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ELUCIDATION OF BIOFILM INHIBITION IN DIFFERENT CLINICAL ISOLATES USING NATURALLY ISOLATED COMPOUNDS FROM *ALBIZIA ODORATISSIMA*

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Abstract:

This study was planned to investigate phytochemical constituents were isolated from the plant Albizia odoratissima and its antibiofilm activity were analysed. Three flavone compounds namely Eupatorin (1),6-methoxy flavone (2),6,2',4'-trimethoxy flavone(3) isolated from the stem bark and structure of the compounds were established based on NMR prediction. Their were no biofilm results in all the three compounds.The antibiofilm activity was evaluated against three reference strains Pseudomonas aeruginosa,Klebseilla pneumonia, Staphylococcus epidermidis by microtitre plate method. Using above reference strains MIC and biofilm inhibition work were done by quantification and SEM. SEM images of the species biofilm inhibition confirmed compound 1,2 and 3 inhibits P.aeruginosa biofilm formation by 93.93%,95.04% and 94.72% at a concentration of 1ng/ml,1ng/ml and 1mg/ml. To compare the results compound 2 showed that greater biofilm inhibition activity against P.aeruginosa at very low concentration of (1ng/ml). The biofilm inhibition of P.aeruginosa of compound 2 on urinary catheter was evaluated using scanning electron microscopic analysis.

Key words: *Isolated flavones · Antibiofilm · Bacteria · SEM · Catheter*

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INTRODUCTION:

A wide variety of microbial infections in the body (~80%) are caused by bacterial biofilm. A collection of microbial communities enclosed by a matrix of extracellular polymeric substance (EPS),which separated by a network of open water channels is called biofilm. Microbial communities adhere to manmade and natural surfaces, such as metals and surgicares, typically at liquid-solid interface [1]. Major clinical importance because more than 60% of the bacterial infections currently treated by physicians in the developed world are considered due to biofilm formation (Fux et al. 2005) [2]. Biofilms have been recognized as being important inhuman disease, and the numbers of biofilm-associated infections are on the rise (Davies 2003) [3]. Different infections due to biofilm forming bacteria such as *Escherichia coli*, *Staphylococcus aureus*, *Klebseilla pneumonia*, *Staphylococcus epidermidis* in body tissues, leading to several diseases. In this work three biofilm forming pathogen such as *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Staphylococcus epidermidis* were used for biofilm inhibition studies. *Pseudomonas aeruginosa* is an opportunistic pathogen that forms biofilms on tissues and other surfaces and it is an important causative agent of a variety of acute and chronic infections, including burn, infections of respiratory tract, urinary tract, eye and ear [4]. Biofilm-associated diseases are common characteristic of *P. aeruginosa* infections is that they are caused by bacterial association with surfaces, either human tissue or indwelling devices [5]. Severe underlying diseases caused by opportunistic pathogen such as *Klebsiella pneumonia* that infects immune compromised patients who are hospitalized or suffering from chronic pulmonary obstruction or diabetes(Allen et al., 1991). These bacterial infections can lead to complications, including urinary tract infections, septicemia and pneumonia in the elderly or in patients with predisposing factors (Williams and Tomas, 1990). Infections due to *K. pneumoniae* are particularly devastating with a mortality rate between 25 and 60% (Ellis, 1998). *K. pneumoniae* is naturally present at low concentrations in the environment, but also in the gastrointestinal tract and natural cavities of humans, and constitute aggregates called biofilms. Furthermore, formation of biofilms by *K. pneumoniae* on urinary catheters and intravenous and prosthetic heart valves has been documentted (Farber and Wolff, 1993; Galdiero et al., 1987; Liu, 1993). This property is considered as an important virulence factor for *K. pneumoniae* (Jagnow and Clegg, 2003) [6]. *Staphylococcus epidermidis* is one of the most commonly isolated bacterial pathogens in hospitals and the most frequent cause of nosocomial infections [7, 8, 9].

S.epidermidis is part of the normal human epithelial bacterial flora but can cause infections when the skin or mucous membrane is injured. *S. epidermidis* can develop into biofilms and become a persistent source of device-associated infections [10]. *S.epidermidis* plays a major role in biofilm-based medical-device-related infections [11]. These bacterial communities pose a critical problem in everyday life, causing many economic and health problems. A biofilm on an indwelling urinary catheter consists of adherent microorganisms, their extracellular products, and host components deposited on the catheter. Catheter Associated Urinary Tract Infections (CAUTIs) are the most common form of hospital acquired infections - HAIs - with more than 560,000 cases/year estimated in the US alone CAUTI is also the leading cause of secondary bloodstream infections. Therefore, alternative sources of antimicrobial substances are required. Currently natural products are a major source of chemical diversity and have provided important therapeutic agents for many bacterial diseases [12] and natural compounds have no side effect and plant molecules are green and safe for prolonged use in the management of diseases.

Plants offer a rich source of antimicrobial agents and other pharmaceuticals (Cowan 1999; Zhao et al. 2005;Li & Vederas 2009). However, of the 500,000 plant species, only 1% has been phytochemically investigated from the perspective of antimicrobial activity (Cowan 1999; Palombo 2009)[13]. As an adaptive evolution, many plant species produce metabolites that can control the growth of microbes and have traditionally been used to treat human diseases, particularly microbial infections[14]. Polyphenols (especially flavonoids) from plants have been acting as potent antimicrobial molecules. Phenolic compounds have proven effective in inhibiting the growth and biofilm formation by pathogenic bacteria Flavonoids are a group of heterocyclic organic compounds that occur widely in the plant kingdom(Havsteen 1983). They are found in fruits, vegetables, nuts, seeds, stems, flowers as well as tea, wine (Middleton and Chithan 1993) and honey (Grange and Davey1990) and represent a common constituent of the human diet (Harborne and Baxter 1999).Many flavonoids that are ubiquitous in the plant kingdom are biologically active in combating diseases due to their diverse beneficial roles in humans.

Herein, for the first time, we have isolated three flavone compounds namely Eupatorin [15-22],6-methoxy flavone [23, 24],6,2',4'-trimethoxy flavone from the stems and stem bark of Albizia odoratissima plant even though it was already isolated by the few groups from different plant sources.

To our best of knowledge there are no reports available for antibiofilm activities of three flavone compounds.Therefore the present study was to investigate the isolation of three flavone compounds for the first time from the stem bark of Albizia odoratissima and also report the antibiofilm effect of active three flavones against three biofilm forming bacteria such as *Pseudomonas aeruginosa, Klebseilla pneumonia* and *Staphylococcus epidermidis* by Microtitre plate method.

MATERIALS AND METHODS:

The NMR spectra for^{1}H and ^{13}C in $CDCl_3$were recorded on a Bruker 300 spectrometer, with TMS. Electrospray ionization mass spectrometry (ESI-MS) analysis was performed in the positive ion mode on a liquid chromatography-ion trap mass spectrometer (LCQ Fleet, Thermo Fisher Instruments Limited, US).The samples were introduced into the ion source by in fusion method at flow rate 1µL/min. The capillary voltage of the mass spectrometer was 33 V, with source voltage 4.98 kV for the mass scale (m/z 150–2000).

Plant material

The stem of Albizia odoratissima was collected from Madurai kamaraj university, Madurai at August 2015. The stems were collected, washed with double distilled water and then dried at room temperature.

Extraction and isolation of compounds

Isolation of compound 1

The stems were collected, washed with double distilled water and then dried at room temperature. The dried stems of the plant(2kg)were first extracted with petroleum ether(2L)using soxhlet apparatus for 2 days. At first petroleum ether is used to remove wax from the plant material.Then extracted with chloroform (2L) for 2 days.The chloroform extract was evaporated to dryness under reduced pressure to yield a crude residue (10g).Its subsequent column chromatography on silica gel (60-120 mesh MERCK) eluted with Pet ether with increasing gradient of chloroform upto(100%) to gave 30 fractions that were monitored by TLC silicgel. The active fractions 13-15 were repeatedly chromatographed on silicagel coumn and then, eluted with chloroform: methanol (90:10) as the developing solvent .Fraction-13, 14 was obtained as a mixture of spot and fraction-15 gave single spot on TLC and it was further purified and recrystallised from methanol to yield yellow colour compound 1 (10mg) and it was characterized by ^{1}H NMR,^{13}CNMR,ESI-MS and FT-IR techniques.

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Isolation of compound 2:

The freshly collected,air dried and powdered stem bark(2kg) was exhaustively extracted with methanol(3x10 litres)for 3 days.The combined methanolic extract was evaporated under reduced pressure at room temperature to yield a crude residue(600g).The whole extract was suspended in water and successivey extracted with n-

Hexane(100g),chloroform(50g),ethyl acetate(70g).The chloroform soluble fraction was subjected to column chromatography over silica gel and eluted with pet ether,pet ether-choroform, chloroform, chloroform-methanol with gradient increase in polarity to yield 10 subfractions.The 8th subfractions were a binary mixture of two compounds that was rechromatographed over silicagel and eluted with pet ether-chloroform(8:2)to afford compound in pure form(20mg) and it was recrystallized from methanol to yield white colour needle shape compound obtained.

Isolation of compound 3:
The stem (2kg) were collected,dried at room temperature,grounded and then successively extracted with pet ether and chloroform. The chloroform extract was concentrated under reduced pressure to yield 100g of yellow viscous mass which was subjected to silic gel chromatography. The column was eluted with petether and then chloroform to give 5 fractions. Fraction 3 was separated by silicagel by eluting with $CHCl_3$-acetone (4:1) to give 4 subfractions. Subfraction 2 which was then purified on column eluting with $CHCl_3$-MeOH (6:1) gave 4 fractions. Fraction 3 contained a flavone which was further recrystallized from methanol, yielded compound 3 (10mg).

Compounds identification:
To determine the structure of this compounds was established by comparison of its spectral data with previous literature values.

Bacterial strains
Pseudomonas aeruginosa, Klebsiella pneumonia, Staphylococcus epidermidis stains were isolated from different biological sources and were used for further biofilm inhibition assays. Bacterial samples were cultured on the following media: Luria bertani (LB) agar/broth medium (HiMedia Laboratories, India).

Preparation of natural compounds
The naturally isolated compounds 1,2 and 3 were dissolved in dimethyl sulphoxide at a concentration of 1mg/ml.

Determination of minimum inhibitory concentration of natural compounds
To determine the minimum inhibitory concentration (MIC) of the natural compounds 1,2 and 3 was evaluated against three bacterial pathogens by the broth microdilution method (Basri and Fan, 2005). The stock solutions of the natural compounds were dissolved in DMSO (Initial concentration, 1mg/mL). The initial test concentration was serially diluted (µg/ml,ng/ml). Each well was inoculated with 200µl of suspension containing 10^6CFU/ml^1 of bacteria. The plates with bacteria were incubated at 37^{0}C for 24 hours. The lowest concentration exhibiting complete inhibition of the microbial growth was taken as the MIC. The tubes were visually examined for the lowest concentration of isolated compound that showed inhibition of microbial growth (indicated by a clear solution) after 24 h .The experiment was performed in duplicate. Bacterial suspensions were used as negative control, while broth containing standard drug(Ganamycin) was used as positive control.

Biofilm inhibition assay
Only those isolates were used in the biofilm-inhibition assay. Test compounds were dissolved in DMSO (1mg/mL), and twofold dilutions were prepared to obtain a final concentration ranging from (mg/ml,µg/ml,ng/ml) in the wells after the addition of the freshly diluted LB broth culture containing 10^6CFU/ml^1 of biofilm-forming isolates per well as shown in Fig. 1

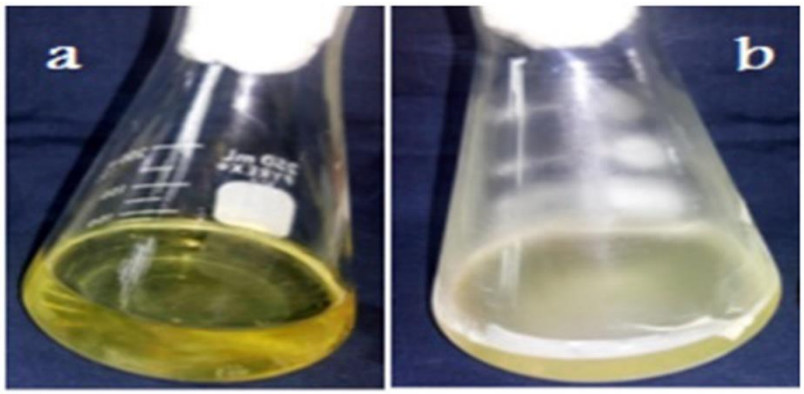

Fig. 1: a-Control (LB broth): b-Biofilm formation.

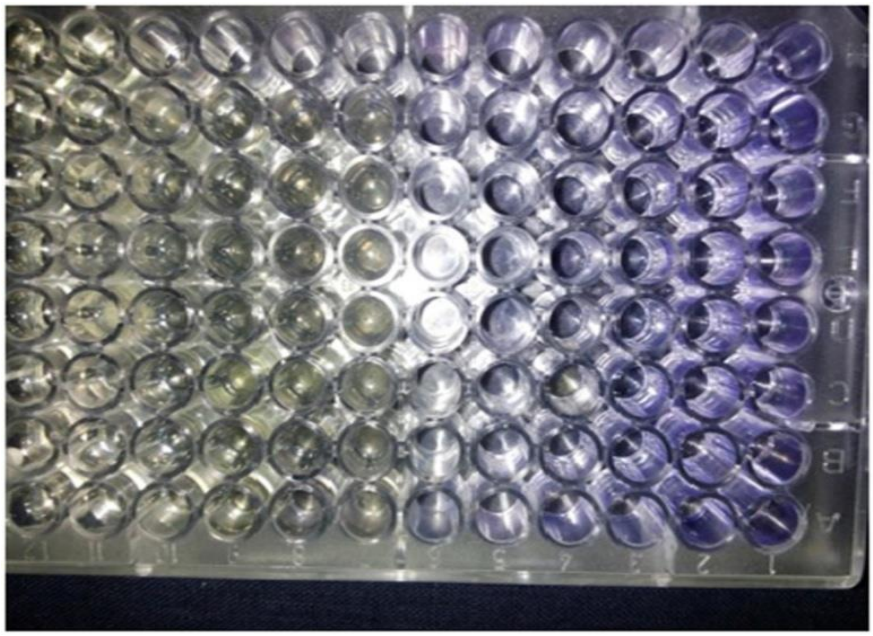

Fig. 2: Biofilm inhibition assay

After incubation at 37^0C for 24 hours, the planktonic suspension and nutrient solution were aspirated and each well was washed three times with 300 µL of sterile physiological saline. The plates were strongly shaken to remove all nonadherent bacteria. The remaining attached bacteria were fixed with 250 mL of 96% ethanol/well and, after 15minutes; the plates were emptied and left to dry. Each well was then stained for 5 minutes with 200µL of 2% crystal violet (CV Gramstain, Merck, Germany). The stain was rinsed off by placing the plates under running tap water. After drying the stained plates, biofilms were visible as purple rings on the sides of each well as shown in Fig. 2

The quantitative analysis of biofilm formation was performed by adding 200 µL of 33 %(v/v) glacial acetic acid (Merck) perwell. The optical density (OD) of the stain was then measured at 595 nm using an enzyme-linked immunosorbent assay reader (Elisa, Germany) as described previously. The inhibitory effect of the compounds on biofilm production was calculated by subtracting the media control. The biofilm inhibitory concentration (BIC) is the concentration of the natural compound at which the biofilm formation was reduced to an absorbance (A595) < 0.5 OD. Each assay for BIC determination was performed in triplicate.The percentage of biofilm inhibition was calculated by the formula [25]

Percentage of inhibition=([Control OD_{595} nm-Test OD_{570} nm]/Control OD_{595} nm)x100

Scanning electron microscopy

Scanning electron microscopy (SEM) was used to observe the architecture of *P.aeuroginosa* biofilms grown in the presence of compound 2 (0.5 × MIC concentration) and to evaluate the eradication of established biofilms treated with compound 2. Sample preparation of biofilms (24 h and preformed) were analyzed by Scanning Electron Microscopy (SEM) (VEGA-3, TESCON) follow-ing standard preparative techniques using CD1CN at 250mg/ml [26]. Briefly, biofilms were formed in 24-well microplates. Each sample was inoculated with a defined volume of overnight culture. Biofilms on the glass pieces were fixed for 2h in solution containing 40%glutaraldehyde. Further the glass pieces were washed with phosphate buffer saline(pH-7) and dehy-drated with alcohol (70% ethanol for 10 min, 95% ethanol for 10 min, and 100% ethanol for 20 min), and allowed to dry prior to gold sputtered.

The scanning electron microscopy images of different biofilm producers shows effective inhibition against three compounds using *P.aeruginosa*. High (MBIC) minimum biofilm inhibitory concentration inferred at ng/ml of all the compounds.To compare the two concentrations, only nano concentrations of compound 2 shows very high inhibition assay. Based on these results the compound-2 was taken for further real sample testing.

Biofilm formation by *P.aeruginosa* on catheter

Biofilms of *P.aeruginosa* were grown on small sterile catheter piece (surface area 1.0 cm2) cut from Foley catheter obtained from JIPMER, manufactured by SISCO latex Pvt.Ltd., (Pudhucherry). The material was sterilized with ethanol (70%) and rinsed with distilled water. Flat square pieces, 1.0 cm in diameter, were obtained by cutting with a sterile surgical knife, the protocol followed by Chandra et al.[27]. A 100µl quantity of the cell suspension was applied to the surfaces of 1.0 cm^2 catheter piece placed in a sterile petri plate. The cells were allowed to adhere for 90 min at 37^0C (adhesion phase). Non adherent cells were removed from the tube by gently washed with 5 ml of PBS.

Coating catheters with natural compounds

Polyurethane triple-lumen central venous catheters (20 cm long and 13 gauge) that had been pretreated with the cationic surfactant tridodecylmethylammonium chloride to enable subsequent bonding of natural compounds. The compounds were prepared with three different concentrations using DMSO. Bio-Guard catheters were dipped in ng/ml of all the compounds for 15 min and were then removed and allowed to dry overnight. Then the processed and fresh catheters were used for biofilm inhibition assay against *P.aeruginosa.*

Statistical analyses

Biofilm inhibition data was statisticaly analysed by using Graph pad prism software [28]. All the experiments were performed in duplicates and data are expressed as mean ± standard deviation. The results were analysed using two-way ANOVA. where homogeneity of variances was not met,the non parametric Dunnett's multiple comparison test was applied. Results were presented as the mean standard deviation and differences were accepted as significant for $p < 0.05$(5% of the significance level).

RESULTS AND DISCUSSION:

Biofilm production assay by microtitre plate test

The biofilm production assay shows higher production after 48h of incubation for three reference strains. Biofilm formation level was significantly more in *P.aeruginosa* when compare to *K.pneumonia* and *S.epidermidis*. *K.pneumonia* shows very low biofilm production while compare with other two strains. From this results biofilm inhibition assay was carried out using different natural compounds in different concentration levels as indicated in Fig. 3.

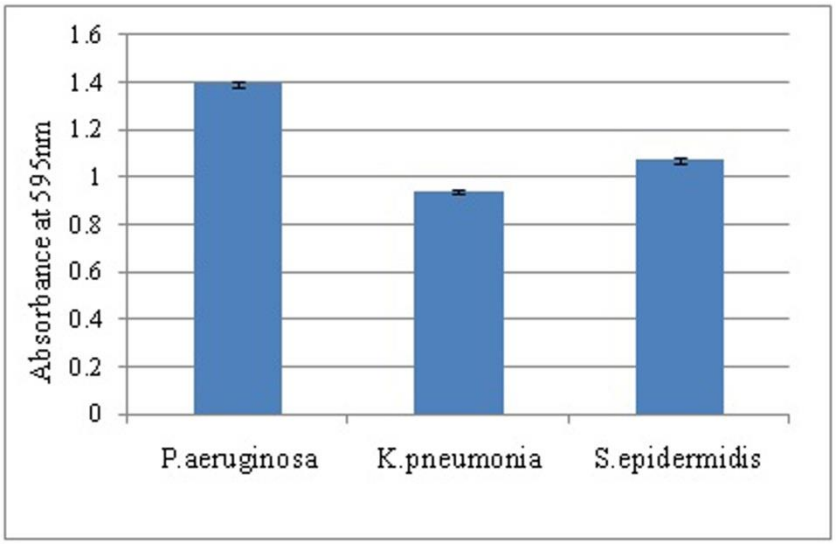

Fig. 3: Biofilm forming ability of reference strains during 48h at 37^0C .The bars on the graph represent mean ± SD of biofilm formation from three independent experiments

Determination of minimum inhibitory concentration of naturally isolated flavones

The results of MIC values for compound 1,2 and 3 tested against different biofilm producers as control are depicted in fig. 4,5 and 6.

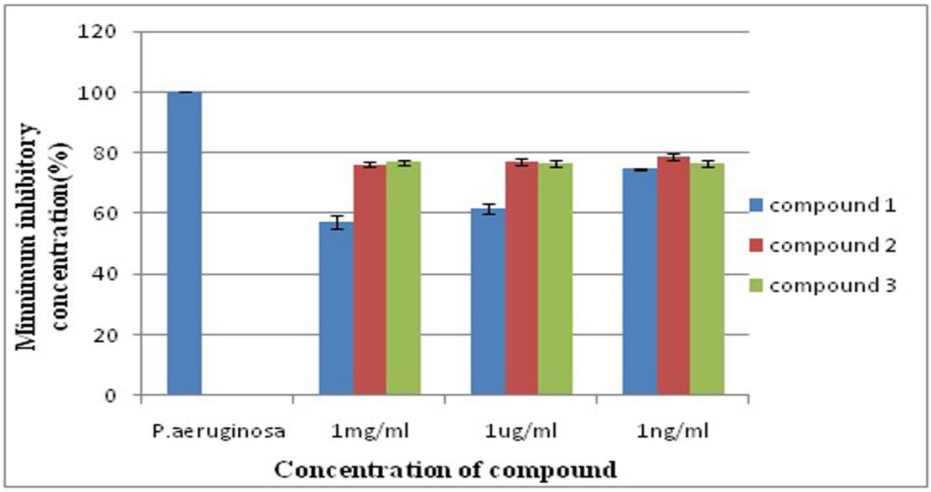

Fig.4: Minimum inhibitory concentration of compound 1, 2, and 3 in the presence of *P.aeruginosa* in a 96-well microtitre plate. Experiments were conducted in duplicate and error bars represent standard deviations

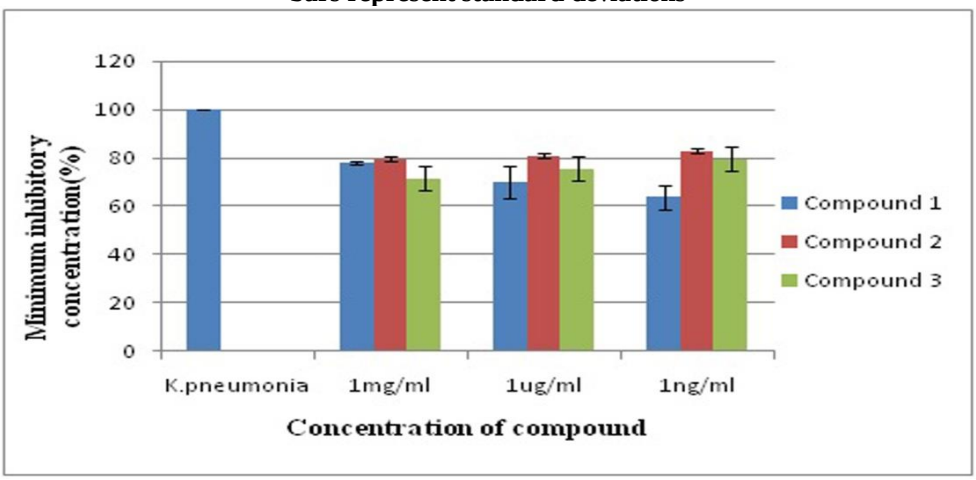

Fig. 5: Minimum inhibitory concentration of compound 1, 2, and 3 in the presence of *K.pneumonia* in a 96-well microtitre plate. Experiments were conducted in duplicate and error bars represent standard deviations

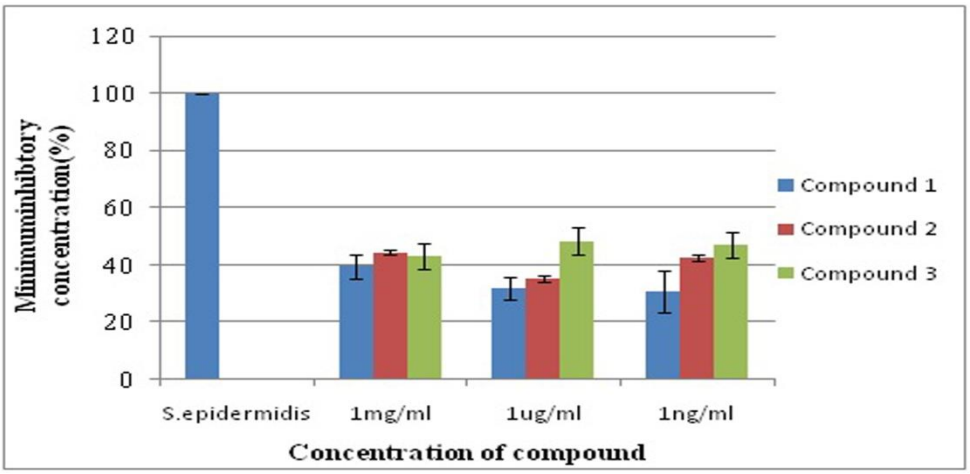

Fig. 6: Minimum inhibitory concentration of compound 1, 2, and 3 in the presence of *S.epidermidis* in a 96-well microtitre plate. Experiments were conducted in duplicate and error bars represent standard deviations

The present experimental data demonstrated that a significant difference in the MIC of the natural compounds was noted as shown in Table 1. The MIC of compound 2 was 82.78 against *K.pneumonia*, whereas that of compound 3 and compound 1 was 79.50 & 77.86, (MIC, compound 2 < 3 < 1). These results confirm that compound 2 and 3 is the most potent antimicrobial compound.

Table 1: Percentage of Minimum Inhibitory Concentration (MIC) of compound 1,2 and 3 in biofilm producing isolates *P.aeruginosa, K.pneumonia* and *S.epidermidis*

Isolates	Compound 1			Compound 2			Compound 3		
	1mg	1µg	1ng	1mg	1µg	1ng	1mg	1µg	1ng
P.aeruginosa	57.10	61.63	74.67	76.28	77.01	78.77	76.86	76.72	76.72
K.pneumonia	77.86	69.97	63.72	79.61	80.94	82.78	71.72	75.71	79.50
S.epidermidis	39.68	32.01	30.55	44.57	35.44	42.46	42.98	48.41	46.95

Biofilm inhibition assay of compounds

The minimum biofilm inhibitory concentration (MBIC) of compounds 1,2 & 3 was determined toward three strains from different sources, selected in the present study due to their strong biofilm-forming ability. Two gram-negative and only one gram positive strain *S.epidermidis* used in this study. The compounds were used in three different concentrations (mg/ml,µg/ml,ng/ml) against three different cultures. As a result compound-1 shows 93% of MBIC with 1ng/ml against *K.pneumonia* and *S.epidermidis* (fig. 7).Compound-2 shows maximum biofilm inhibition (95%) at 1ng/ml against strong biofilm producer *P.aeruginosa*. Results of the present study reveals that the compound 2 inhibited *Pseudomonas aeuroginosa* was at 95%(fig. 8) because one methoxy group present at meta position. In general methoxy group contribute the best biofilm activity because methoxylation is an important molecular feature for membrane penetration [29]. Compound-3 (fig. 9) had an 94.72% of biofilm inhibition against *P.aeruginosa* with concentration of 1mg /ml as depicted in table 2. In compound 1 and 3, biofilm inhibitory activity was less because three methoxy group is present. To compare the previous reported data compounds with three methoxy groups were less active than the compounds with dimethoxy, because their demethoxylation was incomplete.

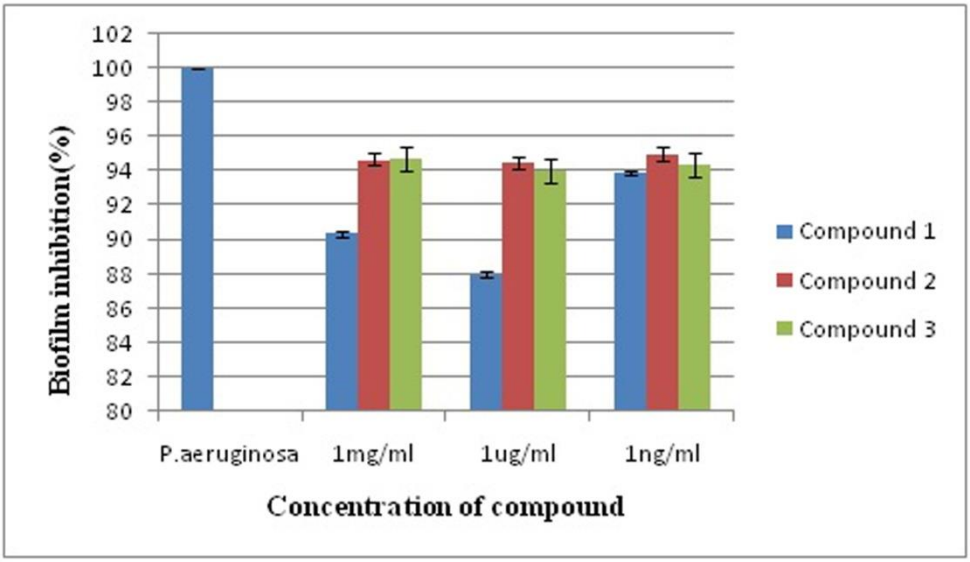

Fig.7: Percentage of biofilm inhibition in *Pseudomonas aeruginosa when* exposed to different concentrations of compound 1,2 and 3.Error bars indicate the standard deviation.

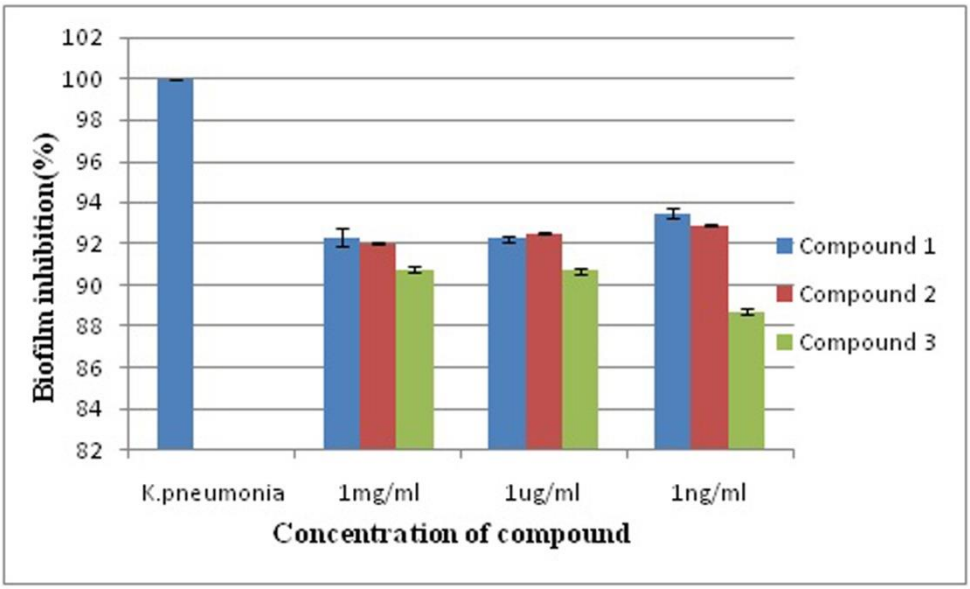

Fig. 8: Percentage of biofilm inhibition in *Klebsiella pneumonia* when exposed to different Concentrations of compound 1, 2 and 3.Error bars indicate the standard deviation.

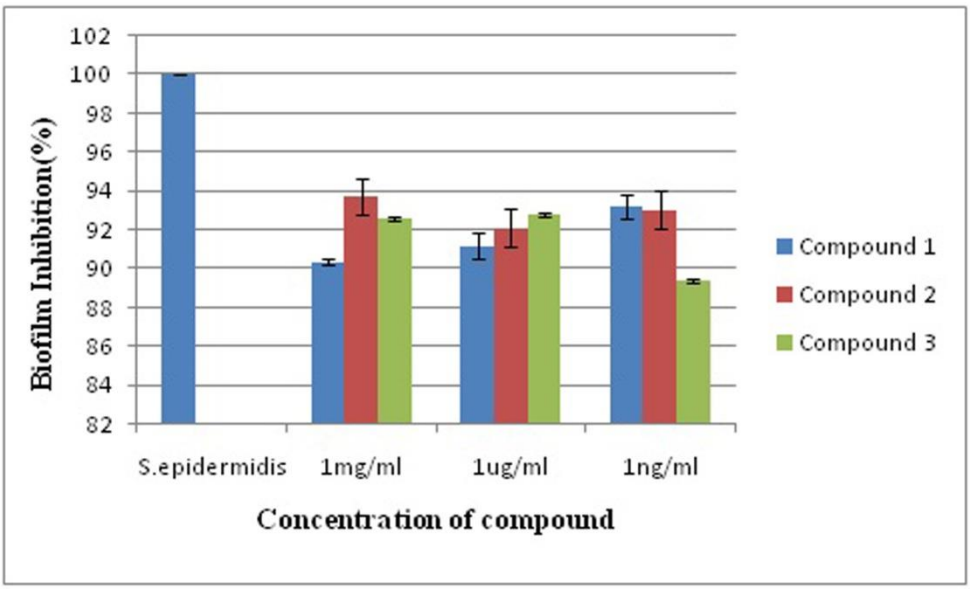

Fig.9: Percentage of biofilm inhibition in *Staphylococcus epidermidis* when exposed to different concentrations of compound 1,2 and 3.Error bars indicate the standard deviation.

Table 2: Biofilm inhibition (%) of three isolates with naturally isolated compounds 1,2 and 3 at different concentration of inhibition values are mean ± SD

Biofilm forming isolates	Compound 1	Compound 2	Compound 3
P. aeruginosa	90.375±0.1767 (1mg/ml) 88.005±0.1909(1ug/ml) 93.93±0.9899(1ng/ml)	94.68±0.2404(1mg/ml) 94.46±0.1626(1ug/ml) 95.00±0.9192(1ng/ml)	94.72±0.4242(1mg/ml) 94.04±0.3676(1ug/ml) 94.39±0.6717(1ng/ml)
K. pneumonia	92.33±0.4313(1mg/ml) 92.25±0.1414(1ug/ml) 93.48±0.2121(1ng/ml)	92.03±0.3040(1mg/ml) 92.52±0.8485(1ug/ml) 92.89±0.0141(1ng/ml)	90.73±0.1909(1mg/ml) 90.72±0.0565(1ug/ml) 88.70±0.1484(1ng/ml)
S. epidermidis	90.38±0.1414(1mg/ml) 91.21±0.6434(1ug/ml) 93.23±0.6363(1ng/ml)	93.73±0.0494(1mg/ml) 92.11±0.0212(1ug/ml) 93.04±0.3676(1ng/ml)	92.57±0.6293(1mg/ml) 92.84±0.9545(1ug/ml) 89.37±0.1060(1ng/ml)

SEM image of all the three compound

The effect of compound 1,2 and 3 upon *P.aeruginosa, K.pneumonia and S.epidermidis* structural morphological changes of three different bacteria were examined by SEM.SEM analysis revealed that in the absence of compounds exhibited at rod-shape,rod- shape and cocci in grape-like clusters with smooth surfaces(micrographs shown in fig.10,11 and 12).SEM of three compound treated with *P.aeruginosa* (Fig. 10)it showed the formation of holes and number of the cell was reduced at three different concentrations and compound 2 can inhibit the adhesion of *P.aeruginosa* and prevent its biofilm formation at 1ng/ml. Fig. 11 Showed that , In treated *K. pneumonia* cells the number of attached bacterial biofilm cells was reduced at all the three different concentrations of three compound when compared with the control but compound 2 more number of cells reduced at 1ng/ml. Considering *S.epidermidis* images inhibition start at 1mg/ml of three compounds and the cell wall was ruptured and holes formed at 1mg/ml of compound 3.

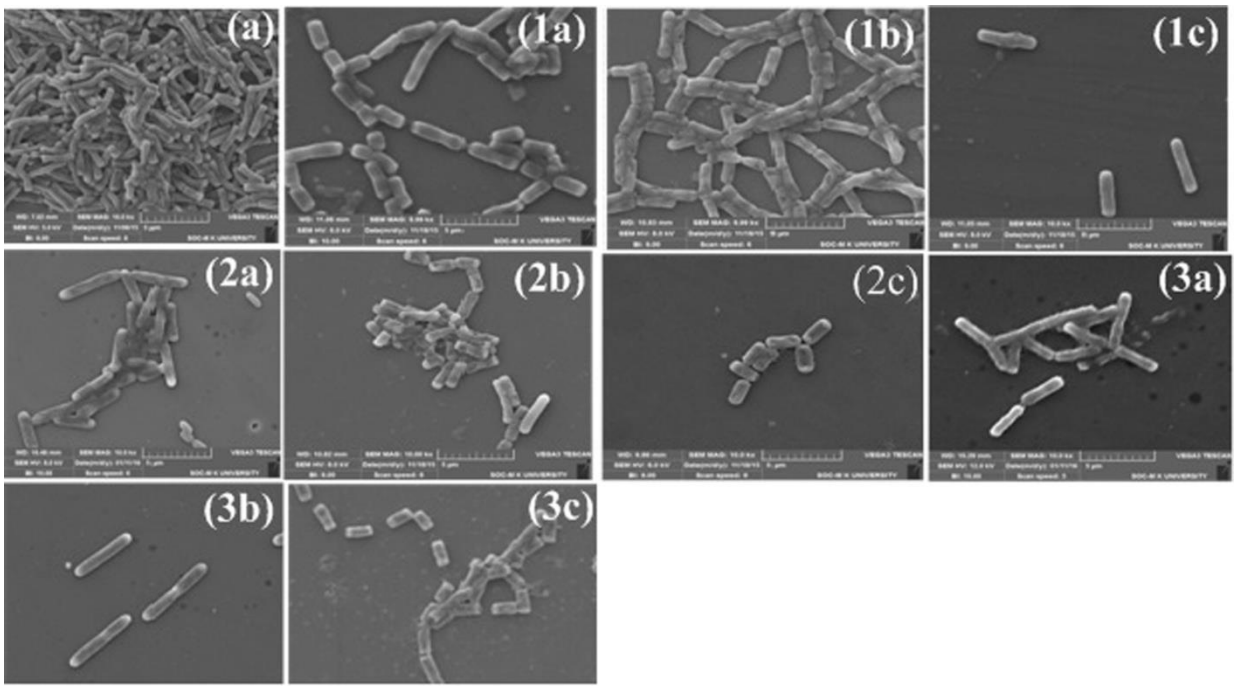

Fig. 10: Scanning electron micrographs of *P.aeruginosa* biofilm inhibition and their treatment with three different concentrations of (a= 1mg, b = 1ug, c= 1ng) compound 1,2 and 3 (a) Control

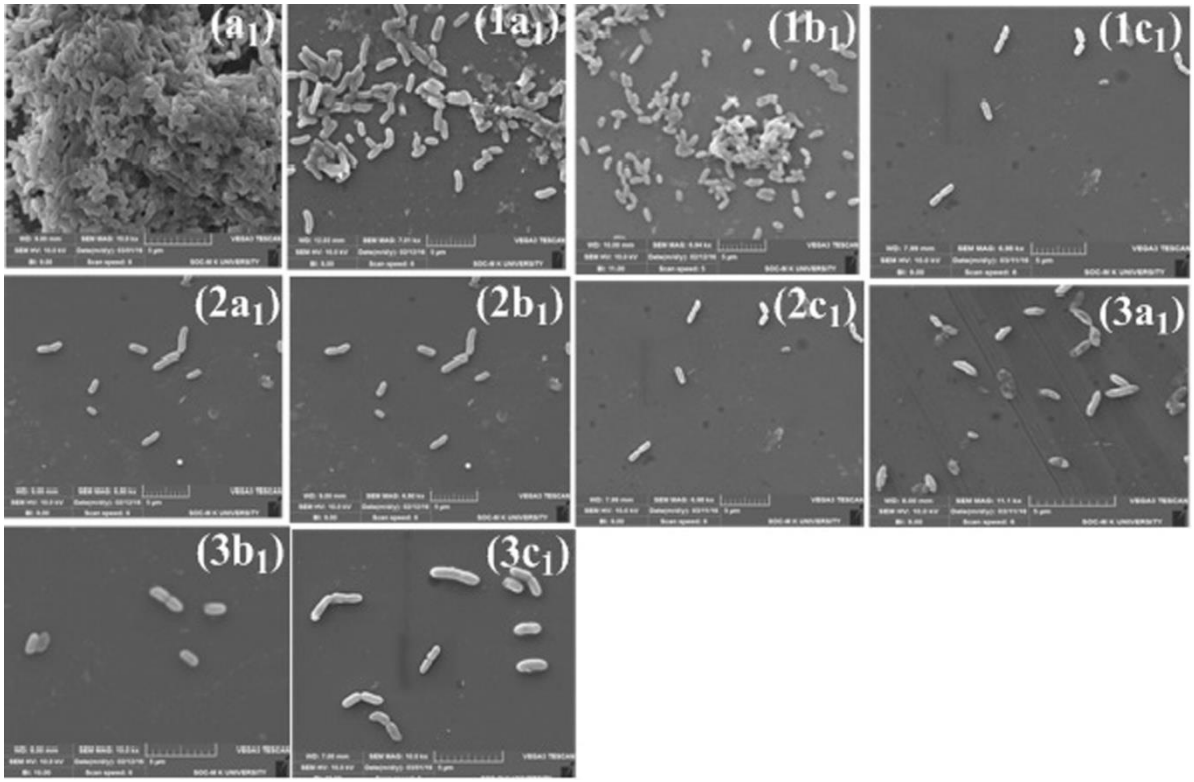

Fig. 11: Scanning electron micrographs of K. pneumonia biofilm inhibition and their treatment with three different concentrations of (a_1= 1mg, b_1 = 1ug, c_1 = 1ng) compound 1,2 and 3 (a_1) Control

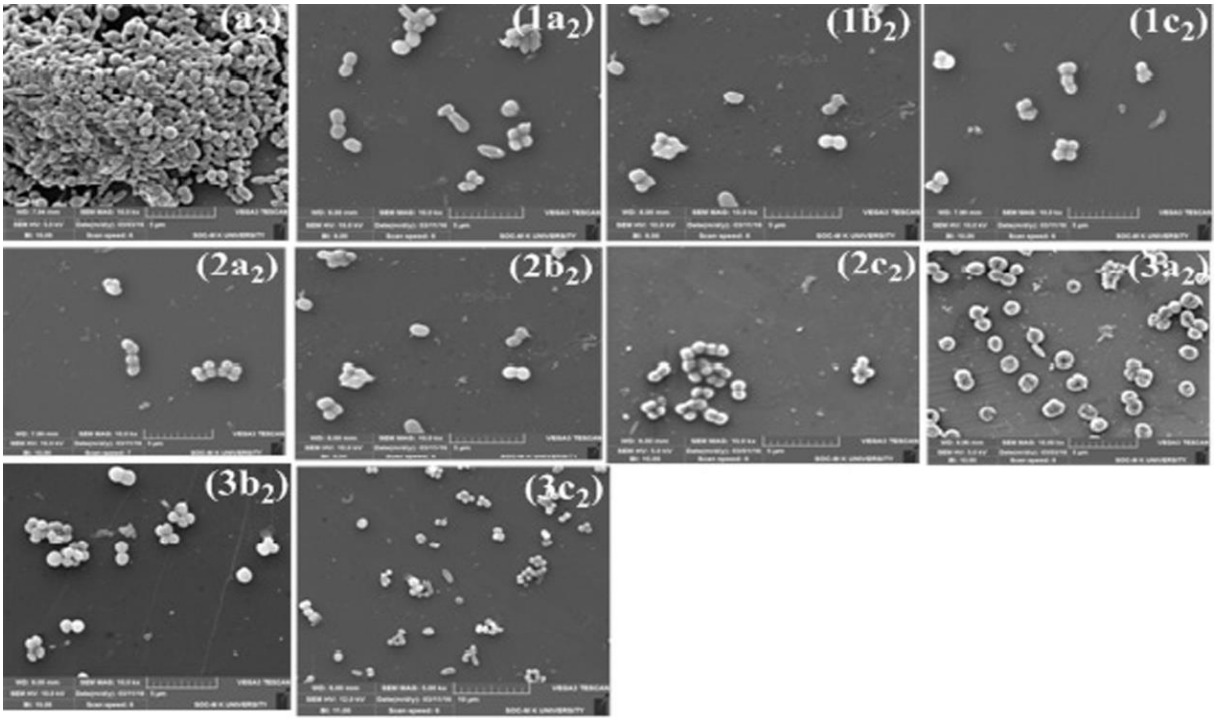

Fig. 12: Scanning electron micrographs of S.epidermidis biofilm inhibition and their treatment with three different concentrations of (a_2= 1mg, b_2 = 1ug, c_2 = 1ng) compound 1,2 and 3 (a_2) Control

Microscopic evaluation of P.aeruginosa biofilms on catheter by SEM

P.aeruginosa biofilm formed after 72 h incubation on catheter piece from each isolate was observed under SEM. Catheter pieces were picked up by sterile forceps from the SDB and gently washed in 5 ml of PBS to remove non adherent cells. The pieces were then placed in a sterilized petri plate. The biofilms were air dried for 3 h. The pieces were then coated with gold and then imaged using a SEM in a high vacuum mode at 8 kV as shown in fig. 13. Scanning electron micrographs of the biofilm were taken at 10 kx magnification.

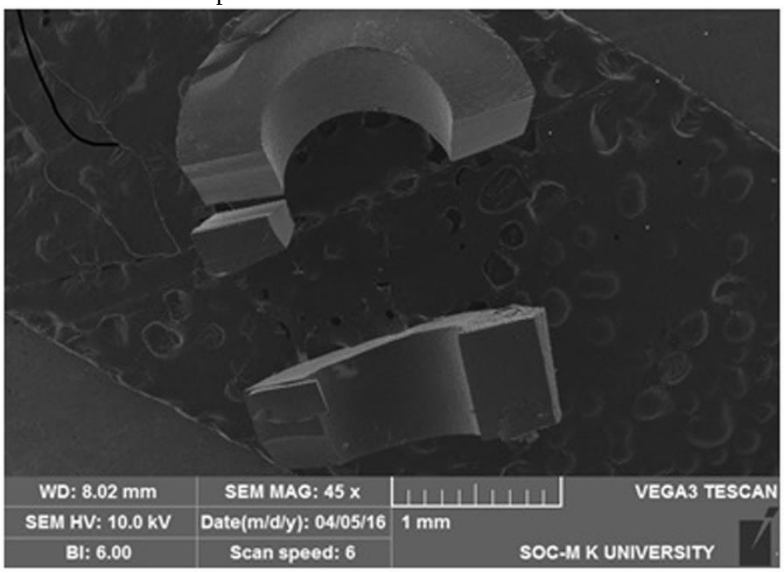

Fig. 13: SEM image of urinary catheter

Colonization in catheters

Bio-Guard catheters used in this study for biofilm inhibition with two different nature of catheters with sample coated and uncoated. When compare to coated and uncoated catheters the uncoated shows effective results. The uncoated samples were treated with the compound-2 with ng/ml concentration as shown in fig.14

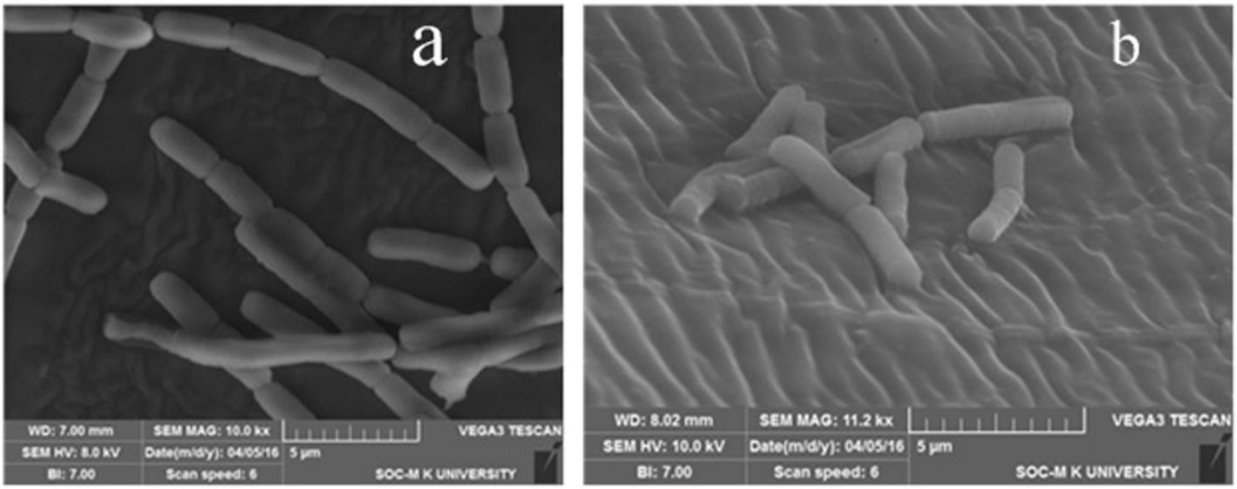

Fig.14: *P.aeruginosa* biofilm growth on catheter by SEM a) control b)in the presence of compound 2

CONCLUSIONS:

In this present study compound 1,2 and 3 were used for the first time as an antibiofilm compound. The results show that all the compounds have potential biofilm inhibition capabilities and compound 2 inhibit the biofilm formation at very low (1ng) concentration level. SEM analysis revealed various morphological changes in all the three bacteria and compound 2 reduce more number of cells at 1ng. Compound-2 inhibits majorly in urinary catheters which shows good impact in medical field. This is the first report of my compound inhibit at low concentration level.

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