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2-Hydroxy-4-methoxybenzaldehyde from *Hemidesmus indicus* is antagonistic to *Staphylococcus epidermidis* biofilm formation

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ABSTRACT

Staphylococcus epidermidis (SE) is an opportunistic nosocomial pathogen that accounts for recalcitrant device-related infections worldwide. Owing to the growing interest in plants and their secondary metabolites targeting bacterial adhesion, this study was intended to uncover the anti-biofilm potential of *Hemidesmus indicus* and its major constituent 2-hydroxy-4-methoxybenzaldehyde (HMB) against SE. The minimum biofilm inhibitory concentration (MBIC) of *H. indicus* root extract and HMB were found to be 500 and 250 $\mu g\ ml^{-1}$, respectively. The results of time-dependent biofilm inhibition and mature biofilm disruption assays confirmed that HMB targets initial cell adhesion. Furthermore, interference by HMB in the expression of adhesin genes (*icaA*, *aap* and *bhp*) and biofilm components was associated with an increased susceptibility of SE to oxidative stress and antibiotics. To conclude, this study reports for the first time HMB as a potential drug against SE biofilms.

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Introduction

The incidence of *Staphylococcus epidermidis* (SE) in device-related and surgical site infections has increased in prevalence, especially in persons with immune deficiency (Gomes et al. 2014). Unlike *S. aureus*, biofilm formation by SE is an important and sole virulence attribute in the onset and development of device-related infections in clinical settings. In SE infections, several factors govern the attachment and accumulation process during biofilm formation. The attachment or adhesion of SE to host surface proteins is facilitated by several cell wall associated proteins such as accumulation-associated protein (Aap), Bap homolog protein (Bhp), *S. epidermidis* surface (Ses) proteins, serine-aspartate repeats (Sdr) and autolysin (AltE) proteins (Bowden et al. 2005). Yet, the integrity of SE biofilms depends on the production of polysaccharide intercellular adhesins, which is controlled by *ica* operon-encoded enzymes, resulting in the formation of robust and multi-layered biofilms (Heilmann et al. 1996).

The available antibiotic therapies target only planktonic cells and leave the biofilm-embedded sessile cells unaffected. Biofilm protection allows the pathogen to cause long-lasting and recalcitrant infections because of its inherent resistance to antibiotics, chemotherapeutics and host immune factors (Reiter et al. 2014). In general, biofilm-mediated resistance to antibiotics worsens the treatment opportunity and contributes to a significant financial burden (Widerström 2016). Hence, the prevention of pathogens from forming biofilms and the disruption of already formed biofilms are therapeutic measures that allow the action of antibiotics or host immune factors to contain the bacterial infections and their severity (Kostakioti et al. 2013). Several approaches have been shown to inhibit bacterial biofilms as potential antivirulent therapy, eg small molecules, vaccines, nanoparticles, phage therapy, matrix degrading enzymes, sub-inhibitory

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concentration of antibiotics and bacterial interference (Darouiche and Hull 2012; Chung and Toh 2014). In spite of several strategies and approaches put forward to target biofilm formation, potential anti-biofilm agents from plant origin are still being sought. Plants are a rich source of structurally diverse bioactive compounds. The awareness of the value of plants in the field of medicine, in the past few decades, has led to a number of plants being screened and their metabolites investigated for their bioactive potential (Harvey 2008; Atanasov et al. 2015; Jamshidi-Kia et al. 2018).

Hemidesmus indicus belongs to the family Apocynaceae, and is commonly called Anantamul (Hindi) or Nannari (Tamil) in the local languages in India. The roots of this plant are used in folk medicine to treat several conditions, including blood diseases, diarrhea, skin diseases and kidney and urinary disorders (Rajan et al. 2011). Furthermore, the root extract has been reported for its antimicrobial, anti-inflammatory, hepatoprotective and antioxidant properties (Prabakan et al. 2000, Ravishankara et al. 2002). The relevance of *H. indicus* in the Indian system of medicine has stimulated research on the phytochemical and pharmacological properties of the plant and derived compounds in the past decades (Mishra et al. 2018; Turrini et al. 2019; Kannappan et al. 2019a). 2-Hydroxy-4-methoxybenzaldehyde (HMB) is an understudied dietary compound identified in *H. indicus*. As indicated in the guidelines for Botanical Drug Development issued by FDA in 2016, HMB can be used as the marker compound in *H. indicus* (FDA 2016). Earlier studies have shown that the presence of phenolic and aldehyde groups in HMB are responsible for its potent antimicrobial activity (Harohally et al. 2017). Due to its unique structural attributes and water-soluble nature, HMB has further attracted interest. The phenolic compound has been reported to have potent antioxidant activity and antiaflatoxigenic properties (Wang et al. 2010; Harohally et al. 2017). It already showed a potent inhibitory activity against *Helicobacter pylori* (Srikanta et al. 2011). Although several studies were performed with *H. indicus* root, none has investigated the efficacy of *H. indicus* and its bioactive molecule HMB in biofilm formation by nosocomial bacterial pathogens. Hence, the aim of this study was to evaluate the potential of *H. indicus* and HMB against biofilm formation by SE.

Materials and methods

Plant materials and extract preparation

The root of *H. indicus* was obtained from a local ayurvedic farm in Karaikudi, Tamil Nadu, India. The detailed procedure for extract preparation and compound identification has been described elsewhere (Kannappan et al. 2019a). The chopped root pieces were washed thoroughly with tap water, shade dried and milled to a fine powder. The soxhlet extraction method was followed to obtain the crude extract. For partial purification, 100 g of powdered *H. indicus* root was successively extracted with varying solvents ranging from non-polar (petroleum ether) to polar (methanol). Subsequently, the methanolic extract which exhibited anti-biofilm activity was filtered with No. 1 Whatman filter paper. The filtrates were dried with a rotatory vacuum concentrator (Christ, RVC 2–18) and stored at room temperature for further use. The residue was weighed and dissolved in methanol at a concentration of 100 mg ml^{-1} which was then used to assess its biofilm inhibitory potential against SE.

2-Hydroxy-4-methoxybenzaldehyde and solutions

Commercial 2-hydroxy-4-methoxybenzaldehyde (HMB) was purchased from Sigma-Aldrich, St Louis, MO, USA (catalog no. 160695). A stock solution of HMB (50 mg ml^{-1}) was prepared in methanol and stored at 4 °C in amber tubes. The working solutions of HMB were prepared by dilution of the stock solutions to the required concentrations in methanol.

Bacterial strain, growth conditions and experimental setup

Methicillin-resistant SE RP62A (ATCC 35984), a biofilm-producing strain, was used in the present study. The strain was obtained from the American Type Culture Collection (ATCC) and stored at −80 °C in a deep freezer with 20% glycerol ($v\ v^{-1}$). It was further maintained at 4 °C in trypticase soya agar (TSA) (HiMedia, Mumbai, India) and cultured in trypticase soya broth (TSB) at 37 °C. For the experimental assays, TSB supplemented with 1% sucrose was used to induce *in vitro* biofilm formation. To obtain a standardized cell suspension to be used as inoculum in the different studies, an overnight culture of SE was adjusted to 0.4 OD at 600 nm (1×10^8 CFU ml^{-1}). Methanol was used as a negative control in all the experiments.

Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) was determined by the double dilution method in Muller

Hinton broth (MHB, HiMedia). HMB was diluted in the wells of microtiter plates (MTP) containing MHB to obtain a final concentration of 32–1,024 μg ml^{-1}. A SE standard cell suspension (1% final concentration in medium) was used to inoculate the MTP containing varying concentrations of HMB. The MTP was incubated for 24 h at 37 °C. After incubation, the wells of the MTP were read at OD_{600nm} using a Multilabel Reader (Molecular Devices, SpectraMax M3, San Jose, CA, USA) (Clinical and Laboratory Standards Institute (CLSI) 2006). MIC was recorded as the lowest concentration of HMB that has an OD value similar to the blank.

Determination of minimal biofilm inhibitory concentration

Minimal biofilm inhibitory concentration (MBIC) was determined using the crystal violet staining method. Briefly, the standard cell suspension of SE was added to the wells of the MTP containing growth medium along with various concentrations of HMB and methanol extract of *H. indicus* (HI). The growth medium in the wells of MTP without HI or HMB was considered as the control. The assay setup was incubated undisturbed for 24 h at 37 °C. After incubation, the MTP was washed twice with sterile phosphate buffered saline (PBS, pH 7.4), in order to remove the planktonic cells. Subsequently, the plates were stained with 0.4% ($w\ v^{-1}$) crystal violet stain followed by washing with sterile PBS or distilled water to remove the non-adhered stain. The processed plates were further allowed to dry. The biofilm-bound crystal violet stain was dissolved with 1 ml of 20% of glacial acetic acid and read at 570 nm using a Multilabel Reader (Kannappan et al. 2017). The percentage biofilm inhibition was calculated as follows:

$$\%\ \text{Inhibition} = ((\text{Control OD}_{570\text{nm}} - \text{Test OD}_{570\text{nm}}) / \text{Control OD}_{570\text{nm}}) \times 100 \qquad (1)$$

The MBIC was determined as the lowest concentration of the test compound that showed the maximum percentage biofilm inhibition without any growth reduction.

In situ visualization of SE biofilms

The microscopic assays were performed according to Kannappan et al. (2017). In order to visualize the SE biofilm cells *in situ*, the bacterium was allowed to grow on MTP bearing 1 × 1 cm glass slides in the presence and absence of HI or HMB at the MBIC for 24 h at 37 °C. After incubation, the glass slides were washed with sterile PBS or distilled water to remove the non-adhered biofilm cells.

For light microscope analysis, the glass slides with biofilm were stained with 0.4% ($w\ v^{-1}$) of crystal violet stain. Then, the slides were examined under a light microscope at ×400 magnification (Nikon Eclipse Ti 100, Tokyo, Japan).

For confocal microscope analysis, the glass slides with biofilm were stained with 0.1% acridine orange stain. The stained glass slides were examined under a confocal laser scanning microscope (CLSM) (LSM 710, Carl Zeiss, Oberkochen, Germany).

For scanning electron microscope (SEM) analysis, the glass slides with biofilm were fixed using glutaraldehyde solution (2.5%) for 3 h in the dark, and subsequently dehydrated using increasing concentrations of ethanol (20, 40, 60, 80 and 100%) for 2 min each. The dehydrated slides were sputter coated with gold and examined under the SEM (VEGA 3 TESCAN, Brno-Kohoutovice, Czech Republic).

Growth curve analysis

A SE standard cell suspension (1% final concentration) was inoculated into 100 ml of growth medium with and without HMB at MBIC. After inoculation, the growth medium OD was read at 600 nm at 4 h time intervals (Packiavathy et al. 2014).

Effect of HMB on different stages of SE biofilm formation

To investigate the effects of HMB on SE biofilm formation, the standard cell suspension was inoculated into the wells of MTP, which were supplemented with HMB at MBIC at every 6 h and incubated at 37 °C for 24 h. After incubation for 24 h, the wells of MTP with biofilms were analyzed by the crystal violet staining method.

In another set of assays, the test pathogen was allowed to form biofilm on MTP at 37 °C for 24 h, and then exposed to HMB. After the initial 24 h incubation, the spent medium with non-adhered cells was removed and the wells were washed with sterile PBS or distilled water. Then, fresh growth medium with and without HMB at different concentrations (1×, 2×, 3×, 4× and 5× MBIC) was added to the wells and further incubated at 37 °C for 24 h. Following incubation, the remaining biofilm biomass was quantified as described by Kannappan et al. (2017).

Effect of HMB on the production of extracellular polymeric substances

The production of extracellular polymeric substances (EPS) was studied with SE biofilms grown in the presence and absence of HMB at MBIC. After harvesting, the cell-free culture supernatants and the cells were collected separately by centrifuging the cell suspension at 12,000 rpm. The collected cells were washed three times with sterile PBS or distilled water and suspended in isotonic buffer containing 10 mM Tris/HCl (pH 8.0), 10 mM EDTA and 2.5% NaCl. The cell-free culture supernatants were suspended in ice-cold ethanol (three times the volume) at 4 °C for 24 h in order to precipitate the cell-bound and cell-free EPS, respectively. Following incubation, the cell-bound and cell-free EPS were separated by centrifugation at 10,000 rpm for 10 min. At the end, the extracted cell-bound and cell-free EPS of the untreated control and the HMB treatment were pooled separately and dried under rotary vacuum evaporator (Christ Alpha 2–4 LD plus, SciQuip Ltd, Wem, UK) (Badireddy et al. 2008). The dried EPS were analyzed by FT-IR and carbohydrate assays as described below.

Equal volumes of dried EPS extracted from untreated control and HMB-treated SE, and potassium bromide (KBr) were mixed to prepare the KBr pellets. The prepared pellets with the untreated control and HMB-treated samples were then analyzed by FT-IR (Nicolet^{TM} iS5, Thermo Scientific, Madison, WI, USA). The IR spectra of SE the biofilms were recorded using the OMNIC software at a range of 4,000–400 cm^{-1} (Schmitt and Flemming 1998).

Fifty micrograms of dried EPS extracted from untreated control and HMB-treated test SE were mixed with equal volumes of 0.9% NaCl and 5% phenol solution. To this mixture, 5 volumes of H_2SO_4 were added and the mixture incubated in the dark for 1 h. Following incubation, the reaction mixture was centrifuged and the supernatant was read at 490 nm spectrophotometrically (Dubois et al. 1951).

Slime production is a qualitative estimation of EPS production and was assessed by the method described by Freeman et al. (1989) with small modifications. Briefly, Congo red agar (CRA) plates (TSB with 5% sucrose and 0.08% Congo red stain) were prepared in the presence and absence of HMB at the MBIC. To visualize the effect of HMB on SE slime production, the bacteria were streaked onto the HMB-free and HMB-supplemented plates. After incubation at 37 °C for 24 h, the plates were observed for the level of coloration. Slime production leads to dark black color colony formation in the biofilm positive samples, whereas a reduction in slime production leads to a reduction in the level of coloration of the colonies to Bordeaux red or red.

Staining of extracellular polymeric substances

SE was allowed to grow on 1 × 1 cm glass slides in the presence and absence of HMB at MBIC for 24 h at 37 °C. After incubation, the glass slides were washed with sterile PBS or distilled water to remove the non-adherent cells. Afterwards, the washed slides were stained with 15 µM propidium iodide (PI) (Molecular Probes, Eugene, OR, USA) for 15 min at room temperature. After 15 min of incubation, the slides were again stained with 50 µg ml^{-1} of Concanavalin A (Con A) conjugated to FITC (Con A-FITC, Sigma-Aldrich) for 15 min. After completely drying, the stained cells were observed under CLSM with excitation and emission wavelengths for PI 568 and 605 nm and for Con A-FITC 488 and 522 nm, respectively (Zeiss LSM Image Examiner, version 4.2.0.121) (Banas et al. 2001).

Effect of HMB on catalase activity in SE

The untreated control and HMB-treated cells were cultured as described above. For the H_2O_2 growth inhibition assay, the cells were swabbed onto TSA plates. The swabbed plates were placed with sterile disks containing 30 µl of 20% H_2O_2 at the center. After incubation at 37 °C for 24 h, the plates were observed for a zone of inhibition (Ramanathan et al. 2018a).

In the tube assay, equal volumes of HMB-treated or untreated SE culture were added to 1% Triton X-100 and 20% H_2O_2. This was mixed well and incubated at room temperature for 15 min. After incubation, the level of oxygen formation was visually compared between the control and treated samples (Savijoki et al. 2014).

Antibiotic penetration through SE biofilms

The protocol of Singh et al. (2010) was followed to assess the penetration of antibiotics through SE biofilms. Fifteen microlitres of standard cell suspensions of SE were placed on a polycarbonate membrane (13 mm diameter; 0.4 mm pore size; Merck Millipore Ltd, Mumbai, India) in TSA supplemented with and without HMB at MBIC along with 1% sucrose and incubated in an upturned position for 24 h at 37 °C.

Table 1. List of primers used for quantitative polymerase chain reaction (qPCR) analysis of biofilm-related genes in *S. epidermidis*.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
aap	GGGCAAACGTAGACAAGGTC	GCTTTCGCTTCATGGCTACT
icaA	TTGATGACGATGCGCCTTTT	CTGCAAGAGATTGACTTCGCT
altE	ATAGAAACGGTGTGGGACGT	ACCTGCACCCCAAGATAAGT
bhp	TGATGACAACGCAACGACAA	TGGTGTTGGACTCGTAGCTT
rplU	TTGTAGGTGGCGACTCAGTT	ATGGTTGACGATGGCCTTTT

After 24 h of incubation, the polycarbonate membrane bearing biofilm cells was transferred to fresh TSA plates swabbed with SE (McFarland standard 0.5). Above the biofilm, a 6-mm nitrocellulose membrane was placed along with an antibiotic disk (rifamycin, 5 µg and tetracycline, 30 µg, vancomycin, 30 µg and amikacin, 30 µg), which was pre-moistened with 20 µl of sterile distilled water. Next, the plates were again incubated for 24 h at 37 °C in order to examine the zone of inhibition.

RNA extraction, cDNA conversion and real-time PCR analysis

The total RNA was isolated from SE treated with and without HMB at MBIC using the hot phenol method with some modifications (Jahn et al. 2008). The isolated RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Foster, CA, USA). The qPCR reactions were carried out as per the manufacturer's instructions using Power SYBR® Green PCR master mix and 7500 Sequence Detection System (Applied Biosystems Inc.). The dataset was normalized with the reference (rplU, ribosomal protein) gene of SE. The relative expression of the target genes was quantified using gene-specific primers listed in Table 1 with the help of the standard formula by Yuan et al. (2006).

Data analysis

All the experiments were carried out in triplicate in three independent experiments, and the statistical analyses were performed using SPSS package (SPSS v20.0; SPSS, Armonk, NY, USA). The results are expressed as means ± standard deviation (SD). Dunnett-ANOVA and the Student's *t*-tests were used to compare the test assays and the controls. * indicates significance at $p \leq 0.05$.

Results

Anti-biofilm activity of HI

The anti-biofilm activity of HI was assessed against SE formed in the MTP. SE grown in the presence of HI (100 to 600 µg ml^{-1}) showed a concentration-dependent reduction in biofilm formation. Spectrophotometric quantification of biofilm formation by SE revealed an 80% reduction at 500 µg ml^{-1} of HI (Figure 1A). No significant activity was observed upon increasing the concentration of HI, and hence, 500 µg ml^{-1} was fixed as MBIC for HI. In addition, the anti-biofilm activity of HI was confirmed using microscopic analyses (Figure 1C).

Determination of MIC and MBIC for HMB

The MIC of HMB against SE was found to be 1,024 µg ml^{-1} (Figure S2 in the online supplemental material). The MBIC of HMB was determined by the crystal violet staining method. Since HMB caused growth reduction at the concentration of 512 µg ml^{-1} (Figure S1), HMB was assessed in SE biofilm formation under concentrations ranging from 50 to 300 µg ml^{-1}. HMB was found to cause 85% inhibition at 250 µg ml^{-1} (Figure 2A). No significant difference was observed between HMB at 250 and 300 µg ml^{-1}. Hence, 250 µg ml^{-1} of HMB was fixed as the MBIC against SE.

Non-lethal effect of HMB on SE growth

To confirm the non-lethality of HMB at MBIC (250 µg ml^{-1}), the growth of SE was monitored in the presence of the anti-biofilm agent. Even after incubation for 24 h, SE supplemented with HMB did not show any significant differences in the growth pattern compared with the control (Figure 2B), indicating a non-lethal effect of HMB on SE growth at the concentrations tested.

Microscopic visualization of SE biofilms

The non-lethal effect on bacterial growth and a promising impact on the development of biofilm architecture determine the efficacy of an anti-biofilm agent. To assess the impact of HMB on SE biofilm architecture, biofilms developed on glass slides exposed and unexposed to HMB at MBIC were analyzed by SEM. As shown in Figure 3, untreated slides displayed a thick lawn of multi-layered biofilm cells embedded in

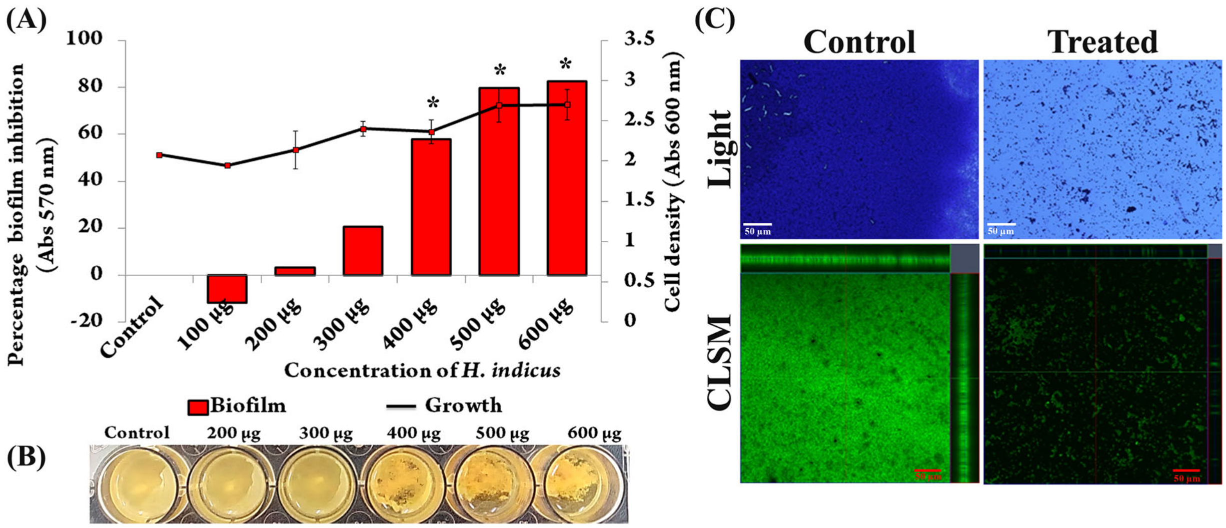

Figure 1. Effect of *H. indicus* on SE biofilm formation. (A) HI shows concentration-dependent biofilm inhibitory activity with 500 µg ml^{-1} as its MBIC; (B) photograph of culture wells showing the aggregation of SE in response to increasing concentrations of HI; (C) microscopic observation of biofilm formed by SE in the presence and absence of HI. The biofilm was stained with crystal violet (upper images) or acridine orange (lower images). The microscopic observations confirm the results of the biofilm biomass quantification assay. Scale bars = 50 µm. Mean values of triplicate independent experiments and SD are shown. * indicates significance at $p \leq 0.05$.

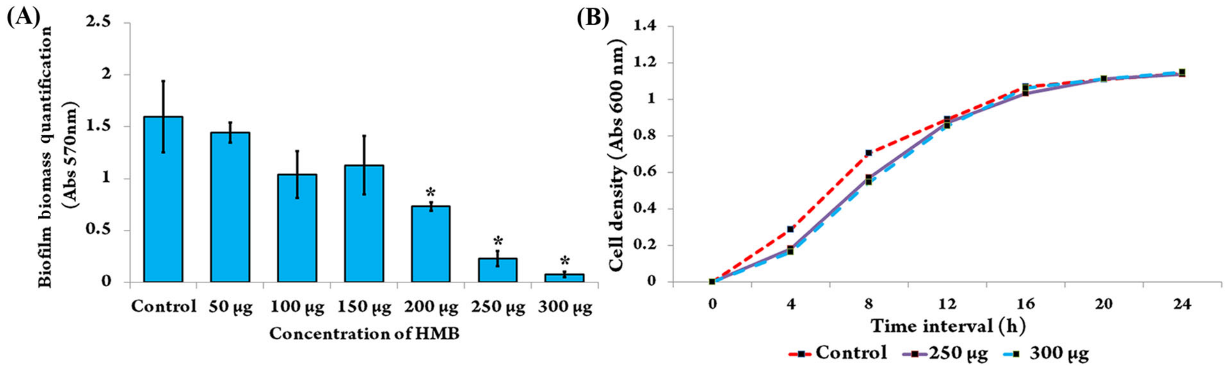

Figure 2. (A) Inhibition of SE biofilm formation by HMB at increasing concentrations ranging from 50 to 300 µg ml^{-1}. HMB concentrations 250 and 300 µg ml^{-1} inhibited almost completely SE biofilm formation. (B) Spectroscopic readings of SE growth in the presence (250 and 300 µg ml^{-1}) and absence of HMB. The concentrations tested showed no significant differences in growth, or in biofilm inhibition compared with control conditions, so the lowest concentration of 250 µg ml^{-1} was fixed as MBIC. Mean values of triplicate independent experiments and SD are shown. * indicates significance at $p \leq 0.05$.

a dense EPS matrix, whereas HMB-treated slides showed a meagre amount of SE cells on the substratum.

Modification of EPS production by HMB

The IR spectra of EPS extracted from SE indicated the presence of fatty acids (3,050–2,750 cm^{-1}), proteins and peptides (1,700–1,500 cm^{-1}) and polysaccharides and nucleic acids (1,300–900 cm^{-1}) (Figure 4A). Compared with the EPS extracted from untreated SE, the attenuation in the absorbance intensity indicated the impact of HMB on EPS components. The absorbance in the aforementioned regions was relatively high in the untreated SE samples. The reduced absorbance in the HMB-treated EPS samples indicates a decrease in the production of EPS. Furthermore, the spectroscopic quantification of EPS polysaccharides revealed a significant drop from the treatment with HMB at MBIC (Figure 4B).

Additionally, the production of EPS was assessed qualitatively by CLSM analysis. Con A stain (green fluorescence) specifically binds to the polysaccharides present in the EPS, whereas PI (red fluorescence) stains the cells. The CLSM micrograph portrayed the overall presence of Con A stain on the control slide, whereas this was substantially reduced upon treatment with HMB (Figure 5).

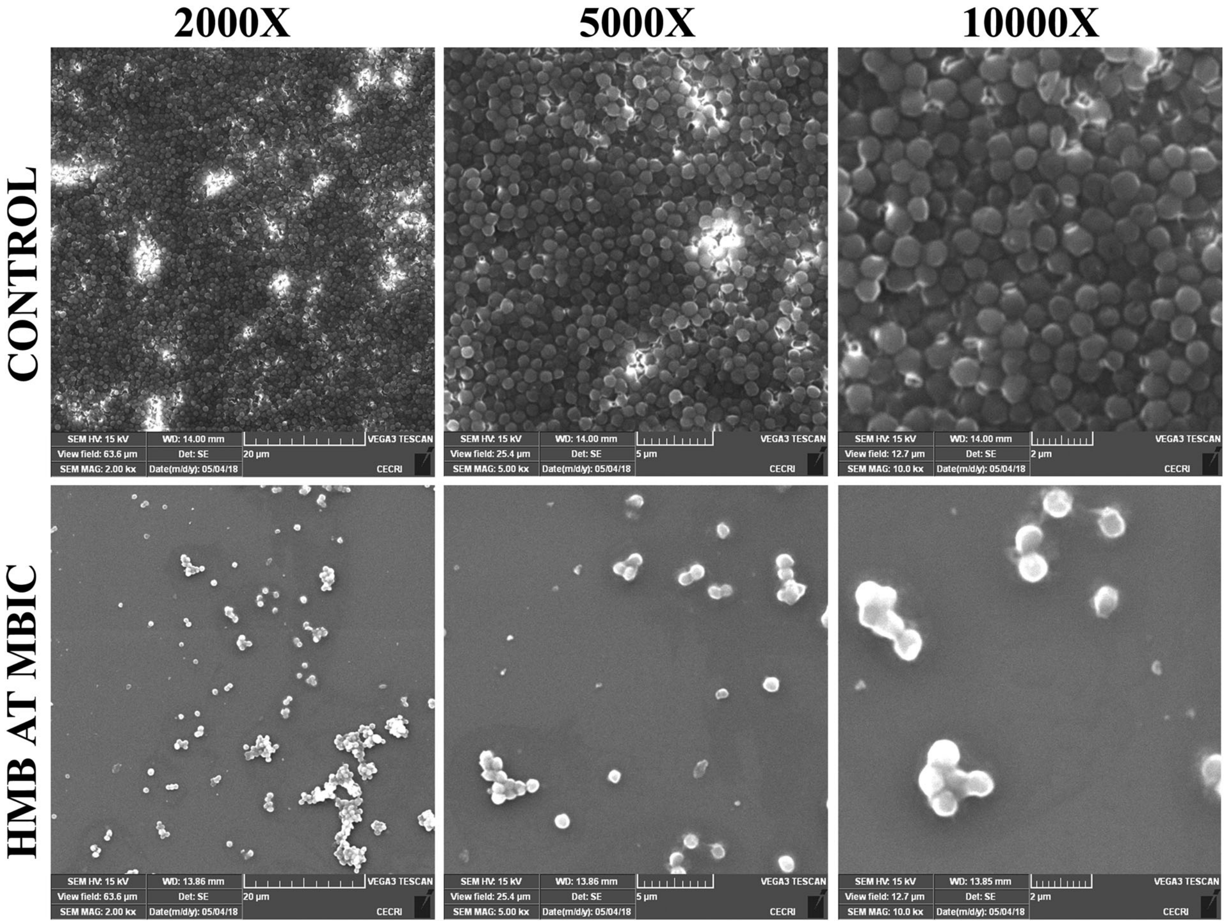

Figure 3. SEM analysis of SE biofilm in the presence and the absence of HMB at MBIC. Control slides showed the ability of SE to form multi-layered biofilm cells, embedded in the EPS matrix. In contrast to the control, this biofilm forming ability was severely reduced upon HMB treatment.

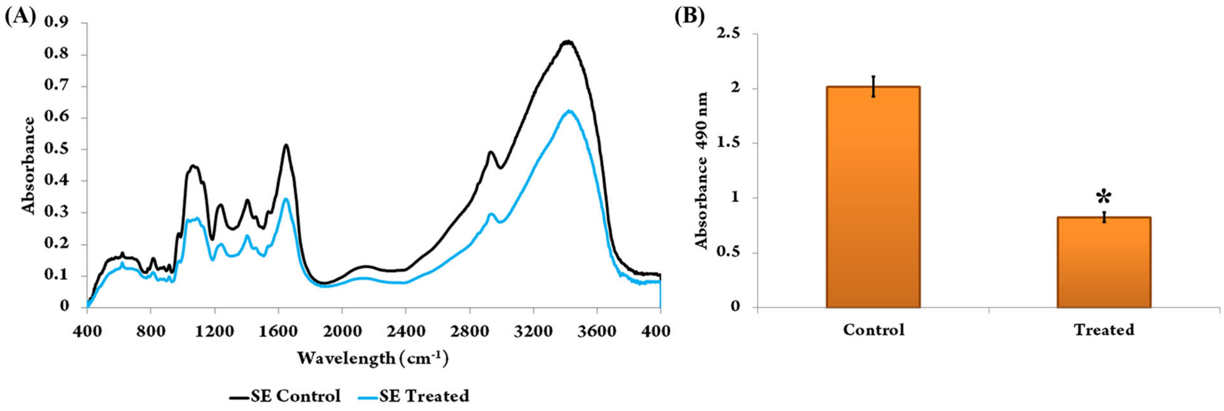

Figure 4. (A) FT-IR analysis of EPS extracted from the SE cells treated with and without HMB at MBIC. The regions corresponding to the polysaccharide and protein components showed a reduced absorbance upon HMB treatment. (B) Inhibitory effect of HMB at MBIC on the EPS component (polysaccharides) of the SE biofilm. Mean values of triplicate independent experiments and SD are shown. * indicates significance at $p \leq 0.05$.

The ability of HMB to inhibit EPS production by SE was further tested with cultures in CRA medium (Figure S2). In the CRA medium, black colonies of SE were evident on the HMB-free agar plates (control). In the case of SE streaked on HMB-supplemented agar plates (treated), the colonies partially failed to turn into the classical black formation.

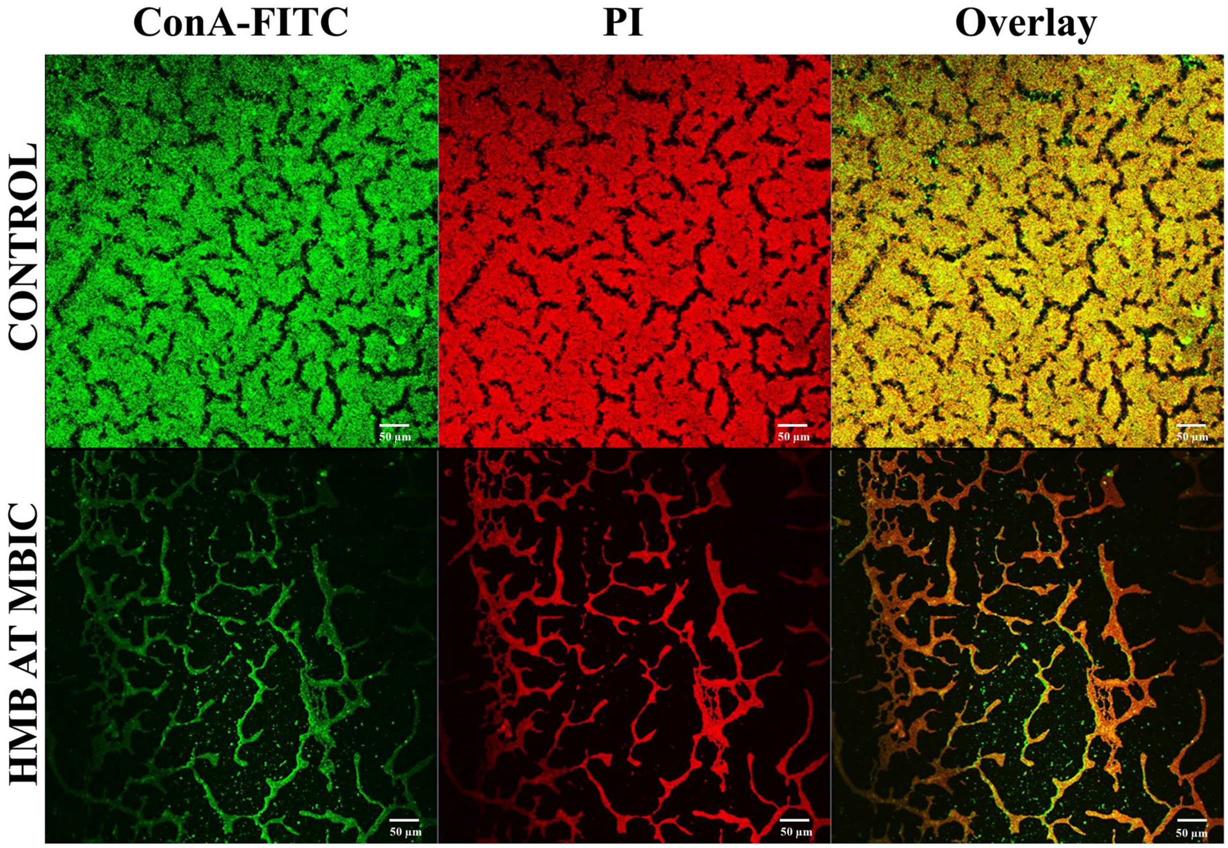

Figure 5. CLSM analysis of EPS production by SE biofilm in the presence and absence of HMB at MBIC. This assay utilizes two stains (ConA-FITC and PI). Con A-FITC stains polysaccharides present in the biofilm cells, whereas PI stains bacterial cells. The overall presence of Con A-FITC stain in the control slide is greatly attenuated in the HMB-treated slide. Scale = 50 µm.

HMB targets the initial adhesion of SE cells when forming biofilms and not preformed biofilms

In order to determine the role of HMB in SE biofilm development, HMB (at MBIC) was added at different times during the biofilm formation process. The results showed that the addition of HMB at 0 h impaired biofilm formation, but when added 6 h or more later no significant impact on biofilm formation was observed (Figure 6B). From the results, it is obvious that HMB targets the initial adhesion of SE and not the preformed biofilms. Further tests were performed to check the effects of HMB on the preformed biofilms (24 h old) under higher concentrations (1×, 2×, 3×, 4× and 5× MBIC). The results again indicated that HMB was unable to eradicate the biofilms, regardless of the concentration used (Figure 6A).

Effect of HMB on SE susceptibility to H_2O_2

The ability of HMB to inhibit cellular catalase was assessed by H_2O_2 disk diffusion and test tube methods (Figure S3). The SE cells treated with HMB were more susceptible to H_2O_2 compared with the untreated SE cells. The H_2O_2 disk in a plate free of HMB inhibited SE growth to a level of 54 mm in diameter, whereas in the plate supplemented with HMB, inhibition reached 61 mm (Figure S3A). The increased diameter in the zone of inhibition is inversely proportional to the level of cell antioxidant protection by catalase.

Furthermore, to confirm the reduced catalase activity resulting from HMB treatment, a tube assay was performed. The tube assay further demonstrated a reduced production of oxygen bubbles from HMB exposure (Figure S3B).

Antibiotic penetration through SE biofilms

The ability of antibiotics in combination with HMB to penetrate and kill the biofilm-embedded cells was analyzed using a biofilm penetration assay. The penetration of tetracycline (inhibition zone of 24 mm in diameter), rifamycin (21 mm in diameter) and amikacin (12 mm in diameter) was retarded in SE control biofilms in comparison with HMB exposed biofilms (Figure 7). The SE biofilms exposed to HMB were more susceptible to tetracycline (29 mm in diameter) than to rifamycin (26 mm in diameter) and amikacin

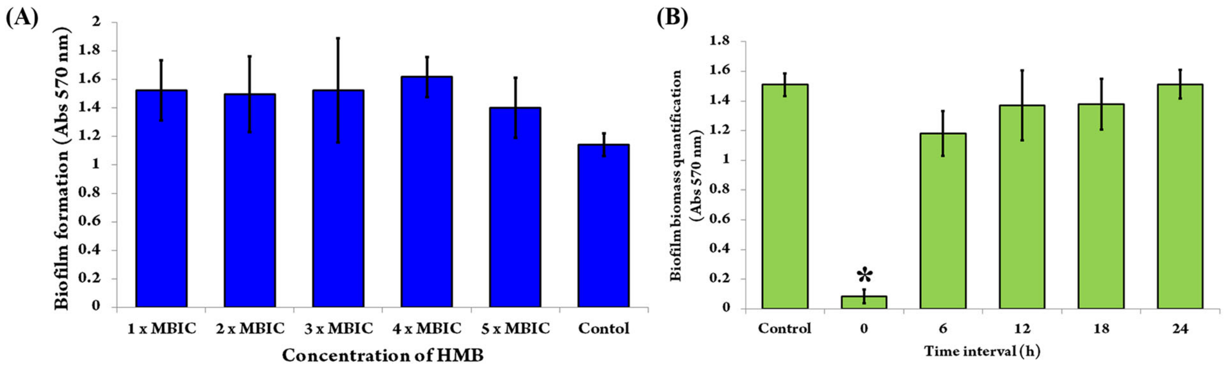

Figure 6. (A) Effect of different HMB concentrations on preformed SE biofilms. HMB failed to eradicate the preformed biofilms of SE at the concentrations tested (0.25, 0.5, 0.75, 1 and 1.25 mg ml^{-1}). (B) Effect of time of HMB addition on SE biofilm formation. HMB at MBIC was added to 24-well MTPs at various time points from 0 to 24 h during incubation. Biofilm quantification by the crystal violet method indicated the biofilm inhibitory capacity of HMB decreased with the maturation stage of the biofilm. Mean values of triplicate independent experiments and SD are shown. * indicates significance at $p \leq 0.05$.

(18 mm). The glycopeptide antibiotic vancomycin showed low potency against SE biofilm cells, alone and combined with HMB.

Analysis of gene expression

Quantitative PCR analysis was carried out to study the influence of HMB on the expression of SE genes associated with biofilm formation (Figure 8). The expression patterns of different genes were compared and it was found that HMB treatment significantly down-regulated the expression of the genes responsible for biofilm maturity and integrity (*icaA*) and intercellular accumulation (*aap* and *bhp*), and up-regulated the expression of the gene responsible for primary attachment *altE*.

Discussion

The emergence of multidrug resistance among clinical pathogens requires novel antimicrobial and antifouling agents that cannot be surpassed by those pathogens. Evidence for plant-derived molecules as novel antimicrobials against multidrug-resistant clinical pathogens is increasing (Packiavathy et al. 2014). In this context, the results obtained in earlier work on HI against biofilm formation of *S. aureus* and *Streptococcus pyogenes* prompted investigation of its biofilm inhibitory potential against SE RP62A (Kannappan et al. 2019a). The GC-MS analysis of successive methanol extracts of HI showed the presence of HMB as one of the prominent components (Figure S4). The authors' group also recently reported that HMB mitigated the expression of virulence factors by *S. aureus* in a *sigB* dependent manner (Kannappan et al. 2019b). Although several medicinal properties such as the antimicrobial, anti-inflammation and antioxidant properties of HI and HMB have been studied, their ability to prevent the biofilm formation by a pathogenic organism as SE has not been studied previously (Prabakan et al. 2000; Ravishankara et al. 2002). Hence, the present study was intended to assess the potential of HI and its bioactive lead HMB in biofilm formation by SE.

HI was found to impair SE biofilm formation without any growth inhibitory effect. Although HI did not target the growth of SE, a report by Saritha et al. (2015) revealed the antibacterial potential and the mode of action of the ethanol extract of HI against the uropathogen *Escherichia coli*. In this study, the HI-derived phytochemical HMB showed anti-biofilm activity against SE in a concentration-dependent manner. The MBIC of HMB was found to be 250 µg ml^{-1}, and this concentration was further used for all the subsequent assays.

The microscopic analysis revealed that SE forms a multi-layered thick lawn of biofilm cells embedded in an EPS matrix. HMB exposure showed poor biofilm development with large surface areas left unoccupied. Similar to these findings, other phytochemical compounds isolated from Indian indigenous medicinal plant extracts have been reported to show anti-biofilm potential against diverse multidrug resistant bacterial pathogens. For example, curcumin from *Curcuma longa* at 100 µg ml^{-1} inhibited biofilm formation by the uropathogens *E. coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Serratia marcescens* by 52, 89, 52 and 76%, respectively (Packiavathy et al. 2014); phytol from *Piper betle* at 10 µg ml^{-1} inhibited biofilm formation by *S. marcescens* to a level of 92%

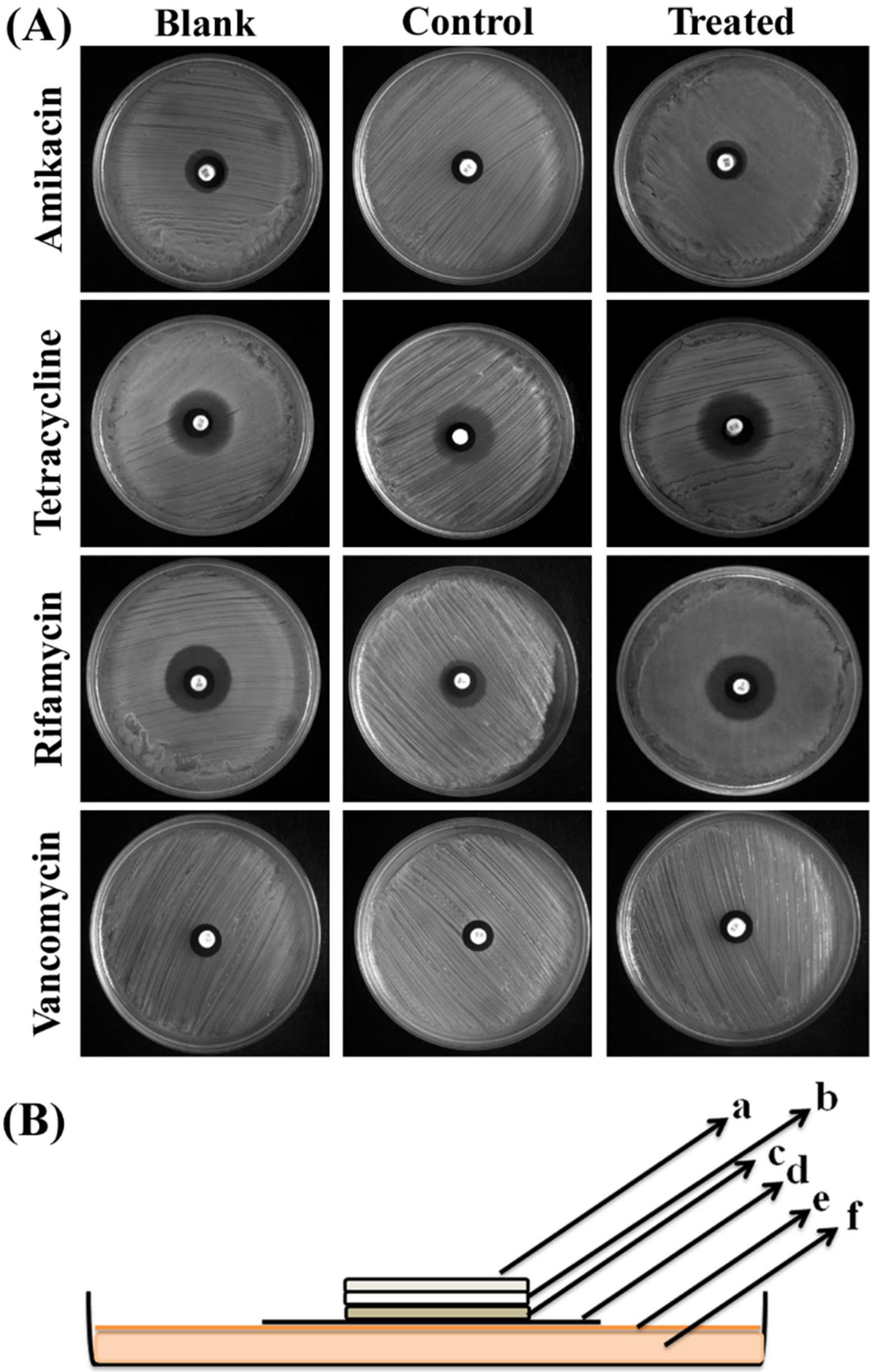

Figure 7. Antibiotic penetration assay. (A) Illustrative plates of SE growth inhibition by antibiotics after penetration through a blank (membrane without biofilm), a control (untreated) and treated biofilms (exposed to HMB). (B) Schematic representation of the experimental setup used to analyze the antibiotic penetration through the biofilms, showing a, 6-mm antibiotic disks; b, 6-mm nitrocellulose membrane; c, SE biofilms grown on black polycarbonate membrane; d, 13-mm black polycarbonate membrane; e, SE culture swabbed in TSA; and f, TSA medium.

(Srinivasan et al. 2016); isosteviol from *Pittosporum tetraspermum* at 100 µg ml^{-1} inhibited biofilm formation by *E. coli* and *P. aeruginosa* to a level of 80% (Al-Dhabi et al. 2015).

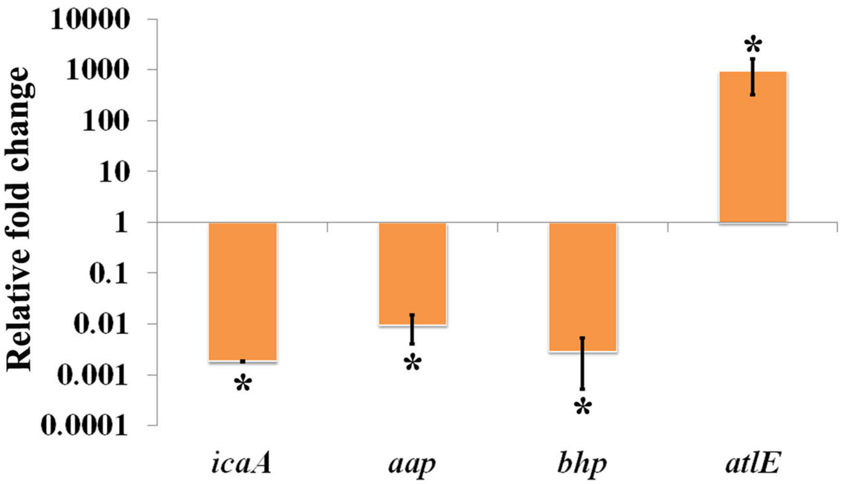

Figure 8. Effect of HMB at MBIC on the relative expression of the genes involved in biofilm formation by SE. Expression was normalized with the ribosomal protein *rplU* gene as reference. Mean values of triplicate independent experiments and SD are shown. * indicates significance at $p \leq 0.05$.

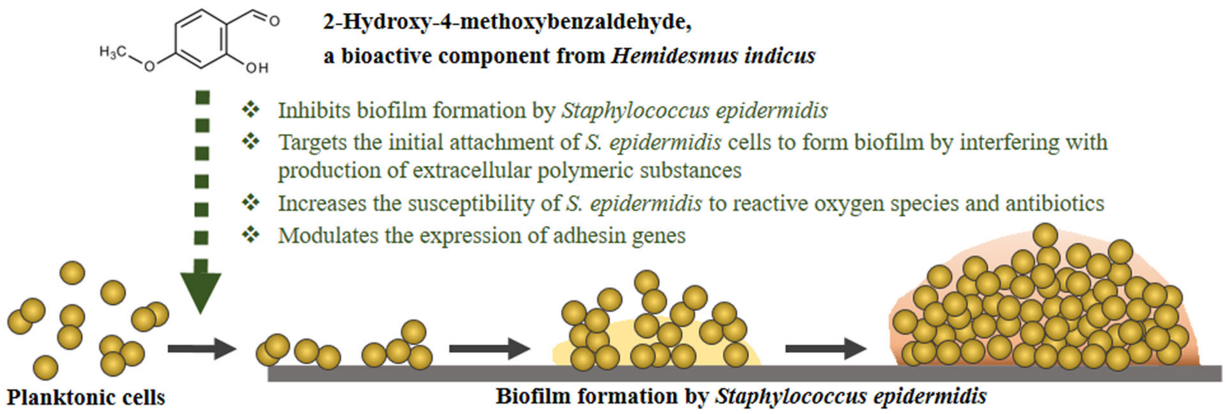

Figure 9. Proposed mechanism for the action of 2-hydroxy-4-methoxybenzaldehyde in SE biofilm formation.

EPS maintains the structural integrity and complexity of a biofilm, playing a critical role in biofilm formation and behavior. EPS is composed of proteins, polysaccharides, nucleic acids and lipids. The level of EPS production by a particular pathogen determines its biofilm forming ability (Brown et al. 2019). Therefore, inhibiting EPS production will lead to inhibition of biofilm formation and thereby render bacterial cells more susceptible to antimicrobials and the host immune system. The results of spectrophotometric and FT-IR analysis of EPS, as well as Con A staining, substantiated the reduction in EPS synthesis upon treatment with HMB. In line with these results, plant derivatives have been consistently reported to inhibit EPS production by other nosocomial pathogens, eg petroselinic acid against *S. marcescens* (Ramanathan et al. 2018b) and α-mangostin against *Acinetobacter baumannii* (Sivaranjani et al. 2018). Other authors have investigated the biofilm forming ability of *Staphylococcus* species using the CRA method (Vasudevan et al. 2003; Sethupathy et al. 2017). In HMB-free CRA plates, growth of SE appeared as black colonies. Indeed, the level of black coloration was reduced to a Bordeaux red color in the slime-producing SE colonies grown in plates supplemented with HMB at MBIC. Previously, sub-MIC of vancomycin was reported to act as a stress factor that initiates cell wall thickening and induces the expression of biofilm-related genes in SE, which ultimately leads to the change in the colony color in CRA plates (Cargill and Upton 2009; Kaiser et al. 2013). In contrast, the present results from the CRA plate assay confirmed that HMB treatment reduced slime production by SE. These results suggest that HMB has the potential to prevent SE biofilm development and may enable the use of HMB to manage SE biofilm-related infections.

Planktonic cells undergo several physiological processes in the biofilm formation process. The first and most important step is the initial adhesion on a surface. This initial step is achieved by both the cell-to-cell interaction and the cell-to-surface or host protein interaction (Kostakioti et al. 2013). Preformed biofilms, by contrast, are a serious problem in bacterial pathogenesis, and are responsible for durable and recalcitrant infections. Unlike planktonic cells, biofilms resist the action of antimicrobials, disinfectants and control measures (Roy et al. 2018). Thus, it is essential that an anti-biofilm agent should not target only the initial cell adhesion but also preformed biofilms. In the present study, biofilm formation by SE was inhibited only when HMB was supplemented at 0 h, after which the compound became ineffective, even at concentrations five-fold higher than the MBIC. Brown et al. (2019) showed that EPS is deposited in the initial stages of microcolony development by *P. aeruginosa* and *S. aureus*. Therefore, based on EPS production and mature biofilm disruption data, it is proposed that HMB interferes with the initial stages of SE biofilm formation.

To prevent damage from reactive oxygen species and host neutrophil attack, aerobic microbes produce antioxidant enzymes such as catalase and superoxide dismutase (Leejae et al. 2013). HMB treatment enhanced the susceptibility of SE to reactive oxygen species, namely to H_2O_2, and enhanced the penetrating and killing ability of antibiotics through biofilms. Recent reports have shown that anti-biofilm phytochemical compounds have the ability to disperse both the initial and preformed biofilm cells (Bai et al. 2019; Zhang et al. 2019), without considering the ability to enhance the activity of reactive oxygen species or antibiotics. Moreover, it has been reported that the protective ability of SE slime was higher against vancomycin and teicoplanin (glycopeptide antibiotics) than against amikacin (Souli and Giamarellou 1998, Mathur et al. 2005). In addition, other works also reported that the susceptibility and penetration of antibiotics such as rifamycin and tetracycline remained unaffected in the presence of SE slime (Sivaranjani et al. 2017). A study by Rogers et al. (2010) stated that a reduction in EPS production by any particular pathogen augments the efficacy of antibiotics. In the present study, HMB interfered with EPS and slime production. Hence, the ability of HMB to increase the susceptibility and penetration of antibiotics such as vancomycin, amikacin, tetracycline and rifamycin through biofilms of SE was assessed. Among the antibiotics tested, vancomycin failed to penetrate the biofilms of SE. That result corroborates previous works, where vancomycin neither killed nor eradicated preformed biofilms even at 64 times the MIC (Qin et al. 2007; Sivaranjani et al. 2017). However, the susceptibility and penetration of other antibiotics, such as amikacin, tetracycline and rifamycin, increased from the treatment with HMB. Therefore, HMB, which was not able to eradicate the preformed SE biofilms, demonstrated a high potential for managing bacterial infection by aiding the actions of reactive oxygen species and antibiotics.

The genetic mechanism regulating biofilm formation depends on several factors such as quorum sensing, environmental factors, and other global regulators (Boles and Horswill 2011). Regulation of *ica* during biofilm formation in SE is a well-known mechanism (O'Gara 2007). The observation of *icaA* gene down-regulation by HMB in the present study also supports these earlier findings and the anti-biofilm activity of the compound. Similar to *icaA*, other genes such as *aap* (accumulation associated protein) and *bhp* (Bap homolog protein), which are responsible to mediate the intercellular accumulation, were also down-regulated. In contrast, the addition of HMB up-regulated the expression of cell wall protein AtlE (autolysin). The mechanism behind the up-regulation of *atlE* is not clear.

To conclude, this work presents HMB from HI as a modulator of SE biofilm formation without targeting bacterial growth (Figure 9). HMB inhibited significantly biofilm-related phenotypes such as EPS formation. The result of time-dependent and mature biofilm eradication assays revealed that HMB essentially targets SE adhesion. Notably, inhibition of biofilm-related phenotypes and down-regulation of adhesion-related genes by HMB seems to augment the susceptibility of SE to reactive oxygen species and antibiotics. Although these findings suggest that HMB is a suitable candidate for treating the onset of SE infection, translational and clinical studies are needed to unravel the molecular mechanism and therapeutic efficacy of HMB in treating biofilm-mediated infections.

Disclosure statement

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