Scientific Research Publishing

Advances in Microbiology, 2021, 11, 117-143
https://www.scirp.org/journal/aim
ISSN Online: 2165-3410
ISSN Print: 2165-3402

In Vitro Efficacy of Clove Oil and Eugenol against *Staphylococcus* spp and *Streptococcus mutans* on Hydrophobicity, Hemolysin Production and Biofilms and their Synergy with Antibiotics

Huma Jafri, Iqbal Ahmad*

Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh, India
Email: *ahmadiqbal8@yahoo.co.in

How to cite this paper: Jafri, H. and Ahmad, I. (2021) *Streptococcus mutans* on Hydrophobicity, Hemolysin Production and Biofilms and their Synergy with Antibiotics. *Advances in Microbiology*, **11**, 117-143.
https://doi.org/10.4236/aim.2021.112009

Received: December 24, 2020
Accepted: February 23, 2021
Published: February 26, 2021

Abstract

The present study aimed to evaluate *Syzygium aromaticum* (clove) plant extract, clove oil and eugenol for their antibacterial activity and their potential to eradicate bacterial biofilms alone and in combination with antibiotics. Anti-bacterial efficacy of *S. aromaticum* extract, clove oil and eugenol was evaluated as minimum inhibitory concentration (MIC) and subsequently sub-MICs was selected for inhibition of virulence factors against test bacterial strains. Biofilm cultivation and eradication was assayed using XTT reduction in 96-well microtiter plate. Checkerboard method was used to study the interaction between essential oils and antibiotics. *Staphylococcus aureus* MTCC3160, *Staphylococcus epidermidis* MTCC435, *Staphylococcus sciuri* (SC-01), *Staphylococcus auricularis* (SU-01) *and Streptococcus mutans* MTCC497 were found strong biofilm former among all the test bacterial strains. The potency of test agents was found in the order of eugenol > clove oil > *S. aromaticum* methanolic extract. Sub-MIC (0.5 × MIC) of clove oil and eugenol showed a significant reduction in cell surface hydrophobicity ($p < 0.05$) and hemolysin production in the test bacterial strains. Eugenol showed no increase in sessile MIC (SMIC) against *S. auricularis* (SU-01), *S. epidermidis* MTCC435 and *S. mutans* MTCC497 compared to planktonic MIC (PMIC). Antibiotics (vancomycin and azithromycin) exhibited upto 1000-folds increased in SMIC compared to PMIC against all the test bacterial strains. Synergy was observed between eugenol and antibiotics (vancomycin/azithromycin) against all the test bacterial strains in both planktonic and sessile mode. Highest synergy was exhibited between eugenol and azithromycin in planktonic mode (FICI value 0.141). Further, microscopy also confirmed the spectacular effect of combina-

tion treatment on pre-formed *S. aureus* MTCC3160 and *S. mutans* MTCC497 biofilms. These findings highlighted the promising role of clove oil and eugenol alone and in combination on pathogenic bacterial biofilms.

Keywords

Syzygium aromaticum, *Staphylococcus* spp, *Streptococcus mutans*, Eugenol, Biofilm Inhibition, Hydrobhobicity, SMIC, Synergy

1. Introduction

Infectious diseases are still a major global cause of mortality and morbidity in humans mainly in developing countries. Many opportunistic and drug resistant pathogenic bacterial species that are widely studied include *Staphylococcus* and *Streptococcus* species [1]. The tendency of these bacteria to cause infections is often related to the expression of several virulence factors, such as cell surface hydrophobicity, hemolysin production and biofilm formation. These factors aid bacteria to invade the host tissue and escape immune defence system [2]. Generally, *Staphylococcus aureus* is frequently responsible to cause bacteremia, pneumonia, deep seated and device related infections. *S. aureus* bacteremia is responsible for significant burden on health care system and accounts about 20 to 30% mortality and morbidity rates [3]. Certainly, the importance of coagulase negative *Staphylococcus* species (*Staphylococcus epidermidis*, *Staphylococcus auricularis* and *Staphylococcus sciuri*) has also increased over the past decades. *S. epidermidis* is opportunistic pathogen as it causes infections in immune-compromised patients. This pathogen is liable to cause infection in healthy person only when it penetrates into the host tissue [4]. The clinical significance of other coagulase negative *Staphylococci* (*S. auricularis* and *S. sciuri*) has been increasing since it can colonize and form biofilms on the host tissues as well as on the medical devices [5] [6] [7]. *S. auricularis* are inhabitants of human skin and mucous membranes. This bacterium has also been linked with various types of infections in immmuno-compromised patients and catheter related blood streams infections, osteomyelitis, bone and joint infections, prosthetic valve endocarditis and wound infections [8]. *S. sciuri* has also been isolated from various infection sites in humans, such as wound infections, soft tissue infections, abscesses, boils, periodontitis and endocarditis [5]. The involvement of *Streptococcus mutans* in oral biofilm associated infections is well known. As long as these bacterial species adhere and form multilayered biofilms on host tissues and different surfaces, it persists and prolongs the infections [9]. Several studies have revealed that the microorganisms in biofilm mode of growth are more tolerant to antibiotics than the planktonic mode [10]. Once biofilm gets established, it becomes difficult to eradicate through common antimicrobial drug therapy. Therefore, new antimicrobials or strategies effective against pathogenic bacterial biofilms are of practical significance in chemotherapy. There are several strategies that can be used to combat

bacterial biofilm infections depending upon nature and site of infection. However, use of antipathogenic and antibiofilm agents which act through modulation of quorum sensing, inhibition of biofilm formation, biofilm degrading enzymes, application of specific bacteriophages, broad spectrum activity of natural products, and nanoparticles are considered as promising strategies [11]. To achieve broad spectrum and enhanced efficacy, combinational therapy may be more effective promising approach to combat pathogenic bacterial biofilm infections. Several advantages may be expected through this approach, such as less chance of emergence of resistance, improved efficacy of single drug in combination and dose related toxicity [12] [13].

Natural products of plant origin such as plant extracts, essential oils and phytochemicals provide infinite opportunities for the discovery of new drugs due to the abundance of chemical diversity [14] [15]. The crude extracts of different parts of the medicinal plants such as root, stem, flower, fruits and twigs have been commonly used for the treatment of microbial infections in traditional system of medicine and modern phytomedicine. *Syzygium aromaticum* extracts and its active compounds are well known for antimicrobial properties against gram positive and negative bacteria [16]. Antimicrobial activity of clove essential oils and eugenol is attributed by disturbing the cell wall and membrane, cell lysis, leakage of cellular contents and inhibition of proton motive force [17] [18] [19]. It is reported that they kill bacteria without promoting the development of resistance [17] [20]. However little is known about anti-infective and antibiofilm activity of *S. aromaticum* and its active compound against pathogenic biofilms and synergy with antibacterial drugs. Therefore, the present study investigated antibiofilm activity of clove essential oil and eugenol alone and in combination with the commonly used antibiotics against the selected strong biofilm forming bacterial strains including *S. aureus*, *S. epidermidis*, *S. auricularis*, *S. sciuri* and *S. mutans* under *in vitro* settings.

2. Material and Methods

2.1. Bacterial Strains Used Under Study

Nine clinical strains of *Staphylococcus* species including *S. aureus* (SAJ-01 to 04), *S. epidermidis* (SEJ-01 to 04) and *S. auricularis* (SU-01) were isolated and or kindly provided by the division of Microbiology, Jawaharlal Nehru Medical College and Hospital, Aligarh Muslim University, Aligarh. *S. sciuri* (SC-01) was isolated from the skin of healthy person in our laboratory by swab culture on nutrient agar (NA) plate. Reference strains of *Staphylococcus* species viz *S. aureus* MTCC3160, *S. epidermidis* MTCC435 and *S. mutans* MTCC497 were procured from Microbial Type Culture Collection, CSIR, IMTECH, Chandigarh, India. The details of above strains are shown in **Table S1**. All the test bacterial strains were identified and characterized by morphological and biochemical tests like gram staining, mannitol salt agar, coagulase test, novobiocin test and nitrate reduction test. The test bacterial strains were maintained on nutrient agar (NA)

slants at 4˚C.

Characterization of bacterial isolates based on 16S rRNA gene sequence analysis:

The partial gene sequencing of 16S rRNA of the *S. sciuri* (SC-01) and *S. auricularis* (SU-01) strains was done commercially at the Macrogen, Inc., Seoul, Korea. The amplified and purified PCR products were sequenced by using 2 primers 785F 5' (GGA TTA GAT ACC CTG GTA) 3' and 907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'. Sequencing was performed by using Big Dye terminator cycle sequencing kit v.3.1 (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA). These sequences were submitted to Gen-Bank sequence database after verification and editing by using Bioedit 7.2.0 software. Next, phylogenetic tree was constructed by using software programme (Neighbor-joining method of Molecular Evolutionary Genetics Analysis (MEGAv6.0)). The evolutionary distance was calculated by using Maximum Composite Likelihood program [21] [22].

2.2. Test Agents Used: *S. aromaticum* Extract, Essential Oil, Eugenol and Antibiotic

A 100 g of air-dried *S. aromaticum* (bud) was collected from local authorized shop and identified by the Department of Botany, Aligarh Muslim University, Aligarh and deposited in the Department of Agricultural Microbiology with No. SA-C/2016/Agric 02. Sample was powdered and soaked in 500 ml of 97% methanol for 5 days with intermittent shaking and filtered out through Whatman filter paper No 1 (Whatman Ltd., UK). Further, clear filtrate was obtained by centrifugation 10,000 rpm for 15 min. The filtrate was concentrated in rotary evaporator at 40˚C and stored at 4˚C until use. The dried extract was further dissolved in 1% DMSO to obtain the required concentration of extract. *S. aromaticum* essential oil (Clove oil) and pure compound (eugenol 99% purity) were purchased from Dabur India Ltd, Delhi, India and Hi-Media Laboratory, Mumbai, India respectively. Essential oil and its active compound (eugenol) were diluted ten times in 1% DMSO. Aqueous stock solution of vancomycin (Cipla, Mumbai, India) and azithromycin (Cipla, Mumbai, India) were prepared at a concentration of 25 mg/ml.

2.3. Gas Chromatography and High Resolution Gas Chromatography-Mass Spectrometry (GC-MS) Analysis of Plant Extract

The composition of *S. aromaticum* methanolic extract was identified by GC-MS analysis. GC-MS analysis was conducted on Shimadzu QP-2010 Plus with Thermal Desorption System TD 20 and separation was obtained in column of 30 m × 0.22 mm × 0.25 μm (Thermo Scientific) at Jawaharlal Nehru University, New Delhi, India. Helium gas was used as a carrier gas while the flow rate of mobile phase was adjusted at 1.21 ml/min. The sample was infused into the

column with a split ratio of 1:10. The linear temperature was attained from 60˚C to 300˚C. Hold time was set at 60˚C for 2 min. The peaks of the sample compounds were identified by using the above system with the reference database NIST libraries.

2.4. Determination of Planktonic Minimum Inhibitory Concentration (PMIC) of *S. aromaticum* Extract, Essential Oil, Eugenol and Antibiotics

The MIC of test agents (*S. aromaticum* plant extract, essential oil, eugenol and vancomycin) for bacterial strains was determined by the broth microdilution technique followed by [23] with some modification. Briefly, overnight grown culture prepared in Brain Heart Infusion Broth (BHIB). 0.1 ml of two-fold serial dilutions of test agents (2 × final concentration) was made in respective medium in the wells of microtiter plates. 0.1 ml of inoculum medium (1×10^6 CFU/ml) was added to each well and incubated at 37˚C for 48 h. Agent free wells were included as a control. Lowest concentration of test extract/essential oil/eugenol/antibiotic inhibiting the visible growth of the bacteria is defined as MIC. The experiment was repeated at least two times with three replicates.

2.5. Biofilm Forming Ability of the Test Bacterial Strains

Standard method of XTT reduction assay using 96-well microtiter plates was adopted as described earlier [24]. Briefly, the strains of *Staphylococcus* and *S. mutans* were grown in NA (glucose 0.25% w/v) and BHIB (sucrose 5% w/v) at 37˚C for 24 h respectively. 0.1 ml of the standardized cell suspension (approx. 1×10^8 of CFU/ml) and equal volume of respective medium was added into the wells of microtiter plates. The plates were incubated at 37˚C for 48 h. Thereafter, non-adherent cells were washed off with sterile PBS. XTT was added in each well to observe oxidation. XTT was used to assay biofilm formation by taking the absorbance at 490 nm using Lab system Multiskan Ex MTP reader. The experiment was repeated at least two times with three replicates. The mean absorbance value was calculated to measure the biofilms quantitatively.

2.6. Assay for Cell Surface Hydrophobicity

The microbial adhesion to hydrocarbons assay was performed to determine the cell surface hydrophobicity (CSH) using the method as adopted by [25] with little modifications. In brief, the bacterial strains were grown in 10 ml of BHIB (pH 7.0) to a shaking incubator at 37˚C for 18 h. The bacterial cells were obtained by centrifugation at 13,000 rpm for 15 min. The pellet were washed three times in PBS and finally suspended in PBS to attain an OD_{600} of 0.5. Two ml of bacterial suspension was mixed with 0.4 ml of xylene and shaken vigorously for 1 min. The absorbance of aqueous phase was determined at 600 nm using UV-visible spectrophotometer (LMSP-V325, India) and the percentage of adherence was calculated as: $\%CSH = \{(1 - A)/A_o) \times 100\}$. Where A and A_o were the absorbance values of aqueous phase at 600 nm before and after exposure to the organic phase.

2.7. Hemolysin Production

Quantitative hemolysin production was determined by using the modified method of [26]. Bacterial strains were grown in 10 ml TSB for 48 h at 37˚C on shaking incubator (200 rpm) until reaching the optical density of 2.5 at 600 nm. The cell supernatant was obtained by centrifugation at 10,000 rpm for 10 min. 0.1 ml of culture supernatant was mixed with equal volume of 2.5% defibrinated sheep blood in PBS buffer (pH 7.0) and incubated at 37˚C for 15 min. The sheep blood was collected from local slaughter shop. The blood cells (unlysed) were peletted by centrifugation at 5500 rpm for 1 min to obtain supernatant. The absorbance of supernatants was read at 543 nm. RBCs treated with Triton X-100 and saline water were served as a positive (100% lysis) and negative control respectively. Percentage of hemolysis was calculated as: Absorbance of the sample-Absorbance of the negative control/Absorbance of the positive control-Absorbance of the negative control.

2.8. Inhibition of Cell Surface Hydrophobicity in the Bacterial Strains

The inhibition of microbial adhesion was determined by using the method as adopted by [25] with little modifications as described previously. Briefly, the cultures of bacterial strains were grown at 0.25 and 0.5 × MIC of essential oils, compounds and drugs. The bacterial strains without any treatment considered as untreated control. Percent reduction in cell surface hydrophobicity of treated strains was calculated over untreated strains. The experiment was repeated at least two times with three replicates and data was recorded as mean values for calculation of percent CSH.

2.9. Inhibition of Hemolysin Production in the Bacterial Strains

The reduction in hemolysin production in the presence of test agents were determined by using the modified method of [26] as described earlier. The bacterial cells were grown in the presence of essential oil, compounds and drugs at sub-MICs (0.25 and 0.5 × MIC) for 48 h at 37˚C on shaking incubator (200 rpm) until reaching the optical density of 2.5 at 600 nm. The absorbance of supernatants was read at 543 nm. The untreated culture supernatant considered as the 100% hemolysis control, and relative percentage of hemolysis was calculated by comparison to untreated control.

2.10. Determination of Eradication of Biofilms by Clove Oil, Eugenol and Antimicrobial Drugs

The sessile MIC (SMIC) of antimicrobial agents for bacterial strains was determined to assess the biofilm eradication by the broth microdilution technique followed by [27]. Briefly, biofilms were allowed to form in microtiter plates as described in previous section. The pre-formed biofilm cells were treated with different concentrations of test agents (clove oil, eugenol, vancomycin (for *Sta-*

phylococcus species) and azithromycin (for *S. mutans*)) prepared in BHIB medium and incubated at 37˚C for 48 h. Agent free wells were included as control. Sessile minimum inhibitory concentration (SMIC) was calculated as the reduction in the mean absorbance of the test clove oil/eugenol treated biofilm to the untreated control and expressed as the concentration eradicating 80% of pre-formed biofilms.

2.11. Assay for Synergistic Interaction between Clove Oil, Eugenol and Antibiotics in Planktonic Mode of Bacterial Growth

The synergy between test agents was determined against the test strains of bacterial species using previously described method [28]. In brief, 0.05 ml of prepared dilution of essential oil was added to the 96-well microtiter plates in the vertical direction and same amount of antibacterial drug was added in horizontal direction to obtain the various test combinations. Further, 0.1 ml of inoculum suspension (1×10^6 CFU/ml) of bacterial species was added to each well followed by incubation at 37˚C for 48 h. The interaction was determined as fractional inhibitory concentrations (FICI). The FICI result was interpreted as synergistic: FICI ≤ 0.5, no interaction > 0.5 - 4.0, and antagonistic > 4.0.

2.12. Assay for Synergistic Interaction between Clove Oil, Eugenol and Antimicrobial Drugs in Sessile Mode of Bacterial Growth

The synergistic effect of essential oils and antibiotics against the bacterial strains was determined by using the checkerboard microtiter assay. Briefly, bacterial biofilms were cultivated in 96-well plates. A two-fold dilution of essential oils and antibiotics were prepared in BHIB. 0.05 ml prepared dilution of essential oils in vertical direction and 0.05 ml of antibiotics (vancomycin for *Staphylococcus* species and azithromycin for *S. mutans*) in horizontal direction was added in the wells. The microtiter plates were observed after 48 h of incubation at 37˚C. The type of interaction was determined by calculating the FICI value as described in the above section.

2.13. Scanning Electron Microscopy (SEM)

The pre-formed bacterial biofilms treatment with combinations of eugenol and antibiotics (vancomycin and azithromycin) were prepared on glass coverslips using the method as described by [24] and processed for scanning electron microscopy (JSM 6510, LV, JEOL, JAPAN). Briefly, treated biofilm cells were washed with PBS and fixed with 5% glutaraldehyde in cocodylate buffer in a graded concentration of ethanol (25%, 50%, 75%, 95% and 100%), immersed in hexamethyldisilazane. The glass disc were dried and mounted on aluminium stubs with silver paint, sputter coated with gold and observed under scanning electron microscope.

2.14. Statistical Analysis

The data was analyzed statistically by one way ANOVA using Duncan's method

(IBM SPSS statistics, version 20). The significance was determined using *P* value less 0.05.

3. Results

3.1. Characteristics of Bacterial Strains and Plant Extract

The test bacterial strains previously obtained and characterized as indicated in the supplementary data (Table S1, Table S2 and Figure S1 and Figure S2). *Staphylococcus* isolates SC-01 and SU-01 were characterized in this study by 16S rRNA gene sequence analysis. The accession numbers of *Staphylococcus scuiri* (SC-01) and *S. auricularis* (SU-01) are KY027071 and KX953265 respectively. The data showed 99% sequence homology of SC-01 with *S. sciuri* DSM20345 and SU-01 with *S. auricularis* ATCC33753 as indicated in supplementary file (Figure S1 and Figure S2). Further, the biofilm forming ability of these bacterial strains was determined on the basis of optical density by XTT reduction assay (Table 1). Biofilm forming ability of the test strains varied from moderate to strong. *S. aureus* MTCC3160, *S. epidermidis* MTCC435, SC-01, SU-01, SAJ-01 and *S. mutans* MTCC497 formed strongest biofilms ($OD_{490} \geq 1$). Among 13 bacterial strains, hemolysin production was detected in eight strains. Similarly, ten (10) strains demonstrated the varying level of cell surface hydrophobicity (CSH). Significant amount of hemolysin production and CSH were noticed in the strains of *S. epidermidis* MTCC435 and *S. sciuri* (SC-01) respectively.

S. aromaticum methanolic crude extract was subjected to GC-MS analysis. It is observed that crude extract of *S. aromaticum* showed the presence of 12 phytocompounds where eugenol, eugenolacetate and caryophyllene were more

Table 1. Production of biofilms and virulence factors in the bacterial strains.

Strains	Biofilm formation (Absorbance at 490 nm)	% Hemolysis	% CSH
SAJ-01	1.041 ± 0.08	ND	49.22 ± 1.74
SAJ-02	0.995 ± 0.04	38.51 ± 2.24	29.25 ± 0.88
SAJ-03	0.452 ± 0.09	74.07 ± 3.28	31.72 ± 1.50
SAJ-04	0.796 ± 0.08	ND	ND
S. aureus MTCC3160	1.288 ± 0.06	38.51 ± 2.24	29.33 ± 1.49
SEJ-01	0.924 ± 0.07	28.30 ± 4.69	65.20 ± 2.18
SEJ-02	0.392 ± 0.05	ND	83.95 ± 2.10
SEJ-03	0.218 ± 0.09	ND	ND
SEJ-04	0.972 ± 0.04	ND	ND
S. epidermidis MTCC435	1.347 ± 0.12	76.15 ± 3.64	45.75 ± 2.02
S. sciuri (SC-01)	1.369 ± 0.06	66.98 ± 3.16	79.21 ± 1.59
S. auricularis (SU-01)	1.382 ± 0.10	51.92 ± 3.89	67.20 ± 0.81
S. mutans MTCC497	1.245 ± 0.05	75.49 ± 4.57	65.70 ± 1.02

ND: Not detected.SA: *S. aureus*; SE: *S. epidermidis.*

predominant. Other detected compounds include eugenol acetate, caryophyllene, gamma.-Sitosterol, olean-12-en-28-oic acid, 2.beta., 3.beta., 23-trihydroxy, caryophyllene oxide, n. Hexadecanoic acid, alpha-Farnesene, benzyl benzoate, vitamin E, octyl cyclodecane and methyl stearate (Table 2 and Figure 1).

3.2. Effect of Test Agents on Bacterial Growth and Virulence Factors

Methanolic extract of clove bud, clove oil and eugenol were assessed to observe the antibacterial potency in term of MIC against the test bacterial strains. The results are presented in Table 3. MIC was ranged from 25 to >6400 µg/ml. MIC

Table 2. List of compounds of *S. aromaticum* methanolic extract identified by GC-MS analysis.

Compounds	% Amount	Retention time
Eugenol	56.17	13.724
Eugenol acetate	20.24	15.974
Caryophyllene	7.96	14.405
gamma.-Sitosterol	1.66	35.874
Olean-12-en-28-oic acid, 2.beta., 3.beta., 23-trihydroxy	1.04	39.792
Caryophyllene oxide	1.89	16.873
n. Hexadecanoic acid	0.53	21.742
Alpha-Farnesene	0.40	15.463
Benzyl benzoate	0.23	19.322
Vitamin E	0.18	33.57
Octyl cyclodecane	0.11	19.565
Methyl stearate	0.08	23.649

Table 3. Minimum inhibitory concentration (MIC) of *S. aromaticum* extract, clove oil and eugenol against bacterial species.

Strains	*S. aromaticum* (methanolic extract)	Clove oil	Eugenol
SAJ-01	6400	200	100
SAJ-02	3200	400	100
SAJ-03	>6400	400	200
SAJ-04	>6400	100	100
S. aureus MTCC3160	800	200	50
SEJ-01	6400	100	200
SEJ-02	6400	100	50
SEJ-03	6400	100	50
SEJ-04	>6400	25	200
S. epidermidis MTCC435	800	200	50
S. sciuri (SC-01)	1600	100	25
S. auricularis (SU-01)	6400	200	50
S. mutans MTCC497	1600	200	200

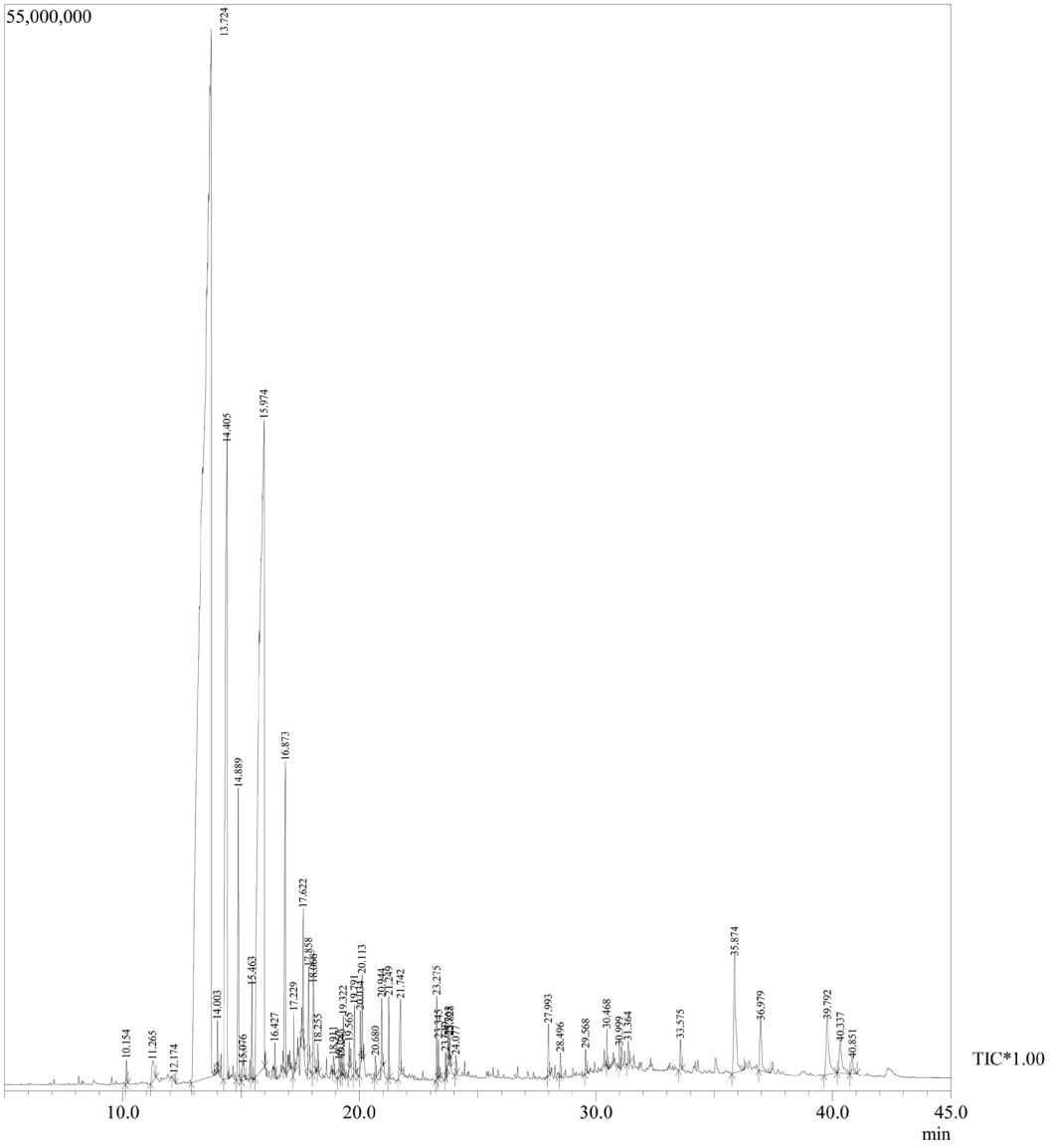

Figure 1. Chromatogram of *S. aromaticum* methanolic extract.

data clearly indicated that eugenol as the most effective in antibacterial potency compared to methanolic clove extract and oil. The clove oil and eugenol were further evaluated for their effects on bacterial virulence factors at sub-MIC values. Cell surface hydrophobicity of test bacterial strains in the presence of sub-MICs of eugenol and clove oil is shown in **Table 4**. Both clove oil and eugenol exhibited anti hydrophobicity potential being higher for eugenol. Eugenol showed similar inhibitory activity on CSH against SC-01 and SU-01. On the other hand, eugenol significantly reduced CSH in *S. mutans* MTCC497 from 68.06 to 8.28% ($p < 0.05$). Antibiotic (control) was found least effective against the test bacterial strains. Similarly, the reduction in hemolysin production was also recorded at sub-MICs of eugenol and clove oil in the test bacterial strains. Significant reduction in hemolysin production was exhibited in the presence of eugenol in *S. sciuri* SC-01 (78%) and *S. epidermidis* MTCC435 (69%) (**Table 5**).

Table 4. Effect of sub-inhibitory concentration of clove oil, eugenol and antibacterial drug on cell surface hydrophobicity of the test bacterial strains.

Test agents (sub-MICs)	Percentage of cell surface hydrophobicity				
	S. epidermidis MTCC435	*S. auricularis* (SU-01)	*S. aureus* MTCC3160	*S. sciuri* (SC-01)	*S. mutans* MTCC497
1) Untreated control	66.97 ± 1.26[a]	88.50 ± 1.80[a]	70.13 ± 1.89[a]	72.75 ± 2.14[a]	68.06 ± 1.75[a]
2) Clove oil					
0.50 × MIC	37.16 ± 1.09[c]	62.31 ± 0.91[c]	41.32 ± 1.41[c]	51.20 ± 3.61[c]	50.58 ± 1.39[b]
0.25 × MIC	48.34 ± 0.92[b]	82.48 ± 0.80[b]	49.86 ± 0.80[b]	62.69 ± 0.65[b]	66.11 ± 1.74[a]
3) Eugenol					
0.50 × MIC	12.91 ± 1.48[c]	11.46 ± 1.15[c]	18.11 ± 2.24[c]	11.17 ± 0.33[c]	8.28 ± 1.71[c]
0.25 × MIC	41.52 ± 0.56[b]	16.10 ± 0.93[b]	38.77 ± 0.33[b]	15.73 ± 0.31[b]	37.00 ± 0.48[b]
4) Antibiotic					
0.50 × MIC	43.31 ± 0.22[c]	49.64 ± 1.07[c]	50.33 ± 1.26[c]	59.16 ± 0.55[b]	56.32 ± 1.37[b]
0.25 × MIC	49.06 ± 0.81[b]	76.64 ± 0.71[b]	66.4 ± 0.83[b]	74.28 ± 0.64[a]	67.13 ± 1.14[a]

Where a, b, c letters indicate meanvalues which are significantly different according to the Duncan's multiple range tests ($p \leq 0.05$). Antibiotics: Vancomycin used for *Staphylococcus* species and azithromycin for *S. mutans.*

Table 5. Effect of sub-inhibitory concentrations of clove oil, eugenol and antibacterial drug on production of hemolysin in the test bacterial strains.

Test agents (sub-MICs)	Percent reduction in hemolysin production				
	S. sciuri (SC-01)	*S. auricularis* (SU-01)	*S. aureus* MTCC3160	*S. epidermidis* MTCC435	*S. mutans* MTCC497
1) Clove oil					
0.5 × MIC	49.12 ± 0.65	44.11 ± 5.54	32.44 ± 2.58	44.08 ± 3.76	48.40 ± 3.88
0.25 × MIC	23.84 ± 4.85	22.20 ± 2.17	15.13 ± 3.99	21.66 ± 2.38	24.43 ± 0.57
2) Eugenol					
0.5 × MIC	78.92 ± 2.81	60.69 ± 0.63	57.43 ± 2.01	69.39 ± 4.62	62.64 ± 1.55
0.25 × MIC	48.64 ± 2.02	40.90 ± 2.16	28.32 ± 2.74	37.74 ± 1.00	39.28 ± 3.13
3) Antibiotic					
0.5 × MIC	38.85 ± 1.90	43.74 ± 0.58	28.60 ± 5.71	42.00 ± 2.98	28.96 ± 1.68
0.25 × MIC	17.37 ± 1.99	12.46 ± 2.67	9.50 ± 1.87	18.95 ± 1.99	12.38 ± 1.76

Antibiotics: Vancomycin used for *Staphylococcus* species and azithromycin for *S. mutans.*

3.3. Biofilm Eradication by Test Agents

The concentration of test agents eradicating more than 80% of pre-formed biofilms was considered as SMIC. Clove oil and eugenol showed no increase or only 2-folds increase in SMIC compared to PMIC. No increase in SMIC of eugenol compared to PMIC was observed against SU-01, *S. epidermidis* MTCC435 and *S. mutans* MTCC497. Similarly, SMIC of clove oil recorded equivalent to PMIC against SU-01. However, antibacterial drug (vancomycin and azithromycin) ex-

hibited 4 to 1000-folds increased in SMIC compared to PMIC against all the test bacterial strains (Table 6).

3.4. Synergistic Interaction between Clove Oil/Eugenol and Antibiotics

Synergy between clove oil/eugenol and antibiotics were tested against the bacterial strains both in planktonic and sessile mode of growth. Various test combinations of essential oils and antibiotics (vancomycin/azithromycin) were tested against the bacterial strains in planktonic mode (Table 7 and Table 8). The combination of clove oil and vancomycin showed indifferent interaction (FICI

Table 6. PMIC and SMIC of essential oils and drugs against bacterial strains.

Strains	Test agents (concentration µg/ml)					
	Clove oil		Eugenol		Antibiotics	
	PMIC	SMIC	PMIC	SMIC	PMIC	SMIC
S. sciuri (SC-01)	100	200	25	50	512	1024
S. auricularis (SU-01)	200	200	50	50	512	1024
S. aureus MTCC3160	200	400	50	100	1024	2048
S. epidermidis MTCC435	200	400	50	50	1024	2048
S. mutans MTCC497	200	400	200	200	128	512

Antibiotics: Vancomycin used for *Staphylococcus* species and azithromycin for *S. mutans* MTCC497.

Table 7. *In vitro* interaction of essential oils with antibiotics against *Staphylococcus* strains in planktonic and sessile mode of growth.

Test gents combination	*S. aureus* MTCC3160				*S. auricularis* (SU-01)			
	A	C	FICI	IT	A	C	FICI	IT
Planktonic mode								
1) CLV/VAN								
CLV	200	50	0.75	I	200	50	0.5	S
VAN	1024	512			1024	256		
2) EUG/VAN								
EUG	50	6.25	0.25	S	50	6.25	0.25	S
VAN	1024	128			1024	128		
Sessile mode								
1) EUG/VAN								
EUG	100	12.5	0.25	S	50	6.25	0.25	S
VAN	2048	256			1024	128		
	S. sciuri (SC-01)				*S. epidermidis* MTCC435			
Planktonic mode								
1) CLV/VAN								

Continued

CLV	100	25	0.312	S	200	50	0.50	S
VAN	1024	64			1024	256		
2) EUG/VAN								
EUG	50	12.5	0.375	S	50	3.12	0.312	S
VAN	1024	128			1024	256		
Sessile mode								
1) EUG/VAN								
EUG	50	12.5	0.375	S	50	12.5	0.375	S
VAN	1024	128			2048	256		

Where A: Alone, C: Combination, IT: Type of interaction, Nature of interaction, I: Indifferent, S: Synergy, FICI: Fractional Inhibitory Concentration Index, CLV: Clove, EUG: Eugenol, VAN: vancomycin.

Table 8. *In vitro* interaction of essential oils with azithromycin against *S. mutans* in planktonic and sessile mode.

Test gents Combination	*S. mutans* MTCC497			
	A	C	FICI	II
Planktonic mode				
1) CLV/AZI				
CLV	200	50	0.5	S
AZI	128	32		
2) EUG/AZI				
EUG	200	3.25	0.141	S
AZI	128	16		
Sessile mode				
1) EUG/AZI	200	12.5	0.187	S
EUG	512	64		
AZI				

Where A: Alone, C: Combination, IT: Type of interaction; Nature of interaction, I: Indifferent, S: Synergy, FICI: Fractional Inhibitory Concentration Index, CLV: Clove, EUG: Eugenol, AZI: Azithromycin.

values 0.75) against *S. aureus* MTCC3160 strains. In contrast, other test staphylococcal strains (*S. epidermidis* MTCC435, SU-01 and SC-01) showed synergy between clove oil and vancomycin with FICI value of 0.312 to 0.5. However, synergistic interaction was exhibited between eugenol and vancomycin against all the test staphylococcal strain in planktonic mode and FICI value was found in the range of 0.25 to 0.375. Eugenol showed maximum synergy with azithromycin against *S. mutans* MTCC497 (FICI = 0.141) in planktonic mode (Table 8).

Similarly, interaction studies on eugenol with antibacterial drug (vancomycin/azithromycin) against pre-formed bacterial biofilms are depicted in Table 7 and Table 8. There was varied extent of synergy between eugenol and vancomy-

cin with FICI index ranged from 0.25 to 0.375 against *Staphylococcus* pre-formed biofilms. No test combinations of oils and drugs exhibited antagonistic effect. Eugenol also showed synergy with azithromycin against *S. mutans* MTCC497 pre-formed biofilms [29].

Furthermore, SEM images indicated the prominent effect of the combine treatment of eugenol and antibiotics on pre-formed bacterial biofilms compared to the untreated control (**Figure 2**).

4. Discussions

Plants and plants products have significant antimicrobial properties against variety of pathogens. Problems such as microbial resistance to currently used antibiotics and lack of development of new antibiotics created an increased interest in the anti-infective herbal medicines. Natural products comprising of plant extracts and phytochemicals have been used to treat various infectious diseases since several decades [30].

The pathogenic bacteria included in the present study were characterized and identified based on cultural, morphological and biochemical characteristics. Among the 13 bacterial strains, 10 clinical and 3 reference strains were identified and characterized by the biochemical, cultural and morphological basis. The strains included in this study were *S. aureus*, *S. epidermidis*, *S. sciuri*, *S. auricularis* and *S. mutans*. *S. sciuri* (SC-01) and *S. auricularis* (SU-01) were identified by 16S rRNA gene sequence analysis as they were showing varied biochemical tests. Production of common virulence attributes such as cell surface hydrophobicity and hemolysin production were studied in the test bacterial strains. These

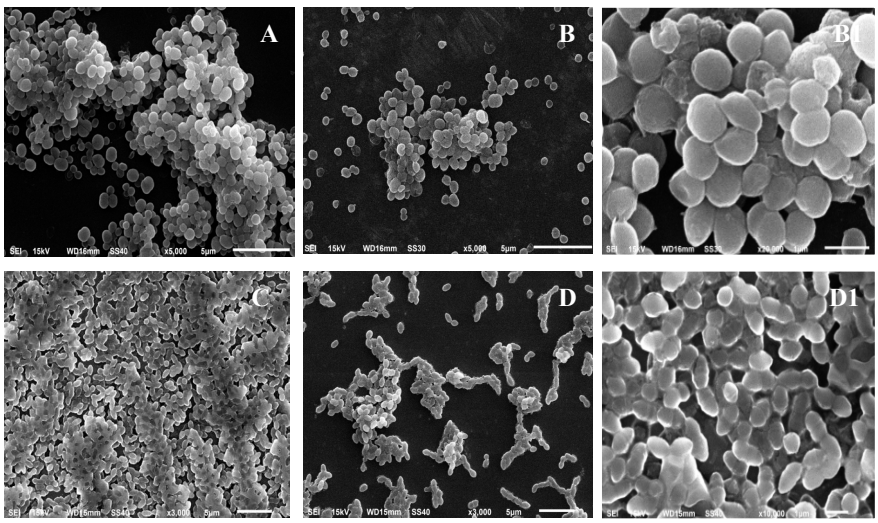

Figure 2. SEM images of pre-formed bacterial biofilm cells treated with combination of eugenol and antibiotics. (A) Untreated *S. aureus* MTCC3160 cells; (B, B1) Eradication of *S. aureus* MTCC3160 biofilm cells by the treatment of combination of eugenol (12.5 µg/ml) and vancomycin (256 µg/ml); (C) Untreated *S. mutans* MTCC497 biofilm cells; (D, D1) Eradication of *S. mutans* MTCC497 biofilm cells by the treatment of combination of eugenol (12.5 µg/ml) and azithromycin (64 µg/ml).

attribute aids in the disruption of the host tissues and also inactivate host antimicrobial mechanisms [31] [32]. Bacterial hemolysin mediate damage to the RBC of the host to meet and satisfy the iron requirement which may further enhance the virulent behavior in staphylococcal and *S. mutans* strains [33] [34]. Hemolysis is an important virulence factor which causes penetration and lysis of the host cells by the bacterial pathogens [34]. The bacterial strains included in this study such as *S. aureus*, *S. auricularis* (SU-01), *S. epidermidis*, *S. sciuri* (SC-01) and *S. mutans* produced above studied virulence factors. Production of cell surface hydrophobicity and hemolysin were detected in *Staphylococci* and *Streptococci* strains. These observations on *Staphylococci* are similar to the reports of other workers [32] [35] [36]. Similarly, production of various virulence factors in *S. mutans* is also reported by many workers [37] [38]. We were concerned with the strains that are pathogenic and exhibit significant virulence traits. Indeed, virulence traits are directly linked with adherence and biofilm development. Interestingly, association of *Staphylococcus* species such as *S. sciuri* and *S. auricularis* from the skin surface were reported in this study which are potentially pathogenic in nature. The pathogenecity of *S. sciuri* and *S. auricularis* were also documented by several other researchers [5] [6] [39].

Biofilm forming ability of the bacteria was evaluated under *in vitro* by XTT reduction assay in 96-well tissue culture plate. About 85% of the test strains developed strong biofilms which has clearly indicated that majority of clinical bacterial pathogens may involve biofilms mode of growth in the disease development in their respective host. Therefore, *in vitro* screening provides an initial idea on intrinsic ability of the strains to form biofilms. In the recent past, many workers have reported the biofilm development by *S. aureus* [40] [41], *S. epidermidis* [42] [43] and *S. mutans* [44] [45], *S. sciuri* [7] [46] and *S. auricularis* [6] [47].

In this study, *S. aromaticum* plant extract, clove essential oil and eugenol were screened for their efficacy against the bacterial pathogens. GC-MS analysis of *S. aromaticum* methanolic plant extract revealed that eugenol was the major compound. Other compounds such as caryophyllene, caryophyllene oxide, n. Hexadecanoic acid and alpha-Farneseneare also reported by other workers [48] [49]. The efficacy of test agents was determined in terms of minimum inhibitory concentration (MIC) so that a range of sub-MICs and above MICs values of active extract/essential oil/eugenol may be carefully selected for their anti-virulence and antibiofilm activities. The methanolic plant extract exhibited moderate to low activity against the test bacterial pathogens. Clove oil and eugenol showed significant antibacterial and antivirulence activity in the staphylococcoal and *S. mutans* strains. It is evident that pure compounds namely eugenol was more effective than essential oil against bacterial pathogens. Antimicrobial activity of *S. aromaticum* plant extract, clove oil and eugenol has been well documented [17] [50] [51] [52].

Since, targeting virulence factors and pathogenicity is considered as promising approach to combat localized and systemic infections [53]. Nowadays, new al-

ternatives have been attempted to attenuate microbial virulence and pathogenicity rather than killing the pathogenic microorganisms. It is believed that targeting virulence and pathogenicity will not result in development of resistance to such agents compared to agents that are used to kill the pathogens [54]. Therefore, agents affecting the production of virulence factors could be used in controlling bacterial infections. In this study, the essential oil of *S. aromaticum* and eugenol showed significant anti-infective activity at sub-MICs. The antimicrobial activity of these oils was further evaluated for their ability to inhibit the production of CSH and hemolysin production in bacterial strains. It is reported that hydrophobic cells have the ability to adhere and escape phagocytosis and these attributes enhance the virulent behavior of these pathogens. Our data clearly showed that eugenol apparently suppress the CSH of test microbial strains. However, inhibition of CSH by the essential oils is not widely studied against the bacterial strains. A report on the inhibition of CSH at sub-MICs of geraniol against *S. aureus* strain has been documented [55]. This is the first report on the inhibition of CSH and hemolysin production in *S. epidermidis*, *S. sciuri*, *S. auricularis* and *S. mutans* strains by clove oil and eugenol.

Furthermore, damage to the host cells is mediated by the production of bacterial hemolysin which is an important virulence factor of these pathogens. Hemolysin aids in the release of iron from the hemoglobin which is used as a nutrient source during the bacterial infections. Our findings suggested the varying level of inhibition of hemolysin production in bacterial strains (*S. aureus*, *S. epidermidis*, *S. sciuri*, *S. auricularis* and *S. mutans*) at sub-MICs of the test essential oils (eugenol and *S. aromaticum*). Eugenol significantly reduced the hemolysin production ($p < 0.05$) at 0.5 and 0.25 × MIC in the test bacterial strains. Similarly, inhibition of hemolysin production in the presence of eugenol and thymol in *S. aureus* strains has also been reported [56]. Thus, anti-hemolysin activity of the test oils may further contribute to the anti-pathogenic action against the bacterial strains. However, anti-hemolysin activity of eugenol was not widely studied in other *Staphylococcus* species and *S. mutans.*

The essential oils are bactericidal at higher concentration, but at lower concentration (sub-MICs) suppression in virulence activity of the microbial pathogens is evident. We found that viability of bacterial strains at sub-MICs of the test oils were not significantly reduced compared to the untreated control (data not shown). This has clearly indicated the inhibition of the virulence factors without significantly affecting the growth of the microbial cells. However, further investigation is needed to know the mechanism of action of the essential oils in the virulence inhibition. In this study essential oil inhibited more than one virulence factors which may further aid in lowering the pathogenicity of the organisms without affecting the growth of the organisms. In this way, chances of development of resistance might be minimized by the treatment of the clove oil and eugenol.

In our study, clove oil and eugenol eradicated the *Staphylococcus* species and

S. mutans biofilm cells. Sessile MIC of clove oil and eugenol was only increased 2-folds against the bacterial strains. Interestingly, no increase in SMIC of eugenol was observed against SU-01, *S. epidermidis* MTCC435 and *S. mutans* MTCC497. However, SMICs of antibiotics (vancomycin/azithromycin) increased upto 1000-folds against bacterial strains. The incidence of increased resistance to vancomycin against *Staphylococcus* species under biofilm mode of growth is also reported by other workers [57] [58] [59]. Similarly, the clinical strains of *S. mutans* under biofilm mode have also showed resistance to various antibiotics [60] [61]. Similarly, eradication of *Staphylococcus* pre-formed biofilms in the presence of essential oils of *Cymbopogon flexuosus*, *Citrus paradisi*, *Thymus vulgaris*, *Cinnamomum verum*, *Rosmarinus officinalis*is reported by other researchers [62] [63] [64]. [65] reported the eradication of *S. aureus* biofilm forming strains (*S. aureus* ATCC29213 and five isolated methicillin resistant strains) in the presence of thymol. [66] investigated the eradication of *S. epidermidis* biofilm by eucalyptus oil. Very few reports are available on the effect of essential oils on pre-formed *S. epidermidis* biofilms. So far no investigation is conducted on the eradication of pre-formed *S. auricularis* and *S. sciuri* biofilms by the essential oils.

Interestingly, essential oils/phytocompounds displayed enhanced bactericidal activity against the bacterial strains in biofilm mode compared to existing antimicrobial drugs. Thus, the cidal mode of action of test agents is quite effective in eradicating pre-formed biofilms. Thus, research on the selection of a new effective treatment in combating bacterial infections; necessitate a search for an agent with different mode of action. Moreover, the agents should not only possess direct antimicrobial activity but also should have synergistic activity with antimicrobial drugs, involve inhibition of expression of virulence factors such as production of cell surface hydrophobicity, hemolysin, adhesion, and aggregation that may further aid in combating microbial infections. As our test oils satisfy all the above characteristics, therefore an attempt has conducted to study the interaction of clove oil/eugenol with antibiotics in planktonic and sessile mode.

Combinational antimicrobial therapy is known for its therapeutic efficacy in controlling various infectious diseases including infection caused by the drug resistant pathogens [67]. It is expected that synergy between plant derived products/phytocompounds and antimicrobial drugs against bacterial pathogens could provide a new formulation for disease treatment [68]. The present study highlights the synergistic interaction between the essential oils with antibiotics against the test bacterial strains under planktonic and biofilm mode. The essential oils of *S. aromaticum* and eugenol have promising antibacterial activity alone against the test bacterial pathogens. Thus, above essential oils were investigated in combination with antimicrobial drugs against the test pathogens. Combination of essential oils (*S. aromaticum* and eugenol) and vancomycin revealed synergistic interaction against coagulase positive and coagulase negative *Staphylococci*. However, clove oil showed indifferent interaction with vancomycin against *S. aureus* MTCC3160. The MIC of vancomycin was reduced upto 8 and

16-folds against *Staphylococcus* species in planktonic and sessile mode. The combination of eugenol with vancomycin exhibited equal synergy against *S. aureus* MTCC3160, *S. sciuri* and *S. auricularis* in planktonic and sessile mode. Very few reports are available on interaction of essential oils with antibiotics against *S. aureus* in planktonic and sessile mode. However, this may be the first report on the interaction of clove oil/eugenol with vancomycin against *Staphylococcus* species such as *S. epidermidis*, *S. sciuri* and *S. auricularis* in planktonic and sessile mode of growth. The interaction between eugenol and vancomycin was reported earlier against *S. aureus* MTCC3160 pre-formed biofilms [69]. Vancomycin alters the cell membrane permeability, inhibits the cell wall biosynthesis and also inhibits the synthesis of ribonucleic acid [70] [71]. The antibacterial activity of eugenol depends on its ability to permeabilize the cell membrane and interact with cellular proteins. Non-specific permeabilization of the cytoplasmic membrane is linked with the leakage of K^+ and ATP from the bacterial cell in the presence of eugenol [72]. Thus, these two antibacterial agents target the different cellular structures and functions which ultimately lead to the lethal effect on the bacterial pathogens. Synergistic interaction between *Zataria multiflora Boiss* essential oil and vancomycin against *S. aureus* is also reported by [73]. Similarly, synergistic interaction between the citral and norfloxacin against methicilin resistant *S. aureus* was also reported by [74]. Other reports on *Carum copticum* essential oil with vancomycin against *S. aureus* [75], essential oil of *Melaleuca armillaris* with cloxacillin against *S. aureus* [76] were also documented. The essential oils of clove and eugenol also showed synergy with azithromycin against *S. mutans* MTCC497 in planktonic mode. However, eugenol showed highest synergy with azithromycin. The MIC of the drug was reduced upto 16-folds against *S. mutans* MTCC497. Azithromycin targets various metabolic processes including protein synthesis, quorum sensing and also biofilm formation [77]. A possible of mode of action of combination of eugenol and antibiotics on *Staphylococcus* and *S. mutans* biofilms seems to have multiple targets on bacterial cells structure including altered cell structure, damaged cell wall, changes in cell membrane permeability and inhibition of protein and ribonucleic synthesis. Additionally, the combination treatment reduces the dose of the antibiotics and thus it helps to overcome the drug toxicity issue. Further, the eradication of biofilms was also confirmed by the scanning electron microscopy by the treatment of combination of eugenol and antibiotics (vancomycin/azithromycin). Microscopy revealed the eradication of *S. aureus* MTCC3160 and *S. mutans* MTCC497 biofilm cells compared to the untreated control at 12.5 μg/ml concentration of eugenol in combination treatment compared to the untreated control. Altered cell morphology and shrinkage of bacterial cells is quite evident from SEM, indicating the possible mode of action of combination of eugenol and antibiotics. Synergy with antibiotics reduced the dose related toxicity issue and also restores the available drug to a state reduced resistance [12]. This clearly suggested that natural products could be used to potentiate the efficacy of antibiotics in appropriate combinations. Moreover, lowering the dose of drugs in combination

treatment may improve the overall efficacy of the treatment.

5. Conclusions

Our findings clearly established that clove oil and its active compounds like eugenol exhibited broad spectrum effect on growth, virulence and biofilms of bacterial strains. Synergy between essential oils and antibiotics is of great practical significance as these combinations can be used to surmount pathogenic biofilms associated infections. However, therapeutic application of combined treatment warrants *in vivo* investigation for its efficacy and safety. Further, molecular understanding and *in vivo* efficacy and safety of such combination needs to be evaluated carefully for future application in therapy.

Acknowledgements

Thanks are due to University grant commission for providing non-net fellowship to H.J. We are also thankful to University Sophisticated Instrumentation Facility (USIF), Aligarh Muslim University, Aligarh, India for SEM analysis. Advanced Instrumentation Research Facility (AIRF), Jawaharlal Nehru University, New Delhi, India is duly acknowledged for GC-MS analysis.

Conflicts of Interest

There is no conflict of interest.

References

[1] Khatoon, Z., McTiernan, C.D., Suuronen, E.J., Mah, T.F. and Alarcon, E.I. (2018) Bacterial Biofilm Formation on Implantable Devices and Approaches to Its Treatment and Prevention. *Heliyon*, **4**, e01067. https://doi.org/10.1016/j.heliyon.2018.e01067

[2] Casadevall, A. and Pirofski, L.A. (2009) Virulence Factors and Their Mechanisms of Action: The View from a Damage-Response Framework. *Journal of Water and Health*, **7**, S2-S18. https://doi.org/10.2166/wh.2009.036

[3] van Hal, S.J., Jensen, S.O., Vaska, V.L., Espedido, B.A., Paterson, D.L. and Gosbell, I.B. (2012) Predictors of Mortality in *Staphylococcus aureus* Bacteremia. *Clinical Microbiol Reviews*, **25**, 362-386. https://doi.org/10.1128/CMR.05022-11

[4] Otto, M. (2009) *Staphylococcus epidermidis*—The "Accidental" Pathogen. *Natures Reviews Microbiology*, **7**, 555-567. https://doi.org/10.1038/nrmicro2182

[5] Stepanović, S., Vuković, D., Trajković, V., Samardžić, T., Ćupić, M. and Švabić-Vlahović, M. (2001) Possible Virulence Factors of *Staphylococcus sciuri*. *FEMS Microbiology Letters*, **199**, 47-53. https://doi.org/10.1111/j.1574-6968.2001.tb10649.x

[6] Szczuka, E., Jabłońska, L. and Kaznowski, A. (2016) Coagulase-Negative Staphylococci: Pathogenesis, Occurrence of Antibiotic Resistance Genes and *in Vitro* Effects of Antimicrobial Agents on Biofilm-Growing Bacteria. *Journal of Medical Microbiology*, **65**, 1405-1413. https://doi.org/10.1099/jmm.0.000372

[7] Naik, M.M., Naik, S.P., Dubey, S.K., Bhat, C. and Charya, L.S. (2018) Enhanced Exopolysaccharide Production and Biofilm Forming Ability in Methicillin Resistant *Staphylococcus sciuri* Isolated from Dairy in Response to Acyl Homoserine Lactone

(AHL). *Journal of Food Science and Technology*, **55**, 2087-2094. https://doi.org/10.1007/s13197-018-3123-0

[8] Piette, A. and Verschraegen, G. (2009) Role of Coagulase-Negative Staphylococci in Human Disease. *Veterinary Microbiology*, **134**, 45-54. https://doi.org/10.1016/j.vetmic.2008.09.009

[9] Becker, K., Heilmann, C. and Peters, G. (2014) Coagulase-Negative Staphylococci. *Clinical Microbiology Reviews*, **27**, 870-926. https://doi.org/10.1128/CMR.00109-13

[10] Olson, M.E., Ceri, H., Morck, D.W., Buret, A.G. and Read, R.R. (2002) Biofilm Bacteria: Formation and Comparative Susceptibility to Antibiotics. *Canadian Journal of Veterinary Research*, **66**, 86.

[11] Khan, M.S.A., Ahmad, I., Sajid, M. and Cameotra, S.S. (2014) Current and Emergent Control Strategies for Medical Biofilms. In: *Antibiofilm Agents*, Springer, Berlin, 117-159. https://doi.org/10.1007/978-3-642-53833-9_7

[12] Vitale, R.G., Afeltra, J. and Dannaoui, E. (2005) Antifungal Combinations. In: *Antifungal Agents*, Humana Press, Totowa, 143-152. https://doi.org/10.1385/1-59259-943-5:143

[13] Khan, M.S.A., Malik, A. and Ahmad, I. (2012) Anti-Candidal Activity of Essential Oils Alone and in Combination with Amphotericin B or Fluconazole against Multi-Drug Resistant Isolates of *Candida albicans*. *Medical Mycology*, **50**, 33-42. https://doi.org/10.3109/13693786.2011.582890

[14] Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K.M. and Latha, L.Y. (2011) Extraction, Isolation and Characterization of Bioactive Compounds from Plants' Extracts. *African Journal of Traditional, Complementary and Alternative Medicines*, **8**, 1-10. https://doi.org/10.4314/ajtcam.v8i1.60483

[15] Newman, D.J. and Cragg, G.M. (2020) Natural Products as Sources of New Drugs over the Nearly Four Decades from 01/1981 to 09/2019. *Journal of Natural Products*, **83**, 770-803. https://doi.org/10.1021/acs.jnatprod.9b01285

[16] Gonelimali, F.D., Lin, J., Miao, W., Xuan, J., Charles, F., Chen, M. and Hatab, S.R. (2018) Antimicrobial Properties and Mechanism of Action of Some Plant Extracts against Food Pathogens and Spoilage Microorganisms. *Frontiers in Microbiology*, **9**, 1639. https://doi.org/10.3389/fmicb.2018.01639

[17] Kavanaugh, N.L. and Ribbeck, K. (2012) Selected Antimicrobial Essential Oils Eradicate Pseudomonas spp. and *Staphylococcus aureus* Biofilms. *Applied and Environmental Microbiology*, **78**, 4057-4061. https://doi.org/10.1128/AEM.07499-11

[18] Xu, J.G., Liu, T., Hu, Q.P. and Cao, X.M. (2016) Chemical Composition, Antibacterial Properties and Mechanism of Action of Essential Oil from Clove Buds against *Staphylococcus aureus*. *Molecules*, **21**, 1194. https://doi.org/10.3390/molecules21091194

[19] Barboza, J.N., da Silva Maia Bezerra Filho, C., Silva, R.O., Medeiros, J.V.R. and de Sousa, D.P. (2018) An Overview on the Anti-Inflammatory Potential and Antioxidant Profile of Eugenol. *Oxidative Medicine and Cellular Longevity*, **2018**, Article ID: 3957262. https://doi.org/10.1155/2018/3957262

[20] Ali, S.M., Khan, A.A., Ahmed, I., Musaddiq, M., Ahmed, K.S., Polasa, H., Ahmed, N., *et al.* (2005) Antimicrobial Activities of Eugenol and Cinnamaldehyde against the Human Gastric Pathogen *Helicobacter pylori*. *Annals of Clinical Microbiology and Antimicrobials*, **4**, 1-7. https://doi.org/10.1186/1476-0711-4-20

[21] Gascuel, O. and Steel, M. (2006) Neighbor-Joining Revealed. *Molecular Biology and Evolution*, **23**, 1997-2000. https://doi.org/10.1093/molbev/msl072

[22] Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. *Molecular Biology and Evolution*, **24**, 1596-1599. https://doi.org/10.1093/molbev/msm092

[23] Zhou, T., Li, Z., Kang, O.H., Mun, S.H., Seo, Y.S., Kong, R., Shin, D.W., Liu, X.Q. and Kwon, D.Y. (2017) Antimicrobial Activity and Synergism of Ursolic Acid 3-O-*α*-L-Arabinopyranoside with Oxacillin against Methicillin-Resistant *Staphylococcus aureus*. *International Journal of Molecular Medicine*, **40**, 1285-1293. https://doi.org/10.3892/ijmm.2017.3099

[24] Jafri, H., Khan, M.S.A. and Ahmad, I. (2019) *In Vitro* Efficacy of Eugenol in Inhibiting Single and Mixed-Biofilms of Drug-Resistant Strains of *Candida albicans* and *Streptococcus mutans*. *Phytomedicine*, **54**, 206-213. https://doi.org/10.1016/j.phymed.2018.10.005

[25] Pérez, P.F., Minnaard, Y., Disalvo, E.A. and De Antoni, G.L. (1998) Surface Properties of Bifidobacterial Strains of Human Origin. *Applied and Environmental Microbiology*, **64**, 21-26. https://doi.org/10.1128/AEM.64.1.21-26.1998

[26] Qiu, J., Zhang, X., Luo, M., Li, H., Dong, J., Wang, J., Leng, B., Wang, X., Feng, H., Ren, W. and Deng, X. (2011) Subinhibitory Concentrations of Perilla Oil Affect the Expression of Secreted Virulence Factor Genes in *Staphylococcus aureus*. *PLoS ONE*, **6**, e16160. https://doi.org/10.1371/journal.pone.0016160

[27] Reiter, K.C., Villa, B., da Silva Paim, T.G., Sambrano, G.E., de Oliveira, C.F. and d'Azevedo, P.A. (2012) Enhancement of Antistaphylococcal Activities of Six Antimicrobials against sasG-Negative Methicillin-Susceptible *Staphylococcus aureus*: An *in Vitro* Biofilm Model. *Diagnostic Microbiology and Infectious Disease*, **74**, 101-105. https://doi.org/10.1016/j.diagmicrobio.2012.05.034

[28] Jafri, H. and Ahmad, I. (2020) Thymus Vulgaris Essential Oil and Thymol Inhibit Biofilms and Interact Synergistically with Antifungal Drugs against Drug Resistant Strains of *Candida albicans* and *Candida tropicalis*. *Journal de mycologie Medicale*, **30**, Article ID: 100911. https://doi.org/10.1016/j.mycmed.2019.100911

[29] Jafri, H., Banerjee, G., Khan, M.S.A., Ahmad, I., Abulreesh, H.H. and Althubiani, A.S. (2020) Synergistic Interaction of Eugenol and Antimicrobial Drugs in Eradication of Single and Mixed Biofilms of *Candida albicans* and *Streptococcus mutans*. *AMB Express*, **10**, 1-9. https://doi.org/10.1186/s13568-020-01123-2

[30] Kim, K.J., Liu, X., Komabayashi, T., Jeong, S.I. and Selli, S. (2016) Natural Products for Infectious Diseases. https://doi.org/10.1155/2016/9459047

[31] Costa, A.R., Batistão, D.W., Ribas, R.M., Sousa, A.M., Pereira, M.O. and Botelho, C.M. (2013) *Staphylococcus aureus* Virulence Factors and Disease.

[32] Čuvalová, Z., Pipová, M., Kantíková, M., Brtková, A. and Fajber, J. (2015) Virulence Factors and Antimicrobial Resistance of Coagulase-Negative Staphylococci Isolated from Drinking Water. *Open Life Sciences*, **10**, 328-338. https://doi.org/10.1515/biol-2015-0034

[33] Woltjes, J., Legdeur-Velthuis, H. and de Graaff, J. (1981) Detection and Characterization of Hemolysin Production in *Streptococcus mutans*. *Infection and Immunity*, **31**, 850-855. https://doi.org/10.1128/IAI.31.3.850-855.1981

[34] Türkyilmaz, S. and Kaya, O. (2006) Determination of Some Virulence Factors in Staphylococcus spp. Isolated from Various Clinical Samples. *Turkish Journal of Veterinary and Animal Sciences*, **30**, 127-132.

[35] Kouidhi, B., Zmantar, T., Hentati, H. and Bakhrouf, A. (2010) Cell Surface Hydrophobicity, Biofilm Formation, Adhesives Properties and Molecular Detection of

Adhesins Genes in *Staphylococcus aureus* Associated to Dental Caries. *Microbial Pathogenesis*, **49**, 14-22. https://doi.org/10.1016/j.micpath.2010.03.007

[36] El-baz, R., Rizk, D.E., Barwa, R. and Hassan, R. (2016) Virulence Factors Profile of *Staphylococcus aureus* Isolated from Different Clinical Sources. *Journal of Microbiology*, **43**, 126.

[37] Lynch, D.J., Villhauer, A.L., Warren, J.J., Marshall, T.A., Dawson, D.V., Blanchette, D.R., Phipps, K.R., Starr, D.E. and Drake, D.R. (2015) Genotypic Characterization of Initial Acquisition of *Streptococcus mutans* in American Indian Children. *Journal of Oral Microbiology*, **7**, 27182. https://doi.org/10.3402/jom.v7.27182

[38] Hasan, S., Singh, K., Danisuddin, M., Verma, P.K. and Khan, A.U. (2014) Inhibition of Major Virulence Pathways of *Streptococcus mutans* by Quercitrin and Deoxynojirimycin: A Synergistic Approach of Infection Control. *PLoS ONE*, **9**, e91736. https://doi.org/10.1371/journal.pone.0091736

[39] Zeman, M., Mašlaňová, I., Indráková, A., Šiborová, M., Mikulášek, K., Bendíčková, K., Pantůček, R., *et al.* (2017) *Staphylococcus sciuri* Bacteriophages Double-Convert for Staphylokinase and Phospholipase, Mediate Interspecies Plasmid Transduction, and Package mecA Gene. *Scientific Reports*, **7**, Article No. 46319. https://doi.org/10.1038/srep46319

[40] Archer, N.K., Mazaitis, M.J., Costerton, J.W., Leid, J.G., Powers, M.E. and Shirtliff, M.E. (2011) *Staphylococcus aureus* Biofilms: Properties, Regulation, and Roles in Human Disease. *Virulence*, **2**, 445-459. https://doi.org/10.4161/viru.2.5.17724

[41] Federman, C., Ma, C. and Biswas, D. (2016) Major Components of Orange Oil Inhibit *Staphylococcus aureus* Growth and Biofilm Formation, and Alter Its Virulence factors. *Journal of Medical Microbiology*, **65**, 688-695. https://doi.org/10.1099/jmm.0.000286

[42] Büttner, H., Mack, D. and Rohde, H. (2015) Structural Basis of *Staphylococcus epidermidis* Biofilm Formation: Mechanisms and Molecular Interactions. *Frontiers in Cellular and Infection Microbiology*, **5**, 14. https://doi.org/10.3389/fcimb.2015.00014

[43] Takenaka, S. and Noiri, Y. (2017) Limitations and Adverse Influences of Antimicrobial Strategy for the Control of Oral Biofilm. *Journal of Dentistry and Oral Biology*, **2**, 1034.

[44] Lombardo Bedran, T.B., Azelmat, J., Palomari Spolidorio, D. and Grenier, D. (2013) Fibrinogen-Induced *Streptococcus mutans* Biofilm Formation and Adherence to Endothelial Cells. *BioMed Research International*, **2013**, Article ID: 431465. https://doi.org/10.1155/2013/431465

[45] Oliveira, W.F., Silva, P.M.S., Silva, R.C.S., Silva, G.M.M., Machado, G., Coelho, L.C.B.B. and Correia, M.T.S. (2018) *Staphylococcus aureus* and *Staphylococcus epidermidis* Infections on Implants. *Journal of Hospital Infection*, **98**, 111-117. https://doi.org/10.1016/j.jhin.2017.11.008

[46] Gajewska, J. and Chajęcka-Wierzchowska, W. (2020) Biofilm Formation Ability and Presence of Adhesion Genes among Coagulase-Negative and Coagulase-Positive Staphylococci Isolates from Raw Cow's Milk. *Pathogens*, **9**, 654. https://doi.org/10.3390/pathogens9080654

[47] Sagar, S. and Kaistha, D.S. (2011) Virulence Profile of an Emerging Coagulase Negative *Staphylococcus auricularis* NC Clinical Isolate. *Journal of Pure and Applied Microbiology*, **5**, 787-792.

[48] Mercy Bastine, C., Mohanapriya, S. and Caroline Jeba, R. (2013) GCMS Analysis of Methanol Extract of Buds of *Syzygium aromaticum*. *Indo American Journal of*

Pharmaceutical Research, **3**, 5485-5491.

[49] Hemalatha, R., Nivetha, P., Mohanapriya, C., Sharmila, G., Muthukumaran, C. and Gopinath, M. (2016) Phytochemical Composition, GC-MS Analysis, *in Vitro* Antioxidant and Antibacterial Potential of Clove Flower Bud (*Eugenia caryophyllus*) Methanolic Extract. *Journal of Food Science and Technology*, **53**, 1189-1198. https://doi.org/10.1007/s13197-015-2108-5

[50] Pulikottil, S.J. and Nath, S. (2015) Potential of Clove of *Syzygium aromaticum* in Development of a Therapeutic Agent for Periodontal Disease: A Review. *South African Dental Journal*, **70**, 108-115.

[51] Atanasova-Pancevska, N., Bogdanov, J. and Kungulovski, D. (2017) *In Vitro* Antimicrobial Activity and Chemical Composition of Two Essential Oils and Eugenol from Flower Buds of *Eugenia caryophyllata*. *Open Biological Sciences Journal*, **3**, 16-25. https://doi.org/10.2174/2352633501703010016

[52] El Atki, Y., Aouam, I., El Kamari, F., Taroq, A., Gourch, A., Lyoussi, B. and Abdellaoui, A. (2019) Antibacterial Efficacy of Thymol, Carvacrol, Eugenol and Menthol as Alternative Agents to Control the Growth of Nosocomial Infection-Bacteria. *Journal of Pharmaceutical Sciences and Research*, **11**, 306-309.

[53] Mühlen, S. and Dersch, P. (2015) Anti-Virulence Strategies to Target Bacterial Infections. In: *How to Overcome the Antibiotic Crisis*, Springer, Cham, 147-183. https://doi.org/10.1007/82_2015_490

[54] Fleitas Martínez, O., Cardoso, M.H., Ribeiro, S.M. and Franco, O.L. (2019) Recent Advances in Anti-Virulence Therapeutic Strategies with a Focus on Dismantling Bacterial Membrane Microdomains, Toxin Neutralization, Quorum-Sensing Interference and Biofilm Inhibition. *Frontiers in Cellular and Infection Microbiology*, **9**, 74. https://doi.org/10.3389/fcimb.2019.00074

[55] Kannappan, A., Sivaranjani, M., Srinivasan, R., Rathna, J., Pandian, S.K. and Ravi, A.V. (2017) Inhibitory Efficacy of Geraniol on Biofilm Formation and Development of Adaptive Resistance in *Staphylococcus epidermidis* RP62A. *Journal of Medical Microbiology*, **66**, 1506-1515. https://doi.org/10.1099/jmm.0.000570

[56] Qiu, J., Feng, H., Lu, J., Xiang, H., Wang, D., Dong, J., Deng, X., *et al*. (2010) Eugenol Reduces the Expression of Virulence-Related Exoproteins in *Staphylococcus aureus*. *Applied and Environmental Microbiology*, **76**, 5846-5851. https://doi.org/10.1128/AEM.00704-10

[57] Manandhar, S., Singh, A., Varma, A., Pandey, S. and Shrivastava, N. (2018) Biofilm Producing Clinical *Staphylococcus aureus* Isolates Augmented Prevalence of Antibiotic Resistant Cases in Tertiary Care Hospitals of Nepal. *Frontiers in Microbiology*, **9**, 2749. https://doi.org/10.3389/fmicb.2018.02749

[58] Zhou, K., Li, C., Chen, D., Pan, Y., Tao, Y., Qu, W., Liu, Z., Wang, X. and Xie, S. (2018) A Review on Nanosystems as an Effective Approach against Infections of *Staphylococcus aureus*. *International Journal of Nanomedicine*, **13**, 7333. https://doi.org/10.2147/IJN.S169935

[59] Craft, K.M., Nguyen, J.M., Berg, L.J. and Townsend, S.D. (2019) Methicillin-Resistant *Staphylococcus aureus* (MRSA): Antibiotic-Resistance and the Biofilm Phenotype. *Medicinal Chemistry Communications*, **10**, 1231-1241. https://doi.org/10.1039/C9MD00044E

[60] William, B., Rwenyonyi, C.M., Swedberg, G. and Kironde, F. (2012) Cotrimoxazole Prophylaxis Specifically Selects for Cotrimoxazole Resistance in *Streptococcus mutans* and *Streptococcus sobrinus* with Varied Polymorphisms in the Target Genes folA and folP. *International Journal of Microbiology*, 2012, Article ID: 916129.

https://doi.org/10.1155/2012/916129

[61] Jubair, H.H. (2015) The Relationship between Biofilm Forming and Antibiotics Resistance of *Streptococcus mutans* Isolated from Dental Caries. *International Journal of Current Microbiology and Applied Sciences*, **5**, 568-574.

[62] Adukwu, E.C., Allen, S.C. and Phillips, C.A. (2012) The Anti-Biofilm Activity of Lemongrass (*Cymbopogon flexuosus*) and Grapefruit (*Citrus paradisi*) Essential Oils against Five Strains of *Staphylococcus aureus*. *Journal of Applied Microbiology*, **113**, 1217-1227. https://doi.org/10.1111/j.1365-2672.2012.05418.x

[63] Ben Abdallah, F., Lagha, R. and Gaber, A. (2020) Biofilm Inhibition and Eradication Properties of Medicinal Plant Essential Oils against Methicillin-Resistant *Staphylococcus aureus* Clinical Isolates. *Pharmaceuticals*, **13**, 369.

[64] Cui, H., Li, W., Li, C., Vittayapadung, S. and Lin, L. (2016) Liposome Containing Cinnamon Oil with Antibacterial Activity against Methicillin-Resistant *Staphylococcus aureus* Biofilm. *Biofouling*, **32**, 215-225. https://doi.org/10.1080/08927014.2015.1134516

[65] Kifer, D., Mužinić, V. and Klarić, M.Š. (2016) Antimicrobial Potency of Single and Combined Mupirocin and Monoterpenes, Thymol, Menthol and 1,8-Cineole against *Staphylococcus aureus* Planktonic and Biofilm Growth. *The Journal of Antibiotics*, **69**, 689-696. https://doi.org/10.1038/ja.2016.10

[66] Haddad, M., Zein, S., Shahrour, H., Elena, K., Chokr, A. and Kanaan, H. (2017) Eucalyptus Oil Prevents *Staphylococcus epidermidis* Biofilm Formation. *European Journal of Pharmaceutical and Medical Research*, **4**, 35-41.

[67] Cheesman, M.J., Ilanko, A., Blonk, B. and Cock, I.E. (2017) Developing New Antimicrobial Therapies: Are Synergistic Combinations of Plant Extracts/Compounds with Conventional Antibiotics the Solution? *Pharmacognosy Reviews*, **11**, 57. https://doi.org/10.4103/phrev.phrev_21_17

[68] Barbieri, R., Coppo, E., Marchese, A., Daglia, M., Sobarzo-Sanchez, E., Nabavi, S.F. and Nabavi, S.M. (2017) Phytochemicals for Human Disease: An Update on Plant-Derived Compounds Antibacterial Activity. *Microbiological Research*, **196**, 44-68. https://doi.org/10.1016/j.micres.2016.12.003

[69] Jafri, H. and Ahmad, I. (2020) Combinational Effect of Essential Oil Compounds and Antimicrobial Drugs on *Candida albicans* and *Staphylococcus aureus* Mixed Biofilms. *Journal of Essential Oil Bearing Plants*, **23**, 697-709. https://doi.org/10.1080/0972060X.2020.1822211

[70] Watanakunakorn, C. (1984) Mode of Action and *In-Vitro* Activity of Vancomycin. *Journal of Antimicrobial Chemotherapy*, **14**, 7-18. https://doi.org/10.1093/jac/14.suppl_D.7

[71] McGuinness, W.A., Malachowa, N. and DeLeo, F.R. (2017) Focus: Infectious Diseases: Vancomycin Resistance in *Staphylococcus aureus*. *The Yale Journal of Biology and Medicine*, **90**, 269.

[72] Hemaiswarya, S. and Doble, M. (2009) Synergistic Interaction of Eugenol with Antibiotics against Gram Negative Bacteria. *Phytomedicine*, **16**, 997-1005. https://doi.org/10.1016/j.phymed.2009.04.006

[73] Mahboubi, M. and Bidgoli, F.G. (2010) Antistaphylococcal Activity of Zataria Multiflora Essential Oil and Its Synergy with Vancomycin. *Phytomedicine*, **17**, 548-550. https://doi.org/10.1016/j.phymed.2009.11.004

[74] Gupta, P., Patel, D.K., Gupta, V.K., Pal, A., Tandon, S. and Darokar, M.P. (2017) Citral, a Monoterpenoid Aldehyde Interacts Synergistically with Norfloxacin against

Methicillin Resistant *Staphylococcus aureus*. *Phytomedicine*, **34**, 85-96. https://doi.org/10.1016/j.phymed.2017.08.016

[75] Talei, G.R., Mohammadi, M., Bahmani, M. and Kopaei, M.R. (2017) Synergistic Effect of *Carum copticum* and *Mentha piperita* Essential Oils with Ciprofloxacin, Vancomycin, and Gentamicin on Gram-Negative and Gram-Positive Bacteria. *International Journal of Pharmaceutical Investigation*, **7**, 82. https://doi.org/10.4103/jphi.JPHI_12_17

[76] Buldain, D., Buchamer, A.V., Marchetti, M.L., Aliverti, F., Bandoni, A. and Mestorino, N. (2018) Combination of Cloxacillin and Essential Oil of *Melaleuca armillaris* as an Alternative against *Staphylococcus aureus*. *Frontiers in Veterinary Science*, **5**, 177. https://doi.org/10.3389/fvets.2018.00177

[77] Parnham, M.J., Haber, V.E., Giamarellos-Bourboulis, E.J., Perletti, G., Verleden, G.M. and Vos, R. (2014) Azithromycin: Mechanisms of Action and Their Relevance for Clinical Applications. *Pharmacology & Therapeutics*, **143**, 225-245. https://doi.org/10.1016/j.pharmthera.2014.03.003

Supplementary File

Table S1. Designation, clinical condition and source of isolation of bacterial strains.

Strains Designation/Name	Identification	Clinical condition	Source
SAJ-01, SAJ-02, SAJ-04 SU-01	*S. aureus* -do- *S. auricularis*	Blood Ear infection Blood	Dept of Microbiology, Jawaharlal Nehru Medical College and Hospital (JNMC), Aligarh, India
SAJ-03 SEJ-01 to 04	*S. aureus* *S. epidermidis*	Eye infection	Kindly provided by Prof. Shamim Ahmad, Dept of Ophthalmology, Jawaharlal Nehru Medical College and Hospital (JNMC), Aligarh, India
SC-01	*S. sciuri*	Skin	Healthy person skin isolate in our laboratory. Department of Agricultural Microbiology, Aligarh Muslim University (AMU), Aligarh, India
S. aureus MTCC3160	Reference strain	-	Microbial Type Culture Collection, CSIR, IMTECH, Chandigarh, India
S. epidermidis MTCC435	Reference strain	-	Microbial Type Culture Collection, CSIR, IMTECH, Chandigarh, India
Streptococcus. mutans MTCC497	Reference strain	-	Microbial Type Culture Collection, CSIR, IMTECH, Chandigarh, India

Table S2. Biochemical characterization of the SC-01 strain.

Biochemical test	Results
Mannitol salt agar	No fermentation of mannotol
Novobiocin sensitivity	Resistant
Catalase	+
Nitrate reduction	+
Gelatin Hydrolysis	–
Starch hydrolysis	–
Urease test	–
Oxidase Test	–

+: indicates positive reaction, –: indicates negative reaction.

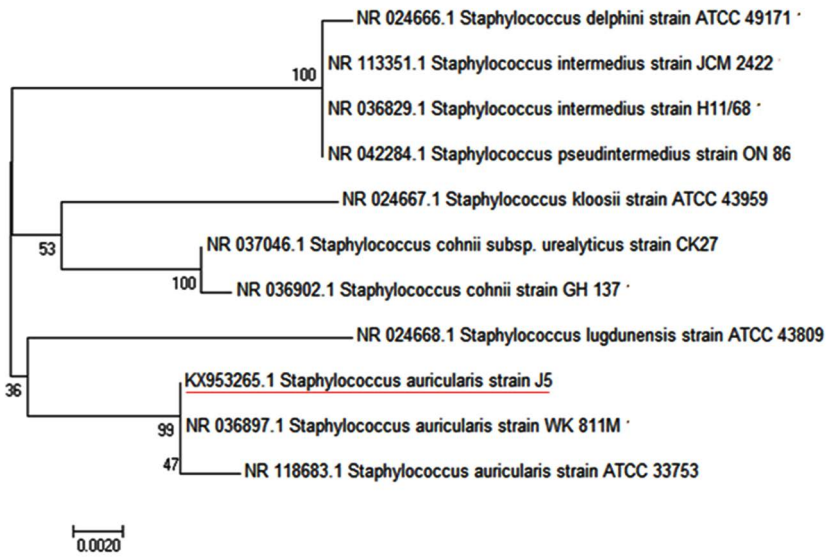

Figure S1. Phylogenetic tree of SU-01 strain (*S. auriuclaris* J5) based on 16S rRNA gene sequence based homology.

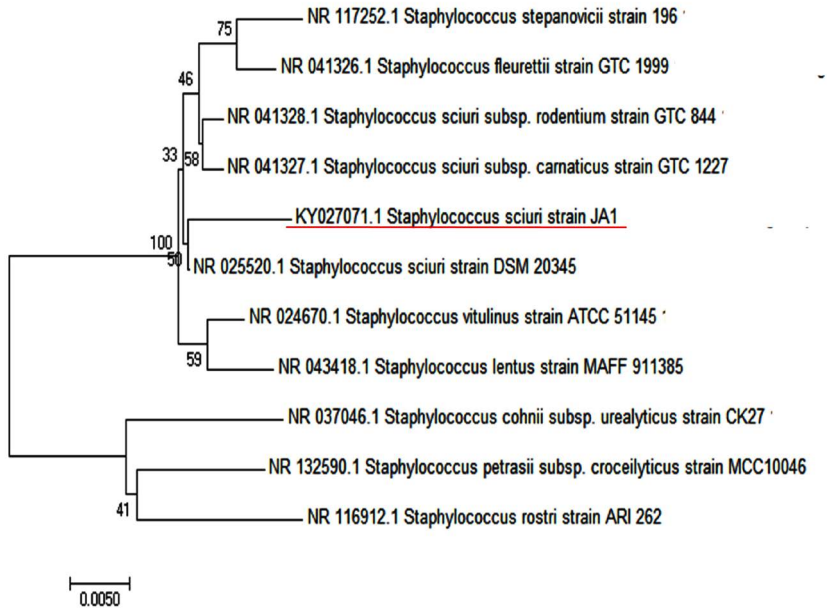

Figure S2. Phylogenetic tree of SC-01 strain (*S. sciuri* JA1) based on 16S rRNA gene sequence based homology.