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Research Article

IN VITRO ANTIBACTERIAL ACTIVITY OF ESSENTIAL PLANT OILS AGAINST BIOFILM FORMING METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

An alarming increase in biofilm forming methicillin-resistant *Staphylococcus aureus* (MRSA) possesses a serious problem in hospital environment demands a renewed effort to seek agents from natural system that are effective against pathogenic bacteria resistant to current antimicrobials. In the study, the distribution of biofilm forming MRSA and the antibacterial activity of essential oils (Eucalyptus, Mint, Turpentine, Neem and Amla) was studied in 58 strains of *S. aureus* isolated from pus samples. Out of 58 clinical samples 22 *S. aureus* were found to be methicillin-resistant and showed a dry black crystalline morphology indicating strong biofilm production and they were screened for the antibacterial activity of five different essential oils by using agar well diffusion method. The results from the agar well diffusion method showed that 4 essential oils could inhibit the growth of biofilm forming *S. aureus* isolates. Among those turpentine oil had strong inhibitory effects with a zone of inhibition ranging from 16.8 ± 1.77 mm to 32.0 ±2.12 mm. Eucalyptus oil shown moderate antibacterial activity against all tested isolates and followed by mint and neem with the average zones of inhibition. The oils at all concentrations showed potent inhibitory activity against the tested *S. aureus* with the exception of amla oil where there were no reports of inhibition. It is known that essential oils are composed of numerous different chemical compounds and their antimicrobial activity might be attributed to several different mechanisms, which could explain the variations in their mode of action. However, more studies are required to find the compounds of essential oils responsible for their antimicrobial activity, since little is known about essential oils and their medicinal property.

Keywords: *Staphylococcus aureus*, MRSA, Biofilm, Antibacterial activity, RAPD

INTRODUCTION

Antibiotic resistance is an important threat to public health on a global scale as it reduces the effectiveness of treatment and increases morbidity, mortality and health care costs [1]. Evolution of highly resistant bacterial strain has compromised the use of new generations of antibiotics [2]. Antibiotic resistance is due to an inherent ability of microorganisms to form surface-attached communities of cells within the extracellular polymeric matrix called biofilms [3]. Microbial biofilms pose a challenge in clinical and industrial settings where the need for sterility is paramount. In response to certain environmental cues, bacteria living in biofilms are capable of using active mechanisms to leave biofilms and return to the planktonic (free-living) state in which sensitivity to antimicrobials is regained [4-6]. Moreover, bacteria within biofilm grow slowly and adopt a phenotype that confers an intrinsic resistance to many antibiotics classes [7] including the β-lactams [8]. The challenge presented by biofilm infections is the remarkable resistance to both host immune responses and available chemotherapies [9,10] and estimates suggest that as many as 80% of chronic bacterial infections are biofilm associated [11]. Consequently, biofilm-associated infections are recalcitrant to antimicrobial therapy and often require surgical intervention to debride infected tissues and/or remove colonized implants.

Chronic nosocomial infections by gram-positive bacteria have become more prevalent in recent years with the increased use of prosthetic biomedical implants. Staphylococcal infections are a major source of patient morbidity and implant failure [12]. *Staphylococcus aureus* causes potentially life threatening nosocomial and community-acquired infections, such as osteomyelitis and endocarditis [13]. The opportunistic pathogen *S. aureus* can form biofilms on many host tissues and implanted medical devices often causing chronic infections [14-17]. The resistance of *S. aureus* is associated with its ability to produce toxins and other extracellular polysaccharides like biofilms. In recent years, multidrug resistant strains have developed. Methicillin-resistant *S. aureus* (MRSA) is a special strain that is resistant to the antibacterial activity of methicillin and other related antibiotics of the penicillin class. Although, MRSA has traditionally been seen as hospital-associated infections, community –acquired MRSA strains have appeared in recent years [18]. Several new strains of MRSA have been found showing antibiotic resistance even to Vancomycin and Teicoplanin; these new evolutions of the MRSA bacteria are called Vancomycin Intermediate- resistant *S. aureus* (VISA) [19]. Community-acquired MDRSA (multidrug resistant *S. aureus*) infections in the absence of identified risk factors have been reported. Many outbreaks of infections due to MDRSA have occurred and it has now become endemic in several centres in the world [20]. Therefore, the current situation of the susceptibility patterns of local strains is essential for the judicious use of alternative drugs for the treatment of infectious diseases from medicinal plants [21].

Essential oils of medicinal plants have been used for hundreds of years of natural medicines to combat a multitude of pathogens, including bacteria, fungi and viruses [22]. Several essential oils confer antimicrobial activity by damaging the cell wall and membrane, leading to cell lysis, leakage of cell contents and inhibition of protonmotive force [23]. In addition, there is evidence that they effectively kill bacteria without promoting the acquisition of resistance [24, 25] and they possess multiple antimicrobial activity i.e., antibacterial [26], antifungal [27], anticancer, antiviral and antioxidant properties [28, 29] against all pathogens [30]. Finally, many essential oils are relatively easy to obtain, have low mammalian toxicity and degrade quickly in water and soil, making them relatively environmentally friendly [31]. For these reasons research is ongoing for new antimicrobial agents, either by the design and synthesis of new agents or through the search of natural plant oils for as yet undiscovered antimicrobial agents [32].

MATERIALS AND METHODS

Collection of essential oils

Five essential oils namely Eucalyptus, Mint, Turpentine, Neem and Amla oils were obtained from the herbal store of Salem district. These oils were selected based on the literature survey and their use in traditional medicine system.

Sample collection

Seventy eight clinical pus swabs were collected from hospitalized patients of various private hospitals in and around Namakkal area from January to February, 2011 using sterile swab saturated with Brain Heart Infusion broth. All the specimens were transported immediately to the laboratory and cultured within 3 to 4 h of collection.

Isolation and characterization of bacteria

The swab specimens were inoculated on various ordinary media: blood agar base, nutrient agar, macconkey agar (Hi Media, India) to obtain discrete colonies. The plates were incubated at 37˚ C for 24 h under aerobic conditions. After 24 h of incubation, the culture plates were examined for recording the appearance, size, colour and morphology of the colonies. Gram stain reaction, catalase test and coagulase test, growth on differential and selective media such as mannitol salt agar, triple sugar iron agar, (Hi Media, India) and other biochemical tests were carried out according to standard techniques [33, 34].

Isolates that are gram positive, cocci, catalase positive, coagulase positive and form yellow colonies on mannitol salt agar were considered *Staphylococcus aureus* in this study.

Antibiotic Susceptibility test

Susceptibility to antimicrobial agents was determined by Disc Diffusion method of Kirby Bauer on Muller-Hinton agar as described by the Clinical and Laboratory Standard Institute (CLSI). The antibiotic discs used (Hi-Media) were Ampicillin, Penicilin-G, Streptomycin, Oxacillin, Amikacin, Gentamicin, Tetracycline, Chloramphenicol, Methicillin and Vancomycin.

Biofilm Production assay

Congo red agar method (CRA)

Congo red agar method [35] had described an alternative method of screening biofilm formation by Staphylococcal isolates; which requires the use of a specially prepared solid medium-brain heart infusion broth (BHI) supplemented with 5% sucrose and Congo red. The medium was composed of BHI (37 g/L), sucrose (50 g/L), agar no.1 (10 g/L) and Congo red stain (0.8 g/L). Congo red was prepared as concentrated aqueous solution and autoclaved at 121°C for 15 minutes, separately from other medium constituents and was then added when the agar had cooled to 55°C. Plates were inoculated and incubated aerobically for 24 to 48 hours at 37°C.

Positive result was indicated by black colonies with a dry crystalline consistency. Weak slime producers usually remained pink, though occasional darkening at the centres of colonies was observed. A darkening of the colonies with the absence of a dry crystalline colonial morphology indicated an indeterminate result. The experiment was performed in triplicates.

Antibacterial screening

Agar well diffusion method

The antibacterial activities of the five essential oils were tested by agar well diffusion method [36]. The culture plates were prepared by pouring 20 ml of sterile Hi-sensitivity (Himedia- M 486) agar medium. The depth of the medium was approximately 4 mm. Three to four similar colonies of pure cultures were inoculated with tryptone soy broth (Himedia- M 323), further, it was incubated at 37˚C for 2-8 h and inoculum size was adjusted to yield uniform suspension containing 10^5-10^6 cells/ml (McFarland's standard). The agar surface of the plates was swabbed in three directions, turning the plates at 60˚ between each swabbing. Confluent growth is desirable for accurate results. Using a 6 mm sterile cork borer, wells were prepared on the swabbed hi-sensitivity agar plates. Five different concentrations of oils were prepared (3, 6, 9, 12 and 15µl) and loaded in appropriate wells. Allowed the plates to stand at refrigerator for 30 min (Pre-diffusion time). The plates were incubated at 37˚C for 16-18 h during which the activity was evidenced by the presence of zones of inhibition surrounding the well. Each experiment was done in triplicate.

Random Amplified Polymorphic DNA Technique

RAPD assays were determined according to Randa (2006) protocol with some modification. The primers was obtained from Sigma, India and used in the PCR comprised primer OPA13 (5'-CAGCACCCAC-3'). RAPD-PCR was carried out in a 20 µl reaction mixture containing 0.5µl of 10 pmol primer, 0.5 µl of Taq DNA polymerase (con. 3U/µl), 2 µl of 10X PCR buffer, 1 µl of DNA template, 1 µl of 25 mM of each deoxynucleotide triphosphate and 15 µl of nuclease-free water. Amplification conditions consisted of denaturation at 94˚ C for 60 sec and 35 cycles of denaturation at 94˚ C for 35 sec, annealing at 33˚ C for 30 sec, extension at 72˚ C for 65 sec and final extension at 72˚ C for 5 min. PCR products were detected in 1% agarose gel. 1 Kb DNA marker was included as molecular size marker. Gels were visualized by staining with EtBr and bands patterns were observed with UV illumination.

RESULT

Among the total of 77 pus swabs collected 58 strains of *S. aureus* were isolated from various pus samples including burns, accidental wounds, and surgical wounds respectively.

Antibiotic Susceptibility test

Antibiotic susceptibility assays revealed that among the 58 isolates, 14 were susceptible to all antibiotics used in this study. All the isolates (100%) were also susceptible to Vancomycin. Higher resistance was observed to Ampicillin and Penicillin-G (75.86%), Streptomycin (67.24%), Oxacillin (65.51%), Amikacin, Gentamicin and Tetracycline (62.06%) and Chloramphenicol (56.89%). Twenty two isolates of *S. aureus* were found to be methicillin-resistant, while the remaining (36) isolates were methicillin-susceptible. Among the isolates studied high resistance was observed against the group of β-lactam antiobiotics.

Biofilm production assay

Congo red agar method (CRA)

Biofilm production by clinical isolates of *S. aureus* is detected by Congo red agar method. Out of 58 clinical isolates of *S. aureus* 22 (37.93%) isolates showed a dry black crystalline morphology indicating strong biofilm production. Twenty eight (48.27%) isolates showed moderate biofilm formation with red or black colonies with or without dry crystalline morphology; eight (13.79%) isolates were weak producers with pink colour colonies which is difficult to differentiate from biofilm negative isolates in the Congo red agar method.

Table 1: Biofilm production by clinical isolates of *Staphylococcus aureus*

Slime test	Congo Red Agar Method	
	Biofilm +	%
Strong biofilm	22	37.93
Moderate biofilm	28	48.27
Weak/non biofilm	8	13.79

Table 2 Antibacterial activity of Essential oils against *Staphylococcus aureus*

Name of the Isolates *Staphylococcus aureus* (Sa)	Zone of Inhibition in mm				
	Eucalyptus oil Mean ± SEM	Mint oil Mean ± SEM	Turpentine oil Mean ± SEM	Neem oil Mean ± SEM	Amla oil Mean ± SEM
Sa 01	14.6 ± 1.66	-	23.4 ± 2.20	19.2 ± 1.56	-
Sa 02	14.0 ± 1.84	21.0 ± 1.41	25.0 ± 1.84	17.8 ± 1.56	-
Sa 03	-	21.0 ± 1.14	26.0 ± 1.84	-	-
Sa 04	17.0 ± 1.41	20.0 ± 1.70	21.0 ± 1.64	21.8 ± 1.35	-
Sa 05	22.4 ± 1.77	29.0 ± 1.70	29.4 ± 2.20	28.0 ± 1.51	-
Sa 06	23.8 ± 1.77	31.4 ± 2.06	30.8 ± 2.26	30.2 ± 1.80	-
Sa 07	21.6 ± 2.20	26.4 ± 1.88	29.2 ± 2.35	27.6 ± 2.63	-
Sa 08	20.4 ± 2.06	27.4 ± 2.20	27.8 ± 2.08	27.0 ± 1.64	-
Sa 09	16.6 ± 1.63	22.4 ± 1.63	-	15.8 ± 1.77	-
Sa 10	-	23.0 ± 1.14	22.6 ± 1.63	20.0 ± 1.22	-
Sa 11	15.0 ± 2.12	18.8 ± 1.56	23.6 ± 1.63	-	-
Sa 12	12.8 ± 1.56	-	20.2 ± 1.56	24.0 ± 1.58	-
Sa 13	17.2 ± 1.77	17.0 ± 1.58	19.0 ± 1.70	15.2 ± 1.06	-
Sa 14	12.2 ± 1.77	15.2 ± 1.56	16.8 ± 1.77	21.4 ± 1.20	-
Sa 15	19.0 ± 1.41	-	23.8 ± 1.98	11.8 ± 1.65	-
Sa 16	26.2 ± 1.93	29.8 ± 2.26	32.0 ± 2.12	30.7 ± 1.26	-
Sa 17	13.8 ± 2.13	21.2 ± 1.46	24.0 ± 1.84	14.6 ± 1.50	-
Sa 18	18.4 ± 1.43	24.2 ± 1.65	22.0 ± 1.41	-	-
Sa 19	15.6 ± 1.63	24.0 ± 1.22	21.0 ± 2.12	-	-
Sa 20	18.0 ± 1.70	20.4 ± 1.36	-	18.4 ± 1.63	-
Sa 21	14.0 ± 1.84	-	19.0 ± 1.70	15.8 ± 1.77	-
Sa 22	-	15.2 ± 1.56	-	15.2 ± 1.06	-

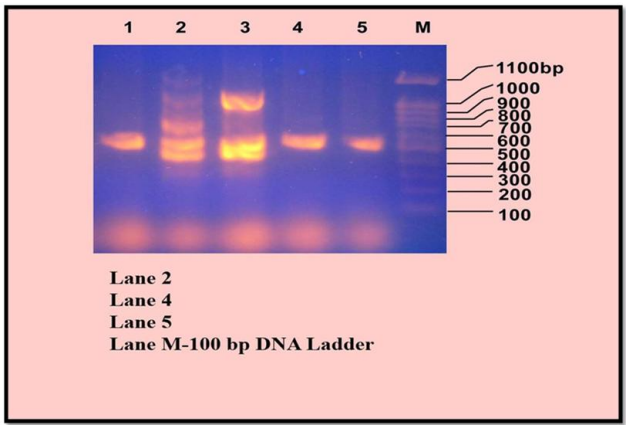

Fig. 1: RAPD Analysis of *Staphylococcus aureus*

Antibacterial activity- Agar Well Diffusion Assay of Essential Oil on Biofilm forming MRSA

Out of 58 clinical samples of *S. aureus* 22 isolates of methicillin resistant and biofilm producing strains were screened for the antibacterial activity of essential oils. Agar well diffusion is one of the most common assays used in the evaluation of antibacterial activity of essential oils. *In vitro* antibacterial properties of the essential oils of Eucalyptus, Mint, Turpentine, Neem and Amla against 22 biofilm forming Methicillin-resistant *S. aureus* (MRSA) exposed at 5 different concentrations were studied. Antibacterial activity of essential oils ranged from no inhibition to complete inhibition against the biofilm forming MRSA isolates. The essential oil of turpentine was effective against all the tested isolates, the mean zone of inhibition ranged from 16.8 ± 1.77 mm to 32.0 ±2.12 mm and this oil shown considerable antibacterial activity against isolates of Sa (*S. aureus*) 16 (32.0 ± 2.12 mm), Sa 06 (30.8 ± 2.26 mm), Sa 05 (29.4 ± 2.20 mm), Sa 07 (29.2 ± 2.35 mm), Sa 08 (27.8 ± 2.08 mm), Sa 03 (26.0 ± 1.84 mm) and Sa 02 (25.0 ± 1.84 mm). Neem and mint oil invariably inhibits all isolates of biofilm forming MRSA; the neem oil was effective against Sa 16 (30.7 ± 1.26 mm), Sa 06 (30.2 ± 1.80 mm), Sa 05 (28.0 ± 1.51 mm), Sa 07 (27.6 ± 2.63), Sa 08 (27.0 ± 1.64 mm), and Sa 12 (24.0 ± 1.58 mm) whereas the mint oil was effective against Sa 06 (31.4 ± 2.06 mm), Sa 16 (29.8 ± 2.26 mm), Sa 05 (29.0 ± 1.70 mm), Sa 08 (27.4 ± 2.20 mm), Sa 07 (26.2 ± 1.88 mm) and Sa 18 (24.2 ± 1.65 mm). Eucalyptus oil shown moderate antibacterial activity against all tested isolates and the average zones of inhibition ranged from 12.2 ± 1.77 mm to 26.2 ± 1.93 mm. Isolates of Sa 16 (26.2 ± 1.93 mm), Sa 06 (23.8 ± 1.77 mm), Sa 05 (22.4 ± 1.77 mm), Sa 07 (21.6 ± 2.20 mm) and Sa 08 (20.4 ± 2.06 mm) were inhibited considerably by eucalyptus oil. Antibacterial activity by agar well diffusion method showed that turpentine oil was most active against biofilm forming MRSA followed by eucalyptus, mint and neem. The oils at all concentrations showed potent inhibitory activity against the tested *S. aureus* with the exception of amla oil where there were no reports of inhibition. On one hand, the growths of tested bacteria in high concentrations of oils were highly inhibited. On the other hand, at low concentrations, a very limited inhibitory effect was observed on the growth of microorganisms in comparison with those witnessed.

Random Amplified Polymorphic DNA Technique

Among the 22 isolates five biofilm forming MRSA strains (Sa 05, Sa 06, Sa 07, Sa 08 and Sa 16) showing higher inhibitory activity against five oils were selected to determine the genetic diversity among *S. aureus* by PCR amplification. Random primers are subjected to optimized conditions for PCR, were applied to all strains. This primer exhibited discriminatory band patterns among the *S. aureus*. The amplified fragments ranging from 100bp to 1200bp. Out of the 5 clinical samples single isolate of *S. aureus* produced number of bands. In our observation 5 types of RAPD patterns were observed.

Two isolates showed only single band (lane 4 and 5). Single common band were observed in all the isolates. The molecular weights of the common bands are nearly 500bp. Single isolates (lane 2) has highest molecular weight bands were observed and that range was 1200bp. This RAPD analysis was clearly indicating the diversity was present in all isolates of *S. aureus*.

DISCUSSION

Staphylococcus aureus is a medically important organism associated with a variety of diseases; some strains can cause chronic infections and gain increased resistance to antimicrobial agents through biofilm formation [37, 38]. Biofilm and multidrug resistance have been identified as virulence factors of great magnitude in *S. aureus* infections in clinical settings. Appearance of resistance against particular antibiotic in a specific region may be due to its frequent and long-term use [39-41]. MRSA represents a major challenge to hospitals in all countries due to the emergence and spread of isolates with decreased susceptibilities to several antibiotic classes, in

addition to methicillin and the other members of the β-lactam family [42]. The result of the present study revealed that a significant number of isolates showed resistance to antibiotics (Penicillin-G, Ampicillin, Streptomycin, Oxacillin, Amikacin, Gentamicin, etc.) that are frequently used. The occurrence of isolates resistant to Streptomycin was less frequent than that observed [43]. There was a higher prevalence of MRSA (37.93%) as compared with those in similar reports in the literature [44, 40, 45 & 41]. Moreover, the resistant proportion was higher in MRSA than in MSSA isolates for various antibiotics as the MRSA generally express resistance to multi drugs [43, 41]. Biofilm infections are a major medical problem with *S. aureus* and coagulase-negative staphylococci, as the leading species responsible for chronic polymer-associated infections [46, 47]. Researchers have investigated the strategies employed by microorganisms to produce biofilms and to understand the pathogenesis. They discovered that biofilm producing bacteria secrete certain chemicals that protect them from disinfectants and antimicrobials and phagocytic host immune systems [38]. Several conventional methods of detecting biofilm production have been established, such as the standard Tube Method [48], plate method [35, 49], and coverslip assay [49] etc. Using the Congo red agar (CRA) plate method for testing biofilms production, only 22 (37.93%) showed black dry crystalline morphology. Slime production has been reported in strains of all *Staphylococcus* spp. associated with the infection of biomedical devices [50]. The CRA plate method is not recommended as a medium for biofilm production in *S. aureus* species as researchers have only recently found that PJA/PNAG (polysaccharide intracellular adhesions/poly N-actyl glucosamine) have little input in the biofilm matrix of *S. aureus* and cannot detected by the CRA method [51]. Similar results have been reported by other authors [49, 52]. These reports suggest that CRA screening cannot be recommended to detect biofilm formation for *S. aureus* isolates.

The activity of natural products, especially essential oils (EO), against microorganisms has been recently confirmed by several studies focusing on antimicrobial activity of EO against planktonic cells. However, bacteria growing in biofilms exhibit a specific phenotype and are often, but not always, more resistant to antimicrobial agents than their planktonic counter parts [53, 54]. Thus it is important to search for natural products that have antibiofilm properties and antimicrobial activity against wound pathogens [55]. In this study, the essential oils of Eucalyptus, Mint, Turpentine, Neem and Amla was evaluated for antibacterial activity against 22 biofilm forming Methicillin-resistant *S. aureus* (MRSA). The result shown that the essential oil possesses some broad-spectrum antibacterial properties, contents of oils is sufficient to inhibit the growth of more than 90% of the tested *S. aureus.* Antibacterial activity of turpentine oil was most active against biofilm forming MRSA followed by neem, mint and eucalyptus. The zone of inhibition of turpentine oil against *S. aureus* isolates ranged from 16.8 ± 1.77 mm to 32.0 ±2.12 mm. The previous studies reported that essential oil from gum of *Pistacia atlantics* Desf. (Turpentine tree) has antimicrobial activity against *S. aureus*, *Escherichia coli* and *Streptococcus pyogenes* [56]. Essential oils rich in phenolic compounds such as *Pistacia* species are widely reported to possess high levels of antimicrobial activity [57]. On the other hand, it should be noted that two major volatile constituents, α-pinene and terpinolene contained in the *Pistacia* species are compounds with interesting antibacterial activity [58]. The antibacterial activity of neem and mint oil ranged from 11.2 ± 1.56 mm to 30.7 ± 1.26 mm. The previous studies accounted that neem oil has a effective antibacterial activity against both gram-positive and gram-negative bacteria [59]. The extract of neem exhibited a pronounced activity against *Bacillus subtilis* (28 mm), high activity against gram-positive organism *S. aureus* (18 mm) and also against the gram-negative bacteria [60]. Similar result was observed [61] that neem oil was effective against *S. aureus* (19 mm), *Salmonella typhi* (17.5 mm), *Escherichia coli* (19.5 mm) and *Pseudomonas aeruginosa* (17 mm). Eucalyptus oil possess a zone of inhibition ranged from 14.2 ± 1.56 mm to 31.4 ± 2.06 mm against the tested *S. aureus* isolates. Eucalyptus oil exhibits an antimicrobial property against bacteria and viruses. The past studies reviewed that eucalyptus oil and its major component, 1,8-cineole, have antimicrobial effects against many bacteria, including *Mycobacterium tuberculosis* and methicillin-resistant *S. aureus* (MRSA), viruses and fungi (including *Candida*) [62]. Based on literature there was an extensive research report on antimicrobial activity of eucalyptus oil a few reports is based on immune-stimulatory, anti-inflammatory [63, 64], anti-oxidant [65], analgesic [66] and spasmolytic effects were reported. The present investigation of antimicrobial activity of essential oils utmost comparably correlated with many research outcomes. In the present study five biofilm forming MRSA strains (Sa 05, Sa 06, Sa 07, Sa 08 and Sa 16) showing high inhibitory activity against five oils were selected to determine the genetic diversity among *S. aureus* by RAPD-PCR amplification. This RAPD analysis was clearly indicating the diversity present in all isolates of *S. aureus*.

CONCLUSIONS

In the present study, essential oils have shown nearly equal antimicrobial effects on both gram-positive and gram-negative bacteria. Turpentine oil was found to be the most effective. However, inhibition zone diameters obtained in well diffusion assays have shown better effectiveness of essential oils against biofilm forming methicillin-resistant *S. aureus* isolates. It may be due to volatile actions of essential oils and due to absence of lipo-polysaccharide layer in gram-positive bacteria that might function as an effective barrier against any incoming bio-molecule [67-74]. There might be another possibility that essential oils may successfully inhibit microbial respiration and increase the plasma membrane permeability, which results in to death of bacterial cells after massive ion leakage [75, 76]. It may also happen due to hydrophilic nature of bacterial cell wall. In the present study, almost all essential oils tested have shown strong antibacterial potential against *S. aureus*. It is known that essential oils are composed of numerous different chemical compounds and their antimicrobial activity might be attributed to several different mechanisms, which could explain the variations in their mode of action [77]. However, more studies are required to find the compounds of essential oils responsible for their antimicrobial activity, since little is known about essential oils and their medicinal property.

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CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

REFERENCES

1. Cassandra L Quave, Lisa RW Plano, Traci Pantuso and Bradley C Bennett. Effects of extracts from Italian medicinal plants on planktonic growth, biofilm formation and adherence of methicillin-resistant *Staphylococcus aureus.* J Ethanopharm 2008;118(3) :418-428.
2. Levy SB. The antibiotic paradox: How the Misuse of antibiotics destroys their curative powers. Perseus Publishing; Cambridge, MA; 2002
3. Davery ME, O' Toole GA. Microbial biofilms: from ecology to molecular genetics. Microbial Mol Boil Rev 2000;64 :847-867.
4. Fux CA, Wilson S, Stoodley P. Detachment characteristics and oxacillin resistance of *Staphylococcus aureus* biofilm emboli in an in vitro catheter infection model. J Bacteriol 2004;186 :4486-4491.
5. Boles BR, Thoendel M, Singh PK. Rhamnolipids mediate detachment of *Pseudomonas aeruginosa* from biofilms. Mol Microbiol 2005;57 :1210-1223.
6. Hall-stoodley L, Stoodley P. Biofilm formation and dispersal and the transmission of human pathogens. Trends Microbial 2005;13 :7-10.

7. Karen E. Beenken, Paul M. Dunman, Fionnuala McAleese, et al. Global gene expression in *Staphylococcus aureus* biofilms. J Bacteriol 2004;186(14) :4665-4684.
8. Lewis K. Persister cells and the riddle of biofilm survival. Biochemistry (Moscow) 2005;70 :267-274.
9. Patel R. Biofilms and antimicrobial resistance. Clin Orthop Relat Res 2005 :41-47.
10. Leid JG, Shirtliff ME, Costerton JW, Stoodley AP. Human leukocytes adhere to, penetrate, and respond to *Staphylococcus aureus* biofilms. Infect. Immune 2002;70 :6339-6345.
11. Davies D. Understanding biofilm resistance to antibacterial agents. Nat Rev Drug Discov 2003;2 :114-122.
12. Blaise R. Boles, Alexander R. Horswill. *agr*- mediated dispersal of *Staphylococcus aureus* biofilms. PLoS pathogen 2008 :4(4).
13. Karthik Sambanthamoorthy, Antony Schwartz, Vijayaraj Nagarajan and Mohamed O Elasri. The Role of *msa* in *Staphylococcus aureus* Biofilm Formation. BMC Microbiology 2008;8 :221.
14. Furukawa S, Kuchma SL, O'Toole GA. Keeping their options open: acute versus persistent infections. J Bacteriol 2006;188 :1211–1217.
15. Parsek MR, Singh PK. Bacterial biofilms: an emerging link to disease pathogenesis. Annu Rev Microbiol 2003;57 :677–701.
16. Harris LG, Richards RG. Staphylococci and implant surfaces: a review. Injury 2006;37(2) :S3–14.
17. Costerton JW. Biofilm theory can guide the treatment of device-related orthopaedic infections. Clin Orthop Relat Res 2005 :7–11.
18. Shakibaie MR, Mansouri S, Hakak S. Plasmid pattern of antibiotic resistance in beta–lactamase producing *Staphylococcus aureus* isolated from hospital in Karman. Iran 2002.
19. Sampathukumar P. Methicillin-Resistant *Staphylococcus aureus*: The latest Health Scare. Moyo Clin Proc 2007;82 :1403-67.
20. Schito GC. The importance of the development of antibiotic resistance in *Staphylococcus aureus*. Clin Microbial Infect 2006;12 :3-8.
21. Ihsan Edan Abdulkareem Alsaimary. Prevalence of β-lactamase producing and non-producing *Staphylococcus aureus* associated with patients in intensive care unit. Int J Med Medi Sci 2012;4(3) :65-74.
22. Rios JL, Recio MC. Medicinal plants and antimicrobial activity. J Ethnopharmacol 2005;100 :80–84.
23. Burt S. Essential oils: their antibacterial properties and potential applications in foods-a review. Int J Food Microbiol 2004;94 :223-253.
24. Ali S, et al. Antimicrobial activities of eugenol and cinnamaldehyde against the human gastric pathogen *Helicobacter pylori*. Ann Clin Microbial Antimicrob 2005;4 :20.
25. Ohno T, et al. Antimicrobial activity of essential oils against *Helicobacter pylori*. Helicobacter 2003;8 :207-215.
26. Ozcan MM, Sagdic L and Ozkan O. Inhibitory effects of spice essential oils on the growth of *Bacillus* species. J Med Food 2006;9 :418-421.
27. Cafarchia C, De-Laurentis N, Milillo MA, Losacco V and Puccini V. Antifungal activity of essential oils from leaves and flower of *Inula viscosa* (Asteraceae) by Apulian region. Parasitologia 2002;44 :153-156.
28. Salehi P, Sonboli A, Eftekha F, Nejad-Ebrahimi S and Yousefzadi M. Essential oil composition, antibacterial and antioxidant activity of the oil and various extracts of *Ziziphora clinopodioidies.* Subsp. rigida (Boiss.) *Rech. f.* from Iran. Biol Pharm Bull 2005;28 :1892-1896.
29. Vardar-Unlu G, F. Candan, A. Sokmen, D. Daferera, M. Polissiou, M. Sokmen, E. Donmez and B. Tepe, (2003). Antimicrobial and antioxidant activity of the essential oil and methanol extracts of *Thymus pectinatus* Fish. Et Mey. *Var. Pectinatus* (Lamiaceae). J Agr Food Chem 2003;51 :63-67.
30. Kalemba D. and Kunicka A. Antibacterial and antifungal properties of essential oils. Curr Med Chem 2003;10 :813-829.
31. Isman MB. Plant essential oils for pest and disease management. Crop Prot 2000;19 :603-608.
32. Cock IE. Antimicrobial Activity of *Aloe barbadensis* Miller Leaf Gel Components. Int J Microbiol 2008;4(2).
33. Forbes B, Sahm DF, Weissfeld AS. Bailey and Scott's Diagnostic Microbiology, Eleventh Edition. Mosby St. Louis; 2002 :24-160.
34. Cowan ST, Steel KJ. Manual for identification of Medical Bacteria. 3rd Edition. Cambridge University Press, pp. 50-140. NCCLS (2002). National Committee for Clinical Laboratory Standards; 2004
35. Freeman DJ, Falkiner FR, Keane CT. New method for detecting slime production by coagulase negative staphylococci. J Clin Pathol 1989;42 :872–4.
36. Okeke MI, Iroegbu CU, Eze EN, Okoli AS and Esimone CO. Evaluation of extracts of the root of *Landolphia owerrience* for antibacterial activity. *Journal of Ethnopharmacology* 2001;78 :119-127.
37. Chen L and Wen YM. The role of bacterial biofilm in persistent infections and control strategies. Int J Oral Sci 2011;3 :66-73.
38. Saitou K, Furuhata K, Kawakami Y, Fukuyama M. Biofilm formation abilities and disinfectant resistance of *Pseudomonas aeruginosa* isolated from cockroaches captured in hospitals. Biocontrol Sci 2009;14 :65-68.
39. Sabour PM, Gill JJ, Lepp D, Pacan JC, Ahmed R, Dingwell R and Leslie K. Molecular typing and distribution of *Staphylococcus aureus* isolates in Eastern Canadian dairy herds. J Clin Microbiol 2004;42 :3449–3455.
40. Moon JS, Lee AR, Kang HM, Lee ES, Kim MN, Paik YH, Park YH, Joo YS and Koo HC. Phenotypic and genetic antibiogram of methicillin-resistant staphylococci isolated from bovine mastitis in Korea. J Dairy Sci 2007;90 :1176–1185.
41. Kumar R, Yadav BR and Singh RS. (2010). Genetic Determinants of antibiotic resistance in *Staphylococcus aureus* isolates from milk of mastitic crossbred cattle. Curr Microbiol 2010;60 :379–386.
42. Islam MA, Alam MM, Choudhury ME, Kobayashi N and Ahmed MU. Determination of minimal inhibitory concentration (MIC) of Cloxacillin for selected isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) with their antibiogram. Bangl. J Vet Med 2008;6(1) :121-126.
43. Wang Y, Wu CM, Lu LM, Ren GWN, Cao XY and Shen JZ. Macrolide-lincosamide-resistant phenotypes and genotypes of *Staphylococcus aureus* isolated from bovine clinical mastitis. Vet Microbiol 2008;130 :118–125.
44. Lee JH. Methicillin (oxacillin)-resistant Staphylococcus aureus strains isolated from major food animals and their potential transmission to humans. Appl Environ Microbiol 2003;69 :6489–6494.
45. Van den Eede A, Martens A, Lipinska U, Struelens M, Deplano A, Denis O, Haesebrouck F, Gasthuys F and Hermans K. High occurrence of methicillin-resistant *Staphylococcus aureus* ST398 in equine nasal samples. Vet Microbiol 2009;133 :138–144.
46. Goetz F. *Staphylococcus* and biofilms. Mol Microbial 2002;43 :1367-1378.
47. Ruzicka F, Hola V, Votava M, Tejkalova R, Horvat R, Heroldova M, Woznicova V. Biofilm detection and clinical significance of *Staphylococcus epidermidis* isolates. Folia Microbial 2004;49 :75-78.
48. Christensen GD, Simpson WA, Bisno AL, Beachey EH. Adherence of slime–producing strains of *Staphylococcus epidermidis* to smooth surfaces. Infect Immun 1982;37 :318–26.
49. Mathur T, Singhal S, Khan S, Upadhyay DJ, Fatma T, Rattan A. Detection of biofilm formation among the clinical isolates of staphylococci: an evaluation of three different screening methods. Indian J Med Microbiol 2006;24 :25-29.

50. O Gara JP, Humphreys H. *Staphylococcus epidermidis* biofilms:importance and implications. J Med Microbiol 2001;50 :582–7.
51. Yasmeen Taj, Farhan Essa, Faisal Aziz, Shahana U. Kazmi. Study on biofilm-forming properties of clinical isolates of *Staphylococcus aureus*. J Infect Dev Ctries 2012;5(6) :403-409.
52. Knobloch JK, Horstkotte MA, Rhode H, Mack D. Evaluation of different detection methods for biofilm formation in *Staphylococcus aureus*. Medical Microbiol Immunol 2002;19 :101-106.
53. Simoes, M. Antimicrobial stratergies effective against infectious bacterial biofilms. Curre Med Chemis 2011;45(3) :2129-2145.
54. Furletti VF, Teixeira P, Obando-pereda G, et al. Action of *Coriandrum sativum* L. essential oil upon oral *Candida albicans* biofilm formation. Eviden. Based. Complem Alter Med 2011;9.
55. Koo H, Gomes BPFA, Rosalen PL, Ambrosano GMB, Park YK and Cury JA. In vitro antimicrobial acitivity of propolis and *Arnica Montana* against oral pathogens. Arch Oral Biol 2000;45(2) :141-148.
56. Ghalem BR and Mohamed B. Essential oil from gum of *Pistacia atlantica* Desf. Screening of antimicrobial activity. Afr J Pharm Pharmaco 2009;3(3) :87-91.
57. Ben Douissa F, Hayder N, Chekir-Ghedira L, Hammami M, Ghedira K, Mariotte AM, Dijoux-Franca MG. New study of the essential oil from leaves of *Pistacia lentiscus* L. (Anacardiaceae) from Tunisia. Flavour Fragrance Journal 2005 :410-414.
58. Tsokou A, Georgopoulou K, Melliou E, Magiatis P, Tsitsa E (2007).Composition and Enantiomeric Analysis of the Essential Oil of the Fruits and the Leaves of *Pistacia vera* from Greece. Molecules 2007;12 :1233-1239.
59. Ravi Kant Upadhyay, Pratibha Dwivedi and Shoeb Ahmad. Screening of antibacterial activity of six plant essential oils against pathogenic bacterial strains. Asi J Med Sci 2010;2(3) :152-158.
60. Sanaa O. Yagoub, Shami El Hai Al Safi, Braaha Ahmed and Asha Z. El Magbol. Antimicrobial activity of some medicinal plants against some gram positive, gram negative and fungi. Docstoc 2010.
61. Tuhin Jahan, Zinnat Ara Begum and Sayeeda Sultana. (2007). Effect of neem oil on some pathogenic bacteria. Bangl. J Pharmacol 2007;2 :71-72.
62. Angela E. Sadlon and Davis W. Lamson, MS. Immune-modifying and antimicrobial effects of Eucalyptus oil and simple inhalation devices. Aletnat Med Revi 2010;15(1).
63. Serafino A, Sinibaldi Vallebona P, Andreola F, et al. Stimulatory effect of Eucalyptus essential oil on innate cell-mediated immune response. BMC Immunol 2008;9 :17.
64. Santos FA, Rao VS. Anti-inflammatory and antinociceptive effects of 1,8-cineole a terpenoid oxide present in many plant essential oils. Phytother Res 2000;14 :240-244.
65. Singh HP, Mittal S, Kaur S, et al. Characterization and anti-oxidant activity of essential oils from fresh and decaying leaves of *Eucalyptus tereticornis*. J Agri Food Chem 2009;57 :6962-6966.
66. Silva J, Abebe W, Sousa SM, et al. Analgesic and anti-inflammatory effects of essential oils of Eucalyptus. J Ethanopharmacol 2003;89 :277-283.
67. Inouye S, Takizawa T and Yamaguchi H. Antibacterial activity of essential oils and their major constituents against respiratory tract pathogen by gaseous contact. J Antimicrob Chemoth 2001;47 :565-573.
68. Canillac N, Mourey A. Antimicrobial activity of the essential oil of *Picea excelsa* on *Listeria*, *Staphylococcus aureus* and coliform bacteria. Food Microbiol 2001;18 :261-268.
69. Dermetzos C, Perdetzoglou DK. Composition and antimicrobial studies of the essential oils of *Origanum calcaratum* Juss. and *O. scabrum* Boiss. et Heldr. From Greece. J. Essent. Oil Res 2001;3 :460-462.
70. Delaquis PJ, Stanich K, Girard B and Mazza G. Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. Int J food Microbial 2002;74 :101-109.
71. Koutsoudaki C, Krsek M, Rodger A. Chemical Composition and Antibacterial Activity of the Essential Oil and the Gum of *Pistacia lentiscus* Var. chia. J Agric Food Chem 2005;53(20) :7681 -7685.
72. Ozçelik B, Aslan M, Orhan I, Karaoglu T. Antibacterial, antifungal, and antiviral activities of the lipophylic extracts of *Pistacia vera*. Microbiol Res. 2005;160 :159-164
73. Kamrani YY, Amanlou M, Esmaeelian B, Bidhendi MS, SahebJamei M. Inhibitory Effects of a Flavonoid-Rich Extract of *Pistacia vera* Hull on Growth and Acid Production of Bacteria Involved in Dental Plaque. Int J Pharmacol 2007;3(3) :219-226.
74. Benhammou N, Bekkara AF, Panovska KT. Antioxidant and antimicrobial activities of the *Pistacia lentiscus* and *Pistacia atlantica* extracts. Afri J Pharm Pharmacol 2008;2 :22–28.
75. Lambert RJ, Skandamis PJ, Coote and Nycas GJ. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. J Appl Microbiol 2001;91 :453-462.
76. Walsh SE, Maillard JY, Russel AD, Catrenich CE, Charbonneau DL and Bartolo RJ. Activity and mechanism of action of selected biocidal agents on gram-positive and negative bacteria. J Appl Microbiol 2003;94 :240-247.
77. Calsamiglia S, Busquet M, Cardozo PW, Castillejos L and Ferret A. Invited review: essential oils as modifiers of rumen microbial fermentation. J Dai Sci 2007;90(6) :2580-2595.