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Antibacterial activity of copper(II) complexes against *Staphylococcus aureus*

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Abstract

Biofilm formation on medical implants is very difficult to overcome, since the bacteria in this form resists host defense mechanism and antibiotic therapy. What is needed is the development of an antibiofouling agent which will prevent the formation of biofilm especially on periprosthetic implants. In this study, the antibiofilm forming potential of two copper(II) complexes namely, $[Cu(bitpy)(dmp)](NO_3)_2$ (**1**) and $[Cu(bitpy)_2](ClO_4)_2$ (**2**) were studied against *Staphylococcus aureus* MTCC – 7443, a soil isolate. From the preliminary investigations, it became clear that the *anti-staphylococcal* activity of complex **2** was better than complex **1** because of the damage the complex caused at membrane level by inhibiting the expression of some extracellular proteins responsible for biofilm formation. Further the antibiofilm forming nature of complex **2** was confirmed by biofilm susceptibility test, SDS – PAGE and microscopic imaging techniques. Complex **2** can therefore be used as one of the antibiofilm forming agent to curb the formation of biofilm on medical implants.

Keywords: *Staphylococcus aureus* MTCC – 7443, Copper(II) complexes, Biofilm, Extracellular proteins

1. INTRODUCTION

Staphylococcus aureus, a gram positive cocci occurring in grape like clusters are ubiquitous and is the most common causative agent of localized supperative lesions in humans. Most strains of *Staphylococcus* possess resistance to β–lactam drugs such as Methicillin, which enhances their importance as human pathogen especially in hospital environment. The cocci causes major problems in food sector by contaminating food products [1], also it is responsible for indwelling device associated infections with biofilm formation on solid supports [2, 3]. Bacterial biofilm is a group of bacterial cells colonizing a surface or substratum with the help of self-produced matrix of extracellular polymeric substances (EPS) [4] which consists of macromolecules such as polysaccharides, proteins, humic substances and uronic acid, collectively known as exopolysaccharides or extracellular polymeric substances (EPS) [5]. Bacteria growing in biofilm escape host immune defense mechanism and are more resistant to antimicrobial agents than their planktonic counterparts [2]. Bacterial anchorage to a suitable surface or substratum is one of the crucial steps in biofilm formation, followed by the production of extracellular polymeric substances (EPS) [6]. The adhesive role of EPS on the cell surface has been studied in gram positive bacteria like *Staphylococcus aureus*.

Designing molecules, which is of biological interest, is the area of research in the field of bioinorganic chemistry. It was reported in the literature that metal complex supersedes organic ligands in their biological activities. [7] Therefore, proper design of ligands and also chelation with suitable metal ions are expected to improve the biological efficacy. Among the various organic ligands benzimidazole (bzim), 1,10-phenanthroline (phen) and their derivatives find potential application as antitumor, antiamoebic, antihistaminic, anthelmintic, antiulcer, antifungal, anticancer and antihypertensive agents [8-14]. It is also known that Cu(II) complexes

of phen, substituent phen/terpyridine (terpy) possess antimicrobial activity and have been employed as antimicrobial agents [15,16]. In this context mixed ligand copper(II) complexes of terpy/phen derivatives have been synthesized, which showed potential anti-proliferative activities towards various cancerous cell lines [17,18]. The objective of this work is to study the antibacterial activity and antibiofouling nature [19] of the two synthesized copper(II) complexes (Complexes **1** and **2**) possessing benzimidazolyl and phenanthroline derivatives and their antifouling activities against *Staphylococcus aureus*. Complex **1** is a mixed ligand complex (bitpy-dmp) and is coordinatively unsaturated. Complex **2** on the other hand, is a bis bitpy complex and is coordinatively saturated. As a result, complex **1** may coordinatively bind to either protein or DNA. On the other hand, complex **2** can only have non-coordinative interaction with protein or DNA. The aim of the study is to understand the effect of these structural differences between the two complexes on their antibacterial and antibiofilm forming activities.

2. MATERIALS AND METHODS

2.1 Materials

Bacterial culture, *Staphylococcus aureus* MTCC – 7443 a soil isolate was obtained as freeze dried powder from microbial type culture collection (MTCC, Chandigarh, India). Growth media (Nutrient broth, Nutrient agar and Luria berteni broth), antibiotic disc and plain paper disc were obtained from Hi–Media (Mumbai, India). Polystyrene 12 well tissue culture plates, Alamar blue and *o*-nitro phenyl-β-D-galactoside (ONPG) were purchased from Biogene, Bangalore, India. All other chemicals and reagents used were of analytical grade obtained from Sigma Aldrich.

92 *2.2 Synthesis of Copper(II) Complexes*

93 *2.2.1 Synthesis of [Cu(bitpy)(dmp)]$(NO_3)_2$.3$H_2$0 (**1**)*

94 The complex **1** was synthesized as per the reported procedure [20]. It was prepared by
95 stirring a methanolic solution of $Cu(NO_3)_2 \cdot 3H_2O$ (0.12 g, 0.5 mmol) with bitpy (0.15 g, 0.5
96 mmol) under room temperature for 15 minute. Subsequently, to the above solution dmp (0.12 g,
97 0.5 mmol) was added and continued stirring for another 15 minute. The reaction mixture was
98 then set aside for slow evaporation. A green solid that separated out upon slow evaporation of
99 the solvent was filtered, and washed with diethyl ether and dried in vacuum. The complex
100 $[Cu(bitpy)(dmp)](NO_3)_2$ was recrystallized from acetonitrile. The authenticity of the complex
101 was confirmed by ESI-Mass spectrometer. Found: C, 67.32; H, 4.68; Cu, 10.05; N, 15.05. Anal
102 Calcd: for $C_{36}H_{29}CuN_7O$: C, 67.64; H, 4.57; Cu, 9.94; N, 15.34.

103 *2.2.2 Synthesis of complex [Cu(bitpy)$_2$]$(ClO_4)_2$. H_2O (**2**)*

104 The complex **2** was synthesized according to the reported procedure [20]. A methanolic
105 solution (50 mL) of $Cu(ClO_4)_2.6H_2O$ (0.18 g, 0.5 mmol) and bitpy (0.35 g, 1 mmol) was refluxed
106 for 30 minute. A green solid that separated out upon slow evaporation of the solvent was filtered,
107 and washed with diethyl ether and dried in vacuum. The complex was recrystallized from
108 acetonitrile-water solution. The authenticity of the complex was confirmed by ESI-Mass
109 spectrometer. Found: C,52.92; H, 3.31; N,14.13. Anal Calcd for $C_{44}H_{34}Cl_2CuN_{10}O_{10}$: C, 52.99
110 %; H, 3.44 % N, 14.05 % .

111 *2.2.3 Culture condition and growth profile*

112 Bacterial strain, *Staphylococcus aureus* MTCC – 7443 obtained as freeze dried powder
113 was transferred to Nutrient broth (Peptone – 5 g/L, Yeast extract – 2 g/L, Beef extract – 1 g/L,
114 and Sodium Chloride – 5 g/L) and incubated at 37 °C for 16-18 h in a shaker. A loop full of

culture from the nutrient broth was streaked onto nutrient agar plate (Peptone – 5 g/L, Yeast extract – 2 g/L, Beef extract – 1 g/L, Sodium Chloride – 5 g/L, and Agar – 15 g/L) and incubated overnight at 37 °C. Plates were refrigerated and used for further studies. The growth pattern of *Staphylococcus aureus* was monitored by increasing the concentration of complex **1** (5, 15, 25, 35, 45, 55, 65, 75, 85 and 95 µg/mL) and complex **2** (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 µg/mL). Bacterial inoculum was prepared by transferring a colony from the agar plate to the nutrient broth and incubated at 37 °C overnight in a shaker. The turbidity of the inoculum at 600 nm was adjusted to 0.1 using Shimadzu UV-160A UV-Visible spectrophotometer. 0.1 mL of bacterial inoculum was added to tubes containing 3 mL of nutrient broth and complexes **1** and **2** were added in varying concentrations and incubated at 37 °C for 24 h. The absorbance of the solution at 600 nm was measured spectrophotometrically.

2.2.4 Anti – Staphylococcal activity of copper complexes

Complex **1** and complex **2** were tested for their anti–*Staphylococcal* activity by Disc diffusion method [21]. Bacterial inoculum was prepared by adjusting the turbidity at 600 nm to 0.5. The agar plates were streaked evenly by dipping sterilized cotton swab into the inoculum and the plates were allowed to dry for 3–5 min. Paper discs (plain and antibiotic impregnated) were placed carefully over the agar plate using flamed forceps. Each complex of 20 µL volume was pipetted out and loaded over the plain disc. The plates were incubated at 37 °C for 24 h and the zone of inhibition was measured.

Further the complexes were tested for the MIC (Minimum inhibitory concentration) by broth dilution method using 12 well polystyrene tissue culture plate [22]. The wells containing NB broth were inoculated with the same inoculum used for measuring the zone of inhibition. Complex **1** of 344 µg/mL concentration was added to the first well of the plate 1 and was serially

diluted until it reached to a concentration of 10.75 µg/mL. In the same manner complex **2** of 124 µg/mL concentration was added to well 1 of plate 2 and was serially diluted to a concentration of 3.87 µg/mL. The plates were incubated at 37 °C for 24 h and the absorbance was measured at 600 nm using Tecan Infinite M 200 Elisa reader. The MBC (Minimum bactericidal concentration) was recorded by spot inoculating nutrient agar plate using plate 1 and 2 used for MIC measurements. The nutrient agar plates were incubated at 37 °C for 24 h and the MBC was recorded as the concentration which resulted in no growth of the bacterium.

2.2.5 Membrane damage assay

The effect of complexes on the integrity of cell membrane was analyzed by measuring the A_{260} value of the intracellular material that gets released upon interaction with the complex [23]. Bacterial inoculum was prepared by transferring a loopful of culture from the nutrient agar plate to the nutrient broth and incubated overnight at 37 °C. The culture was centrifuged at 10000 rpm for 10 min, harvested, washed and resuspended in 0.01 mol L^{-1} PBS solution. Finally the absorbance of the solution was adjusted to 0.7 at 420 nm. Complexes of 1.5 mL of varying concentration were added to 1.5 mL of bacterial inoculum. The release of intracellular material at different time interval was recorded by measuring the absorbance at 260 nm using Shimadzu UV-160A UV-Visible spectrophotometer.

2.2.6 Inner membrane permeabilization assay

In this assay the release of cytoplasmic β – galactosidase (Enzyme produced by live bacteria when it encounters the substrate ONPG - *o*-nitro phenyl-β-D-galactoside in the culture medium) was measured [24]. The bacterial inoculum was prepared by harvesting the log phase bacteria grown in nutrient broth containing 2 % lactose. The harvested cells were washed and resuspended in 0.01mol L^{-1} PBS solution and the absorbance of the cell suspension was adjusted

to 1.2 at A_{420}. Complexes (1.6 mL) with varying concentration were added to 1.6 mL of bacterial inoculum. To this 150 µL of 30 mM ONPG was added and mixed well. Increase in A_{420} indicates, the production of *o*–nitrophenol over time which was recorded using electronic absorption spectra.

2.2.7 Biofilm susceptibility test by Alamar blue method

This test was performed in 96-well non-tissue culture treated microtitre plate. The wells of the titer plate were filled with nutrient broth and inoculated using bacterial culture (0.1 absorbance at 600 nm) so that the final volume comes to 100 µL. Plates were incubated at 37 °C for 24 h without shaking. After incubation 50 µL of the suspension was discarded from all the control and test wells and 50 µL of the complex of varying concentration was added (Complex **1** – 15, 35, 55, 75, 95, 115 µg/mL) and Complex **2** – 10, 15, 20, 25, 30, 35 µg/mL). The plates were incubated at 37 °C for 24 h with shaking. After incubation 5 µL of Alamar blue was added to the wells and the plate was incubated at 37 °C for 1 h with gentle shaking. Absorbance at 570 and 600 nm was recorded using Tecan Infinite M 200 Elisa reader. Positive and negative controls were maintained along with the test [25]. The percent reduction of Alamar blue was calculated as follows

$$\%\ Reduction\ of\ Alamar\ blue = \frac{(\varepsilon_{ox})\lambda_2 A\,\lambda_1 - (\varepsilon_{ox})\lambda_1 A\,\lambda_2}{(\varepsilon_{red})\lambda_1 A'\,\lambda_2 - (\varepsilon_{red})\lambda_2 A'\lambda_1} \times 100$$

Here, ε_{ox} = Molar extinction coefficient of Alamar blue in oxidized form (blue)

ε_{red} = Molar extinction coefficient of Alamar blue in reduced form (pink)

A = Absorbance of test wells

A′ = Absorbance of negative control well

λ_1 = 570 nm

λ_2 = 600 nm

ε_{ox} = 117216 at 600 nm and 80586 at 570 nm

ε_{red} = 14652 at 600 nm and 155677 at 570 nm

2.2.8 DNA Isolation and Binding

To isolate the bacterial DNA the nutrient medium (Luria Bertani broth – Casein enzymic hydrolysate – 10 g/L, Yeast extract – 5 g/L, sodium chloride – 10 g/L) was inoculated and incubated at 37 °C for 24 h. 2 mL of bacterial culture was aspirated and centrifuged at 10,000 rpm for 10 min, the supernatant was discarded. To the pellet 0.5 mL of lysis buffer (100 mM Tris pH – 8.0, 50 mM EDTA, 50 mM lysozyme) 0.5 mL of saturated phenol was added and incubated at 55 – 60 °C for 10 min in a water bath. After incubation, the tubes were centrifuged at 10,000 rpm for 10 min and the supernatant was carefully aspirated. To the supernatant equal volume of chloroform: isoamyl alcohol (24:1) and 1/20th volume of 3 M sodium acetate (pH 4.8) was added, centrifuged at 10,000 rpm for 10 min. The supernatant was collected and 3 volume of chilled ethanol was added to precipitate the DNA, which was collected by centrifugation. This was dried and dissolved in tris buffer (10 mM tris pH – 8.0) and refrigerated for further use [26].

DNA cleavage was analyzed by agarose gel electrophoresis. Copper(II) complexes **1** and **2** were dissolved in DMSO and were added to the bacterial DNA. This was incubated at 37 °C for 2 h, after incubation bromophenol blue dye was added and the test and control samples were loaded carefully into the wells. This was electrophoresed for 30 min and the stained gel was illuminated under UV lamp and gel documented.

2.2.9 Extracellular Protein assay and Detection of Biofilm protein – SDS PAGE

The effect of copper(II) complexes on biofilm formation was investigated from the ECP of *S. aureus*. The bacterium was treated with three different concentration of complex **1** (15, 35,

and 55 µg/mL) and complex **2** (15, 35 and 55 µM/mL) and incubated for 24 h. The extracellular proteins were isolated by centrifuging the 24 h old culture at 10000 rpm for 10 min. The supernatant was collected and the presence of extracellular proteins was calculated by Bradford method [27]. For protein gel analysis copper(II) complex treated and untreated ECP of *S.aureus* was electrophoresed at 180 V on a 12 % (W/V) polyacrylamide gel using standard protocol [28]. Protein bands were visualized using coomassie brilliant blue G – 250.

2.2.10 SEM and Confocal Microscopy

The effect of copper(II) complexes on the biofilm architecture of *S. aureus* was visualized by scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). Six well tissue culture plate containing 13 mm glass coverslip was used for biofilm formation. To each well of the plate, culture medium (Nutrient broth) was added and inoculated with an inoculum of 0.1 absorbance at 600 nm. Complexes of varying concentrations were added to the test wells and the plate was incubated statically at 37 °C for 24 h. After incubation the medium was carefully aspirated and the wells along with the coverslips were washed twice with 0.01 mol L^{-1} PBS. For SEM, the biofilm adhered to the coverslip were fixed with 2 % gluteraldehyde in 0.01 mol L^{-1} PBS for 30 min, then washed twice with 0.01 mol L^{-1} PBS and dehydrated using graded ethanol series for 10 min [29]. After critical point drying, the coverslip was sputter coated and examined using FEI Quanta 200 Environmental Scanning Electron Microscope (SEM). For CLSM, the coverslip was stained with 100 µL of acridine orange (0.01 % W/V) for 10 min in dark at room temperature. After staining the coverslip was gently washed twice with 0.01 mol L^{-1} PBS, dried and observed using confocal laser scanning microscope with Leica confocal software for three dimensional imaging and biofilm intensity measurements [30].

2.2.11 Statistical Analysis

A Kruskal-Wallis test was carried out to check the significant difference among the concentration levels of metal complexes. The software used was SPSS version 22.

3. RESULTS AND DISCUSSION

3.1. Growth profile

The growth profile of *S. aureus* MTCC – 7443 was monitored for 24 h spectrophotometrically in the absence and presence of varying concentration of complexes. From the Fig. 1, it could be seen that as the concentration of the complexes increases, absorbance measured at the wavelength of 600 nm showed a clear decline. Out of the two complexes tested for their inhibitory potential, complex **2** at a concentration of 8 µg/mL was able to inhibit the bacterial growth to the maximum, whereas complex **1** was able to inhibit the bacterial growth to maximum at a higher concentration of 25 µg/mL. This indicates that complex **2**, was able to inhibit the bacterial growth at a minimum concentration than complex **1**, which is a mixed ligand copper(II) complex, which showed growth inhibition at higher concentration. From the growth profile pattern results of the complexes tested it is seen that complex **2** which is a bis ligand copper(II) complex possesses good inhibitory potential at a minimum concentration than complex **1** which is a mixed ligand copper (II) complex. The nature of the ligand is known to influence the redox property of the metal complex, which is one of the parameters that influence the toxicity of the metal complex towards microbes and cells [31]. The ligands associated with a metal complex also determine the DNA as well as protein binding ability of the metal complex [32]. Ligands also determine the ability of metal complexes to bind or rupture cell membranes [33]. Hence, the better inhibitory property of complex **2** than that of complex **1** may be attributed to the nature of ligands coordinated to the central metal ion in this complex. It is of interest to

note that the nature of the ligand coordinated to the metal ion has been shown to have significant effect on the aggregation of protein [34].

3.2 Antistaphylococcal activity

Growth inhibition by Disc diffusion method was carried out for complex **1**, complex **2** and commercial antibiotics. In this method, the zone of inhibition or clearance of bacterial growth around the disc impregnated antibiotic/complex was monitored. The zone of inhibition for complexes **1** and **2** at a concentration of 18 µg/20µL and 12 µg/20 µL was found to be 11.5 mm and 18.9 mm, respectively (Table 1). The minimum inhibitory concentration (MIC) value for complex **1** and **2** was found to be 40 µg/mL and 12 µg/mL. The minimum bactericidal concentration (MBC) was calculated from the MIC recorded culture plate by spot inoculation on nutrient agar plate, which was kept for overnight incubation. After incubation the MBC was noted as the concentration, which showed no visible growth on the agar plate, and it was found to be 172 µg/mL for complex **1** and 62 µg/mL for complex **2**. Complex **2** at a relatively lower concentration was able to inhibit the growth of *S. aureus, which* is evident from the results obtained with zone of inhibition, MIC and MBC. These results along with the growth profile pattern revealed complex **2** to be a better antibacterial agent at lower concentration compared to complex **1**.

3.3 Integrity of cell membrane

Membrane damage was studied by measuring the absorbance of the intracellular compounds at 260 nm that gets released upon cell membrane damage caused by the copper(II) complex upon interaction with the bacteria [23]. Complex **1** treated bacterial suspension showed an increase in the A_{260} value initially for a time period of 90 min then followed a decline with increase in time of exposure (Fig. 2a) whereas complex **2** treated bacterial suspension showed an

increase in A_{260} value with an increase in the concentration and time of exposure (Fig. 2b). For complex **1**, the A_{260} values for 120 min of exposure time were found to be 0.066, 0.168 and 0.163 at 35 µg/mL, 45 µg/mL and 65 µg/mL of the complex, respectively. Complex **2** caused more damage to the bacterial cell membrane by giving an A_{260} value of 0.282, 0.421 and 0.483 at 5 µg/mL, 15 µg/mL and 25 µg/mL of the complex for an exposure time of 120 min. Complex **2** can have better interaction with the cell membrane of the bacteria because the ligand in this complex posses free N and NH groups, which can form hydrogen bonds with the peptidoglycan layer of the bacteria. This may be the reason for the better *staphylococcal* membrane damaging activity of this complex than complex **1**, which is in agreement with the antistaphylococcal activity results [35].

3.4 Permeabilization assay

In this assay the release of cytoplasmic β – galactosidase upon interaction with the copper(II) complexes was studied. Interaction with copper complexes makes the bacterial cells permeable and the cell permeability was measured by providing a chromogenic substrate o-nitrophenyl-β-D-galactoside (ONPG) into the medium. The release of cytoplasmic β-galactosidase was assayed by measuring hydrolysis of the chromogenic substrate, o-nitrophenyl-β-D-galactoside (ONPG) to o-nitrophenol. The amount of o-nitrophenol (yellow colour) formed can be measured by determining the absorbance at 420 nm. This is an indirect measurement of cell permeability with respect to the release of β – galactosidase enzyme. It was found that complex **1**, upon interaction with the bacterial suspension showed an increase in the release of β – galactosidase with the maximum being released after 2 h (Fig. 3a). The release of the enzyme for complex **2** was also found to be maximum after 2 h of interaction, but found to be very less when compared to complex **1** (Fig. 3b). Increase in the release of the enzyme indicates an

increase in the permeability of the inner membrane. In this case complex **1**, with an increase in the concentration showed an increase in the release of the enzyme, making the bacterial membrane permeable to its entry than complex **2**. Complex **1** can coordinatively bind to the cell membrane. On the other hand complex **2**, which is coordinately saturated, cannot bind coordinatively to the cell membranes. This molecule can enter inside the cell through passive diffusion. This difference between the two complexes on their action on the cells may be responsible for their effect on the permeabilization of the cell membrane. Results were analyzed using Kruskal-Wallis test and were found to be significantly different as P value was less than 0.05.

3.5 Biofilm Susceptibility

The susceptibility of the bacterial biofilm to varying concentration of the complex was studied by calculating the percentage reduction of Alamar blue spectrophotometrically. When observed visually wells containing low concentration of the complex appeared pink and with high concentration the wells remained purple in colour. Complex **1** of maximum concentration (115 µg/mL) gave a percentage reduction of 66 (Fig. 4), percentage reduction of 13 was obtained with complex **2** of maximum concentration (35 µg/mL). From the % reduction values obtained for different concentration of complexes **1** and **2**, it could be concluded that the complex **2** at a concentration of 35 µg/mL and above can be used as an antibiofouling agent to control the formation of biofilm.

3.6 DNA Cleavage

DNA Cleavage activity of complex **1** and **2** was studied by agarose gel electrophoresis method. On comparing with the control (Fig. 5, lane 4) the DNA cleavage efficiency of complex **1** is partial at a concentration of 10 µg/mL (lane 3) and at a higher concentration of 20 µg/mL

(lane 2) the mobility of the genomic DNA was retarted, due to fair permeabilization of the complex into the cell. Complex **2** at a concentration of 5 µg/mL was not able to cleave the DNA (lane 1), this could be attributed due to poor permeabilization and DNA binding of the complex. It has been shown previously that complex **1** brought about DNA cleavage when treated with plasmid DNA, whereas complex **2** brought about DNA condensation [18, 20].

3.7 Extracellular protein assay and SDS - PAGE

The supernatant from the untreated and complex treated biofilm samples were analyzed for the extracellular proteins by Bradford method and lyophilized for analyzing the proteins by SDS – PAGE. It was found that complex **1** treated culture suspensions were able to express extracellular proteins in higher amounts than complex **2** treated suspensions (Table. 2). From the Fig. 6 it could be seen that the expression of protein pattern of complex **1** treated bacterial suspension at a concentration of 35 µg/mL (lane 5) is similar to that of the control (lane 3). With higher concentration of 55 µg/mL, only few proteins were expressed (lane 2). For complex **2** treated bacterial biofilm suspension, expression of most of the proteins was inhibited at a concentration of 35 and 55 µg/mL (lane 4 and 1). The reason for this is that complex **2** possesses good membrane damaging potential, thereby preventing the expression of most of the extracellular proteins responsible for biofilm formation.

3.8 Scanning Electron Microscopy

The damage caused by the complexes to the bacterial cell membrane was further analyzed by SEM. On comparing the morphology of the control cells (Fig. 7a) with cells treated with 80 µg/mL solution of complex **1**, it was found that the complex **1** treated cells showed changes in the morphology such as shrinkage of the cells due to partial damage to the cell wall (Fig. 7b). The cells treated with 60 µg/mL solutions of complex **2** showed huge alteration in the

morphology with irregular cell wall, as it is clearly seen from Figure 7c. And also the cells were not seen in clusters as it was seen in the control. From the images of complex treated bacterial cells it becomes evident that the mechanism of cell destruction could be membrane damage.

3.9 Confocal Microscopy

The biofilm architecture and the intensity of the metal complex treated and untreated samples were monitored by CLSM. It could be seen from Fig. 8a, that in the untreated control sample the biofilm formation was thick and dense, whereas samples treated with 80 µg/mL of complex **1** showed a slight decrease in the biofilm growth and thickness (Fig. 8b). This could be attributed to the fact that complex **1** can only cause minimal inhibitory effect on the extracellular proteins responsible for biofilm formation. In case of samples treated with 60 µg/mL of complex **2**, a huge difference in the growth and thickness of the biofilm was observed (Fig. 8c) which could be due to the interference of the complex with the extracellular proteins.

4. CONCLUSION

Antibiofouling activity of the two copper(II) complexes was studied with the biofilm forming bacteria *Staphylococcus aureus* MTCC - 7443, a soil isolate. From the preliminary investigations it could be concluded that the complexes **1** and **2** had an inhibitory effect on the growth of S. *aureus*, with complex **2** showing better antistaphylococcal activity than complex **1**. Complex **2** was found to be less permeable causing severe damage at the membrane level, leading to cell content leakage and ultimately kill the bacterium. Since the complex **2** was less permeable it had no effect on the DNA cleavage, but showed adverse effect on the expression of extracellular proteins responsible for biofilm formation as proteins are considered as the major determinants in monitoring the antibiotic efficacy against biofilm [21]. Complex **1** was found to be more permeable, with fair DNA cleavage activity at high concentration. As the complex **1**

was more permeable into the cell it caused less damage at the membrane level, allowing minimal expression of extracellular proteins responsible for biofilm formation. The Alamar blue biofilm susceptibility test and the microscopic studies reveal that complex **2** can be employed as an antibiofouling agent in implant associated infections after further study on the toxicity of the complex using *in-vivo* models.

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480 **Figure Captions**

481 Figure 1. Growth of *S. aureus* in the presence of varying concentration of complex **1** and **2**

482 Figure 2. Release of intracellular material absorbing at 260 nm from *S. aureus* suspensions
483 a) Treated with complex **1**, b) Treated with complex **2** at varying concentration
484 and time

485 Figure 3. Release of cytoplasmic β – galactosidase by *S. aureus* a) Treated with complex **1**
486 b) Treated with complex **2** at varying concentration and time

487 Figure 4. Percent reduction of Alamar blue by *S. aureus* biofilm treated at varying
488 concentration of complex **1** and **2**

489 Figure 5. DNA binding pattern of *S. aureus* untreated DNA (lane 4), DNA treated with
490 complex **1** of 10 µg/mL (lane 2); complex **1** of 20 µg/mL (lane 3) and complex **2**
491 of 5 µg/mL (lane 1)

492 Figure 6. SDS – PAGE of extracellular proteins (ECP) from *S. aureus* biofilm showing
493 protein bands for untreated biofilm (lane 3), biofilms treated with 35 and 55
494 µg/mL of complex **1** (lane 5 and 2) and complex **2** (lane 4 and 1)

495 Figure 7. SEM images (10000x) of *S. aureus* (a) Control (b) Complex **1** treated (c)
496 Complex **2** treated

497 Figuer 8. Confocal images of *S. aureus* (a) Control (b) Complex **1** treated (c) Complex **2**
498 treated

499 **Tables**

500 Table 1. Antibiotic sensitivity pattern of Complexes and commercial antibiotics for *S.aureus*

501 Table 2. Extracellular proteins (ECP) produced by *S. aureus*

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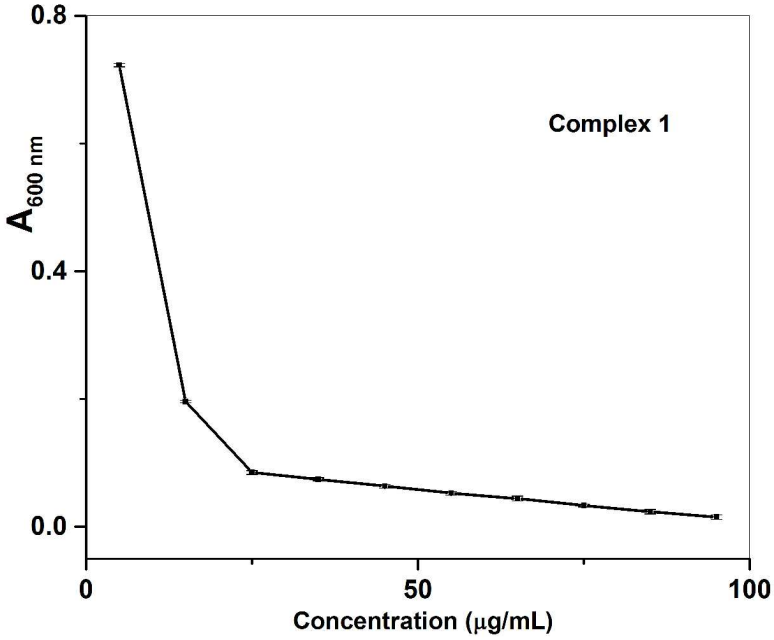

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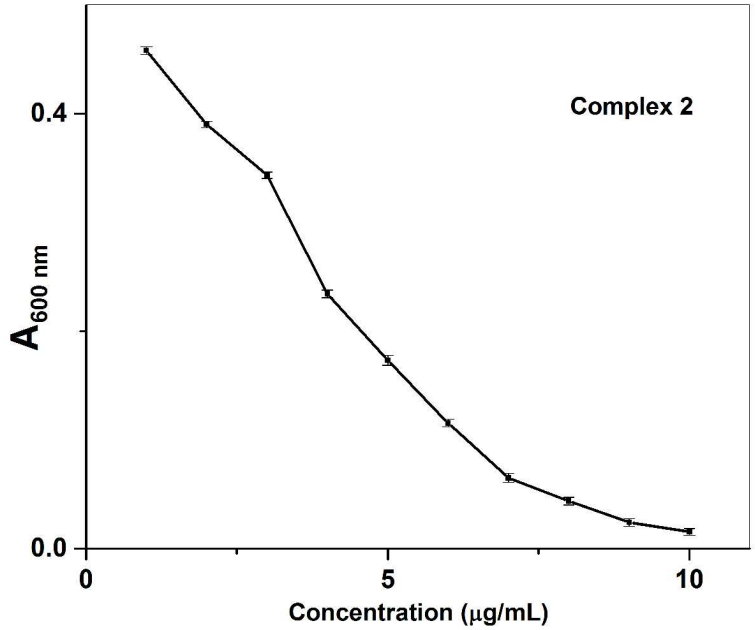

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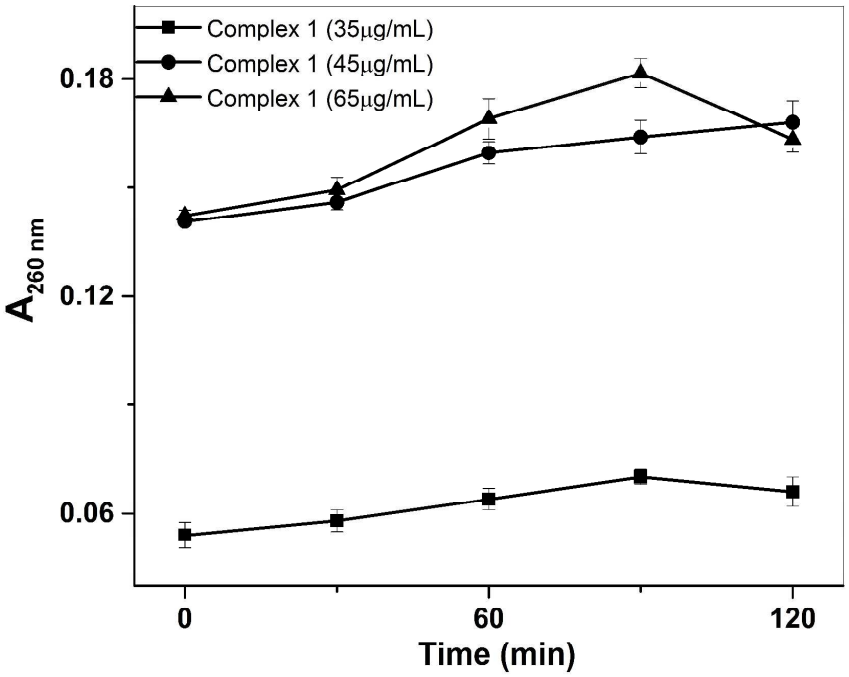
517 Results were found significantly different and analyzed using nonparametric anova.

518 **Figure 1.**



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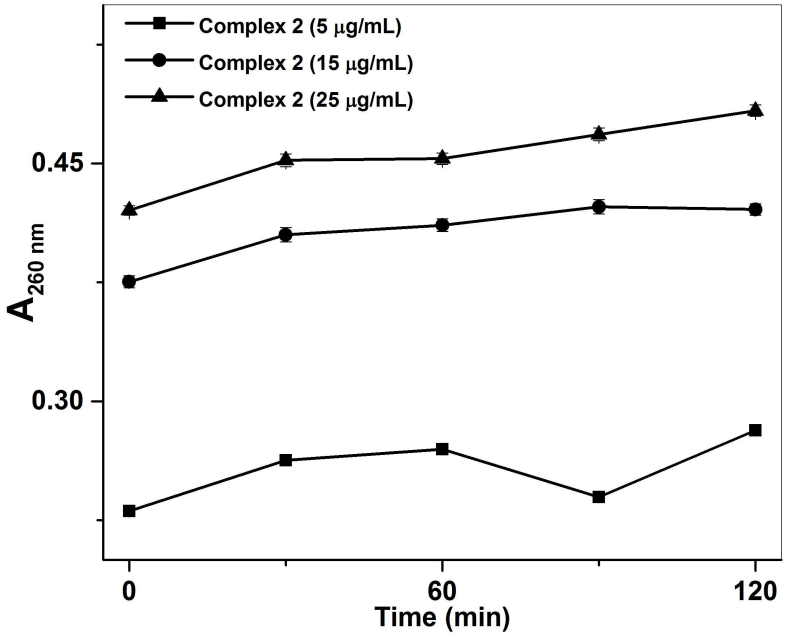

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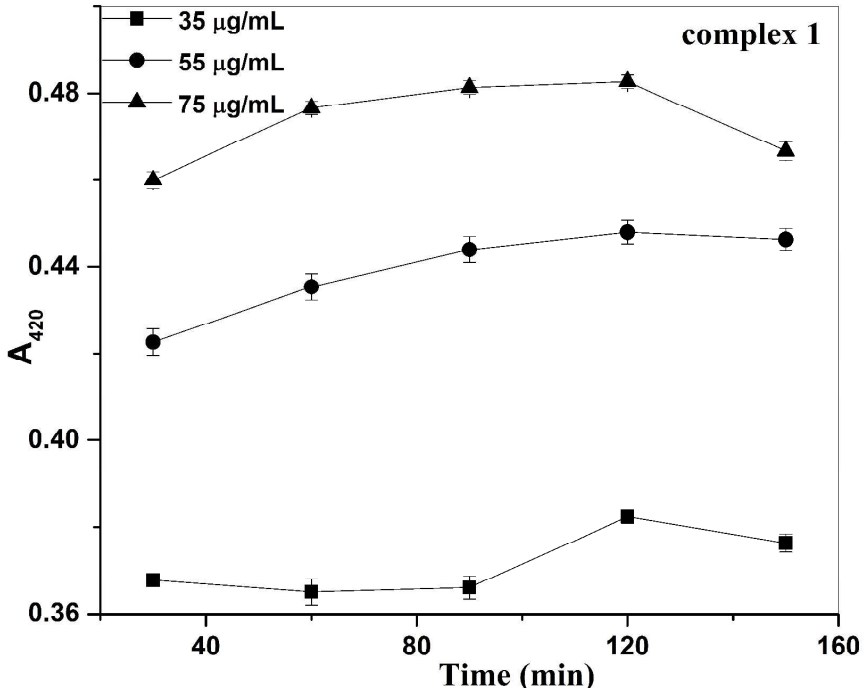
521 Results were found significantly different and analyzed using non parametric anova.

522 **Figure 2.**



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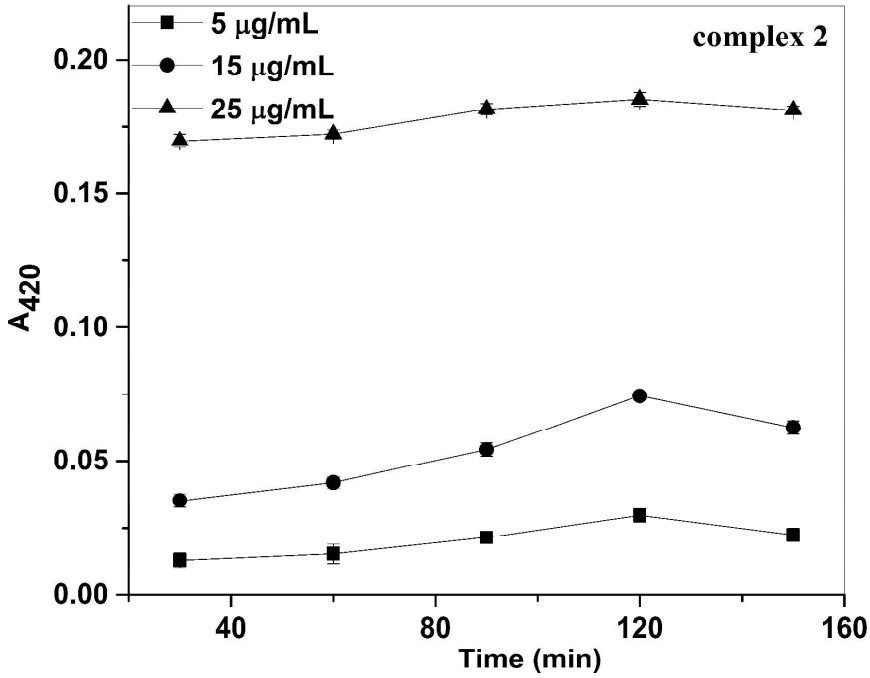

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526 **Figure 3.**

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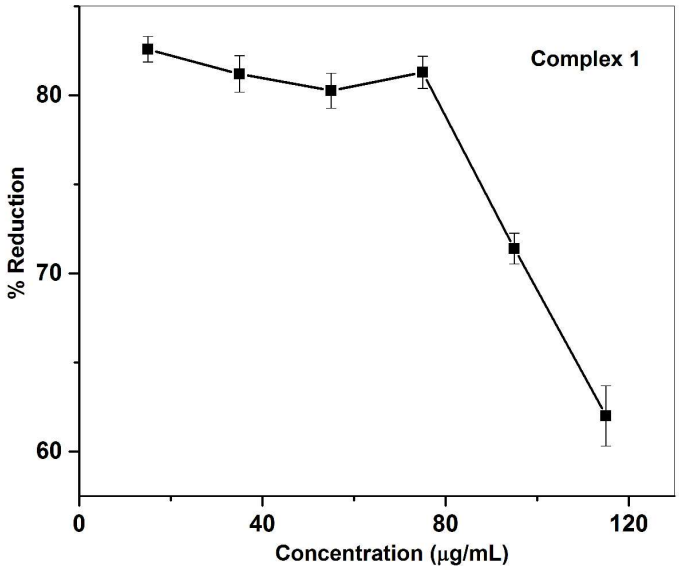

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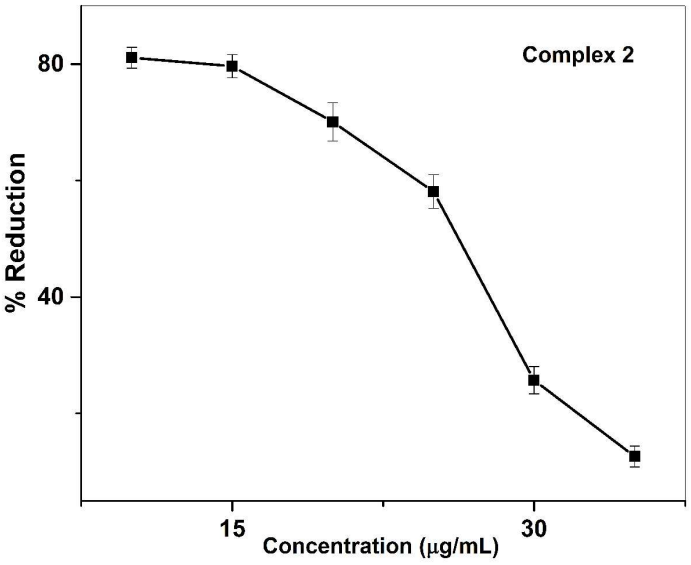

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530 **Figure 4.**

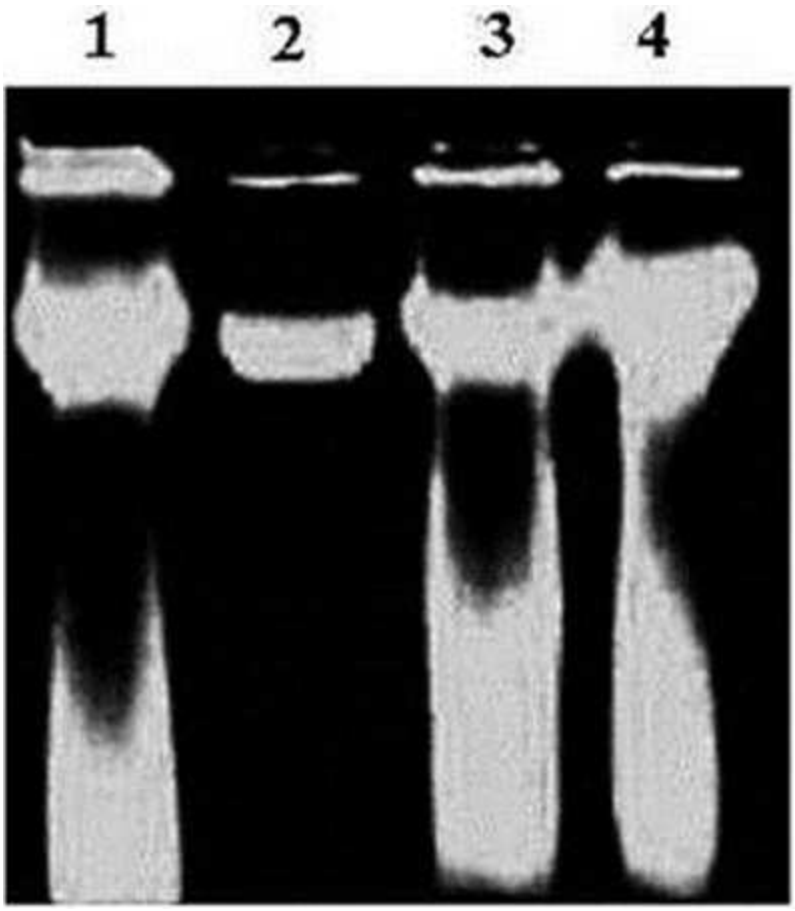
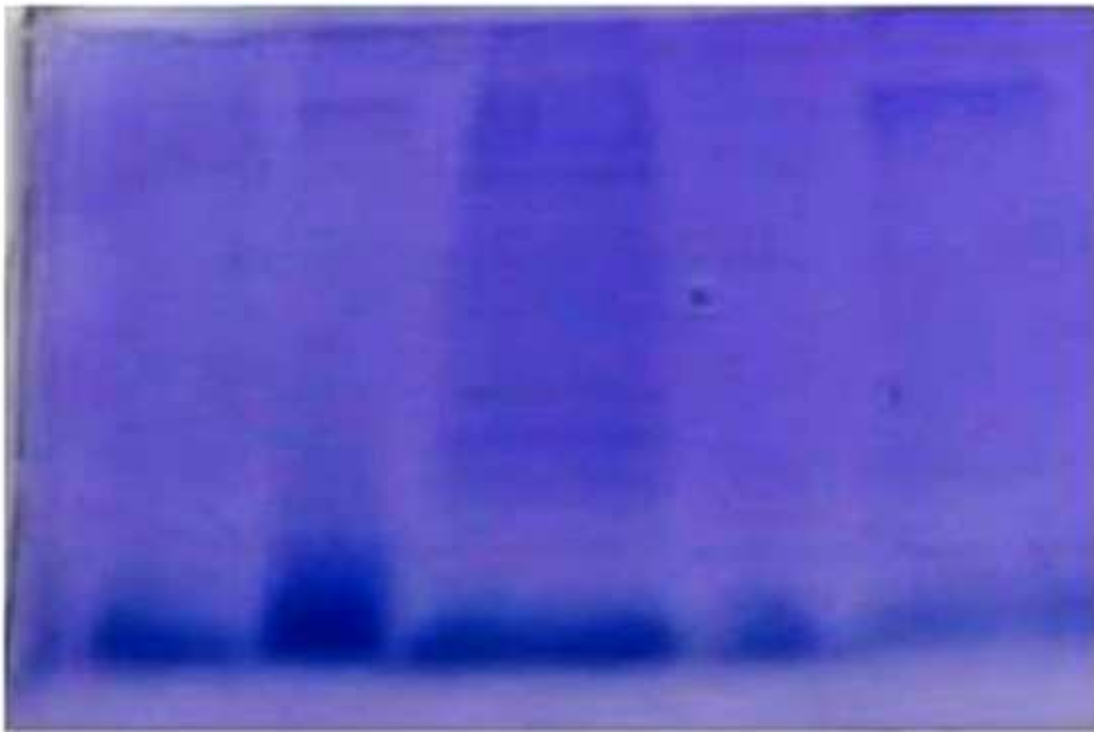


Figure 5.



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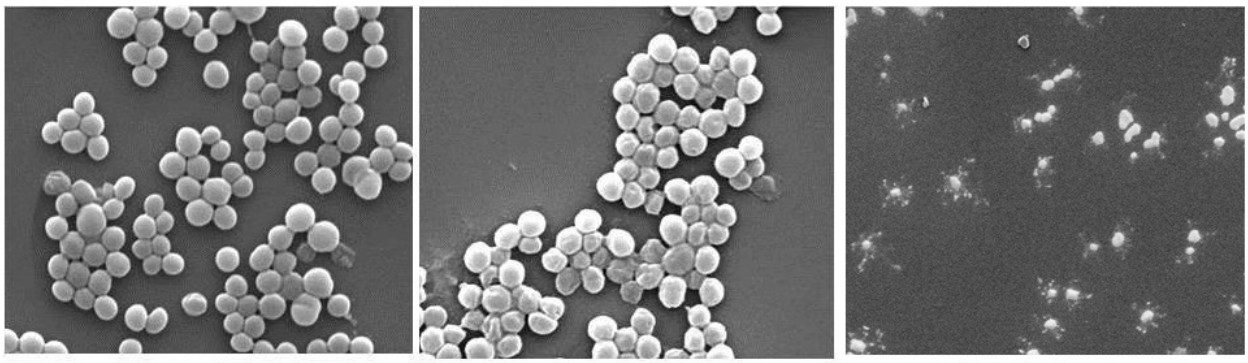

Figure 7.

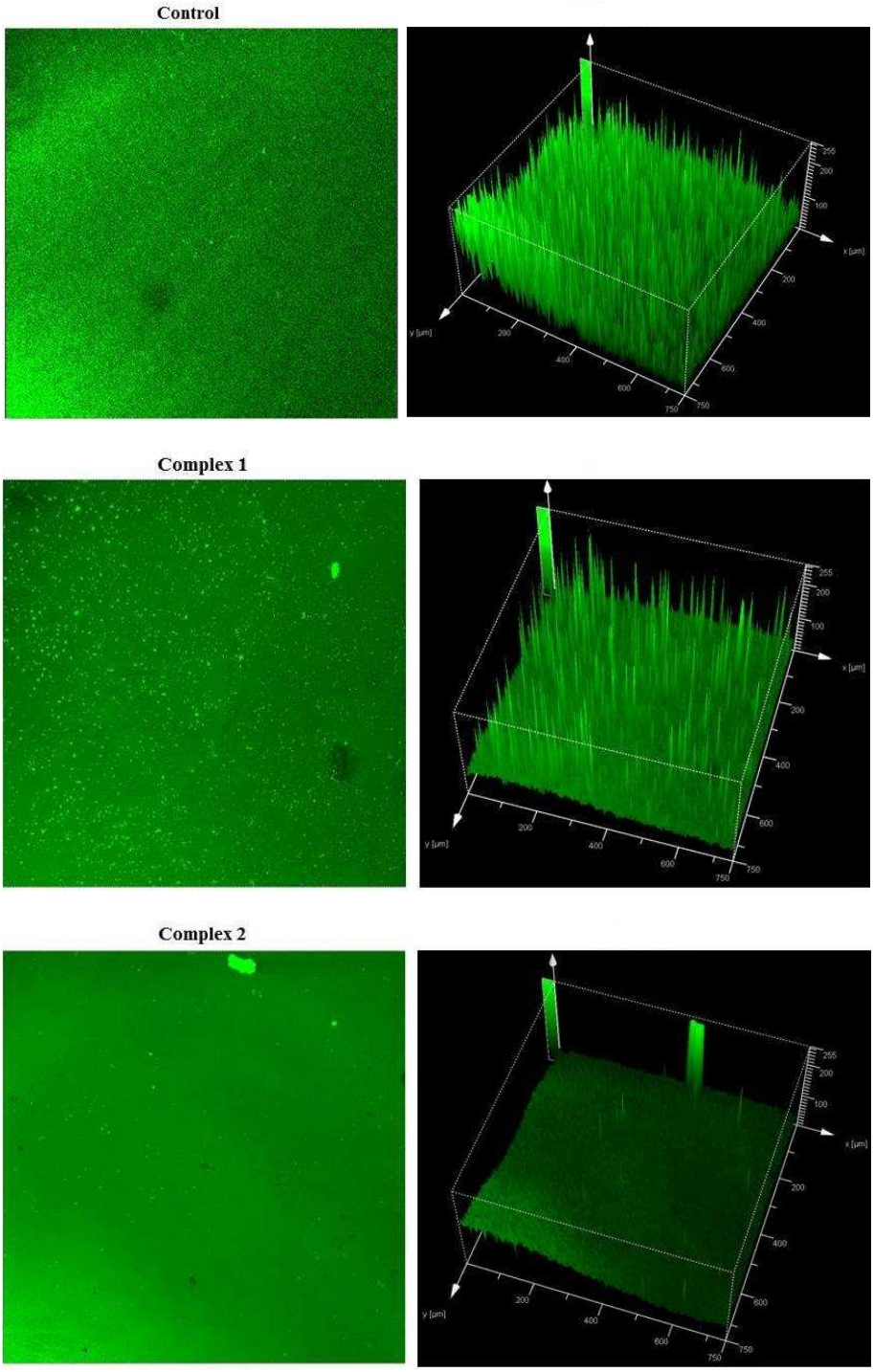

Figure 8

Table 1. Antibiotic sensitivity pattern of Complexes and commercial antibiotics for *S.aureus*

Antibiotic	Zone of Inhibition (mm)
Complex **1**	11.5 ± 0.5
Complex **2**	18.9 ± 0.2
Kanamycin	25.9 ± 0.2
Cephataxime	21.6 ± 0.5
Methicillin	12.7 ± 0.6
Ampicillin	21.8 ± 0.8

Table 2. Extracellular protein (ECP) produced by *S. aureus*

Concentration (µg/mL)	Extracellular Protein (ECP)	
	Complex 1	Complex 2
15	14.5 ± 0.39686	5.34 ± 0.33843
35	8.22 ± 0.41016	4.62 ± 0.25027
55	4.42 ± 0.26889	2.22 ± 0.39716

Synopsis for GA

- $[Cu(bitpy)_2]^{2+}$ showed better *antistaphylococcal* activity than $[Cu(bitpy)(dmp)]^{2+}$. SEM and confocal microscopy showed the damage caused by complex **2** is more effective than **1**. Complex **2** can be better used as anti-biofouling agent