., 1992)
Exfoliative toxin A (<i>eta</i>)	ETA1-F ETA2-R	5'-GCAGGTGTTGATTAGCATT-3' 5'-AGATGTCCTATTTTGCTG-3'	93	(Sila et al., 2009)

by 24 hours incubated 0.5 MacFarland inoculum and oils with 1:1 ratio with dimethyl sulfoxide (DMSO) were poured in each well and the solvent also used as negative control. Culture plates were incubated at 37°C for 18-24 hours and after incubation, the diameter of zones of inhibition around the wells were measured in millimeter by ruler (Malathy et al., 2021).

Minimum Inhibitory Concentration (MIC) of plant essential oils

Minimum concentration of essential oil having ability to inhibit visible growth of *S. aureus* was evaluated by performing antimicrobial assay such as MIC. A volume of 100µL nutrient broth was pipetted in each row from 1st to 12th wells of 96 well flat bottom micro-titration plate. Two fold serial dilution of 100µL plant EO's such as *S. aromaticum*, *C. verum*, *E. globulus* and *F. assa-foetida* were made from 1st to 10th well each and 100µL volume was discarded from 10th well. Bacterial suspension of 100µL (0.5 MacFarland) was pipetted from 1st to 11th wells each then optical density of micro-titration plate was taken at 630nm at 0 time and 24 hours after the incubation at 37°C. After this, the same MIC procedure was repeated for *E. globulus* fractions in n-hexane, chloroform, n-hexane + chloroform and ethyl-acetate organic solvents (Argyri et al., 2021).

Cytotoxicity analysis of plant essential oils

Cytotoxicity assay of *E. globulus* oil n-hexane fraction was performed using Baby Hamster Kidney 21 (BHK-21) cell line which was grown in Glasgow minimum essential medium (GMEM) having 8-10% Fetal Calf Serum (FCS). Using 96 wells flat bottom micro-titration plate, 1×10⁵ BHK-21 cells were inoculated per 300µL GMEM in each well and after cell monolayer formation, 2 fold serial dilutions of *E. globulus* oil n-hexane fraction of essential oil were prepared followed by incubation at 37°C under 5% CO₂ concentration. After washing with sterile PBS, equal volume of 1% crystal violet solution and 3% formalin were used as staining solution and after washing stained cell line (96 well plate) was placed for overnight air drying. In each well, 50µL DMSO was added and optical density was taken at 570 nm by ELISA plate reader (Maurya et al., 2021). Using following formula, cell survival percentage was calculated as:

$$\text{CSP} = \frac{\text{Test O.D} - \text{Negative control O.D}}{\text{Live cell control O.D} - \text{Negative control O.D}} \times 100$$

GC/MS analysis of *E. globulus* oil

E. globulus oil fraction in n-hexane organic solvent was subjected for GC-MS analysis following the method of Azadmanesh and his fellows with minor modifications and for this, CARBOWAX capillary column along with helium as carrier gas was used. Sample injector was heated at 260°C and essential oil fraction made in 3 different solvents combinations were injected at 1µL/minute rate. Active compounds in tested sample were detected by comparison of retention time with standard compound (Azadmanesh et al., 2021).

Statistical analysis

Data obtained from antibiotic sensitivity, MIC and agar-well diffusion assay was analyzed through One way analysis of variance (ANOVA) followed by Duncan's multiple range test as post hoc by using Statistical package for social sciences (SPSS) Version 20.0 and level of significance (p-value) of 0.05 was chosen for statistical analysis.

RESULTS

Biochemical identification

S. aureus isolates were identified as gram-positive cocci in clusters, positive for catalase, beta hemolysis and oxidase negative. Only 2 isolates were observed coagulase positive.

Phylogenetic analysis

S. aureus phylogenetic analysis (16S r RNA gene sequence) was performed through the Maximum Likelihood method, bootstrap consensus tree inferred from 1000 replicates by using 33 16S rRNA sequences from NCBI data base and 10 Pakistan *S. aureus* (colored star) sequences submitted to GenBank. Accession numbers were retrieved from NCBI i.e. MW344063.1, MW344064.1 and MW344065.1. *S. aureus* MW344064.1 and MW341439.1 are 98% related to each other and both are 15% evolutionary related to MW344065.1 and MW341440.1, MW345972.1 35% homologous to MW 344063.1 and MW341438.1 and both are 98% evolutionary related to each other (Figure 1).

Coagulase, methicillin and exfoliative toxin A gene amplification

S. aureus isolates confirmed at molecular level (n=03) were subjected to *coa*, *mecA* and *eta* gene amplification. Out of three, 2 isolates were observed positive for coagulase gene. All three isolates were positive for *mecA* gene and one isolate was observed positive for *eta* gene (Figure 2). As our 3 isolates were positive for *mecA* gene that clearly indicate towards MRSA.

Antibiotic sensitivity

Confirmed *S. aureus* isolates (n=3) were subjected to antimicrobial susceptibility pattern and their zone of inhibitions were compared with clinical laboratory standard institute (CLSI). The highest mean zone of inhibition was recorded for amoxicillin clavulanate as mean ZOI±S.D. (18.67±2.08mm) while lowest mean zone of inhibition was recorded for gentamicin (1.67±2.88mm). As statistically, amoxicillin clavulanate antibiotic differ significantly ($p<0.05$) from other group of antibiotics. The tested isolates were resistant to all the antibiotics as compared to CLSI-2020 standards (Table 2).

Plant essential oils antimicrobial activity against *S. aureus*

The antimicrobial pattern of selected plant essential oils were tested for *S. aureus* isolates with accession numbers MW344063.1, MW344064.1 and MW344065.1 by well diffusion method and zone of inhibitions were recorded. The highest zone of inhibition was measured for *C. verum* (22.67±1.52^c mm) followed by *E. globulus* (18.67±2.51^b mm), *S. aromaticum* (12.67±2.51^a mm) and lowest for *F. assa-foetida* (9.67±0.57^a mm) plant oil. Statistically, *C. verum* plant essential oil zone of inhibition differed significantly ($p<0.05$) with other three plant essential oils and non-significance difference ($p>0.05$) was observed among *S. aromaticum* and *F. assa-foetida* zones of inhibition.

Minimum inhibitory concentration (MIC) of plant essential oils was determined against *S. aureus* isolates. Mean MIC of *E. globulus*, *C. verum*, and *F. assa-foetida* differed non-significantly ($p>0.05$) and *S. aromaticum* differed significantly ($p<0.05$) from other plant essential oils. Lowest mean MIC was recorded for *E. globulus* (0.33±0.11^a mg/mL) and highest mean MIC was observed against *S. aromaticum* (10.03±3.47^b mg/mL) essential oil (Table 3).

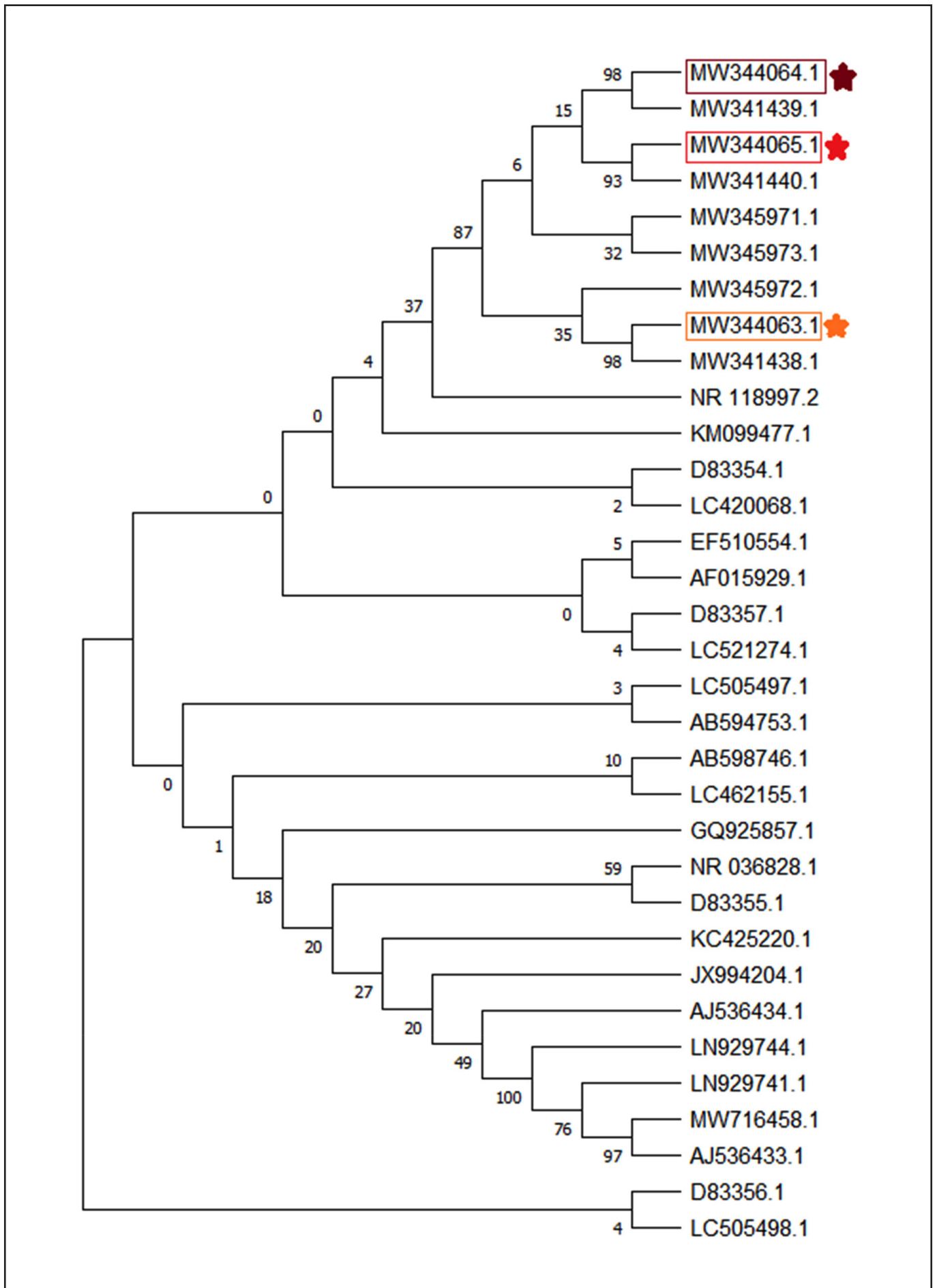


Figure 1. Phylogenetic analysis based on 16S rRNA gene sequences of *S. aureus*.

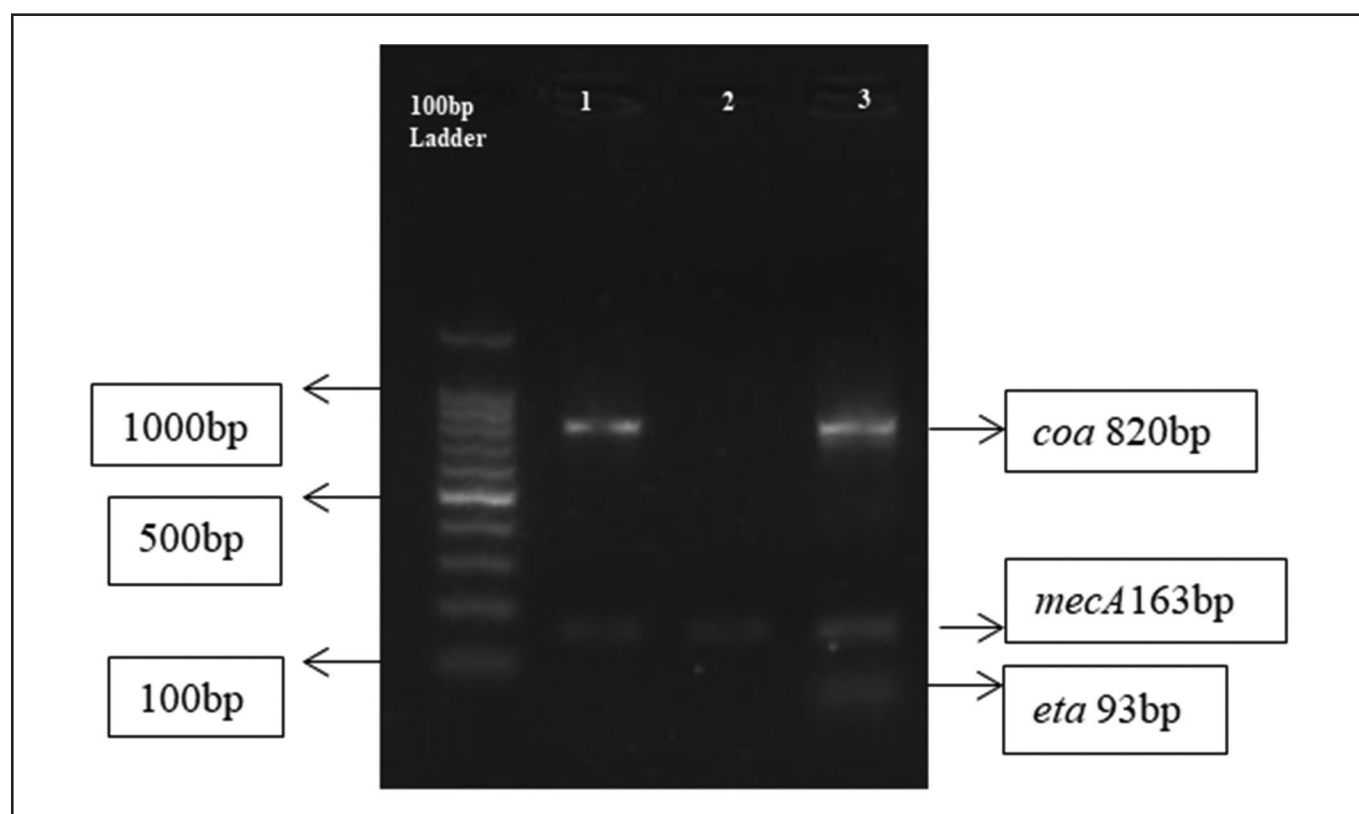


Figure 2. Methicillin resistant, coagulase and exfoliative toxin genes based Multiplex PCR. Lanes 1, 2 and 3: multiplex PCR product of Methicillin resistant *Staphylococcus aureus* isolates 1, 2 and 3.

Table 2. Antibiotic sensitivity of *S. aureus* isolates

Sr. No	Antibiotics	Conc. (μg)	CLSI Standards (mm)			Mean ZOI \pm S.D.
			Resistant	Intermediate	Sensitive	
1	Cefoxitin	30	$R \leq 21$	–	$S \geq 22$	$12.00 \pm 2.00^{c,d}$
2	Ampicillin	10	$R \leq 21$	–	$S \geq 22$	$12.67 \pm 3.05^{c,d}$
3	Amoxicillin	25	$R \leq 21$	–	$S \geq 22$	$11.33 \pm 1.52^{c,d}$
4	Penicillin	10	$R \leq 28$	–	$S \geq 29$	$13.33 \pm 0.57^{c,d,e}$
5	Augmentin	30	$R \leq 21$	–	$S \geq 22$	$18.67 \pm 2.08^{d,e}$
6	Ciprofloxacin	5	$R \leq 15$	I=16 – 20	$S \geq 21$	$9.67 \pm 1.15^{b,c}$
7	Erythromycin	15	$R \leq 13$	I=14 – 22	$S \geq 23$	$3.33 \pm 5.77^{a,b}$
8	Gentamycin	10	$R \leq 12$	I=13 – 14	$S \geq 15$	1.67 ± 2.88^a

Value with different superscripts (a, b, c, d, e) differ significantly ($p < 0.05$), with similar differ non-significantly ($p > 0.05$) and vice versa.

Table 3. MIC of plant essential oils against MRSA

Sr. No	Plant Essential oils	MIC Values (mg/ml)			Mean MIC \pm S.D.
1	<i>Syzygium aromaticum</i>	12.04	12.04	6.02	10.03 ± 3.47^b
2	<i>Eucalyptus globulus</i>	0.40	0.20	0.40	0.33 ± 0.11^a
3	<i>Cinnamomum verum</i>	0.41	0.41	0.82	0.54 ± 0.23^a
4	<i>Ferula assa-foetida</i>	6.29	0.78	0.78	2.61 ± 3.18^a

Antimicrobial activity of *Eucalyptus globulus* essential oil fractions

As *E. globulus* plant essential oil exhibited lowest MIC value and was further subjected for fractionation by column chromatography to study the effective antimicrobial pattern. Four fractions of *E. globulus* such as n-hexane, chloroform,

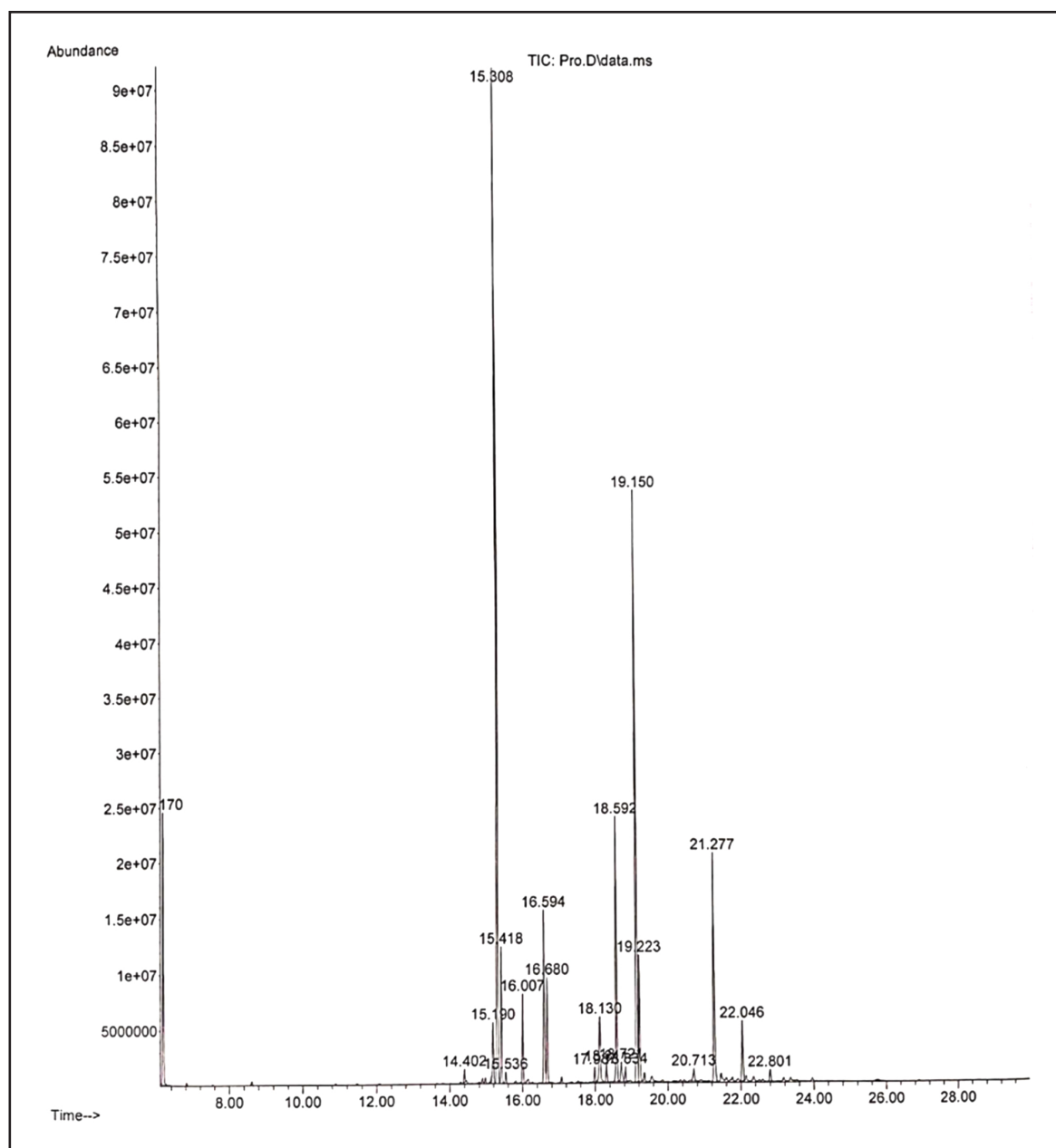
n-hexane + chloroform and ethyl-acetate were obtained. Lowest mean MIC was recorded for n-hexane fraction (10.04 ± 5.80 mg/mL) and highest mean MIC was recorded for chloroform fraction (83.83 ± 36.30 mg/mL). Statistically, mean MIC of n-hexane fraction differs significantly with other three fractions and chloroform, n-hexane + chloroform and ethyl-acetate differ non-significantly with each other.

Cytotoxicity of n-hexane fraction of *E. globulus* essential oil

The lowest MIC was recorded for *E. globulus* n-hexane fraction as compared to chloroform, n-hexane + chloroform and ethyl acetate fractions. *E. globulus* n-hexane fraction was processed for cytotoxicity assay. Cytotoxicity was evaluated on BHK21 cell line and cell survival percentage was 51.7% at 54.87mg/mL concentration and this concentration relates to the exact MIC concentration exhibited by oil fraction.

Table 4. GCMS analysis of *E. globulus* n-hexane fraction

Compounds	Retention time	Percentages
Bicyclo[4.1.0]heptane	14.4	0.33
Benzene	15.1	1.9
Benzene	15.3	29.9
Cyclohexano	15.4	3.8
γ -Terpinene	16	2.55
Cyclohexene	16.5	5.22
Benzene	16.6	3
Trans-4-methoxy thujane	18.1	3
Anisole, 2-isopropyl-5-methyl	18.5	8
Bicyclo[3.1.0]hexane, 6-isopropylidene-1-methyl	19.1	17.7
3-Cyclohexene-1-methanol, $\alpha,\alpha,4$ -trimethyl-, acetate	19.2	3.8
2,4-Octadienoic acid, 3-methyl	20.7	0.5
Octane	21.2	7.7
Methoxycitronellal	22	2
Longifolene	22.8	0.44

**Figure 3.** Chromatogram of *E. globulus* oil n-hexane fraction by GC-MS analysis.

GC-MS analysis

Through GC-MS analysis in n-hexane fraction of *E. globulus* essential oil, benzene was found 29.9% abundant as active compound. Bicyclo [3.1.0] hexane, 6-isopropylidene-1-methyl in n-hexane fraction of *E. globulus* was found second abundant (17.7%) active compound (Table 4, Figure 3).

DISCUSSION

Clinical and diagnostic laboratories mostly use manual, automated phenotypic and commercial bacterial identification methods (Church et al., 2020). Gram staining, colony characters, growth requirements, metabolic and enzymatic reactions fall into phenotypic methods of identification, but these methods have been altered due to environmental stress and evolution (Ochman et al., 2005). Identification through biochemical test results can be compromised by inexperience and technologist's bias. Ribosomal RNA 16S gene sequence based genotypic identification has emerged as an accurate, objective and reliable method for bacterial identification with defining taxonomic relationships among bacteria (Alown et al., 2021). Our results were in agreement to the findings of Krimmer and his co-workers that 16S rRNA gene sequencing is quite efficient and economic method than whole gene sequencing for bacterial species confirmation at molecular level (Krimmer et al., 1999).

The MRSA, one type of nosocomial pathogen is spreading probably due to continues overuse of broad spectrum beta lactam antibiotics (Fatholahzadeh et al., 2009). The MRSA was identified through methicillin resistant gene amplification. In present study, characterized isolates (n=3) were positive for containing resistance genes. Present study results were in agreement to Algammal et al. (2020). Coagulase enzyme is main virulence factor of *S. aureus* leads to the clotting of plasma in host. It causes conversion of fibrinogen to fibrin and fibrin production shields the bacteria from phagocytosis. In laboratory, coagulase test has been done as standard phenotypic test and researchers implement the coagulase gene identification test as accurate defined test. Coagulase is highly polymorphic because it contains palindromic sequences (variable region based on length) at 3' end (81 bp tandem repeat) (AL-Zengena et al., 2019). Due to these sequences, difference in amplified products was observed when amplification was performed using same primer sets (Schwarzkopf, 1995). But in present study by using same primer set, amplicons with similar band size were obtained after amplification and 2 out of 3 isolates were positive for coagulase (*coa*) gene. These results were in contrast to study of Javed and his colleagues (Javid et al., 2018). Exfoliative toxin (ET) of *S. aureus* is responsible for causing blisters in bullous impetigo and in disseminated form as staphylococcal scaled skin syndrome (SSSS) (Lange et al., 2020). ET has 3 iso-forms such as ETA, ETB and ETD. In the present study, only one isolate was observed positive for ETA (*eta*) gene among 3 *S. aureus* isolates.

The *S. aureus* infections especially MRSA is difficult to treat due to resistant to various antibiotics (Algammal et al., 2020). According to CLSI-2020 manual, level of bacterial resistance is grouped in to sensitive, intermediate and resistance. Cefoxitin, ampicillin, amoxicillin, penicillin and amoxicillin clavulanate fall in beta lactam antibiotic group and *S. aureus* exhibited resistance to beta lactam antibiotics. Previously similar pattern was observed by Tyasningsih and his co-workers (Tyasningsih et al., 2019). As, these antibiotics disrupt the cell wall synthesis (peptidoglycan). Bacteria produced beta lactamase which break up the beta lactam ring so beta lactam antibiotic become unable to bind to penicillin binding proteins (PBPs) (Effendi, 2009).

Essential oils are extensively used in pharmaceuticals, cosmetics and in beverage industry and known as natural plant products. Herbs chemical extracts and oils are used as medicine to treat various bacterial infections. The reason behind their usage is emerging bacterial resistance to commercial synthetic antibiotics. Clove oil exhibits antimicrobial activity against *S. aureus*, *K. pneumonia*, *E. coli* and *E. faecium* (Weber et al., 2004). In this study, *F. assa-foetida* EO inhibited *S. aureus* at 2.61±3.18mg/mL concentration while Sharopov and coworkers found that *F. assa-foetida* EO in 0.5–4 µL/mL concentration inhibited *S. aureus* growth (Sharopov et al., 2019). The *C. verum* EO exhibited zone of inhibition against *S. aureus* growth with 22.67±1.52 mm and 0.54±0.23 mg/mL MIC value was recorded. The antibacterial activity of *C. verum* oil is due to the active component cinnamaldehyde (Wijesinghe et al., 2021). The *E. globulus* EO exhibits prominent antibacterial activity against gram positive microorganisms including *S. aureus*. *E. globulus* EO showed second highest inhibition zone against *S. aureus* isolates. Among solvent fractions of *E. globulus*, n-hexane fraction proved promising against *S. aureus*. Present study results were in agreement to results of Pereira and his colleagues (Pereira et al., 2014) and Ali and his coworkers (Ali et al., 2021).

Essential oils are used for the therapy of various microbial infections and for this purpose, their *in-vitro* and *in-vivo* evaluation is mandatory. Cytotoxicity assays are performed for this purpose on several types of eukaryotic cell lines. It was concluded that cytopathic effects of essential oils are dependent on concentration; higher the concentration higher will be the cytotoxicity. *E. globulus* cytotoxicity was ascertained by brine shrimp lethality test (BSLT) (Akolade et al., 2012) and results were observed in agreement to Falah and his colleagues (Falah et al., 2021) that when concentration was increased, increase in cytotoxic effect was observed. For cytotoxicity testing of *E. globulus* oils, MIC concentration was used. In this present study, cell survival increased (92-98%) with the decrease of oil concentration (1.85-1.89mg/mL).

The GC-MS is gold standard test for chemical composition analysis of different organic and inorganic compounds, extracts, oils and nanoparticles (Behbahani et al., 2019). Eucalyptus plant essential oils are used as folk medicines as anti-inflammatory, analgesic and antipyretic tonics. *E. globulus* fresh leave essential oil was analyzed through GC/MS and twenty components were identified. The highest percentage of 48.6 was recorded for 1,8-cineole as active component (Daroui-Mokaddem et al., 2010). Nearly 45 components from *E. globulus* fruit oil were identified through GLC/MS. Aromadendrene has been found in abundance (31.17%) followed by 1,8-cineole 14.55% and globulol 10.69% (Mulyaningsih et al., 2010). Mulyaningsih and his colleagues have founded 1,8-cineole as second abundant active component in essential oil that is in contrast to Daroui-Mokaddem and his co-workers findings. Compound 1,8-cineole was found 77.02% in essential oil as 1st active chemical in agreement to Daroui-Mokaddem and his colleagues study (Daroui-Mokaddem et al., 2010). In present study, *E. globulus* essential oil n-hexane fraction was subjected to GC/MS analysis and benzene was found 29.9% abundant as compared to other components as active compound followed by Bicyclo [3.1.0] hexane, 6-isopropylidene-1-methyl 17.7% as 2nd active component in essential oil fraction. As cytotoxicity test was performed to evaluate the safety index of n-hexane oil fraction so benzene can be considered safe if used within reported concentration. These findings were in contrast to the previous studies (Thappa et al., 1990).

E. globulus essential oil and its n-hexane fraction are better alternate for treatment in antibiotic resistant cases of MRSA (Takahashi et al., 2004). Due to less cytotoxicity, this oil can be the alternative therapeutic option for the treatment of MRSA infections via oral route or transdermal application.

Conflict of interest

The authors declare that they have no conflict of interests.

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