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Complete List of Authors:	Parai, Debaprasad; University of Kalyani, Department of Microbiology Islam, Ekramul; University of Kalyani, Department of Microbiology Mitra, Jayati; Regional Institute of Ophthalmology, Department of Pathology Mukherjee, Samir Kumar; University of Kalyani, Department of Microbiology
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Authors: Debaprasad Parai¹, Ekramul Islam¹, Jayati Mitra² and Samir Kumar Mukherjee^{1*}

Address: ¹Department of Microbiology, University of Kalyani, Kalyani 741235, India.

²Department of Pathology, Regional Institute of Ophthalmology, Kolkata 700073 India.

Email addresses:

Debaprasad Parai: debaprasad.bio@gmail.com

Ekramul Islam: ekramul.rs@gmail.com

Jayati Mitra: tanmayermail@gmail.com

Samir Kumar Mukherjee: dr.samirmukherjee@gmail.com

***Corresponding Author:** Prof. Samir Kumar Mukherjee

Address for correspondence: Department of Microbiology, University of Kalyani, Kalyani 741235, India.

Email: dr.samirmukherjee@gmail.com

Tel: +91-9433136617

Fax: +91-3325828282

Effect of Bacoside A on growth and biofilm formation by *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Debaprasad Parai¹, Ekramul Islam¹, Jayati Mitra² and Samir Kumar Mukherjee^{1*}

¹Department of Microbiology, University of Kalyani, Kalyani 741235, India.

²Department of Pathology, Regional Institute of Ophthalmology, Kolkata 700073, India

Abstract

The goal of this study was to evaluate the antibiofilm and antimicrobial activities of Bacoside A, a mixture formulation of phytochemicals from *Bacopa monnieri* against *Staphylococcus aureus* and *Pseudomonas aeruginosa* which are known to form biofilm as one of their virulence traits. The antimicrobial effects of Bacoside A were tested using the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays. Cell membrane disruption assay was performed to find its possible target site. MTT assay, crystal violet assay and microscopic studies were performed to assess the antibiofilm activity. Bacoside A showed its antimicrobial activity against both the test organisms in their planktonic and biofilm states. It could significantly remove ~88–93% bacterial biofilm developed on microtiter plate at sub-minimum inhibitory concentration of 200 µg mL⁻¹. Biochemical and microscopic studies suggested that the eradication of biofilm might be due to the loss of extracellular polymeric substances and change in cell membrane integrity of the selected bacterial strains treated with Bacoside A. These results indicate that Bacoside A might be considered as an antimicrobial having ability to disrupt biofilms, thus it could alone or in combination with other therapeutics be useful to treat biofilm related infections caused by opportunistic bacterial pathogens.

Keywords: Bacoside A; Biofilm eradication; Extracellular polymeric substances; *Staphylococcus aureus*; *Pseudomonas aeruginosa*

Introduction

In most natural environments, microbes are commonly found in close association with surfaces and interfaces in the form of multicellular aggregates glued together with the slime they secrete. Biofilms are such clusters of microbial cells that are attached to a substratum like rock surfaces, living and dead plant or animal tissues, and synthetic polymers, ceramics and metal alloys by creating a protective matrix with extracellular polymeric substances (Stoodley et al. 2002; Hall-Stoodley et al. 2004). The mature biofilm could provide shelter by aggregating the bacteria, and thus protect them against desiccation, starvation and mechanical dispersion (Ahimou et al. 2007; Flemming et al. 2007). Extracellular polymeric substances (EPS) account the major proportion (~50–90%) of the total organic matter in a biofilm matrix (Vu et al. 2009). It has been suggested that EPS matrix protects bacteria from phagocytosis and opsonisation, and also prevents the access of antibiotics to the bacterial cells of the biofilm community and thus play a crucial role in human health (Costerton et al. 1999; Stephens 2002; Høiby et al. 2010). In fact, biofilm-associated bacterial infections are estimated to comprise more than 60% of all bacterial infections (Lewis 2001). Among the bacteria that are found to develop biofilm as one of the virulence factors, the Gram-negative bacterium *Pseudomonas aeruginosa* and Gram-positive bacterium *Staphylococcus aureus* have received most attention. *P. aeruginosa* is the most frequent cause of severe chronic infection in patients with cystic fibrosis whereas *S. aureus* are the most frequent causes of nosocomial infections on indwelling medical devices (Donlan 2001; Balasubramanian et al. 2013).

Due to the inappropriate use of conventional antibiotics and development of multidrug resistant (MDR) bacterial pathogenic strains, interest in natural products, notably phytochemicals, as alternative therapeutic agents is growing up (Cowan et al. 1999; Abreu et al. 2012). The major subclasses of phytochemicals are phenolics, alkaloids, terpenoids and

other essential oils constituents, lectins and polypeptides, and polyacetylenes (Cowan 1999; Newman et al. 2000). These groups are well-documented for their antimicrobial properties when present in curcumin, reserpine, resveratrol, quercetin, andrographolides etc. (Hu et al. 2013; Dusane et al. 2014; Su et al. 2014; Wen et al. 2014).

Bacopa monnieri is a common herb found in tropical countries and has a long history as a medicinal plant (Singh and Dhawan 1997). Extract of *B. monnieri* has been reported to contain mainly alkaloids, tetracyclic triterpenoids, flavonoids, saponin, bacosides and phytosterols (Roodenrys et al. 2002). *B. monnieri* extract showed a variety of medicinal effects against anxiety, gastrointestinal discomfort, skin disorders, epilepsy, pyrexia and analgesia (Aguiar and Borowski 2013). It could be easily predicted that extract of *B. monnieri* might have enormous versatility of medicinal implementation though very little significant work has been done so far on its antimicrobial activity (Khan et al. 2010; Mathur et al. 2010; Emran et al. 2015). Bacoside A, known to be the main bioactive constituents of *B. monnieri* extract, is a mixture of bacoside A3, bacopaside II, bacopasaponin C and bacopaside X, all are triterpenoid saponins having jujubogenin or pseudojujubogenin as their aglycone moiety (Ramasamy et al. 2015). Terpenoids have been reported to result in membrane disruption by interfering with the lipid moiety, thus alter membrane permeability which leads to the death of the treated bacteria (Trombetta et al. 2005; de León and Moujir 2008; Jasmine et al. 2011).

Considering the above background this article mainly focuses on the antimicrobial activity of Bacoside A on *P. aeruginosa* MTCC 2488 and *S. aureus* MTCC 96, two moderately pathogenic strains, targeting its effect on biofilm control.

Materials and methods

Materials

Nutrient Broth (NB), Brain-Heart Infusion (BHI) broth, Congo red and crystal violet dye were purchased from HiMedia, India. Sterilized flat bottomed 24 and 96 wells microtitre

plates were procured from Tarsons, India. Absolute ethanol, HPLC grade methanol and glacial acetic acid were bought from Merck, India. Paraformaldehyde, Bacoside A, Dimethyl sulfoxide (DMSO) and 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich, India. Circular glass coverslips (diameter 12 mm; thickness 0.13–0.17 mm) and 0.2 μm RanDisc[™] syringe filters were bought from Blue Star©, India and Rankem, India, respectively.

Bacterial strains and culture conditions

Bacterial strains, *Staphylococcus aureus* MTCC 96 and *Pseudomonas aeruginosa* MTCC 2488 were obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India. Both these strains were featured as human pathogen and were of biosafety level 2 strains according to U.S. Public Health Service Guidelines. *S. aureus* MTCC 96 and *P. aeruginosa* MTCC 2488 were cultured in Brain-Heart Infusion (BHI) and Nutrient Broth (NB), respectively, at 37°C in an incubator with 180 rpm mechanical shaking (MaxQ 6000, Thermo Scientific, Ohio, USA) to obtain 16 h grown cell suspension.

Minimum inhibitory concentration of Bacoside A

MIC of Bacoside A was determined against *S. aureus* MTCC 96 and *P. aeruginosa* MTCC 2488 following a standard microdilution protocol based on Clinical and Laboratory Standards Institute [CLSI] guidelines (Jorgensen and Ferraro 2009). Bacterial suspension of 20 μL ($\sim 5 \times 10^5$ colony forming units [CFU] mL^{-1}) was inoculated in a 96 wells microtitre plate containing 200 μL medium including varying concentrations (50–600 $\mu\text{g mL}^{-1}$) of Bacoside A separately in each well and grown at 37°C for 24 h with 100 rpm shaking. Positive control was prepared with cells in media without Bacoside A and negative control was set only with sterile broth, both having same proportions of 1% DMSO (v/v). Growth responses of the bacterial strains were determined after 24 h by measuring the optical density at 600 nm in a microtitre plate reader (Multiskan® EX, Thermo Scientific, Finland). MIC

was defined as the lowest concentration of the antimicrobial compound that inhibited bacterial growth ($OD_{600} < 0.05$) after 24 h of incubation at specified temperature. The incubated broth from each well that showed no growth was further inoculated onto agar plate without any antimicrobial and incubated at same temperature for 24 h to determine the minimum bactericidal concentration (MBC) by drop method. MBC was defined as the lowest concentration that killed test bacteria. The MIC of Bacoside A for the test bacterial strains was calculated from three independent experiments, each having three replicas.

Bacterial time-kill curves of Bacoside A

Time-kill curves were obtained after treating *S. aureus* MTCC 96 and *P. aeruginosa* MTCC 2488 cells at their mid-log phase ($OD_{600} = 0.4$) with $200 \mu\text{g mL}^{-1}$ Bacoside A. The concentration of $200 \mu\text{g mL}^{-1}$ was chosen as a sub-lethal concentration of Bacoside A after determining the MIC and MBC from the previous experiment. Untreated cell culture only with same proportions of 1% DMSO (v/v) was considered as control. Optical density of the culture was taken at 60 min intervals in a UV-Vis spectrophotometer (Evolution 201, Thermo Scientific, Austria). The viable cell number was calculated by counting the number of bacterial colonies developed on the plate and expressed as CFU mL^{-1} .

Membrane damage assay

To study the effect of the phytochemical on the bacterial cell membrane integrity, the membrane damage assay was performed by estimating the amount of nucleic acids released from the cell (Chen and Cooper 2002). Both bacterial strains were cultured in the respective liquid media as mentioned earlier by taking a loopfull of inoculum from the agar plate and incubated overnight. The culture was centrifuged at $5600 \times g$ for 10 min, then the supernatant was discarded and the cell pellet was washed with 0.01 mol L^{-1} phosphate buffer saline (PBS, $\text{pH} = 7.4$) solution. Cell pellet was then re-suspended in respective liquid media in a way to set the optical density to 0.8 at 420 nm and the different concentrations ($50 \mu\text{g mL}^{-1}$, $100 \mu\text{g}$

mL^{-1} and $200 \mu\text{g mL}^{-1}$) of Bacoside A was added to bacterial suspension. Control set was without Bacoside A, but with 1% DMSO (v/v) as mentioned earlier. Two mL of sample was removed from each tube at 30 min intervals and immediately sieved with $0.2 \mu\text{m}$ syringe filters to discard the bacterial cells. The released amount of DNA and RNA in the filtrate was measured at 260 nm in a UV-Vis spectrophotometer (Evolution 201, Thermo Scientific, Austria).

Antibiofilm activity of Bacoside A

Scanning electron microscopy

The effect of Bacoside A on the biofilm architecture was visualized by SEM images. Glass coverslips as biofilm substratum were sterilized with NaOCl solution (0.5%, v/v) and rinsed thoroughly with sterilized Milli-Q grade water. To initiate biofilm development, sterilized glass coverslips were immersed in 24 wells plate containing 1 mL of respective growth media. Media was inoculated with the respective bacterial cell suspension ($\text{OD}_{600} = 0.1$) and incubated for 24 h at 37°C (Goswami et al. 2014). The developed bacterial biofilm were subsequently treated with $200 \mu\text{g mL}^{-1}$ of bacoside A and were incubated for another 24 h at 37°C under static condition. Control set was the biofilms treated only with same proportions of 1% DMSO (v/v). After 24 h of treatment, the media from the well was aspirated and the glass coverslips were washed with sterile PBS. Coverslips were then fixed with 2% freshly prepared depolymerized paraformaldehyde for 30 min. Ultrapure paraformaldehyde powder was dissolved in PBS (0.01 mol L^{-1}) and was subsequently heated at 55°C adding few drops of 10 N sodium hydroxide to get a clear solution of depolymerized paraformaldehyde. The coverslips were washed twice with PBS (0.01 mol L^{-1}) and dehydrated with increasing concentration series of ethanol (50%, 60%, 70%, 80%, 90% and 100%) for 10 min in each concentration (Kwieciński et al. 2009). Images were captured by Environmental Scanning Electron Microscope (EVO[®] LS 10, Zeiss, Germany) and analyzed

by smartSEM Graphical User Interface software. The imaging conditions were with 5–10 kV acceleration potential, 10–15 mm working distance, 50–100 pA probe current, and 6000X magnification.

Biofilm quantification by crystal violet assay

Biofilm biomass was quantified following the method of Stepanović et al. (2007). Bacterial cells were grown for 16 h in respective media, and 200 μL culture of each bacterial strain was inoculated to 20 mL of media and incubated at 37°C. Diluted 200 μL bacterial cell suspension ($\text{OD}_{600} = 0.1$) from mid-log phase of that culture was then added to a sterile flat bottom microtiter plate and incubated for 24 h to allow biofilm formation. After 24 h of incubation the used media was discarded from the wells and the respective fresh media containing different concentrations of Bacoside A (50–200 $\mu\text{g mL}^{-1}$) were added to the wells maintaining three replicas for each treatment. Positive control was prepared with biofilm without Bacoside A and negative control was set only with sterile broth, both having same proportions of 1% DMSO (v/v). The plate was subsequently incubated at 37°C for another 24 h under static condition. The content of the each well was aspirated and wells were vigorously shaken with 200 μL PBS (0.01 mol L^{-1}) to wash out all non-adherent bacteria. The remaining attached bacteria were fixed with 200 μL of methanol for 15 min. The plates were then stained with 200 μL of 2% crystal violet for 5 min at room temperature. Biofilm matrices on the plate surface were slowly rinsed thrice by submerging the plate in a tub of sterile de-ionized water. The microtiter plate was left to dry at room temperature for 60 min by placing it upside down on a stack of tissue paper. The dye bound to the adherent cells was re-solubilized with 200 μL of 33% (v/v) glacial acetic acid and the solubilised dye was transferred to a new microtiter plate. Absorbance was measured at 570 nm in a microtiter plate reader (Multiskan® EX, Thermo Scientific, Finland). All assays were performed in triplicates.

Biofilm viability test by MTT assay

S. aureus MTCC 96 and *P. aeruginosa* MTCC 2488 were grown overnight in a sterile 96 wells microtiter plate to develop biofilm (Müsken et al. 2010) and then different concentrations ($50\text{--}200\ \mu\text{g mL}^{-1}$) of Bacoside A was added following the method as described in crystal violet assay. Positive and negative control sets were prepared as described earlier. The spent media was dispensed from the wells and washed with PBS to remove planktonic bacteria. The wells were air dried and $200\ \mu\text{L}$ fresh media with MTT reagent ($0.5\ \text{mg mL}^{-1}$) was added to each well of the microtitre plate and incubated in dark for 2 h at 37°C . After incubation the MTT solution was discarded and immediately $200\ \mu\text{L}$ of DMSO was added to the wells to solubilize the formazan crystals formed in the wells, mixed thoroughly and left for 15 min incubation period at 25°C (Saising et al. 2012). At the end of incubation the absorbance of the solution was measured at a wavelength of 570 nm using a microtiter plate reader (Multiskan® EX, Thermo Scientific, Finland). All assays were performed in triplicates.

Congo red staining for qualitative estimation of extracellular polymeric substances

S. aureus MTCC 96 and *P. aeruginosa* MTCC 2488 biofilm were grown overnight in sterile 24 wells plate containing circular glass coverslips as described earlier. The developed bacterial biofilm were subsequently treated with $200\ \mu\text{g mL}^{-1}$ of bacoside A and were incubated for another 24 h at 37°C under static condition. Biofilm treated only with 1% DMSO was considered as control. Coverslips were taken out of the wells and washed with PBS ($0.01\ \text{mol L}^{-1}$) to remove non-adherent substances or planktonic cells and were air dried. The cover slips were then stained with $20\ \mu\text{L}$ of 1% Congo red under dark for 30 min and observed under fluorescence microscope (Motic BA410, Hong Kong) at 490 nm (Goswami et al. 2014).

A confocal laser scanning microscope (Leica TCS SP8, Leica Microsystems, Germany) was used to acquire images of biofilms samples following a standard protocol (Jeon et al. 2009). Samples were prepared following the steps as mentioned in fluorescence microscopy. Images were recorded at an excitation wavelength of 488 nm and an emission wavelength of 600–630 nm by detection of the fluorescence from Congo red. Image stacks through Z axis were collected from random points of the biofilms and analyzed by Leica Application Suite X (LAS X) software to get a 3D view of the biofilm matrix.

Statistical analysis

Descriptive statistical analyses were performed using GraphPad Prism version 5.00 (GraphPad Software, San Diego, USA) considering each set as one treatment regime with three replications including respective control set. The data are presented as mean $\pm$ standard deviation (SD). Intergroup differences were calculated using analysis of variance (ANOVA). *P* value of < 0.05 was considered statistically significant.

Results

Determination of antibacterial activity of Bacoside A

Bacoside A was found to inhibit the growth of both strains within the concentration range of 100–600 $\mu\text{g mL}^{-1}$. It was observed that 100 $\mu\text{g mL}^{-1}$ ceased ~50% of the bacterial growth, and above 200 $\mu\text{g mL}^{-1}$ concentration, growth was completely ceased but killed ~90% of cells (Fig. 1a), which corroborated with earlier findings on *S. aureus* (Emran et al. 2015). Thus 200 $\mu\text{g mL}^{-1}$ was considered as the sub-MIC value for the further investigations. The MBC was recorded as 400 $\mu\text{g mL}^{-1}$ where not a single viable colony was obtained on agar plate. The time-kill curves of Bacoside A against both *S. aureus* MTCC 96 and *P. aeruginosa* MTCC 2488 are presented in Fig. 1b to show the cessation of growth of both the strains upon treatment.

Effect of Bacoside A on bacterial membrane

In many instances membrane serves as the architectural support for protecting the cell against any biocidal challenge (Chen and Cooper 2002). Thus, measuring the release of intracellular components would be a good parameter to assess damage in membrane integrity, if any. In this study, damage in membrane integrity for both the strains was increased with the increasing concentration of Bacoside A and its exposure time (Fig. 2). As a result, absorbance (A_{260}) of the released intracellular components, mostly nucleic acids, increased in the treated sets for both the bacterial strains.

Antibiofilm activity of Bacoside A

Scanning Electron Microscopic study

The control cells of *S. aureus* MTCC 96 showed matured biofilm with multilayered cells along with densely cell-cell attachment as visualized in SEM images (Fig. 3a). But the Bacoside A treated cells were found to lose such attachments and allocated into small discrete clusters (Fig. 3b). Evidence of significant eradication of *P. aeruginosa* MTCC 2488 biofilm was also established by the SEM images when treated with $200\text{ }\mu\text{g mL}^{-1}$ of Bacoside A. The treated cells showed shrinkage, apparent surface smoothness disruption and loss of cell-cell adhesion (Fig. 3d). On the other hand, control set showed organized cluster of pseudomonad cells (Fig. 3c).

Crystal violet and MTT assay for studying biofilm eradication

Crystal violet assay suggested that treatment of *S. aureus* MTCC 96 and *P. aeruginosa* MTCC 2488 with Bacoside A resulted in removal of biofilm formed by those strains (Fig. 4). Dispersion of biofilm increased with increasing Bacoside A concentration and it achieved ~90% in both the strains when treated with sub-MIC dose ($200\text{ }\mu\text{g mL}^{-1}$). Results from MTT assay showed that the percentage of viable bacterial cells within biofilm declined with increasing dose of Bacoside A (Fig. 5). It is significantly declined to ~10% in comparison to the positive control set for both the strains when treated with sub-MIC dose.

ANOVA analysis also suggested that both the data at 200 $\mu\text{g mL}^{-1}$ were significant ($P < 0.001$) compared to positive control.

Microscopic study of biofilm matrix

The function of the EPS matrix is to provide cohesion between bacterial cells, thus securing the formation of a well structured biofilm. Fluorescence images of Congo red stained biofilm showed a notable loss of EPS matrix network eventually the adherence both in *S. aureus* MTCC 96 and *P. aeruginosa* MTCC 2488 after Bacoside A treatment (Fig. 6). Similar results were found in CSLM images, which showed the changes in topography of the developed biofilm when treated with 200 $\mu\text{g mL}^{-1}$ of Bacoside A for both *S. aureus* MTCC 96 and *P. aeruginosa* 2488 (Fig. 7a-d). LAS X software analysis showed that the average thickness of *S. aureus* biofilm was higher than that formed by *P. aeruginosa* (Fig. 7a, c) in untreated regime, however, upon treatment remnant biofilm showed nearly same thickness ($\sim 2 \mu\text{m}$) for both the strains (Fig. 7b, d).

Discussion

Emergence of antibiotic resistance in planktonic bacteria and of their biofilm counterpart is currently guiding the interest of researchers towards alternative drug sources, mostly from immense natural diversity (Abreu et al. 2012; Taylor 2013). Primarily the secondary metabolites from the plants have shown the antimicrobial effects and are being used as a major source of drugs having minor or no side effects (Newman and Cragg 2012). In this study, Bacoside A has shown its potential as an antimicrobial and antibiofilm agent against two most opportunistic pathogenic bacteria entities of human concern, namely *S. aureus* and *P. aeruginosa*. The test phytochemical could eliminate a good percentage of bacterial biofilm of those selected bacterial strains from the biofilm substratum. Bacoside A could be considered as an effective antimicrobial agent as it gave the MIC value $< 400 \mu\text{g mL}^{-1}$ (Simões et al. 2009). Qualitatively SEM images suggested high eradication of bacterial

biofilm after treating with Bacoside A, which was quantitatively determined by crystal violet assay. MTT assay further established the mortality percentage of bacterial cells within a treated biofilm.

Triterpenoid saponin might have a role in alteration of membrane permeability when it comes in contact with bacterial membrane lipids and thus helps to create a channel for the following treated drugs (Trombetta et al. 2005; Arabski et al. 2009). Here, damage of cell membrane might be due to the detergent-like activity of saponin present in Bacoside A, which was hypothesized by several earlier studies on antimicrobial and anti-biofilm properties of other plant saponins (Khanna et al. 2008; Sadowska et al. 2014; Ye et al. 2015).

An essential requirement to develop mature biofilm is cell-cell cohesion as well as high cell density (Costerton et al. 1999). In addition, EPS has a very important role in the biofilm-mode of bacterial life as it aids intense interaction and more persistency to the biofilm cells (Høiby et al. 2010). SEM, fluorescence microscopy and CLSM images revealed the loss of such phenomena including changes in cell morphology when the mature biofilms were treated with Bacoside A. Congo red staining of Bacoside A treated sample showed a clear evidence of biofilm dispersion due to loss of EPS. Earlier reports also suggested EPS production inhibition lead to eradication of bacterial biofilm (Pandit et al. 2015). So, Bacoside A might play another role through the loss of EPS component which eventually lead to the dispersion of bacterial biofilm. Such observations were also documented earlier with terpenoids and saponins against mature candida biofilm (Pires et al. 2011; Sadowska et al. 2014).

Thus it could be concluded that Bacoside A has both antimicrobial and antibiofilm effects on *S. aureus* MTCC 96 and *P. aeruginosa* MTCC 2488. This phytochemical might alter the bacterial cell membrane permeability and interact with intracellular components in planktonic stage. Within a mature biofilm it could detach and disperse bacterial cells from the

substratum. Although there are many reports available on the antimicrobial properties of plants extracts, very little research has been done so far on the antibacterial and antibiofilm activities of Bacoside A. This is the first report on the antibiofilm activity of Bacoside A against such opportunistic bacterial pathogens. Considering the activity of Bacoside A against pathogenic bacterial strains, it shows a promise in the perspective of new antibacterial drug development.

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Legends of Figures

Fig. 1. Antibacterial effect of Bacoside A on planktonic cells of *S. aureus* MTCC 96 and *P. aeruginosa* MTCC 2488. (a) MIC of Bacoside A after 24 h of incubation; growth responses presented as a measure of OD value at 600 nm at different concentrations. Data are the mean of three replicas with $\pm$ SD. (b) Time-kill curve [viable cell number versus treatment time] of the test bacterial strains treated at mid-log phase with 200 $\mu\text{g mL}^{-1}$ Bacoside A and compared with untreated cells. Data are the mean of three replicas with $\pm$ SD bars. * = $P < 0.05$; *** = $P < 0.001$; ** = $P < 0.01$; *** = $P < 0.001$: significant compared with respective controls.

Fig. 2. Effect of Bacoside A on cell membrane. Release of intracellular materials, targeting nucleic acids, from treated cells of (a) *S. aureus* MTCC 96 and (b) *P. aeruginosa* MTCC 2488 with the function of time as a measure of absorbance at 260 nm. In both cases cells were treated with different concentrations of Bacoside A. Data are the mean of three replicas with $\pm$ SD bars. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$: significant compared with respective controls.

Fig. 3. Scanning electron microscopy (SEM) images of biofilm. (a) Control and (b) treated biofilm of *S. aureus* MTCC 96; (c) Control and (d) treated biofilm of *P. aeruginosa* MTCC 2488. For both the strains, treatment was done with 200 $\mu\text{g mL}^{-1}$ Bacoside A for 24 h and control biofilms were incubated with 1% DMSO for 24 h. Scale bar represents 2 μm .

Fig. 4. Effect of Bacoside A on bacterial biofilm as measured by crystal violet assay. Biofilm eradication percentage compared to respective positive control in (a) *S. aureus* MTCC 96, and (b) *P. aeruginosa* MTCC 2488. In both the strains, developed biofilms were treated with different concentrations of Bacoside A for 24 h or 1% DMSO as positive control. Data are the mean of three replicas with $\pm$ SD bars. *** = $P < 0.001$: significant compared with respective controls.

Fig. 5. Viability assay of preformed biofilm cells treated with Bacoside A. Percentage of cell viability compared to respective positive control, (a) *S. aureus* MTCC 96 and (b) *P. aeruginosa* MTCC 2488. The developed biofilms were treated with different concentrations of Bacoside A for 24 h or 1% DMSO as positive control; cell viability was determined by MTT assay. Data are the mean of three replicas with $\pm$ SD bars. ** = $P < 0.01$; *** = $P < 0.001$: significant compared with respective controls.

Fig. 6. Congo red stained fluorescence microscope images of biofilm. The biofilm was treated for 24 h either with 200 $\mu\text{g mL}^{-1}$ Bacoside A or 1% DMSO as control and then the

biofilm matrix was stained with 1% Congo red. (a) Control and (b) treated biofilm of *S. aureus* MTCC 96; (c) Control and (d) treated biofilm of *P. aeruginosa* MTCC 2488. Scale bar represents 25 μm .

Fig. 7. CLSM of bacterial biofilm before and after Bacoside A treatment. The developed biofilm was treated for 24 h with 200 $\mu\text{g mL}^{-1}$ Bacoside A or 1% DMSO as control and then the biofilm matrix was stained with 1% Congo red for CLSM image analysis. (a) Control and (b) treated biofilm of *S. aureus* MTCC 96; (c) Control and (d) treated biofilm of *P. aeruginosa* MTCC 2488. All four images have two separate views- oblique 3D view (shown in upper part) and vertical view (shown in lower part).

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Fig. 1.

127x192mm (600 x 600 DPI)

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Fig. 2.

95x142mm (600 x 600 DPI)

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Fig. 3.

166x148mm (300 x 300 DPI)

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