Inhibitory efficacy of geraniol on biofilm formation and development of adaptive resistance in *Staphylococcus epidermidis* RP62A

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**Abstract**

**Purpose.** The current study has been designed to delineate the efficacy of geraniol (GE) on biofilm formation in *Staphylococcus epidermidis* as well as the effect of subinhibitory concentrations of GE on the development of adaptive resistance.

**Methodology.** Biofilm biomass quantification assay was performed to evaluate the antibiofilm activity of GE against *S. epidermidis*. Microscopic observation of biofilms and extracellular polymeric substance (EPS), slime and cell surface hydrophobicity (CSH) production were also studied to support the antibiofilm potential of GE. In addition, *S. epidermidis* was examined for its adaptive resistance development upon continuous exposure of GE at its subinhibitory concentrations.

**Results/Key findings.** The MIC of GE against *S. epidermidis* was 512 µg ml\(^{-1}\). Without hampering the growth of the pathogen, GE at its sub-MICs (50, 100, 150 and 200 µg ml\(^{-1}\)) exhibited a dose-dependent increase in antibiofilm activity. The minimal biofilm inhibitory concentration (MBIC) of GE was found to be 200 µg ml\(^{-1}\) with a maximum biofilm inhibition of 85 %.

Disintegrated biofilm architecture, reduced EPS, slime and CSH production validated the antibiofilm efficacy of GE. Although the action of GE on preformed biofilm is limited, a 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay and live/dead cell staining method revealed reduction in the viability (47 %) of biofilm inhabitants at 2×MIC concentration. Sequential exposure of *S. epidermidis* to the sub-MICs of GE resulted in poor development of adaptive resistance with diminished biofilm formation.

**Conclusion.** The present study highlights the potential of GE as a suitable candidate for the control of biofilm-mediated *S. epidermidis* infections.

**INTRODUCTION**

Nosocomial infections are the fourth leading cause of morbidity and mortality in clinical settings, among which, 60 % of the infections are associated with medical devices [1]. The ability of pathogenic bacteria to adhere to biotic and abiotic surfaces is the major intrinsic factor accounting for device-related infections globally. Formation of multifaceted biofilm by micro-organisms is a multi-tone procedure, where the bacterial cells entrench themselves into a self-secreted extracellular polymeric substance (EPS) matrix mainly composed of polysaccharides, proteins and nucleic acids [2].

Staphylococci are considered as the most frequent cause of nosocomial infections worldwide. *Staphylococcus epidermidis* is a coagulase negative staphylococcus that represents the most prevalent and persistent species found in human skin and mucosal microflora. *S. epidermidis* has been considered non-pathogenic for decades, but in recent years they started to be regarded as one of the etiological opportunistic pathogens causing biofilm-related infections in clinical settings [3]. Unlike other staphylococci, *S. epidermidis* does not produce a wide array of toxins and degradative enzymes. The strong adhering potential of this pathogen aids in colonization on implanted medical devices such as prosthetic joint, intracardiac devices, vascular graft, central
nervous system shunt, etc., and foreign bodies like central venous catheters. Such adherence and subsequent coloniza-
tion of \textit{S. epidermidis} on medical devices are tough to treat, which often leads to the need to remove infected biomedical implants [4].

The ability of pathogens to form biofilms has led to multi-
drug resistance. This structure-based resistance is not only due to the exchange of resistance markers within the biofilm cells, but also because of limitations posed by EPS, such as poor penetration and off-target binding of the administered antibacterial agents. In addition to the presence of antibiotic resistance markers and biofilm-mediated resistance, persisters are the small phenotypic variant subpopulation present in the biofilm, which can tolerate the effect of antibiotics without undergoing any genetic change [5]. Altogether, these characteristics make biofilm-embedded bacterial cells far more resistant to antibiotics and other antibacterial agents. Due to the biofilm-mediated health burdens caused by pathogenic organisms, identifying alternative treatment strategies that target a specific step in the biofilm development stage has become imperative and believed to be a promising control measure. In this context, exploring natural compounds, especially the well-researched plant derivatives such as essential oil (EO) and EO-related phytochemicals have gained interest and were proven to have a wide range of medicinal properties [6]. Although the plant-derived EO and its components have already been proven for their efficiency as antimicrobial agents against a variety of drug-resistant organisms, the research on EO and its components against antivirulence and antibiofilm agents is on the rise. For instance, eugenol against the virulence of \textit{S. aureus} and \textit{Candida albicans}, limonene against \textit{Streptococcus pyogenes} biofilm formation and virulence factor production have been evaluated [7–9]. Similarly, several EO components have been investigated for their effect on preformed biofilms of staphylococcal species [10].

Geraniol (GE), an acyclic monoterpenic alcohol, is a major component in the EO of lemongrass, lime, ginger, lavender, etc. GE has been approved by the United States Food and Drug Administration (FDA) as a flavouring agent in food materials [11]. Although GE has been studied for its antimicrobial, antioxidant, anti-inflammatory, antitumor and other biological activities, its efficacy as an antibiofilm agent and its impact on the development of adaptive resistance on \textit{S. epidermidis} during continuous exposure remains unexplored [12–14]. Hence, the present study has been designed to determine the efficacy of GE against \textit{S. epidermidis} biofilm formation and to investigate it on adaptive resistance development in \textit{S. epidermidis} upon prolonged exposure.

\section*{METHODS}

\subsection*{Bacterial strain and culture conditions}

A biofilm-producing strain, \textit{S. epidermidis} RP62A (ATCC 35984) was used in this study. The test pathogen was maintained in Todd–Hewitt agar (THA) (Hi-Media, Mumbai, India) and cultured in Todd–Hewitt broth (THB). For the biofilm assay, THB supplemented with 1 \textit{\%} sucrose was used to induce the \textit{in vitro} biofilm formation. For standard cell suspension, overnight culture of the test pathogen was adjusted to 0.4 OD at 600 nm (1 × 10^9 c.f.u. ml^{-1}). Since GE was dissolved in ethanol, ethanol was used as a negative control.

\textbf{GE}

GE was purchased from Sigma-Aldrich, St. Louis, MO, USA (catalogue no. 163333). Stock solution of GE was prepared in ethanol and stored at 4 °C.

\subsection*{Determination of MIC}

The MIC for GE was determined through the broth dilution method as described by the Clinical and Laboratory Standards Institute [15]. Briefly, 1 \text{\%} of the test strain was added to 1 ml of Muller–Hinton broth (MHB) (Hi-Media, Mumbai, India) containing serially diluted GE at concentrations ranging from 512 to 32 µg ml^{-1} and incubated at 37 °C for 24 h. The MIC was recorded as the lowest concentration of GE that showed complete inhibition of bacterial growth.

\subsection*{Effect of GE on \textit{S. epidermidis} biofilm formation}

GE was tested for its potential activity on the biofilm formation of \textit{S. epidermidis}. Briefly, 1 \text{\%} of the test pathogen was added to 1 ml of THB along with GE at subinhibitory concentrations (50, 100, 150, 200, 250 and 300 µg ml^{-1}), and the cells were allowed to grow as biofilms in the wells of a 24-well micro titre plate (MTP) at 37 °C for 24 h. After incubation, the planktonic cells were removed and the biofilms adhered on the wells were washed twice with PBS of pH 7.4. The biofilm cells adhered to the cells were stained with crystal violet (0.4 \text{\%} w/v) solution. The adhered stain in the biofilm cells was eluted with 95 \text{\%} ethyl alcohol and quantified at OD_{570}\text{\,nm} by a UV-visible spectrophotometer (Hitachi U-2800, Tokyo, Japan). The minimal biofilm inhibitory concentration (MBIC) of GE was determined as the least concentration that produced maximum biofilm inhibition in \textit{S. epidermidis} [16].

\subsection*{XTT assay}

The XTT [2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide] reduction assay was performed to determine the metabolic activity of the cells, which is considered directly proportional to the number of viable cells [17]. Briefly, the untreated and GE-treated cells in the MTP wells were washed with PBS and resuspended with the same. Twenty-five microlitres of freshly prepared XTT-menadione solution containing XTT (Sigma Aldrich, St. Louis, MO, USA) and menadione (Hi-Media, Mumbai, India) in the ratio of 12.5:1 were added to the 200 µl of untreated and GE-treated cell suspension and incubated at 37 °C for 8 h in the dark. After incubation, the cells were pelleted by centrifugation and the supernatant was read at OD_{490}\text{\,nm}. Sterile PBS incubated along with XTT-menadione was used as blank.
**In situ microscopic visualization**

**Light microscopic analysis**
The effect of GE on *S. epidermidis* biofilm formation was qualitatively assessed by a light microscope as described elsewhere [16]. The test bacterium was grown in the presence and the absence of GE at MBIC as before in a 24-well MTP containing 1 ml of THB and glass slides (1 × 1 cm) by incubation at 37°C for 24 h in the presence and the absence of GE at MBIC. After incubation, the slides were washed with sterile PBS and stained with crystal violet (0.4 % w/v). The excess stain on the slides was washed and air dried. The slides were then observed under a light microscope (Nikon Eclipse Ti 100, Tokyo, Japan) at ×400 magnification.

**Confocal laser scanning microscopic (CLSM) analysis**
For CLSM analysis, the slides were washed with sterile PBS and fixed with 2.5 % glutaraldehyde solution for 3 h in the dark, followed by dehydration using increasing concentration of ethanol (20, 40, 60, 80 and 100 %) for 2 min each. The dehydrated slides were sputter coated by gold and examined under SEM (VEGA 3 TESCAN, Czech Republic) [9].

**Effect of GE on EPS**

**EPS quantification**
EPS estimation was carried out by following the method described earlier [19]. Briefly, the test pathogen in the presence and the absence of GE at MBIC was grown in MTP containing 1 ml of THB at 37°C for 24 h. After incubation, the planktonic cells were removed and the biofilms adhered to the MTP wells were washed with 0.9 % NaCl. To the NaCl mixture, an equal volume of 5 % phenol and five volumes of H2SO4 were added and incubated in the dark for 1 h. After incubation, the reaction mixture was centrifuged at 10,000 r.p.m. for 10 min and the supernatant was read at OD490 nm.

**EPS staining**
Concanavalin A (Con A) conjugated to FITC (Sigma Aldrich, St. Louis, MO, USA) was used to detect the EPS production in the biofilm of *S. epidermidis*. The protocol performed by Banas et al. [20] was followed with little modifications. The test pathogen, in the presence and the absence of GE at MBIC was grown as biofilms on the glass slides for 24 h at 37°C. The slides were washed with sterile PBS and stained with 15 µM propidium iodide (PI) (Molecular Probes, Eugene, OR, USA) for 15 min at room temperature. The slides with PI were washed with sterile PBS and further stained with 50 µg ml⁻¹ of Con A-FITC for 15 min. The stained slides were imaged using CLSM (Zeiss LSM Image Examiner, version 4.2.0.121). The excitation and emission of PI and Con A-FITC were 568 and 605 nm and 488 and 522 nm, respectively.

**Fourier transform infrared (FT-IR) spectroscopic analysis**
The bacterial cell pellet was subjected to FT-IR analysis to observe the changes in the cellular components upon treatment with GE. Briefly, 2 mg of the cell pellet was mixed with 100 mg of potassium bromide (KBr) to prepare the KBr-cell pellet. The IR spectra for the prepared KBr-cell pellet were recorded with a FT-IR system (Nicolet i5S, Thermo Scientific, Madison, WI, USA). A total of 64 scans were taken with 4 cm⁻¹ resolution. The spectrum was scanned in the range of 4000–400 cm⁻¹. The IR spectra were plotted as absorbance and analysed using OMNIC software [21].

**Mature biofilm disruption assay**
The test pathogen was allowed to form biofilm for 24 h on the MTP as described above. After incubation, the spent growth media were discarded and fresh THB was added along with GE at different concentrations (MBIC, MIC and 2 × MIC) and further incubated at 37°C for 24 h. After incubation, biofilm biomass quantification and XTT reduction assay were carried out as explained above.

**Live/dead staining**
The protocol of the mature biofilm disruption assay was followed for the live/dead experiment as well with slight modifications. The test pathogen was allowed to form biofilm for 24 h on the 1 cm² glass slides in the MTP. At the end of incubation, the glass slides were washed in sterile PBS and transferred to a fresh MTP containing THB along with GE at different concentrations (MBIC, MIC and 2 × MIC) and incubated at 37°C for 24 h. The glass slides were then washed with sterile PBS thrice and stained with 1 µM concentration of PI and SYTO 9 (Live/Dead BacLight bacterial viability kit, Molecular Probes, Eugene, OR, USA, catalogue no. L7012). The stained slides were then visualized under CLSM and processed with Zeiss LSM Image Examiner (version 4.2.0.121). The excitation and emission of PI and SYTO 9 were 535 and 617 nm and 485 and 498 nm, respectively [22].

**Slime production**
To determine the slime production, a phenotypic method using congo red agar (CRA) plates was performed. CRA plates with and without GE at MBIC were prepared as described earlier [23]. To visualize the effect of GE on slime production, the test pathogen was streaked on the GE-free and GE supplemented CRA plates and incubated at 37°C for 24 h.

**Microbial adherence to hydrocarbons (MATH) assay**
The effect of GE on CSH was determined by the MATH assay [18]. The cell suspension of GE-treated and untreated *S. epidermidis* (1.0 OD at 600 nm) was prepared in PBS. To the 2 ml of cell suspensions, an equal volume of toluene was added and vortexed for 2 min. The cell suspension was kept undisturbed until the separation of toluene and aqueous
phases. The toluene phase was then removed and the aqueous phase was read at OD_{600} nm. The hydrophobicity index (HI) or % of CSH production was calculated using the formula:

$$HI = \frac{1}{2} \left( \frac{OD \text{ after vortexing}}{OD \text{ before vortexing}} \right) \times 100$$

**Adaptation of S. epidermidis to GE**

To study the effect of GE on the test pathogen, successive passage was carried out by following the method of Apolônio et al. [24]. The test pathogen was inoculated in THB+1 % sucrose supplemented with 50 µg ml$^{-1}$ of GE and incubated at 37 °C for 24 h. The S. epidermidis culture was again subcultured in the fresh THB+1 % sucrose supplemented with GE. The bacterium was sequentially exposed to a single concentration for 3 days by repeated subculturing followed by exposure to higher concentrations of GE until the bacterium stopped growing. The pathogen growth was read at OD_{600} nm every day. Bacterial culture grown without GE acted as a control. Simultaneously, the biofilm biomass was also quantified during the continuous exposure as described above.

**Statistics**

All the assays were performed independently in biological triplicate. Values are expressed in mean±SD and the statistical analysis was performed using SPSS package (SPSS v20.0; SPSS, Armonk, NY, USA). One way ANOVA was used to compare the control and treated samples, with a P-value less than 0.05 being significant.

**RESULTS**

**Determination of MIC**

GE showed growth inhibition in a concentration-dependent manner and the MIC was found to be 512 µg ml$^{-1}$. Hence in the present study, GE was used at its sub-MIC to evaluate its potential activity against S. epidermidis biofilm formation.

**Quantification of biofilm biomass in the MTP assay**

In the biofilm biomass quantification assay, a concentration-dependent increase in the antibiofilm activity of GE was observed. It showed 6, 17, 67 and 85 % reduction in biofilm formation at 50, 100, 150 and 200 µg ml$^{-1}$, respectively (Fig. 1). Since GE at 250 µg ml$^{-1}$ and above showed growth reduction, 200 µg ml$^{-1}$ was taken as its MBIC and the same was used for all further assays.

**Assessment of bacterial viability using the XTT assay**

Further, to confirm the non-antibacterial nature of GE at its sub-MIC, the XTT reduction assay was carried out. The result of the XTT reduction assay clearly revealed that GE at its MBIC inhibits only the biofilm formation but not the viability of S. epidermidis (Fig. 1).

**In situ analysis of biofilm formation**

In the light microscopic images, control slides showed a thick and continuous lawn of biofilm, whereas GE-treated slides showed a discontinuous and poor biofilm development (Fig. 2a). Further, CLSM analysis showed disintegrated and loosened biofilm structures in GE-treated samples in contrast to the untreated control (Fig. 2b). COMSTAT analysis also evidenced the reduction of parameters, such as biofilm biomass and average thickness, and an increase in the surface-to-volume ratio upon GE treatment (Table 1). SEM analysis was also performed to elucidate the antibiofilm efficacy of GE, in which the control slide clearly showed multi-layered cells entrenched in EPS, while the GE-treated slides showed reduced EPS and meagre biofilm architecture (Fig. 2c).

![Fig. 1. Effect of GE on the inhibition of biofilm formation (MTP assay) and metabolic activity (XTT reduction assay) of S. epidermidis. Mean values of triplicate independent experiments and SD are shown. * indicates significance at P≤0.05.](image-url)
Effect of GE on EPS

EPS plays a significant role in maintaining the biofilm architecture. Exposure of *S. epidermidis* to GE displayed significant reduction in EPS production (Fig. 3). At MBIC, GE inhibited the production of EPS to the maximum of 69%. In addition to EPS quantification, the CLSM micrograph revealed the overall presence of Con A stain (green) in the overlay image of the control slide, whereas such an appearance was drastically reduced upon treatment with GE (Fig. 4).

MATH assay

The effect of GE at its MBIC on the CSH of *S. epidermidis* was assessed. In the MATH assay, GE-treated cells showed a significant difference in CSH production (Fig. 3).

FT-IR analysis

FT-IR analysis was performed to monitor the changes in the cellular components of *S. epidermidis* upon treatment with GE. Variation in the spectral region corresponding to the polycaccharide (1000–1200 cm⁻¹) and carboxylate group (1373–1405 cm⁻¹) in GE-treated cells highlights the impact of GE on the cellular components of *S. epidermidis* (Fig. 5).

Slime production

Similar to EPS, slime production is also an important trait associated with biofilm formation. Since GE inhibits biofilm formation of *S. epidermidis*, the ability of GE to interfere with slime production was determined using the CRA plate assay. The level of black colouration is directly proportional to the level of slime production. The outcome of the assay clearly showed the ability of GE to reduce the slime production of *S. epidermidis* compared to the untreated control (Fig. 6).

Mature biofilm disruption assay

Biofilms, once formed, are highly tolerant to stress and resistant to several antibiotics and host immune responses. Hence, the effect of GE on killing and/or eradicating the preformed biofilm was studied. The results of the biofilm biomass quantification assay clearly revealed that GE did not have the ability to dislodge the preformed *S. epidermidis* biofilms (Fig. 7b). In addition, the XTT reduction assay showed that GE at 2×MIC (1024 µg ml⁻¹) was potent enough to reduce the viability of biofilm-embedded *S. epidermidis* cells to 47% (Fig. 7b). Further, observation of live/dead analysis through CLSM validated the results of the XTT reduction assay and the mature biofilm disruption assay (Fig. 7a).

Habituation of *S. epidermidis* to GE neither induce resistance nor biofilm formation

To evaluate the development of adaptive resistance in *S. epidermidis*, *S. epidermidis* was subjected to sequential exposure to GE from subinhibitory to lethal concentrations. From the results, it is clear that *S. epidermidis* was not able to mount a resistance response during the continuous exposure to the sub-MICs of GE (Fig. 8a). At the same time, the exposure of the test pathogen to the sub-MICs of GE resulted in poor biofilm development (Fig. 8b).

DISCUSSION

Biofilm formation is an important non-specific resistance mechanism developed by microorganisms. Although the known antimicrobials are potent enough to target planktonic cells, the sessile bacterial cells in the form of biofilms with a self-produced EPS matrix resist the penetration of antimicrobials and avoid host immune responses. The inherent ability of pathogenic micro-organisms to form biofilms has serious clinical complications, which give rise to several chronic infections [5, 25]. As biofilm formation is considered to play a key role in *S. epidermidis* pathogenesis, it has been suggested that targeting biofilm formation would...
be one such effective treatment strategy to counteract the S. epidermidis biofilm-mediated problems faced in clinical settings [4]. The present study explores the potential of GE, an acyclic monoterpene alcohol present in diverse plant species, against S. epidermidis biofilm formation. The result of MIC determination demonstrates that GE possesses significant antibacterial activity against S. epidermidis, similar to the results reported earlier [26]. Hence, GE at its sub-MIC was evaluated against S. epidermidis biofilm formation. GE at the tested concentrations inhibited the biofilm biomass in a concentration-dependent manner. Since 200 µg ml⁻¹ of GE inhibited 85% biofilm biomass production, the same has been taken as MBIC. The XTT reduction assay revealed that GE acts as an ideal antibiofilm agent at its MBIC with no sign of negative effect on the metabolic activity of S. epidermidis, a property much expected for an anti-infective agent. In our study, visible reduction in microcolony formation and loosened biofilm architecture were apparent in the presence of GE at MBIC as observed through light, confocal and scanning microscopy. The results clearly indicated that the treatment of S. epidermidis with GE resulted in the formation of frail biofilms. Similar to these findings, the EO components, such as limonene and phytol, were reported to inhibit the biofilm formation and virulence factors’ production of the nosocomial pathogens Streptococcus pyogenes and Serratia marcescens, respectively [9, 27].

**Fig. 3.** Effect of GE at MBIC on S. epidermidis EPS and CSH production. Mean values of triplicate independent experiments and SD are shown. * indicates significance at $P \leq 0.05$.

**Fig. 4.** CLSM analysis of EPS production in S. epidermidis biofilm in the presence and absence of GE at MBIC. Con A-FITC stains polysaccharides. PI stains bacterial cells. Overlay image showing overall presence of Con A-FITC stain in the control slide compared to that of the GE-treated slide.
Biofilm maturation is another important step in the staphylococcal biofilm life cycle. Development of microcolonies into a mature biofilm results in persistent and recalcitrant infections. Thus, it is suggested that any agent interfering with the biofilm structure has great potential in the control and prevention of biofilm-mediated infections [28]. Hence, the efficiency of GE was tested by adding it to a preformed biofilm. However, no significant activity was observed at the tested concentrations. Therefore, the obtained results revealed that GE is active only at the initial stages of biofilm formation (attachment and early aggregation) but not on the mature biofilms. Since biofilms pose an inherent barrier to the penetrating solute particles [5], GE was also assessed for its penetrating ability on the preformed biofilm. The reduction in metabolic activity of biofilm cells as evident from the XTT reduction assay and live/dead staining validated the permeability potential of GE through the preformed biofilms of *S. epidermidis*. The results of the present study are in line with the previous report made with other *Staphylococcus* spp., wherein 2–4×MIC of *Melaleuca alternifolia* EO and its components reduced the metabolic activity of the cells to about 90% in 24 h–old biofilm [29]. Earlier, Adukwu *et al.* [30] and Kwiecinski *et al.* [31] reported similar activity where the grapefruit oil and tea tree oil have failed to eradicate the preformed biofilms of *S. aureus*. But in contrast to our results, both oils increase the metabolic activity of the *S. aureus* cells within the biofilm and it was suggested that it could be the result of stress response development.
The level of biofilm formation by a particular pathogen depends on the production of CSH and EPS. CSH mediates the initial adherence of the bacterium to a solid substratum, whereas the EPS ensures the bacterial existence by protecting it against the invading biocides and acts as an energy depository for biofilm inhabitants [32]. Based on the observations made in this study, it is inferred that GE reduces both the CSH and EPS production. To further confirm the modifications made by GE, CLSM and FT-IR analyses were performed. Results of CLSM analysis with Con A staining clearly portrayed that GE targets the *S. epidermidis* biofilm formation by means of inhibiting the EPS production. Further, FT-IR analysis resulted in the reduction of spectral region corresponding to the polysaccharide (1000–1200 cm\(^{-1}\)) and carboxylate group (1373 and 1405 cm\(^{-1}\)) in the GE-treated sample that could be attributed to the decrease in the EPS production in GE-treated cells. To substantiate the fact, the results of CLSM and SEM micrographs also showed reduction in EPS upon exposure to GE. Our results are in agreement with the results of Packiavathy et al. [33], wherein curcumin effectively inhibited the EPS production of *Vibrio* spp.

Slime plays an important role in the persistence of *S. epidermidis* infections. Younger et al. [34] reported the correlation between slime production and the persistence of clinically significant bacterial infection. Likewise, slime production in *S. epidermidis* has been shown to impede the cell-mediated immunity [35]. In the CRA plate assay, black colour formation with consistent dry crystalline colonies is a typical feature of slime-producing coagulase negative staphylococci and the same was observed in the *S. epidermidis* untreated control. In comparison to the control, *S. epidermidis* on the GE-supplemented plate showed a reduced level of colouration.

The development of adaptive resistance among bacterial pathogens is a global concern. Despite the availability of a number of reports on the use of GE against several clinical and food borne multi-drug-resistant pathogens [36, 37], the effect of GE on the pathogens during continuous exposure is still unclear. In this study, the continuous exposure of *S. epidermidis* to GE has been assessed and found that there is no development of resistance. In addition, continuous exposure to GE at sub-MIC resulted in poor biofilm development. Very few reports have attempted to study the susceptibility of bacterial strains following continuous exposure to EO components. In 2014, Apolónio et al. [24] reported the use of citral and eugenol as antistaphylococcal and antilisterial biofilm agents and further highlighted their efficacy with regard to the development of drug resistance. Similarly, McMahon et al. [38] in 2007 reported the use of tea tree oil as an antistaphylococcal agent but the adaptation of the test pathogens to tea tree oil resulted in increased resistance against the known antibiotics. The results of the present study might be attributed to the multi-targeted mode of action of GE towards the test bacterial pathogen.

In conclusion, the present work, for the first time, delineates the antibiofilm efficacy of GE against *S. epidermidis*. Significant reduction in the biofilm formation, EPS and slime production validated the efficacy of GE against *S. epidermidis*. 

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Fig. 7. Effect of GE on the preformed biofilm of *S. epidermidis*. (a) CLSM visualization of live/dead stain showing the effect of GE at tested concentrations on the viability of the biofilm cells. (b) Graph representing the efficacy of GE in eradicating the biofilm cells quantified by biofilm biomass quantification (MTP assay) and on the metabolic activity of biofilm cells (XTT reduction assay). GE at 2×MIC showed no statistically significant difference in terms of biofilm biomass quantification. But, it reduces the viability of the biofilm-embedded cells to a maximum of 47%.
In addition, results of CSH production, FT-IR analysis and the mature biofilm eradication assay suggested that GE plausibly targets the adherence of S. epidermidis, an initial step in biofilm development. Sequential exposure of S. epidermidis to the sub-MICs resulted in poor development of adaptive resistance to the compound with diminished biofilm formation. Thus, the findings of the present study revealed the potential of GE as a suitable candidate for the control and prevention of biofilm-mediated S. epidermidis infections.

Conflicts of interest
The authors declare that there are no conflicts of interest.

References


