

# In vitro activity of alpha-mangostin in killing and eradicating *Staphylococcus epidermidis* RP62A biofilms

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**Abstract** Alpha-mangostin ( $\alpha$ -MG) has been reported to be an effective antibacterial agent against planktonic cells of many Gram-positive bacteria. However, the antibiofilm potency of  $\alpha$ -MG remains unexplored till date. In this study, the antibiofilm and mature biofilm eradication ability of  $\alpha$ -MG against *Staphylococcus epidermidis* RP62A (ATCC 35984) biofilms were evaluated. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of  $\alpha$ -MG against *S. epidermidis* RP62A were found to be 1.25 and 5  $\mu$ g/mL, respectively.  $\alpha$ -MG exhibited a phenomenal concentration dependent rapid bactericidal activity ( $>4$ -log reduction within 5 min). In a multi-passage resistance analysis using *S. epidermidis*, no development of resistance to  $\alpha$ -MG as well as antibiotics was observed in its habituation.  $\alpha$ -MG at its 1/2 MIC effectively inhibited the initial biofilm formation of *S. epidermidis*, which was further confirmed through scanning electron microscopic (SEM) analysis that portrayed a lucid reduction in the aggregation and the spread of biofilm. The crystal violet staining and viable cell quantification results confirmed the eradication of preformed immature and mature biofilms of *S. epidermidis* by  $\alpha$ -MG in a concentration dependent manner. Besides, the biofilm eradication ability was also confirmed through SEM and live/dead BacLight staining using confocal laser scanning microscopy (CLSM). Thus, the present study exemplifies that  $\alpha$ -MG

could plausibly assist to eliminate biofilm infections associated with multidrug-resistance staphylococci.

**Keywords** *Staphylococcus epidermidis* · Alpha-mangostin · Biofilm · Resistance · Bactericidal

## Introduction

*Staphylococcus epidermidis* beyond its local homeostasis characteristics on human skin as a commensal flora, it has been well recognized for the most frequent cause of health care-associated bloodstream infections and biomaterial-associated infections (Otto 2012; Ziebuhr et al. 2006). *S. epidermidis* are the utmost notable member of coagulase-negative staphylococcal group (CoNS), as nearly 80% of CoNS isolates were *S. epidermidis*. It is ranked first and third in causing native valve infective endocarditis (NVIE) and prosthetic valve infective endocarditis (PVIE), respectively. Despite its lesser virulence, it has been well adapted to colonize and establish highly structured, multilayered robust biofilms on any implanted biomaterial surfaces such as vascular catheters, prosthetic joints, and artificial heart valves (Qin et al. 2007).

In *S. epidermidis*, biofilm formation is the most imperative virulence trait making this commensal inhabitant as hazardous to implanted medical device usage. In the intricate architecture of biofilm, bacterial cells encase themselves and escape from host defense mechanisms as well as antimicrobial agents, which eventually leads to chronic and refractory infections (Lefebvre et al. 2016; Nilsson et al. 2016; Falsetta et al. 2012). Besides, *S. epidermidis* infections associated with biofilms persist until the removal or subsequent replacement of implantable medical devices, resulting in further distress and cost (Donlan and Costerton 2002). Furthermore, this

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primary pathogenic trait—the biofilm formation has also been envisaged for the increased emergence of antibiotic resistance in several strains of clinical settings.

In the prevailing antimicrobial therapy, *S. epidermidis* in particular methicillin-resistant *S. epidermidis* (MRSE) are being treated with the last resort of antibiotics viz., vancomycin, daptomycin, and linezolid, which affect the planktonic cells rather than targeting the sessile cells of biofilm architecture (Roper et al. 2000; Parra-Ruiz et al. 2012; Reiter et al. 2014). This has hastened and widened the quest for the discovery of novel antimicrobial agents from natural sources with proficiency to penetrate biofilm and target sessile cells of such recalcitrant biofilm-forming dreadful pathogen.

Alpha-mangostin ( $\alpha$ -MG), a natural xanthanoid found predominantly as secondary metabolite in pericarps of Mangosteen (*Garcinia mangostana* Linn) also referred as “queen of fruits” (Ibrahim et al. 2016). Hitherto, plethora of reports on  $\alpha$ -MG have well demonstrated its broad spectrum pharmaceutical significance including anti-inflammatory, anti-tumor, cardioprotective, anti-diabetic, antibacterial, antifungal, antiparasitic, antioxidant, antiobesity and antibacterial activities (Ibrahim et al. 2016). Although, the mechanism of bactericidal action of  $\alpha$ -MG on planktonic cells of *Staphylococcus aureus* has been extensively investigated by Koh et al. (2013) and studies related to antibiofilm and biofilm eradicating efficacy of  $\alpha$ -MG on bacterial pathogens are scarce. Therefore, the current study was aimed to uncover the in vitro bactericidal efficacy of  $\alpha$ -MG on both planktonic and sessile cells of methicillin-resistant *S. epidermidis* (RP62A; ATCC 35984).

## Materials and methods

### Bacterial strain, culture media, and conditions

*S. epidermidis* RP62A (ATCC 35984) was purchased from the American Type Culture Collection (ATCC, Manassas, USA) and was maintained in tryptic soy broth (TSB; Himedia, India) at  $-80\text{ }^{\circ}\text{C}$  with 30% glycerol. For biofilm assay, TSB was supplemented with 0.25% glucose (TSBG) and incubated at  $37\text{ }^{\circ}\text{C}$ .

### Determination of minimum inhibitory concentration and minimum bactericidal concentration

The minimum inhibitory concentration (MIC) was determined by microdilution method in Mueller Hinton broth (MHB, HiMedia, India) (CLSI 2006). Vancomycin (HiMedia, India) and  $\alpha$ -MG (Sigma, St. Louis, MO, USA) was diluted in a 24-well microtitre plate (MTP) to obtain final concentrations ranging from 40 to 0.156 and 10–0.156  $\mu\text{g}/\text{mL}$ , respectively. An aliquot of the bacterial suspension (10  $\mu\text{L}$ ,  $1 \times 10^6$  CFU/

mL) was used to inoculate and incubated at  $37\text{ }^{\circ}\text{C}$  for overnight. The MIC was recorded as the lowest concentration of  $\alpha$ -MG that inhibits the visible growth of bacteria. For minimum bactericidal concentration (MBC), 100  $\mu\text{L}$  from MIC assay MTP wells with no visible bacterial growth were spread on Mueller Hinton agar (MHA) plates and incubated at  $37\text{ }^{\circ}\text{C}$  for 24 h. MBC was recorded as the lowest concentration of  $\alpha$ -MG which allowed to grow less than five colonies on each plate.

### Kinetics of killing planktonic cells

A 1: 200 dilution ( $1 \times 10^6$  CFU/mL) of the overnight culture of *S. epidermidis* was used to inoculate 20 mL of fresh MHB. Concentrations of  $\alpha$ -MG from 0.3125  $\mu\text{g}/\text{mL}$  (1/4 MIC) to 10  $\mu\text{g}/\text{mL}$  ( $8 \times$  MIC) were added to MHB and incubated for 12 h at  $37\text{ }^{\circ}\text{C}$  with shaking (160 rpm). For every 5 min of time interval, 100  $\mu\text{L}$  of the culture from each tube was serially diluted, spread on MHA plates and incubated at  $37\text{ }^{\circ}\text{C}$  for 24 h. After incubation, the viable bacterial cells (colony forming unit—CFU) were counted and the rate of killing was determined by plotting CFU/mL against time (D’Arezzo et al. 2012; Liu et al. 2014).

### $\alpha$ -MG habituation of *S. epidermidis* cells

TSBG (10 mL) supplemented with  $\alpha$ -MG (0.156  $\mu\text{g}/\text{mL}$ ) were inoculated with 100  $\mu\text{L}$  of standardized cell suspension ( $1 \times 10^7$  CFU/mL) and incubated at  $37\text{ }^{\circ}\text{C}$  for 24 h. In parallel, the non-habituated cells (bacteria were grown in TSBG without  $\alpha$ -MG) were maintained as control. After incubation, non-habituated and  $\alpha$ -MG habituated cells were recovered by centrifugation ( $8000 \times g$  for 10 min), washed twice with phosphate buffer saline (PBS; pH 7.4), and resuspended in PBS to form standardized cell suspension as above. The non-habituated and  $\alpha$ -MG habituated cells were sub-cultured daily for 4 days in fresh TSBG with and without  $\alpha$ -MG at same concentration. At each successive passage, the bacterial growth was evaluated by absorbance at 600 nm. After four sequential passages at same concentration of  $\alpha$ -MG, cells were transferred to TSBG at one fold increasing concentration of  $\alpha$ -MG (0.3125  $\mu\text{g}/\text{mL}$ ). This process was repeated until the  $\alpha$ -MG habituated cells stops growing (Apolonio et al. 2014).

### Penetration of antibiotics through $\alpha$ -MG habituated and non-habituated biofilms

A 20  $\mu\text{L}$  drop of standardized cell suspension (0.5,  $10^7$  CFU/mL) of control and  $\alpha$ -MG habituated cultures were used to seed on black, polycarbonate membranes (PCM) (13 mm diameter; 0.4 mm pore size (Merck Millipore Ltd., India)) and placed on MHA plates. The plates were incubated upturned at  $37\text{ }^{\circ}\text{C}$  for 48 h. For every 24 h of incubation, the membrane-

carried biofilms (MCBs) were transferred to fresh TSA plates. The MCBs were then washed with PBS by shaking at 160 rpm for 1 min to eliminate non-adherent cells. The MCBs were transferred to MHA plates swabbed with control and  $\alpha$ -MG habituated *S. epidermidis* to McFarland standard 0.5, where it gives a confluent lawn of growth following incubation. The nitrocellulose membrane (6 mm diameter; 0.4 mm pore size) was placed over the surface of each MCBs along with pre-moistened antibiotic disc (amikacin, 30  $\mu$ g; tetracycline, 30  $\mu$ g and vancomycin, 30  $\mu$ g). In parallel, the control assemblies were made with sterile membranes and antibiotic discs. The plates were incubated at 37 °C for 24 h and zones of growth inhibition were documented. The clear zones of control assemblies were considered as 100% penetration (retardation) which is used to find out the percentage of antibiotics penetration through biofilms (Singh et al. 2010). Experiments were performed in duplicate.

### Assessment of biofilm biomass

The  $\alpha$ -MG at 1/16, 1/8, 1/4, 1/2, and 1  $\times$  MIC was added to TSBG containing bacterial suspension of  $10^6$  CFU/mL and incubated for 24 h at 37 °C. After incubation, growth OD was measured at 600 nm. The free floating planktonic cells in the MTP wells were discarded and the plates were washed thrice with sterile PBS. The adhered sessile cells were stained with 0.4% (w/v) crystal violet (CV). Subsequently, the excess stain was washed thrice with sterile distilled water. One milliliter of 20% glacial acetic acid was added to solubilize stained biofilm cells and it was measured spectrophotometrically at 570 nm using multilabel reader (Molecular devices, SpectraMax M3, USA) (Cramton et al. 1999; Packiavathy et al. 2013).

### Assessment of bacterial viability using XTT assay

The cellular viability was quantified using the tetrazolium sodium 3'-{1-[(phenylamino)-carbonyl]-3-4-tetrazolium}-bis(4-methoxy-6-nitro)-benzene sulfonic acid hydrate (XTT) reduction assay described by Gowrishankar et al. (2014). The sodium salt of XTT was dissolved in sterile PBS (1 mg/mL) and menadione was dissolved in acetone (1 mM). XTT/menadione solution was prepared freshly prior to each experiment at the ratio of 12.5:1 of XTT and menadione, respectively. After incubation, both planktonic and biofilm cells were removed from the vehicle control (VC) and treated ( $\alpha$ -MG and vancomycin) wells. The bacterial cells were washed thrice with sterile PBS and resuspended in 175  $\mu$ l of the same buffer. Twenty-five microliters of XTT/menadione solution was added to this cell suspensions and incubated dark at 37 °C for 8 h. Cellular viability was measured spectrophotometrically through the reduction of XTT into orange colored formazan at 490 nm.

### Impact of $\alpha$ -MG against immature and mature biofilm

The effect of  $\alpha$ -MG on immature (6 h old) and mature (24 h old) biofilms of *S. epidermidis* was assessed (Stepanovic et al. 2007). Overnight cell suspension (10  $\mu$ L,  $10^6$  CFU/mL) was used to inoculate 1 mL of TSBG in MTP wells and incubated statically at 37 °C for 6 h (immature) and 24 h (mature). After incubation, the medium containing non-adherent bacterial cells was removed and 1 mL of fresh TSBG with  $\alpha$ -MG and vancomycin at the concentrations of 16  $\times$ , 8  $\times$ , 4  $\times$ , 2  $\times$  and 1  $\times$  MIC were added to separate wells and incubated further for 16 h at 37 °C. After incubation, biofilm biomass and metabolic activity of viable cells were quantified as per the above mentioned protocols.

### Scanning electron microscopic analysis of immature biofilms

*S. epidermidis* was statically incubated for 6 h in the wells of MTP with 1  $\times$  1 cm glass slides at 37 °C, then  $\alpha$ -MG and vancomycin were added at the concentration of 16 $\times$  MIC in fresh TSB medium and incubated for another 16 h at 37 °C. After incubation, non-adherent cells were removed and sessile cells in the glass slides were subjected to scanning electron microscopic (SEM) analysis. The biofilms on glass slides were flooded with 2.5% glutaraldehyde for 12 h and washed in 0.1 M sodium acetate buffer (pH 7.3). Biofilms were dehydrated in a graded ethanol series from 30, 50, 60, 70, 90, and 95% for 10 min each, followed by rinsing thrice with 100% ethanol. Specimens were dried and immediately sputter coated with metal. Biofilms were visualized by using a Hitachi S-3000H scanning electron microscope (Hitachi, Japan) (Liu et al. 2015).

### Observation of *S. epidermidis* biofilms by confocal laser scanning microscopy

The effect of  $\alpha$ -MG and vancomycin on bacterial viability in mature (24 h old) biofilms of *S. epidermidis* ATCC 35984 was evaluated by confocal laser scanning microscopy (CLSM) (Sivaranjani et al. 2016). After incubation, VC and treated biofilms were stained with SYTO9 and propidium iodide (PI) (Live/Dead staining, Molecular Probe) and visualized by CLSM (Model: LSM 710, Carl Zeiss, Germany).

### Statistical analysis

All experiments were performed in triplicate. Data were expressed as arithmetic mean  $\pm$  standard deviation and analyzed with Dunnett-ANOVA test. Data regarding the penetration of antibiotics through biofilms was alone analyzed using two-tailed, paired *t* test using statistical software package

(SPSS Chicago, IL, USA). *P* values of 0.05 were considered statistically significant.

## Results

### Effect of $\alpha$ -MG on growth of *S. epidermidis* planktonic cells

The MIC of  $\alpha$ -MG and vancomycin against *S. epidermidis* was found to be 1.25 and 10  $\mu\text{g}/\text{mL}$ , respectively. The MBC of  $\alpha$ -MG and vancomycin for *S. epidermidis* was 5 and 20  $\mu\text{g}/\text{mL}$ , respectively.

The rapid killing efficacy of  $\alpha$ -MG on planktonic cells of *S. epidermidis* was investigated by performing time kill curve assay. As anticipated,  $\alpha$ -MG displayed rapid concentration-dependent killing of *S. epidermidis* cells at concentrations above  $4\times$  MIC (5  $\mu\text{g}/\text{mL}$ ) and  $2\times$  MIC (2.5  $\mu\text{g}/\text{mL}$ ) of  $\alpha$ -MG, achieving 6 log and 4 log reduction of viable count within 5 min of exposure time, respectively. A less than 2 log reduction of viable counts was achieved while treating with  $1\times$  MIC (1.25  $\mu\text{g}/\text{mL}$ ) of  $\alpha$ -MG (Fig. 1a). On the other hand, vancomycin displayed no significant reduction in viable cells even after 6 h of incubation with vancomycin at  $2\times$ ,  $4\times$  and  $8\times$  MIC (Fig. 1b).

### Impact of $\alpha$ -MG habituation on development of resistance

Antibiotic resistance was defined as  $>4$ -fold increase from its original MIC value (Farrell et al. 2011). In order to evaluate the acquisition of resistance to  $\alpha$ -MG on *S. epidermidis*, subsequent passage was performed. The *S. epidermidis* cells grown at the concentrations of  $\alpha$ -MG from 0.156 to 1.25  $\mu\text{g}/\text{mL}$ , no obvious growth inhibition was observed up to  $16^\circ$  passage. While the  $\alpha$ -MG concentration was increased to 2.5  $\mu\text{g}/\text{mL}$ , the bacterial cell growth was inhibited gradually in  $17^\circ$  and  $18^\circ$  passage and complete visible growth inhibition was observed at  $19^\circ$  passage. Through this experiment, it was concluded that even the continuous exposure of  $\alpha$ -MG did not develop any resistance in *S. epidermidis*, in which 1-fold

increase of MIC was observed for  $\alpha$ -MG (i.e., MIC at  $1^\circ$  passage 1.25  $\mu\text{g}/\text{mL}$  and MIC at  $19^\circ$  passage 2.5  $\mu\text{g}/\text{mL}$ ) (Fig. 2). Besides, the influence of  $\alpha$ -MG habituation on the susceptibility of *S. epidermidis* planktonic cells to antibiotics such as ampicillin, amikacin, tetracycline, rifampicin, gentamycin, vancomycin, and erythromycin was evaluated. The  $\alpha$ -MG habituated planktonic cells did not induce resistance to the tested antibiotics (Table S1).

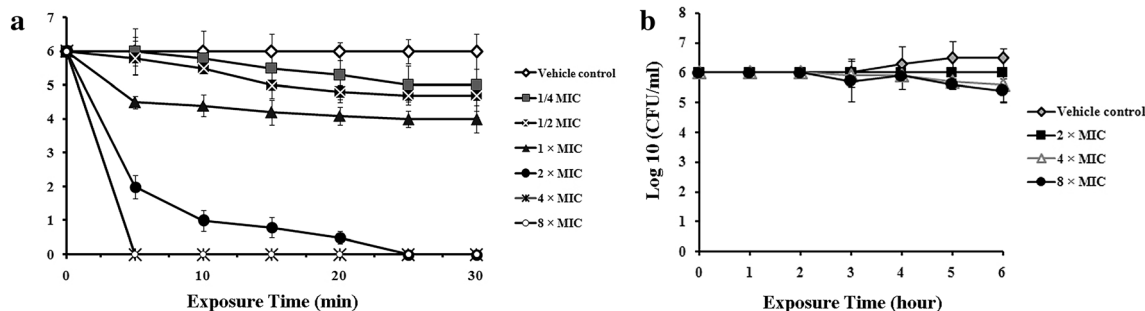
Concomitantly, the impact of  $\alpha$ -MG habituated *S. epidermidis* biofilms was also assessed using biofilm penetration assay. The penetration of glycopeptide antibiotic-vancomycin was significantly reduced through  $\alpha$ -MG habituated and non-habituated biofilms in comparison with control assemblies. In contrast, no significant difference was observed in the penetration of tetracycline and amikacin through  $\alpha$ -MG habituated and non-habituated biofilms (Fig. 3). The diameter of the zone of inhibition in  $\alpha$ -MG habituated and non-habituated biofilms was similar when compared with their respective control assemblies (Table 1). Hence, it is appropriate to state that  $\alpha$ -MG habituation does not induce the resistance in planktonic as well as biofilm cells of *S. epidermidis*.

### Effect of $\alpha$ -MG on *S. epidermidis* biofilm formation

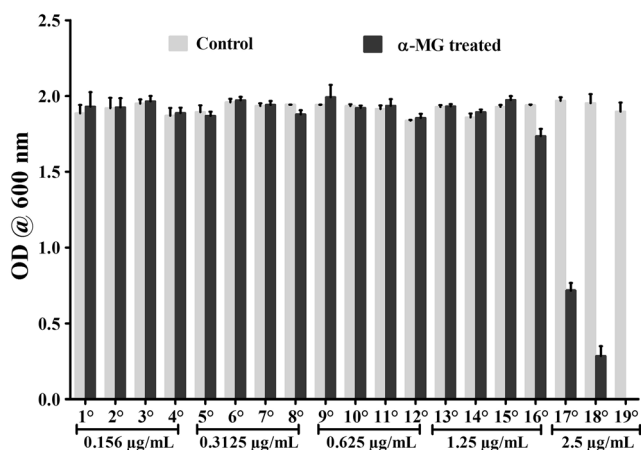
A visible growth reduction was observed at  $1\times$  MIC of  $\alpha$ -MG. At  $1/2$  MIC, the growth was decreased up to 40% and biofilm was inhibited up to 80%. A less pronounced inhibitory effect on growth and biofilm formation (30–40%) was observed at  $1/4$  to  $1/16$  MIC treatment of  $\alpha$ -MG (Fig. 4). Furthermore, SEM micrograph clearly revealed the structurally complex and dynamic multilayered matrix architecture of *S. epidermidis* biofilm in VC, whereas the  $\alpha$ -MG ( $1/2$  MIC) treated micrographs absolutely lacks such depiction of matrix structures (Fig. 5).

### Effect of $\alpha$ -MG and vancomycin on immature biofilms

As a prelude to assess the inhibition of biofilm formation of *S. epidermidis*, the ability of  $\alpha$ -MG at MIC to disrupt the immature biofilm was examined. Outcome of this assay

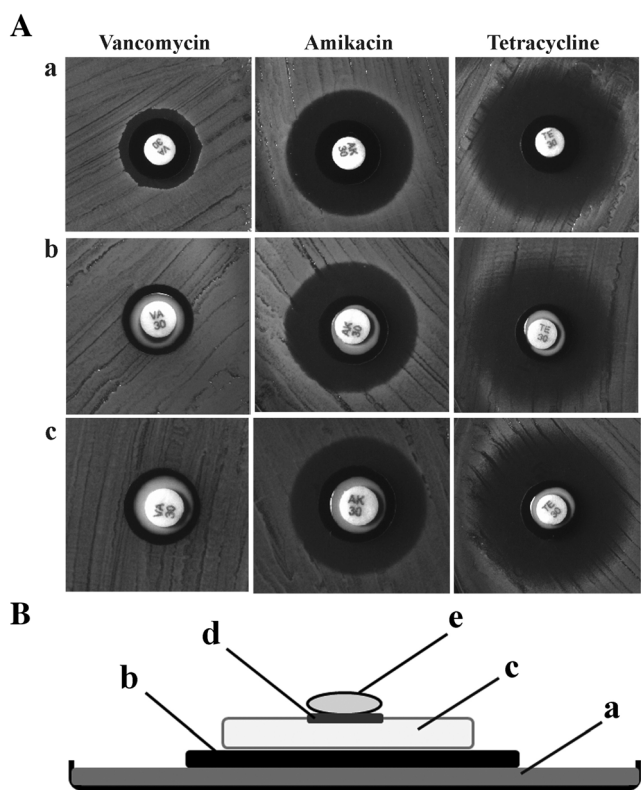


**Fig. 1** Time kill kinetics curve of *S. epidermidis* using  $\alpha$ -MG (a) and vancomycin (b) as comparator. The bacterial growth killing was determined by plotting survival colonies (CFU/mL) against time



**Fig. 2** Subsequent passage resistance selection studies of  $\alpha$ -MG on non-habituated (control) and  $\alpha$ -MG habituated *S. epidermidis* cells

demonstrated that  $\alpha$ -MG at 1 $\times$  and 2 $\times$  MIC was not sufficient enough to disrupt the immature biofilm. The same observation was also found with vancomycin (1 $\times$  and 2 $\times$  MIC) treated bacterial biofilms. Thus, the compound at increasing concentrations was used to assess its



**Fig. 3** **A** Picto-graphical depiction of the antibiotics penetration through control assemblies (*a*), non-habituated (*b*), and  $\alpha$ -MG habituated (*c*) *S. epidermidis* biofilms as determined on MHA plates by the zone of bacterial growth inhibition. **B** Schematic representation of the experimental setup used to analyze the antibiotics penetration through the biofilms. (*a*) MHA plates, (*b*) 13-mm black polycarbonate membrane, (*c*)  $\alpha$ -MG habituated/non-habituated *S. epidermidis* biofilms grown on black polycarbonate membrane, (*d*) 6-mm nitrocellulose membrane, and (*e*) 6-mm antibiotic discs—tetracycline, vancomycin, and amikacin

biofilm disrupting efficacy. The increasing concentrations of  $\alpha$ -MG from 4 $\times$ , 8 $\times$  and 16 $\times$  MIC significantly eradicated the immature biofilms accordingly by 55, 73, and 93%. On the other hand, vancomycin at 4 $\times$ , 8 $\times$ , and 16 $\times$  MIC disrupted the biofilm only to the level of 10, 26, and 36%, respectively. The XTT assay also confirmed the results of immature biofilm disruption assay, as  $\alpha$ -MG significantly killed the sessile cells of *S. epidermidis* in a dose dependent manner.  $\alpha$ -MG at 4 $\times$ , 8 $\times$ , and 16 $\times$  MIC effectively reduced the viability of preformed biofilm cells by 40, 71, and 97%, whereas vancomycin showed 20, 32, and 38%, respectively (Table 2). The SEM micrograph of VC and vancomycin treated at 16 $\times$  MIC clearly depicted highly structured, multilayered, and robust biofilm matrix formation by exopolysaccharides. In contrast, the micrographs of  $\alpha$ -MG (4 $\times$ , 8 $\times$ , and 16 $\times$  MIC) treatment portrayed concentration-dependent disruption in the preformed immature biofilms. The results of the SEM analysis clearly depicts that concentration dependent bactericidal activity was observed when the immature biofilms were treated with  $\alpha$ -MG at 4 $\times$ , 8 $\times$ , and 16 $\times$  MIC respectively (Fig. 6). At 4 $\times$  MIC of  $\alpha$ -MG, the bacterial cells were killed effectively and the three dimensional structure of biofilm was also reduced. However, live cells with intact cell membrane were often observed at 4 $\times$  MIC of  $\alpha$ -MG treated biofilm cells. Furthermore, biofilms treated at 8 $\times$  and 16 $\times$  MIC of  $\alpha$ -MG evidently decreased the viable bacterial load as well as the aggregation in the matrix structure while this was not found in vancomycin treated biofilms even at 16 $\times$  MIC.

#### Effect of $\alpha$ -MG and vancomycin on mature *S. epidermidis* biofilms

At the tested concentrations (8 $\times$  and 16 $\times$  MIC),  $\alpha$ -MG was proficient enough to disrupt the preformed biofilms and effectively killed the biofilm embedded cells. In contrast, vancomycin was found ineffective in killing the sessile bacterial cells of the mature biofilm (Table 3). On the other hand, the bactericidal effect of  $\alpha$ -MG on 6 h old biofilms was very high when compare to 24 h old biofilms. The bactericidal activity of  $\alpha$ -MG on mature (24 h old) biofilms was also assessed by CLSM with Live/Dead staining. CLSM micrographs of  $\alpha$ -MG treated with increasing concentration displayed an elevated level of PI intensity and low intensity of green fluorophore due to SYTO9, which clearly validated the phenomenal concentration dependent killing efficacy of  $\alpha$ -MG on mature biofilms. Nevertheless, vancomycin even at 16 $\times$  MIC was inefficient in killing the sessile cells of *S. epidermidis* RP62A (Fig. 7).

**Table 1** Penetration of antibiotics through the control assemblies, non-habituated and  $\alpha$ -MG habituated biofilms of *S. epidermidis* ATCC 35984

Antibiotics	Concentration ( $\mu\text{g}$ )	Non-habituated <i>S. epidermidis</i> biofilms				$\alpha$ -MG habituated <i>S. epidermidis</i> biofilms			
		Control assembly zone diameter (mm)	Zone diameter (mm)	% retardation	<i>P</i> value	Zone diameter (mm)	% retardation	<i>P</i> value	
Vancomycin	30	18 $\pm$ 0.10	13	>27.77 $\pm$ 0	S ( <i>P</i> 0.05)	13	>27.77 $\pm$ 0	S ( <i>P</i> 0.05)	
Amikacin	30	23.08 $\pm$ 0.67	23 $\pm$ 0.5	0.35 $\pm$ 0.40	NS ( <i>P</i> > 0.05)	23.08 $\pm$ 0.67	0	NS ( <i>P</i> > 0.05)	
Tetracycline	30	32.58 $\pm$ 0.28	31.33 $\pm$ 0	3.8 $\pm$ 0.85	NS ( <i>P</i> > 0.05)	32.16 $\pm$ 0.57	1.2 $\pm$ 0.89	NS ( <i>P</i> > 0.05)	

The clear zones of control assemblies (mean + SD) on MHA plates were considered as representative of 100% penetration which used to find out the percentage retardation of antibiotics penetration through biofilms

*S* significant, *NS* non-significant

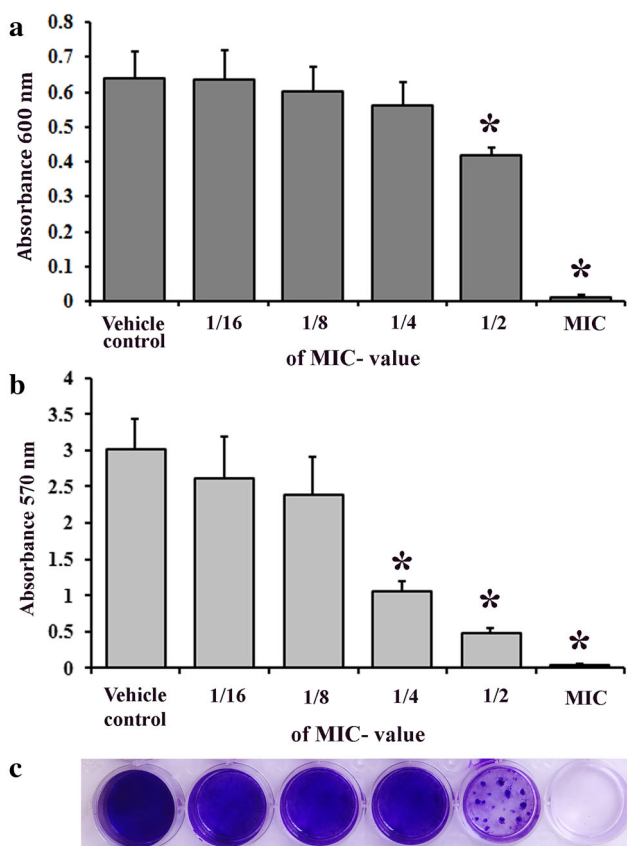
## Discussion

Since 1980s, plethora of scientific reports have envisaged  $\alpha$ -MG as a potent antimicrobial agent with rapid killing efficacy even at very low MIC values (Sakagami et al. 2005; Koh et al. 2013) against various Gram-positive pathogens, particularly methicillin-resistant *S. aureus* (MRSA; MIC value of 1.95  $\mu\text{g}/\text{mL}$ ) (Chin and Kinghorn 2008) and vancomycin resistant Enterococci (VRE; MIC value of 6.25  $\mu\text{g}/\text{mL}$ ) (Sakagami

et al. 2005). The present study also demonstrates a rapid bactericidal activity of  $\alpha$ -MG against the planktonic cells of *S. epidermidis* RP62A with a very low MIC and MBC values of 1.25 and 5  $\mu\text{g}/\text{mL}$ , respectively. A phenomenal rapid bactericidal efficacy of  $\alpha$ -MG was certain, as it readily killed the planktonic cells of *S. epidermidis* at 2 $\times$  MIC of  $\alpha$ -MG to the level of 4- $\log_{10}$  reduction with in 5 min. This is in par with the findings of Koh et al. (2013), wherein they reported that  $\alpha$ -MG exhibits a rapid bactericidal efficacy (3- $\log_{10}$  reduction within 5 min) in methicillin-resistant *Staphylococcus aureus* (MRSA) via disrupting the integrity of cytoplasmic membrane leading to the loss of intracellular components. Earlier reports on antibacterial activity of  $\alpha$ -MG against *Bacillus cereus* (Koh et al. 2013), *Streptococcus pneumoniae* (Coyle and Rybak 2001) and *Streptococcus mutans* (Nguyen and Marquis 2011) have also demonstrated a similar fashion of killing action. Therefore, it is envisaged that  $\alpha$ -MG could plausibly target the cytosolic membrane of *S. epidermidis* to cause rapid death.

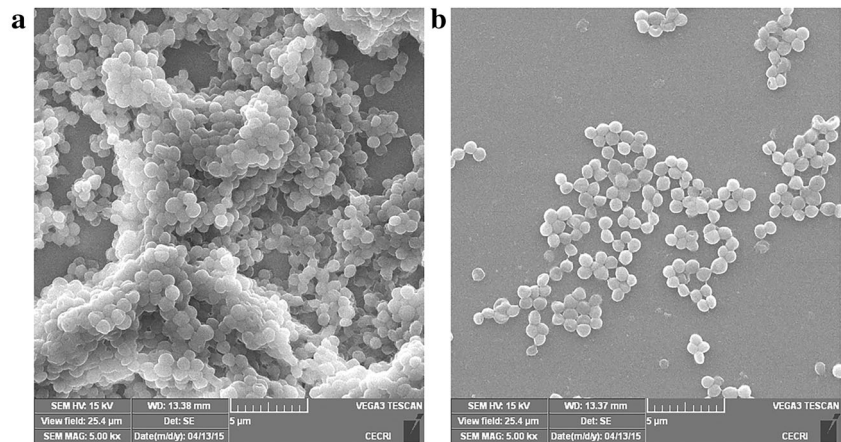
Nevertheless, the action of vancomycin even after 6 h of incubation was in vain to achieve 4- $\log_{10}$  reduction in viable bacteria count. A similar kind of observation was also reported by Coyle and Rybak (2001), who signifies that even after 300 min of vancomycin exposure at higher concentration, a 3- $\log_{10}$  reduction in viable *S. pneumoniae* was unachievable. It has also been broadly accepted that vancomycin exhibits an inherently slower mechanism of action by binding to the peptidoglycan component, D-Ala- D-Ala terminus and inhibits the crosslinking of peptidoglycan through transpeptidation reaction (Beauregard et al. 1995).

$\alpha$ -MG is also believed to pose lower risk for selective pressure, as it mimics the chemical structure of meta-phenylene ethynylene (mPE), a membrane targeting cationic antimicrobial peptide that exhibit rapid bactericidal activity and shows a lesser possibility toward rising resistance to Gram-positive bacteria (Tew et al. 2006). Our result signifies that  $\alpha$ -MG elicit rapid bactericidal activity as it targets the bacterial membrane (Koh et al. 2013) and hence, resistance is prevented in both planktonic and biofilm cells by averting the mutational possibility of membrane structure.



**Fig. 4** Effect of  $\alpha$ -MG on *S. epidermidis* ATCC 35984 growth and biofilm formation. Assessment of growth (OD 600) (a), biofilm biomass quantification (OD 570) (b), and representative photographs showing CV stained biofilm cells attached on MTP wells (c)

**Fig. 5** Effect of  $\alpha$ -MG on biofilm formation of *S. epidermidis* ATCC 35984 was analyzed by using SEM. Control (a) and  $\alpha$ -MG at 1/2 MIC (b)



In addition,  $\alpha$ -MG also exhibits a significant biofilm inhibitory efficacy at sub-MICs. SEM result also elucidates that 1/2 MIC of  $\alpha$ -MG treatment effectively reduced the three dimensional structure of biofilm in comparison to untreated control. The bacterial growth OD and CV quantification of biofilm confirmed that the biofilm inhibitory potential of  $\alpha$ -MG is directly proportional to the inhibition of bacterial growth. This could be explained by “inoculum effect,” which means the effect of any bactericidal agents turn to be less effective when the bacterial cell density is higher than the amount of bactericidal agent available per bacterial cells (Udekwa et al. 2009). Besides, several studies suggested that sub-inhibitory concentrations of various antibiotics, including nafcillin, tetracycline, and vancomycin increase the biofilm formation of staphylococci (Frank et al. 2007; Hsu et al. 2011; Kuehl et al. 2009). Hence,  $\alpha$ -MG with its initial biofilm inhibitory efficacy against *S. epidermidis* at sub-inhibitory concentrations, it would not fall into the above said class of antibiotics.

Even though the planktonic cells are highly sensitive to most of the known antibacterial agents, cells residing inside the biofilms are extremely robust to eradicate. As reported by Claessens et al. (2015), the routine practice of determining bacterial antibiotic susceptibility for planktonic cells is not a better insight for the response of sessile communities. Hence, in the current study, CV and XTT quantification methods are used to assess the effects of  $\alpha$ -MG and vancomycin on in vitro preformed biofilms of *S. epidermidis* (Claessens et al. 2015). The CV and XTT quantification assay results revealed that  $\alpha$ -MG showed profound killing and eradicating potential against immature biofilms of *S. epidermidis*. The SEM micrographs of immature biofilms treated with  $\alpha$ -MG have also depicted the better killing efficacy with morphological disruption of sessile cells; however, no such effect was observed in micrographs of vancomycin treated samples. All these results confirmed the low killing efficacy of vancomycin toward sessile cells of *S. epidermidis* unlike the planktonic cells which were

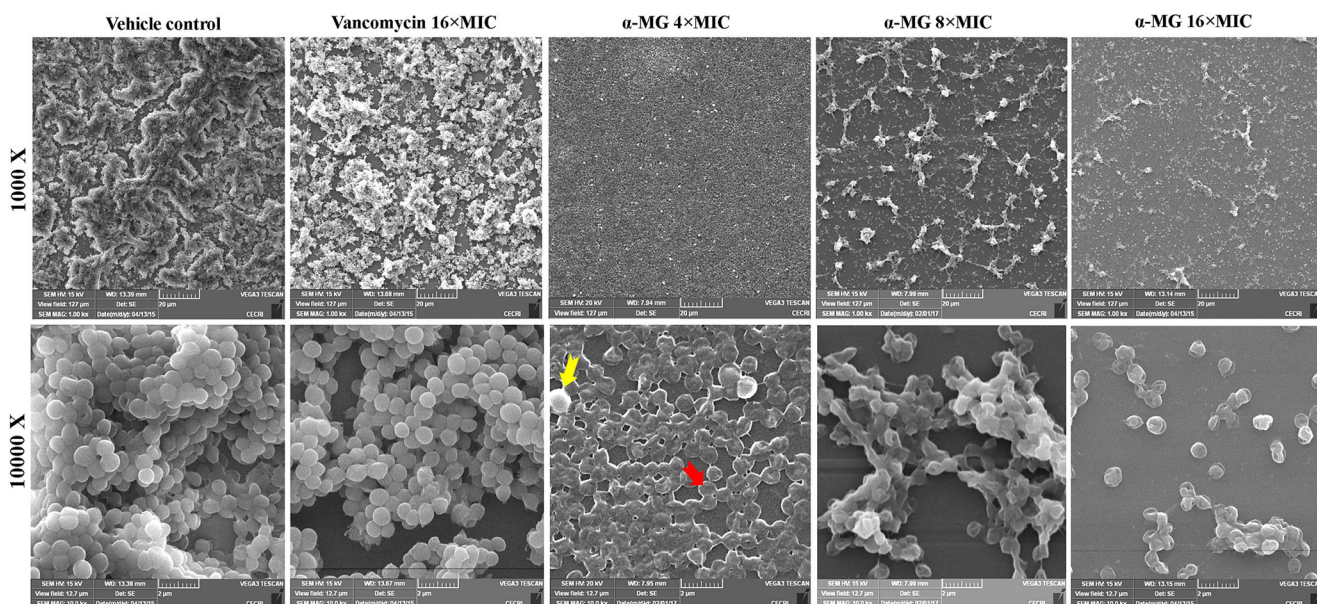
**Table 2** Effect of  $\alpha$ -MG and vancomycin on immature (6 h old) biofilms of *S. epidermidis* ATCC 35984

		Vehicle control	1 × MIC	2 × MIC	4 × MIC	8 × MIC	16 × MIC	
Biofilm biomass (CV staining)	OD@ 570 nm	$\alpha$ -MG	2.77 ± 0.30	2.60 ± 0.30	2.51 ± 0.28	1.22 ± 0.11*	0.74 ± 0.04*	0.18 ± 0.08*
		VAN	2.77 ± 0.30	2.69 ± 0.31	2.65 ± 0.29	2.47 ± 0.27	2.02 ± 0.17	1.75 ± 0.18
	% of inhibition	$\alpha$ -MG	NA	6.2 ± 2.5	9.2 ± 4.1	55.7 ± 2.2*	73.0 ± 2.9*	93.6 ± 2.4*
		VAN	NA	2.9 ± 1.0	4.2 ± 1.2	10.9 ± 3.2	26.9 ± 1.7	36.9 ± 2.2
Metabolically active cells (XTT reduction assay)	OD @ 490 nm	$\alpha$ -MG	1.67 ± 0.16	1.62 ± 0.15	1.57 ± 0.18	1.01 ± 0.16*	0.48 ± 0.14*	0.05 ± 0.03*
		VAN	1.67 ± 0.16	1.69 ± 0.15	1.65 ± 0.17	1.33 ± 0.17	1.14 ± 0.17	1.03 ± 0.15
	% of inhibition	$\alpha$ -MG	NA	2.7 ± 2.1	6.3 ± 1.7	40.0 ± 3.8*	71.9 ± 5.9*	97.1 ± 1.6*
		VAN	NA	NI	1.4 ± 1	20.2 ± 3.6	32.1 ± 4	38.3 ± 2.6

Biofilm biomass was quantified by crystal violet staining method at 570 nm. Metabolically active cells were quantified at 490 nm by XTT reduction method

NA not applicable, NI no inhibition

\*Differences were considered significant at  $P < 0.05$



**Fig. 6** Scanning electron micrograph of immature (6 h old) biofilms of *S. epidermidis* ATCC 35984 treated with  $\alpha$ -MG and vancomycin. *Yellow arrows* indicate the live cells with intact cell membrane and *red arrows* indicate the dead cells with cell membrane damage

sensitive to vancomycin. This result falls in line with the findings of Tenover et al. (2001), indicating that vancomycin failed to disrupt the mature biofilm of *S. epidermidis* even at the highest concentration. Moreover, the conventional antibiotic regimens of vancomycin treatment are not sufficient to diminish biofilms associated infections. It is a well-known fact that mature biofilms are extremely robust to all kinds of antibiotics. Therefore, in this study, the potential of  $\alpha$ -MG in disrupting and killing the mature biofilm embedded cells was evaluated. Although  $\alpha$ -MG is an effective bactericidal agent for inhibiting the onset of biofilm formation and immature biofilms, the killing activity on mature (24 h old) biofilms was comparatively low which proved that the bactericidal activity on biofilms solely depend on the age of biofilms.

Furthermore, current treatment strategies also relies on the combinatorial therapy as one of the effective treatment strategies in which Chusri et al. (2016) reported that  $\alpha$ -MG exhibited minimal antibacterial and antibiofilm activity when compared to that of polyherbal formula (*Curcuma longa*, *Areca catechu*, *Oryza sativa* and *G. mangostana*) viz., Ya-SaMam-Phlae (YSMP). Hence, this earlier study puts forth an idea that the  $\alpha$ -MG reported in the present study can be further evaluated for its antibacterial and antibiofilm activity with other well-known phytochemicals to maximize its activity.

There are several reports available on the phyto-compounds in inhibiting the staphylococcal biofilms. The phyto-compounds such as berberine, carvacrol, oregano, thymol, black pepper, cananga, myrrh oils, demethylfruticuline

**Table 3** Effect of  $\alpha$ -MG and vancomycin on mature (24 h old) biofilms of *S. epidermidis* ATCC 35984

			Vehicle control	1 × MIC	2 × MIC	4 × MIC	8 × MIC	16 × MIC
Biofilm biomass (CV staining)	OD@ 570 nm	$\alpha$ -MG	2.77 ± 0.14	2.78 ± 0.14	2.77 ± 0.12	2.62 ± 0.11	1.71 ± 0.08	1.45 ± 0.14*
		VAN	2.77 ± 0.14	2.88 ± 0.19	2.84 ± 0.26	2.85 ± 0.21	2.77 ± 0.15	2.81 ± 0.12
	% of inhibition	$\alpha$ -MG	NA	NI	NI	5.1 ± 1.1	38 ± 0.9	47.6 ± 2.5*
		VAN	NA	NI	NI	NI	NI	NI
Metabolically active cells (XTT assay)	OD @ 490 nm	$\alpha$ -MG	2.21 ± 0.41	2.28 ± 0.37	2.09 ± 0.40	1.89 ± 0.35	1.64 ± 0.31	1.00 ± 0.25*
		VAN	2.21 ± 0.41	2.26 ± 0.38	2.27 ± 0.46	2.27 ± 0.42	2.24 ± 0.43	2.23 ± 0.42
	% of inhibition	$\alpha$ -MG	NA	NI	5.8 ± 0.7	14.4 ± 3.7	25.9 ± 1.4	55.1 ± 2.7*
		VAN	NA	NI	NI	NI	NI	NI

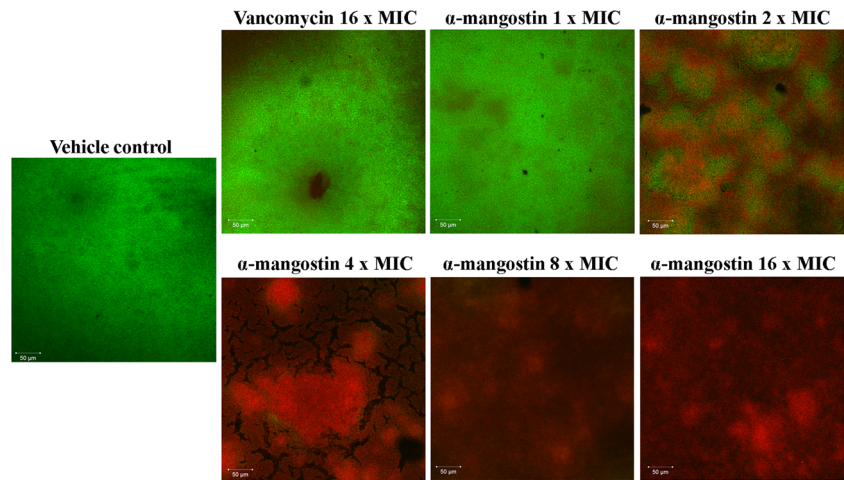
Biofilm biomass was quantified by crystal violet staining method at 570 nm. Metabolically active cells were quantified at 490 nm by XTT reduction method

NA not applicable, NI no inhibition

\*Differences were considered significant at  $P < 0.05$



**Fig. 7** Bactericidal effects of  $\alpha$ -MG and vancomycin on mature biofilm (24 h old) of *S. epidermidis* observed in CLSM using Live/Dead BacLight viability staining (SYTO9/PI). Bacterial cells stained with green fluorescence (SYTO9) are viable and with red fluorescence (PI) are dead



A, and fructulane A have been frequently reported for their bactericidal and antibiofilm activity on staphylococcal biofilms (Lee et al. 2014; Nostro et al. 2007; Schito et al. 2011; Wang et al. 2009). In addition, tea tree oil (1%) was reported to inhibit the metabolism of *S. aureus* biofilm (Kwiecinski et al. 2009). Although tea tree oil is an effective bactericidal agent against wide range of bacteria, administration of sub-lethal concentration of tea tree oil lead to the development of resistance against many of the antibiotics (McMahon et al. 2007). Nevertheless, the treatment of  $\alpha$ -MG does not pose any risk of resistance development in both planktonic and biofilms cells of *S. epidermidis* which is evident through in vitro analysis. Further, in vivo analysis using animal models such as rats/mouse on the effect of  $\alpha$ -MG for its bactericidal activity on both planktonic and sessile cells of *S. epidermidis* RP62A is the need of the hour. However, plethora of reports are available on the in vivo potential of  $\alpha$ -MG that potentiate the multifarious activity of  $\alpha$ -MG such as renoprotective activity against cisplatin induced nephrotoxicity via inhibiting the catalase production, nitrosative/oxidative stress, renal dysfunction, structural damage and increase in mRNA levels of tumor necrosis factor in rat animal model (Perez-Rojas et al. 2009). In another study, Sampath and Vijayaraghavan (2007) reported that  $\alpha$ -MG has a protective effect against lipid-peroxidation and antioxidant defense system during isoproterenol-induced myocardial infarction in rats. Gopalakrishnan et al. (1980) reported the efficiency of  $\alpha$ -MG in inhibiting the immunocytadherence and systemic anaphylaxis in rats and guinea pigs. The intraperitoneal and oral routes administration of  $\alpha$ -MG showed significant anti-inflammatory response in both normal and bilaterally adrenalectomized rats (Shankaranarayan et al. 1979).  $\alpha$ -MG has also been reported for its cytotoxic effect on head and neck squamous carcinoma (HNSCC) cell lines, human melanoma SK-MEL-28 cell line, epidermoid carcinoma of the mouth (KB) cells, breast cancer (BC-1) cells, human leukemia cell line HL60 and other leukemia cell lines (K562, NB4 and

U937) (Suksamram et al. 2006; Kaomongkolgit et al. 2011; Wang et al. 2011; Matsumoto et al. 2003). Similarly, Shih et al. (2010) reported that  $\alpha$ -MG have anti-metastatic effect on phorbol 12-myristate 13-acetate induced matrix metalloproteinases (MMP-2/MMP-9) expression in human lung adenocarcinoma A549 cells. Kosem et al. (2013) have studied the non-toxic nature of  $\alpha$ -MG (4000  $\mu$ g/mL) in human gingival fibroblast and recognized as safe to treat oral candidiasis due to its strong fungicidal and non-toxic nature.

To sum up, the present study demonstrates  $\alpha$ -MG as an effective antibacterial agent against both planktonic and sessile cells of *S. epidermidis* RP62A without posing any risk for the development of resistance. Further, in vivo investigation using eukaryotic model system together with translational studies is needed to understand the underlying molecular mechanism of action of  $\alpha$ -MG against sessile population as well as their efficacy to treat biofilm-mediated infections.

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#### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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