PROJECT COMPLETION REPORT

Notes:
1. 10 copies of the Project Completion Report (PCR) should be sent within one month of the completion or termination of the project.
2. The PCR should be in bound form.
3. Cover page should include the title of the project; file number, names and addresses of the investigation.

1. Title of the project: **Study of microbial biofilm inhibition by traditionally used medicinal plants of Assam and Arunachal Pradesh and their role on extracellular polymeric substances (EPS) secretion.**

2. Principal Investigator(s) and Co-Investigator(s): **Dr. Manabendra Mandal**

3. Implementing Institution(s) and other collaborating Institution(s): **Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur , Assam**

4. Date of commencement: 19 November 2013

5. Planned date of completion: 19 November 2014

6. Actual date of completion: 23 December 2015

7. Objectives as stated in the project proposal:

- Screening of the plants traditionally used for treatment of wounds, skin diseases and other infections.
- Determination of the phytochemical constituents in the plant extract.
- Checking the antimicrobial and antibiofilm activity (mainly on *S. aureus* and *P. aeruginosa*, *C. albicans* biofilm inhibition and removal) of different extract.
- EPS Isolation and characterization of the *S. aureus* and *P. aeruginosa*, *C. albicans* biofilms before and after treatment of plant extract.
- Antimicrobial, biofilm inhibition and biofilm removal property of the active fractions.
- Docking studies of the isolated compound with the biofilm associated proteins in *S. aureus P. aeruginosa* and *C. albicans*.
- Cytotoxicity assay of the bioactive plant extracts.

8. Deviation made from original objectives if any, while implementing the project and reasons thereof:

Following objectives are remain uncompleted and work is going on:

- EPS Isolation and characterization of the S. aureus and P. aeruginosa, C. albicans biofilms before and after treatment of plant extract.

Justification: Due to the shortage of funding and instrument the complete purification of the plant extract still not be completed.

Additional to the above mentioned objective we have added a new objective;

Synthesis of Green silver nanoparticles to combat biofilm formation

9. Experimental work giving full details of experimental set up, methods adopted, data collected supported by necessary table, charts, diagrams & photographs.

Collection of Sample and processing:

On the basis of traditional knowledge the plant samples were collected from the different part of Assam. Fresh leaves of the plant species were plucked from the tree. Leaves were first washed with sterile water, then surface sterilized by 70 % ethanol followed by bleaching with 5 % aqueous sodium hypochlorite solution. Finally leaves were again washed with sterile double distilled water to get rid of any impurities. After surface sterilization, the healthy leaves were dried at room temperature to maintain their natural phytoconstituents. The dried leaves were ground to fine powder and stored at -20° C till further use. Plant was identified by the Botanical survey of India, Shillong, India

Extraction and Fractionation

Powder of leaves (500 g) was soaked in different solvents for 24 h under continuous stirring. They were then centrifuged at 5000 rpm for 20 min, and the supernatant was concentrated using a vacuum evaporator and lyophilized to yield a crude extract. The dried extract was resuspended in appropriate volume of methanol and bio-assay guided partial fractionation of extract was carried out using thin-layer chromatography (TLC) and column chromatography. The solvents were developed by TLC plates (Merck, Germany) using hexane, ethyl acetate and n-butanol as eluent and visualized under UV light. In column chromatography a series of fractions with n-hexane, ethyl acetate and n-butanol were eluted. Each fraction was concentrated, lyophilized and subjected to antibiofilm assay.

Phytochemical Analysis of plant extract:

The bioactive fraction of *Syzygium cumini* leaf extract is subjected to test for the presence of secondry metabolite by using different reagent as described by S.Goweri et al. 2010.

Growth curve analysis

The growth curve for *Staphylococcus aureus* and *Pseudomonas aeruginosa*, cultivated in the presence and absence of active purified fraction (EA) from *Syzygium cumini* leaves was used for studying antibacterial activity. Briefly, test inoculums (100 µl of 10^6 to 10^7 CFU/ml) were added to the test tube already containing EA in 10 ml of MHB. The test tubes were incubated at 37°C and the OD were recorded at 600 nm at 2 h intervals up to 24 h.

In vitro biofilm inhibition assay: Tissue culture plate method (TCP)

To determine the efficiency of EA in biofilm inhibition of *S.aureus* and *P.aeruginosa*, TCP method was used as described elsewhere (Gupta et al., 2014). Briefly, wells of polystyrene 96-well-flat bottom tissue culture plates (sterile) were filled with 10^6 to 10^7CFU/ml bacterial culture in 100 µL of tryptic soy broth (TSB). To the culture, 50µl of EA at concentration of 125 µg/ml to 900 µg/ml were added. The 96 well plates were incubated for 24 h at 37° C. After incubation period the content of each well was gently removed. The wells were washed thrice with phosphate buffer saline (PBS pH 7.4) to remove free-floating planktonic bacteria. Biofilms formed by adherent organisms in plate were fixed by keeping the plate at 60°C for 1 h and stained with crystal violet (0.1%, w/v). Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying. After drying, 100% ethanol was added to the wells and the optical densities (OD) of stained adherent bacteria were determined with a microplate reader (Thermo Scientific Multiskan GO) at 570 nm. The biofilm inhibition was calculated based on the solubility of the retained dye in wells by using the formula-

% Biofilm inhibition= $[(OD_{control} - OD_{test}) / OD_{control}) \times 100$ where control OD is the absorbance which is obtained without addition of EA.

Scanning electron microscopy: biofilm visualization

To determine the effect of the EA on biofilm formation, scanning electron microscopy (SEM) was carried out in cover slip. Briefly 10^6-10^7 CFU/ml bacterial cell culture was added in a 24 well tissue culture plate and a coverslip is kept on the bottom of the plate. EA at the concentration of 900 µg/ml was added in the corresponding wells. Biofilm formation was allowed on the surface of the coverslip for 24 h. After the incubation the biofilm formed on the cover slip from treated and untreated wells

were fixed with 2% glutaraldehyde overnight. The fixed samples were then dehydrated in a graded series of ethanol. The formed biofilm were analyzed by scanning electron microscopy .

Pyocyanin Analysis

Overnight grown *P. aeruginosa* culture was diluted to 1:100 into 10 mL fresh LB medium. EA at the final concentration 125, 300, 600 and 900µg/ml were added to the culture. After 24 h of aerobic growth with shaking at 37°C. Cells were separated from culture by centrifugation at 10000 rpm for 15 min. Culture supernatant were passed through 0.22 µm syringe filters (Millipore) and cell-free culture supernatant was extracted by addition of 4.5 ml of chloroform to 7.5 ml of culture supernatant. At this point the colour changes to green blue. 3 ml of bottom blue layer was mixed with 1.5 ml of 0.2M HCl. The resulting pink color indicated the pyocyanin production and it was measured at 520 nm by UV-Vis spectrophotometer. (Thermo Fischer Scientific, Evolution 201)

Effect of EA on swimming and swarming motility

The motility assay was done by method described by Vattem et al. 2007 with slight modification. Different concentrations of EA were mixed with swarm agar plate and swim agar plate and were poured into plates and point inoculated with *P. aeruginosa* and incubated at 37° C for 48 h. The extent of swarming and swimming was determined by the diameter of swarm and swim in compared with control.

Staphyloxanthin Biosynthesis Inhibition Assay

For in vitro pigment inhibition studies, *S. aureus* was cultured in TSB (5 ml) in the presence of inhibitor compounds for 24 h, in duplicate. Prior to assay, the bacteria were centrifuged and washed in PBS. Staphyloxanthin was extracted with MeOH, and the OD was determined at 450 nm using a spectrophotometer (Thermo Fischer Scientific, Evolution 201).

GC-MS Analysis of partially purified extract

The bioactive components of EA fraction were identified by gas chromatography–mass spectrometry (GC-MS). A volume of 1 µl of EA was injected into GC-MS (PerkinElmer, USA) coupled to a Clarus 500 Mass Spectrometer mass detector under electron impact ionization (70 EV). The stepped temperature program was held at 80°C for 2 min, increased from 80°C to 250°C at 10°C per minute, and held for 5 min. The total run time was 30 minutes. The carrier gas used in the analysis was helium with a flow rate of 1 ml/min. Interpretation of mass spectra or peaks were identified by computer searching in a commercial mass spectral reference library (Wiley NIST).

Molecular docking studies

The possible molecular interaction between the bioactive compounds in EA of *S. cumini* and QS signaling protein was determined using the Molegro Virtual Docker (MVD) [Molegro APS]. Molecular docking simulation was carried out against RhlG/NADP active-site complex (PDB ID: 2B4Q), LasR-TP4 complex (PDB ID: 3JPU) and Pseudaminidase from *Pseudomonas aeruginosa* (PDB ID: 2W38). Initially MVD was used for the binding cavity prediction of said proteins. 0.8 Å resolution discrete grid covering the protein was created by a sphere of radius 1.4 Å placed to check any overlap with any of the spheres. Then each accessible grid point was checked to confirm that it is a part of a cavity or not. Finally the connected regions were determined if they are neighbors. Cavities found were then ranked according to their volume (Thomsen and Christensen, 2006). On the other hand, the two-dimensional structure of the bioactive compounds determined using GC-MS were converted to their corresponding three-dimensional structures using ChemOffice 2010 (ChemOffice 2010, Cambridge Soft, USA). For docking simulations and the energy of these compounds were optimized using MM2 force field methods (Ulrich and Norman, 1982) and saved as sybyl mol2 file format using ChemOffice 2010. A maximum iteration of 1,500 with a simple evolution size of 50 and a minimum of 20 runs were performed for each compound. The best pose of each compound was selected for docking scores and protein-ligand interaction analyzed.

In vitro cytotoxicity evaluation by MTT assay

The murine monocytic macrophage cell line (RAW 264.7) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% foetal bovine serum (FBS). Initially, 1×104 cells were grown in each well of a 96-well plate at 37° C and 5% CO2 for 24 hrs. To determine the effect of EA on cell morphology and viability, the cells were treated with different concentration of EA for 24 hrs followed by analysis with inverted microscope (Zeiss, Model AXIOVERT A1). The wells were treated with MTT solution for 4 h and kept at 37°C in a cell culture incubator (Eppendorf Galaxy 170). MTT was reduced to an insoluble dark purple formazan by mitochondrial dehydrogenases in metabolically active cells. The formazan crystals were dissolved in denaturing buffer (SDS, HCl and isopropanol) and the absorbance was read at 580 nm in 96 well plate reader (Thermo Scientific Multiskan GO). The cell viability was calculated by using the formula:

Viable cell (%)=(OD of drug treated sample/ OD of untreated sample)×100

Formation and characterization of green silver nanoparticle for enhancement of antibiofilm activity:

Ten milliliters of leaf broth (*s.cumini* and *P.guajava*) was added to 90 ml of 1×10^{-3} M aqueous $AgNO_3$ solution for the reduction of Ag+ ions in a 250 ml conical flask and the formation was monitored periodically using UV-VIS spectroscopy. The silver nanoparticles obtained from the solution were

purified by repeated centrifugation at 12,000 rpm for 20 min followed by dispersion of the pellet in deionized water to remove biomolecules such as proteins, free silver ions, and secondary metabolites. The structure and composition of the freeze dried purified silver nanoparticles were characterized by EDX (energy-dispersive X-ray spectroscopy) equipped with a scanning electron microscope their crystallinity using an X-ray diffractometer employing CuKα radiation, the possible participation of bioactive functional groups in capping and stabilization of bio-reduced Ag+ ions by KBr pellet method in a Fourier transform infrared, and the size and morphology of silver nanoparticles using TEM , a 10 µl AgNP sample was placed on a carbon-coated copper grid and was allowed to dry at room temperature overnight. The sample-loaded copper grid was placed under an IR-lamp for 15 minutes to ensure complete dryness.

Statistical analysis.

Statistical analyses were performed using Graph pad prism software version 5.0 (GRAPHPAD Software, San Diego, CA). P values were calculated using a one-way analysis of variance (ANOVA) test and multiple comparison test on Graph Pad Prism.

Results

Plant samples collected and their activity against bacterial biofilm

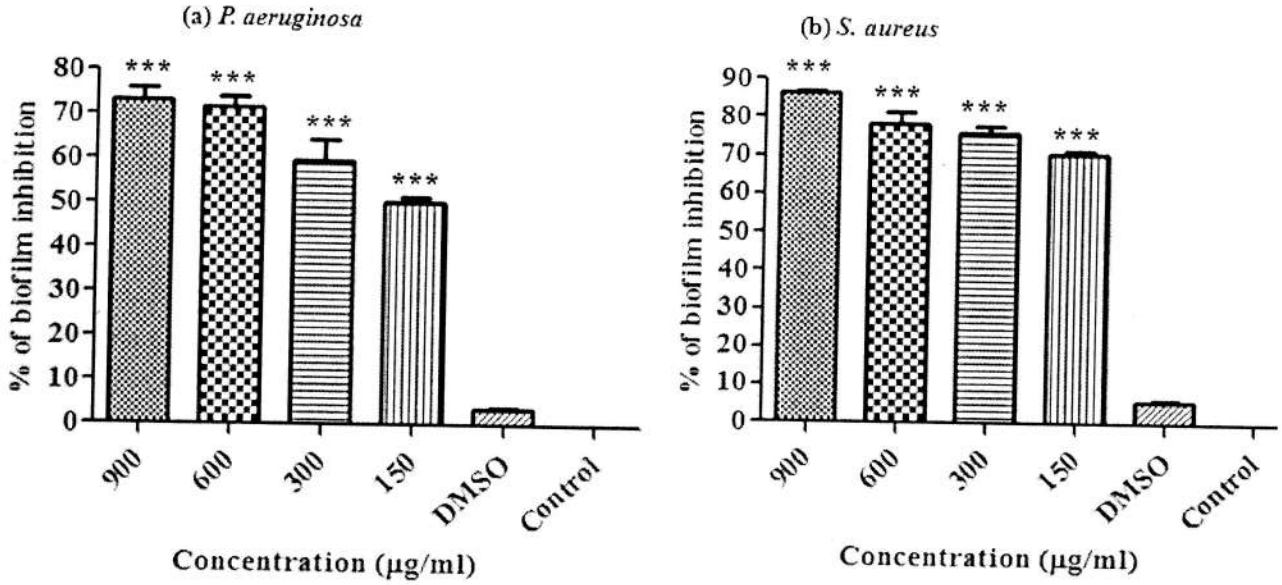
Total 13 different plant samples have been collected and 8 different solvents were used for the preparation of extract. Herbarium of each sample has been prepared. Among the total extract screened the hydro methanol (70% methanol) extract of *S.cumini* leaf showing maximum antibiofilm activity. Thus the hydro methanol extract of *Syzygium cumini* was used to further bioactive guided fractionation.

Phytochemical constituent in the bioactive plant extract and partial purified compound

Test of secondary metabolite	Reagent used	Hydro-methanol extract	Ethyl acetate fraction
Alkaloids	Wagner's reagent	+	–
Saponins	Sodium bicarbonate	+	–
Tanins	Ferric chloride	++	++
Phenolic acids and Flavonoids	Magnesium ribbon	+++	+++
Steroids	Chloroform and Sulfuric acid	+	+
Carbohydrate	Fehling's reagent	+	–
Tarpenoids	Chloroform and Sulfuric acid	–	–

Effect of partially purified fraction on biofilm formation

We tested the effect of EA on biofilm formation in the tissue culture plate by monitoring the binding of the crystal violet to adherent cells to the plate. The present study revealed the increase in biofilm inhibition with an increase in EA concentration.



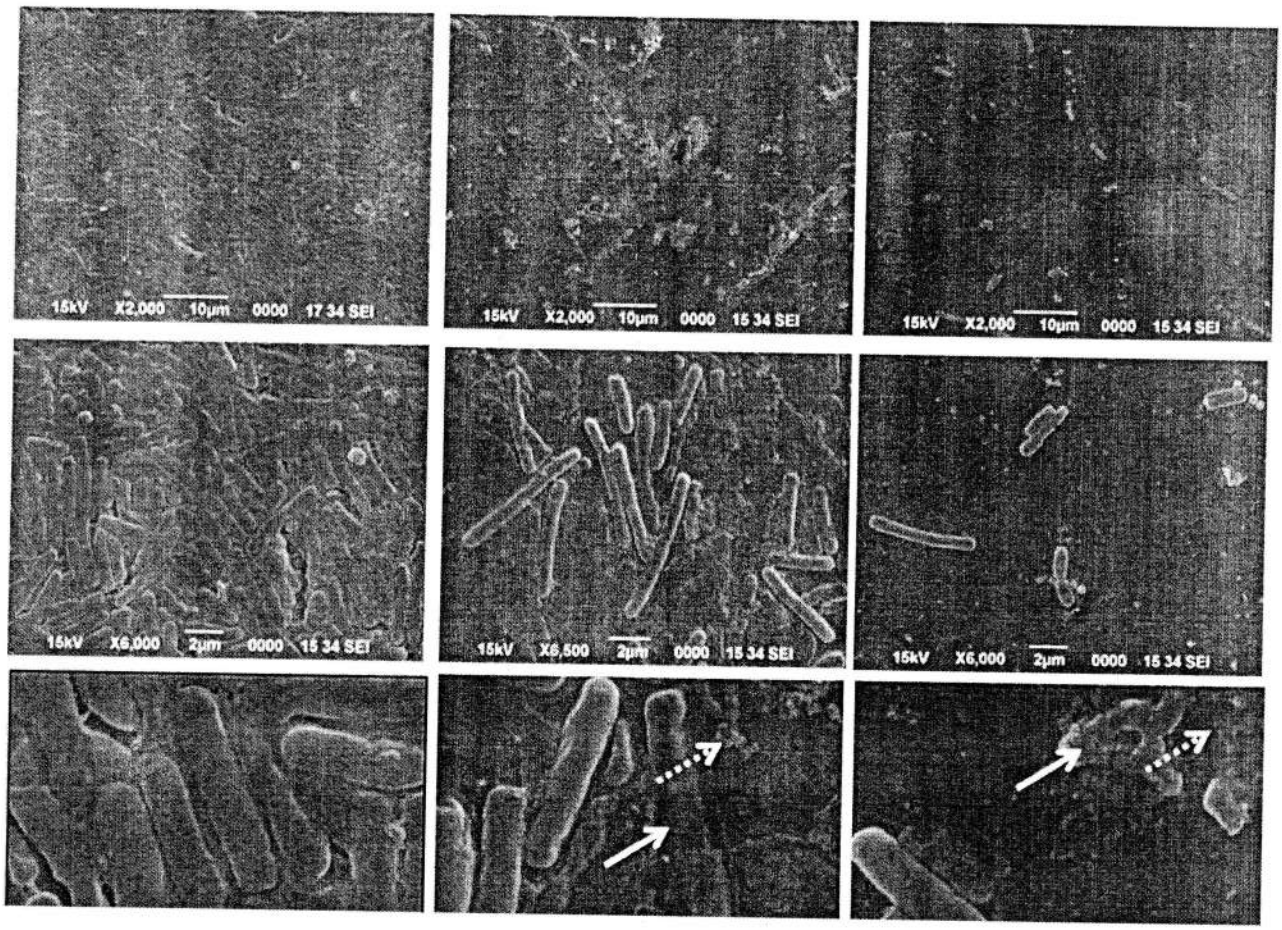


Fig. Scanning electron microscopy images of the biofilms by *P.aeruginosa* upon coverslip. Untreated biofilms (control for biofilm formation)A,B,C at different magnification. Extract treated biofilms : ME (D,E,F); EA (G,H,I).Solid

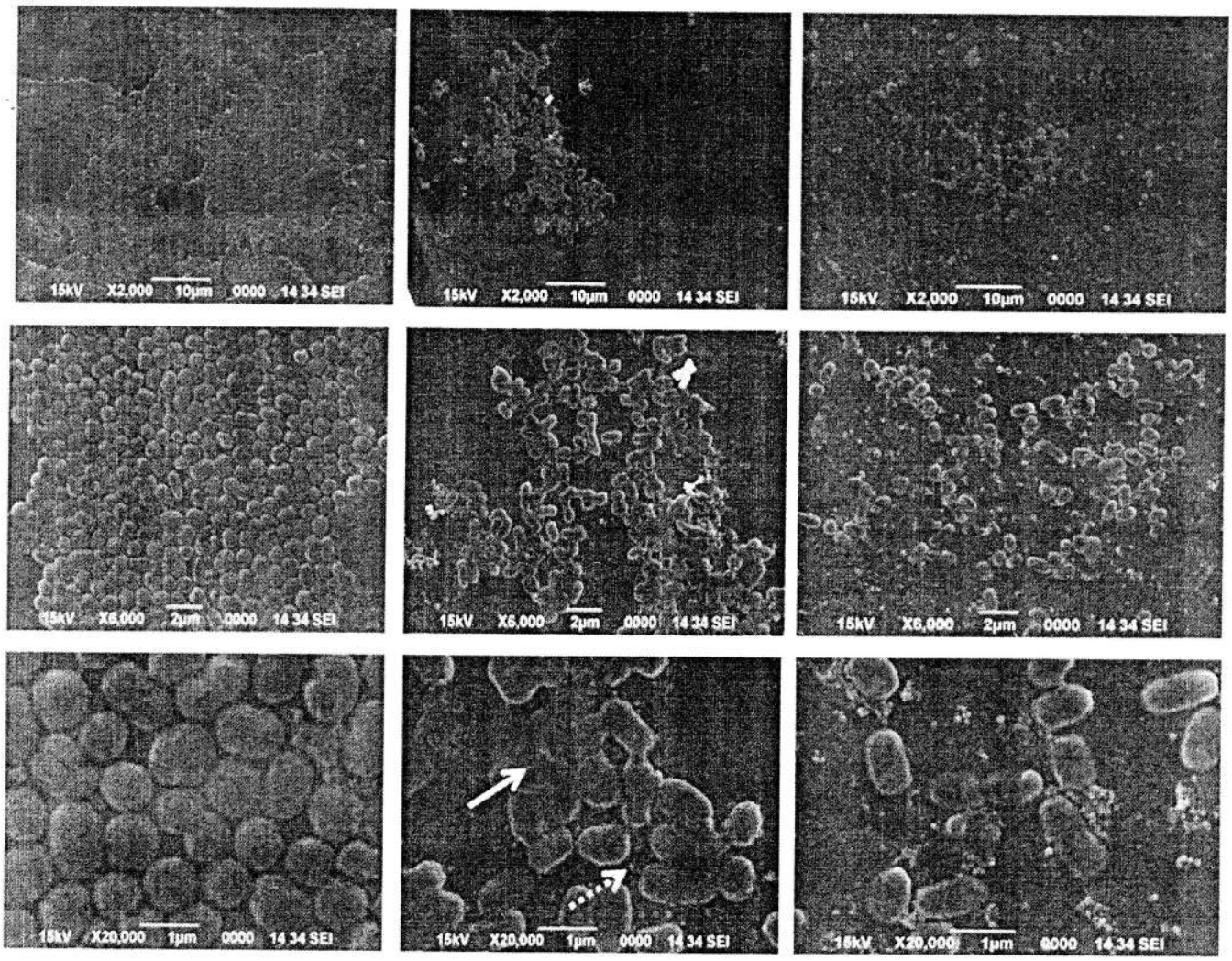
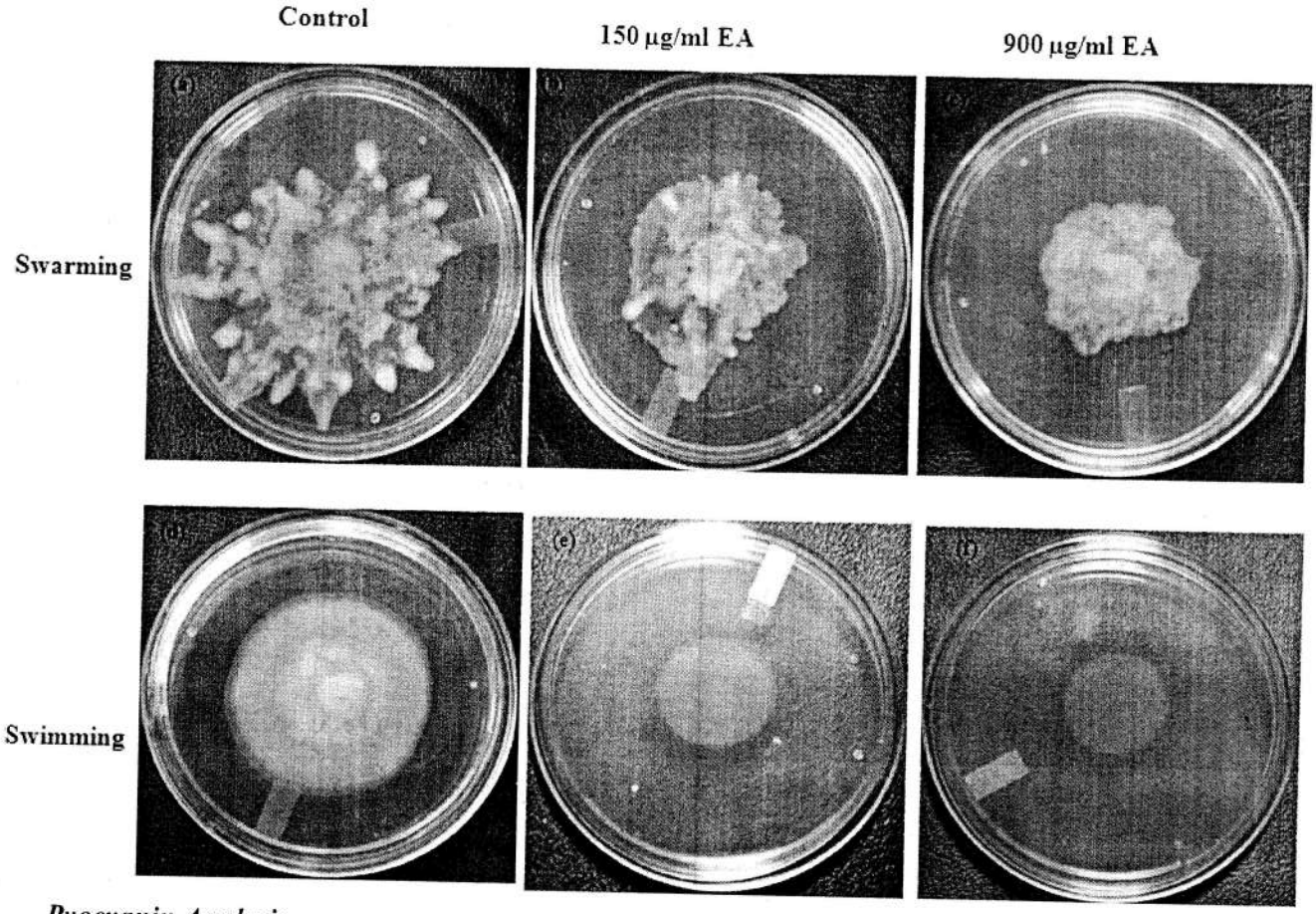


Fig. Scanning electron microscopy images of the biofilms by *S. aureus* upon coverslip. Untreated biofilms (control for biofilm formation)A,B,C at different magnification. Extract treated biofilms : ME (D,E,F); EA (G,H,I).Solid arrows: deformation ,dotted arrows: matrix overproduction.

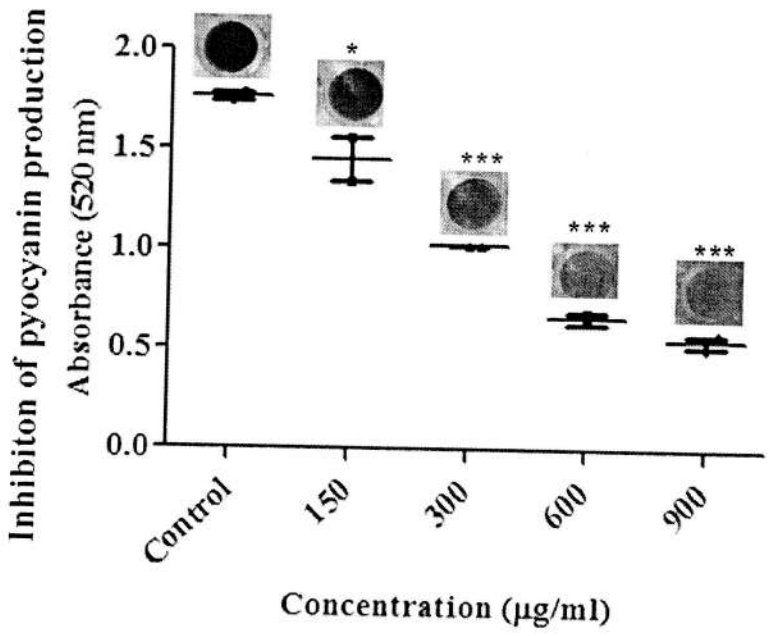
Effect of EA on swimming and swarming motility

Motility in *P. aeruginosa* is appendages mediated movement which enables bacteria to move or to adhere to the surface. This adherence leads to the formation of biofilm and also virulence of the bacteria. In the present study EA reduced the swarming motility and swimming motility of *P. aeruginosa.*



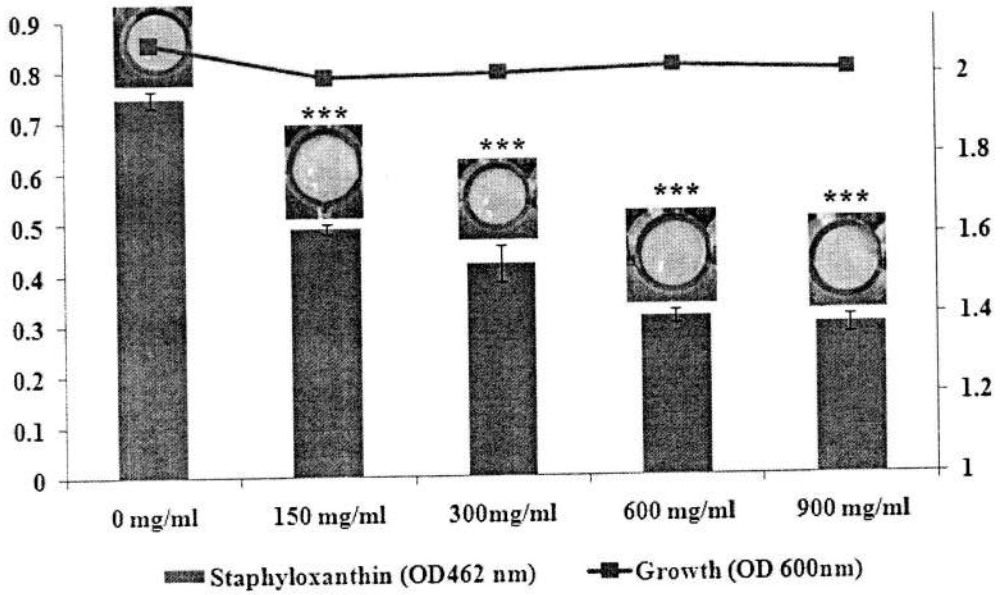
Pyocyanin Analysis

The relative concentration of pyocyanin was measured spectrophotometerically at the wavelength 520 nm. Pre treatment with EA (concentration 150, 300, 600, 900 µg/ml) produced significant reduction in pyocyanin concentration in a dose dependent manner.



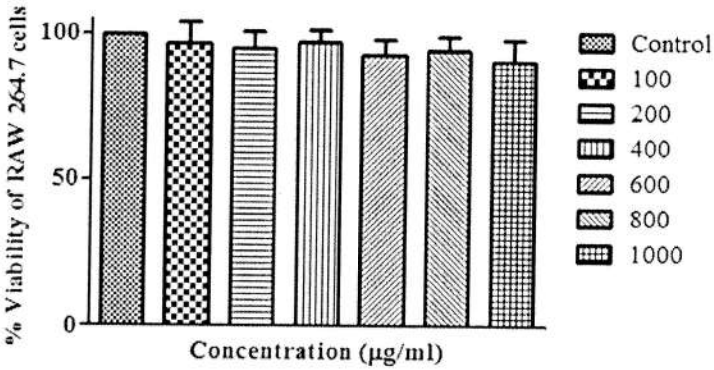
Staphyloxanthin Biosynthesis Inhibition Assay

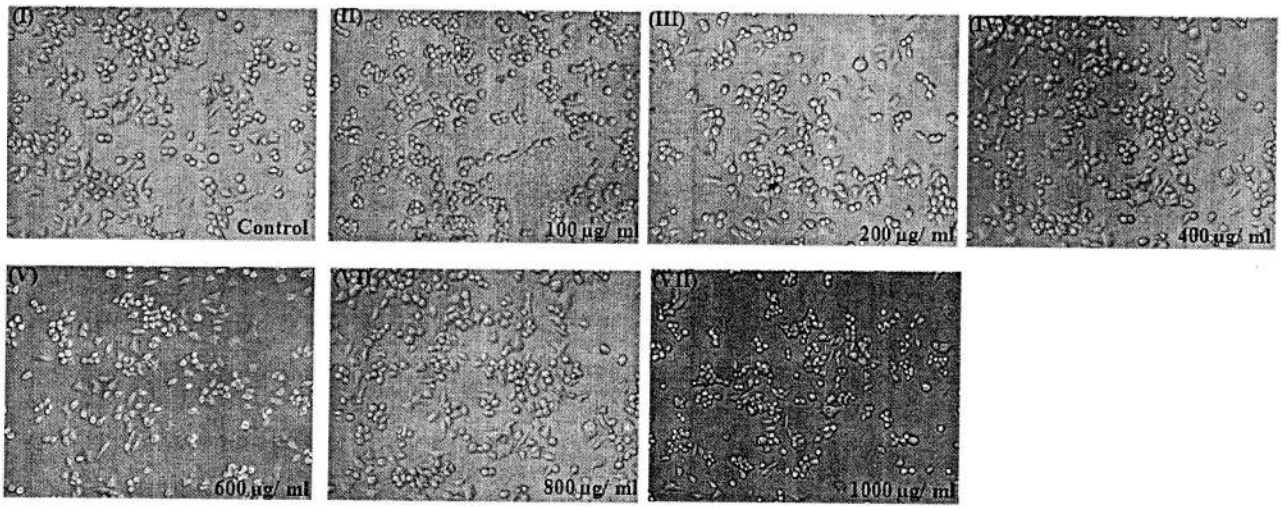
The golden colour carotenoid pigment (staphyloxanthin) is a virulence factor of pathogen *S. aureus.* We examined whether EA could inhibit the synthesis of this pigment. The results indicated that the treated cells possessed less pigment compared to the untreated cells.



Cytotoxicity testing of extract

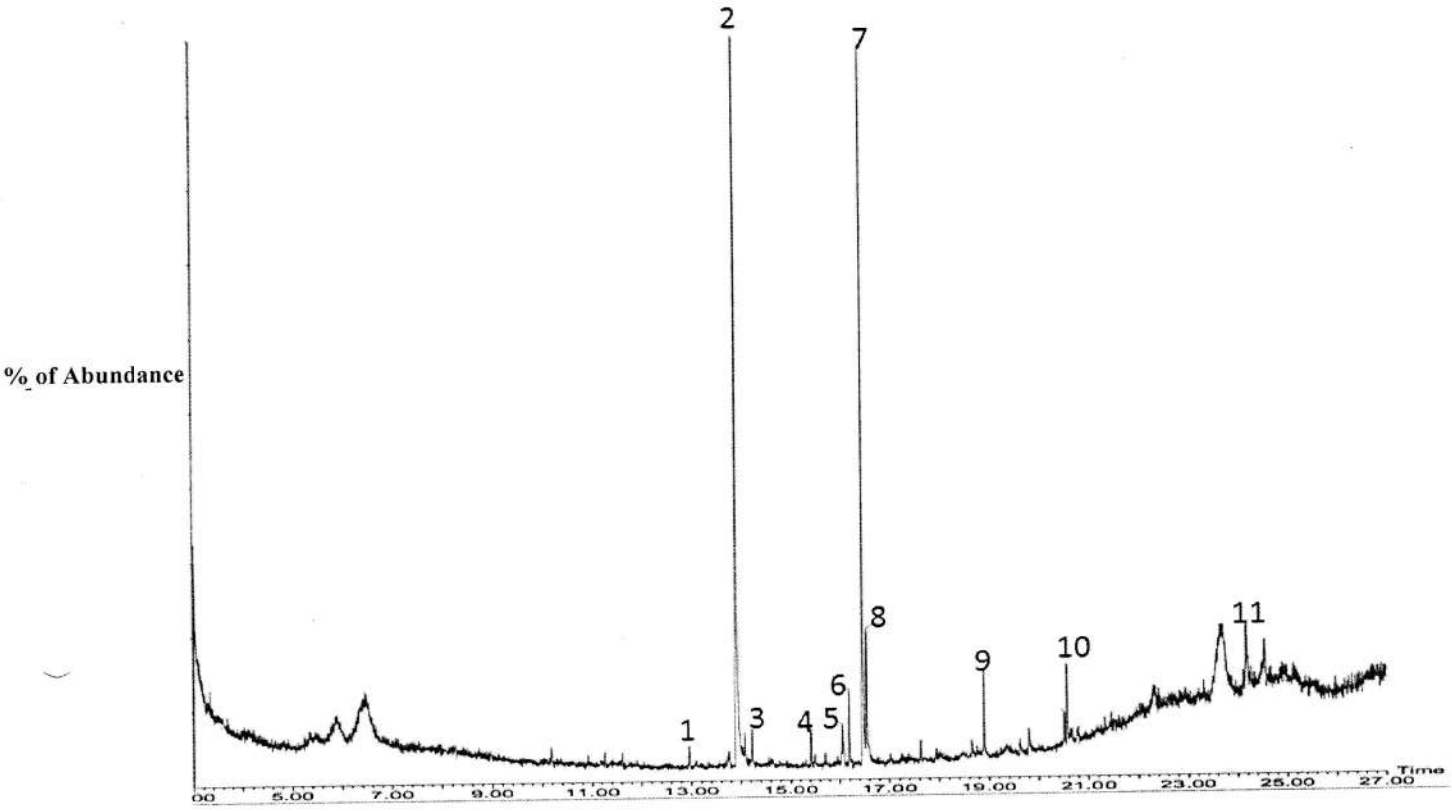
The in-vitro toxicity of EA was accessed by using RAW 264.7 cell line. The stability of the cells is a good indicator of compounds for screening of cytotoxicity. Fig. below represents the percentage of viable RAW cells treated with different concentration of extract. All concentration ranging from 100 to 1000 µg/mL, did not show any significant effect on cell viability. Cells treated with different concentrations of EA were observed for their morphological changes using inverted microscope and it was observed that the cells did not show any visible morphological changes on treatment with EA at different concentrations.





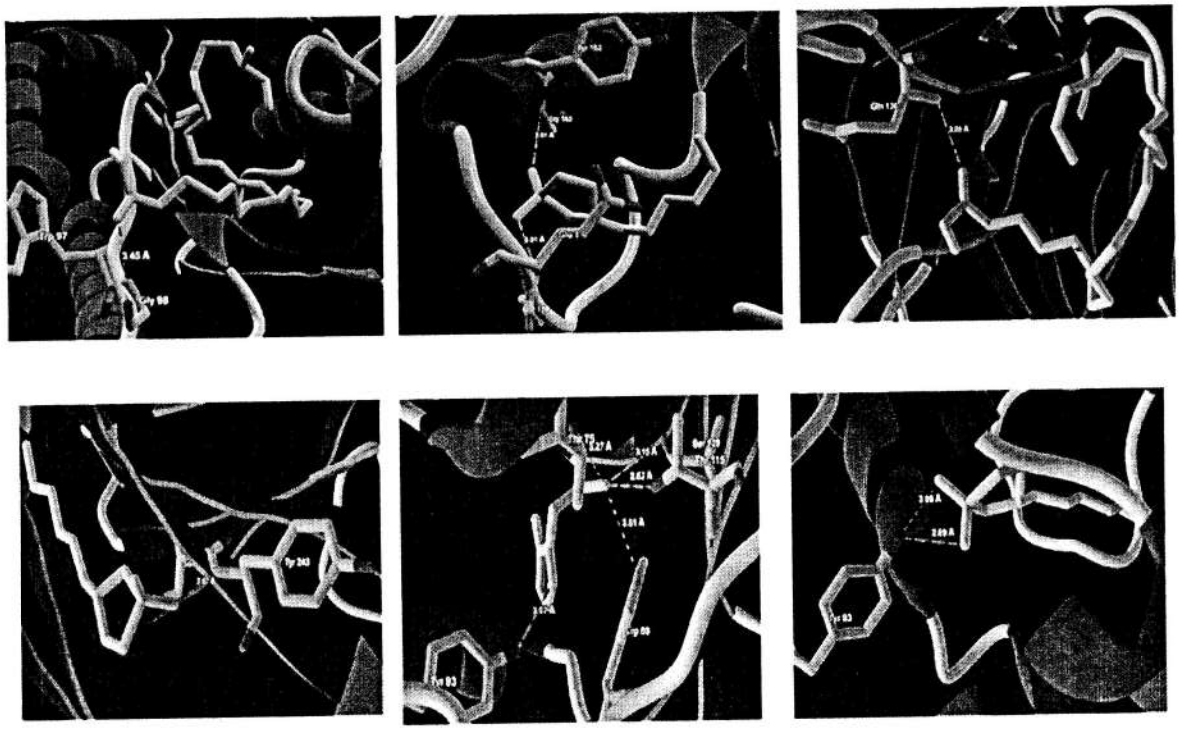
Identification of bioactive compounds and molecular docking

GC–MS analyses of the EA resulted in the identification of 11 components . GC-MS analysis of the EA resulted in the identification of 12 bioactive components. Among the phytochemicals, 3-N-Hexylthiane s-s-dioxide, 3-N-Hexylthiolane s s-dioxide, Dodecane 1-Fluoro- and Heptacosanoic acid were the most abundant compounds (Figure below). Interestingly, the molecular docking carried out against the compounds obtained from the GC-MS result shown in Table 1(a) (PDB ID: 2B4Q) Table 1(b) (PDB ID: 2W38) and Table 1(c) (PDB ID: 3JPU) revealed that Compound 9 (Heptacosanoic acid); compound 2 (3-N-Hexylthiane s, s-Dioxide) and Compound 11 (3-Methyl 2-(2-Oxopropyl) Furan) exhibiting favourable molecular interaction as evidenced from the Rerank score, MolDock score and H bond energy. The snaps shots of the molecular interaction are shown in Fig. The study confirmed the potentially rich anti-biofilm agents in *S. cumini* partially purified extract



Retention time (min)

Fig. GC–MS chromatogram of antibiofilm EA fraction of *S. jambolanum* leaves the compounds and their retention time(parenthesis)1. DODECANE, 1-FLUORO(12.963); 2. 3-N-HEXYLTHIANE, S,S-DIOXIDE(13.913);3. DODECANE, 1-FLUORO-(14.228); 4. DODECANE, 1-FLUORO-(15.424); 5. 5,6-DECADIEN-3-YNE, 5,7-DIETHYL-(16.059); 6. DIHYDRO-CIS-.ALPHA.-COPAENE-8-OL(16.194); 7. 3-N-HEXYLTHIOLANE, S,S-DIOXIDE(16.469); 8. DODECANE, 1-FLUORO-(16.549); 9. HEPTACOSANOIC ACID, 25-METHYL-, METHYL ESTER(18.895); 10 . BICYCLO[4.1.0]HEPTANE, 7-PENTYL- (20.506); 11 . 3-METHYL-2-(2-OXOPROPYL)FURAN (24.182)



Ligand	MolDock Score[a]	Rerank Score[b]	Interaction[c]	HBond[d]	LE1[e]	LE3[f]
Comp 9	-107.74	-75.08	-152.68	-1.59	-3.48	-2.42
Comp 2-7	-91.94	-75.03	-91.14	-3.66	-7.07	-5.77
Comp 5	-102.55	-71.50	-94.04	0.00	-7.32	-5.82
Comp 1-3-4-8	-90.39	-70.65	-88.24	0.00	-6.95	-5.43
Comp 6	-78.98	-66.75	-90.43	-2.50	-4.94	-4.17
Comp 10	-77.69	-65.90	-83.21	0.00	-6.47	-5.49
Comp 11	-77.01	-63.22	-74.58	-0.98	-7.70	-6.32

Ligand	MolDock Score[a]	Rerank Score[b]	Interaction[c]	HBond[d]	LE1[e]	LE3[f]
Comp 9	-146.39	-98.95	-160.73	-1.35	-4.72	-3.19
Comp 2-7	-92.26	-74.62	-87.61	-2.50	-7.10	-5.74
Comp 1-3-4-8	-91.85	-73.92	-88.41	0.00	-7.07	-5.69
Comp 11	-81.23	-66.68	-79.78	-4.77	-8.12	-6.67
Comp 5	-92.15	-64.64	-88.09	0.00	-6.58	-4.62
Comp 10	-77.19	-63.96	-81.84	0.00	-6.43	-5.33
Comp 6	-77.70	-63.73	-89.49	-2.80	-4.86	-3.98

Ligand	MolDock Score[a]	Rerank Score[b]	Interaction[c]	HBond[d]	LE1[e]	LE3[f]
Comp 2-7	-99.4	-80.2	-95.3	-2.1	-7.6	-6.2
Comp 11	-74.4	-72.5	-75.1	-2.8	-7.4	-6.2
Comp 1-3-4-8	-95.4	-68.3	-89.4	0.0	-7.3	-6.0
Comp 10	-80.8	-67.3	-84.9	0.0	-6.7	-5.6
Comp 6	-70.4	-41.7	-81.7	-2.3	-4.4	-2.6
Comp 5	-99.0	-37.4	-94.1	0.0	-7.1	-2.7
Comp 9	-112.6	21.1	-145.8	-2.2	-3.6	0.7

Characterization of AgNPs

UV-visible spectra showed the characteristic plasmon peak of silver at around 442 nm for *S. cumini* mediated synthesized AgNPs and at 482 nm for *P. guajava* mediated AgNPs . The absorbances were recorded for AgNPs with a final concentration of 0.1 mM $AgNO_3$. Generation of nanoparticles was monitored from 0 to 72 h and increase in absorbance was observed during this period.

The elemental composition of EDX data revealed the presence of silver in the formed AgNPs. Apart from the silver ions there are other signals of carbon, oxygen and sulphur which could be the residual presence of plant extracts. Lattice parameters of the prepared nanoparticles were studied by XRD technique. The pattern shows the formation of face centered cubic (fcc) silver, which could be confirmed by the peak positions at 2θ values 38.3, 44.6, 64.2 and 74.1 (JCPDS 89-3722). These correspond to the characteristic Bragg's reflection planes (111), (200), (220) and (311).

This was further supported by TEM analysis, where maximum particle density was observed within 10-15 nm for *S. cumini* mediated synthesized AgNPs and at 50-60 nm for *P. guajava* mediated AgNPs.

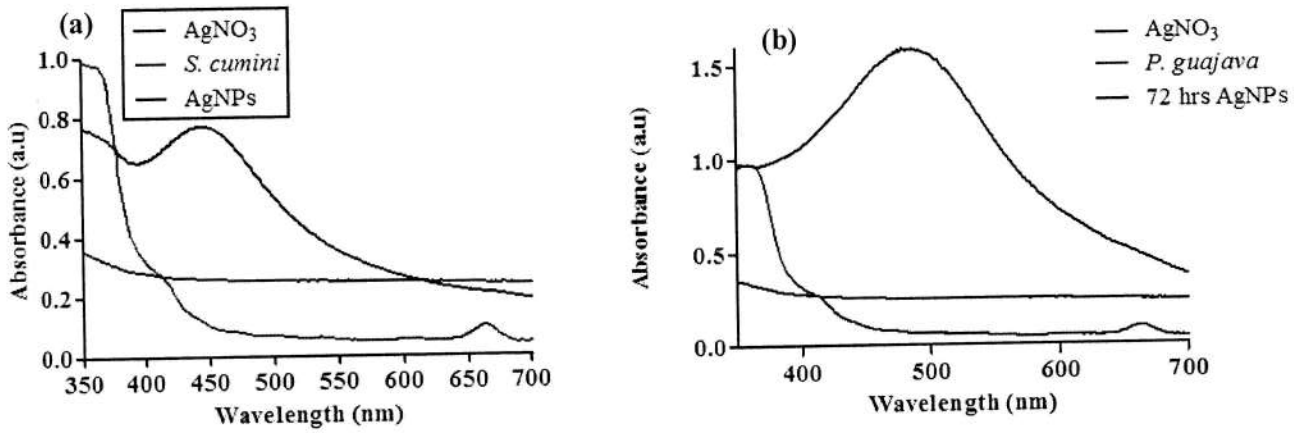


Figure UV-visible spectrum of AgNPs from *S. cumini* (a) and *P .guajava* (b). 1 mM$AgNO_3$ in aqueous (blue); Plant extract (red) and AgNPs (black). The curve of AgNPs and aqueous solution of $AgNO_3$ are recorded after a period of 72 hrs incubation.

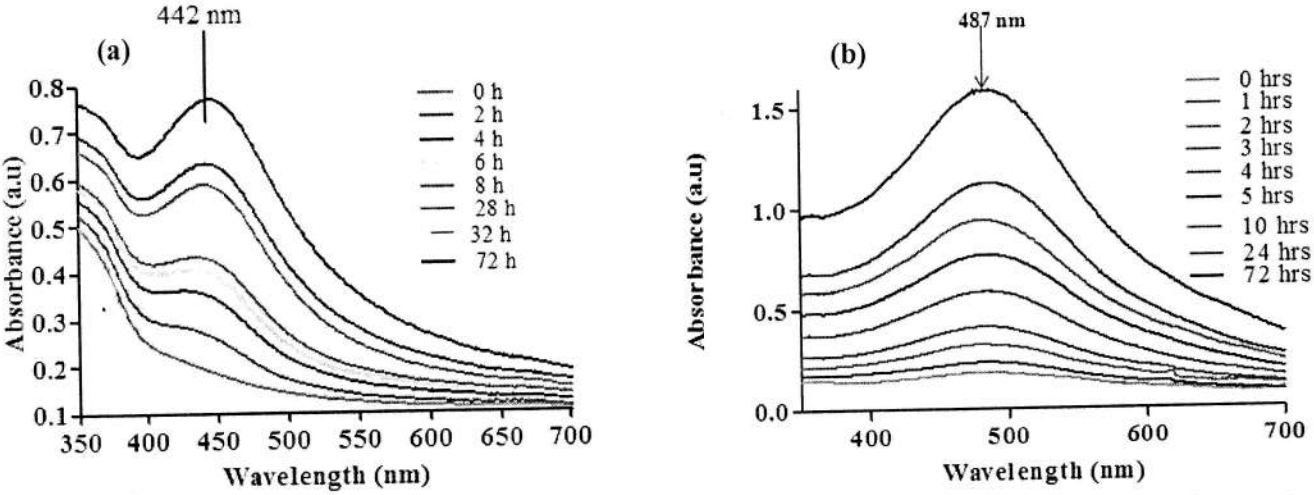


Figure. Absorption spectra of time dependent formation of AgNPs obtained from *S.cumini*(a) and *P .guajava* (b)

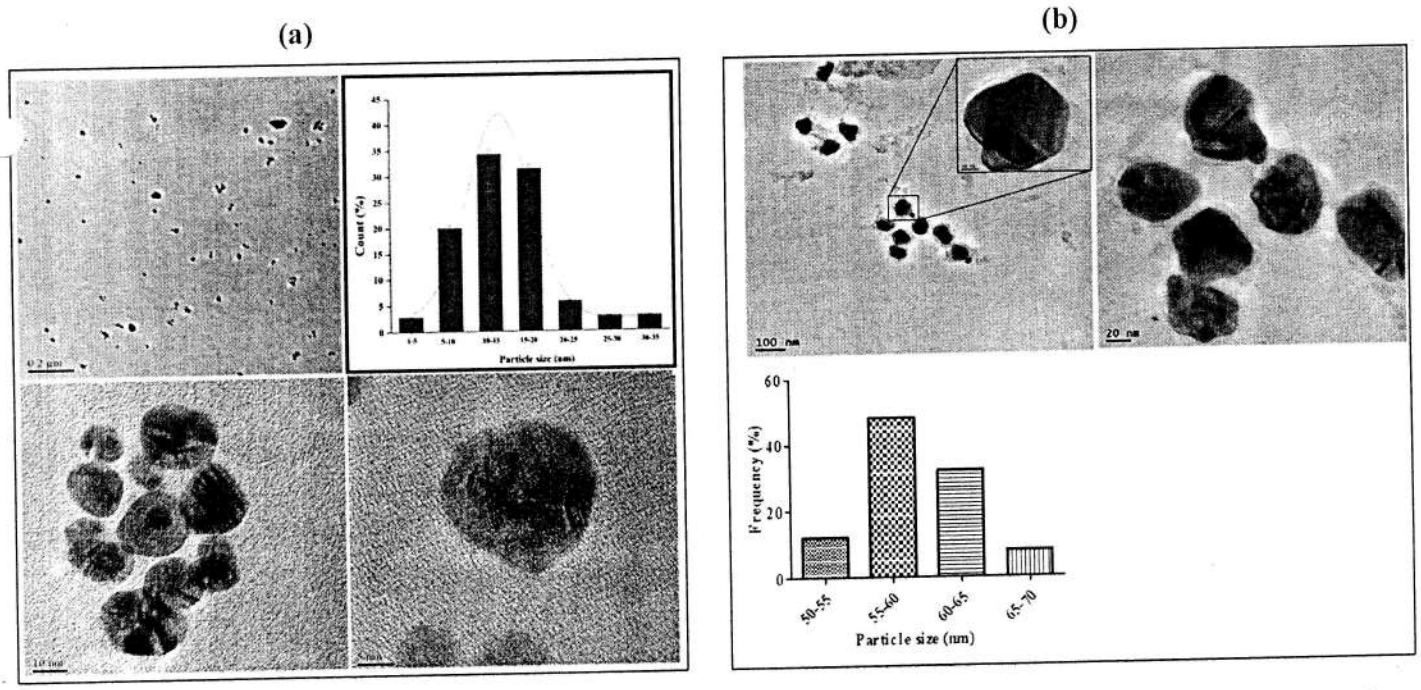


Figure: TEM micrographs of phytosynthesized silver nanoparticles from *S. cumini* (a) and *P. guajava* (b), histogram frequency for the particle size distribution derived by counting over multiple images

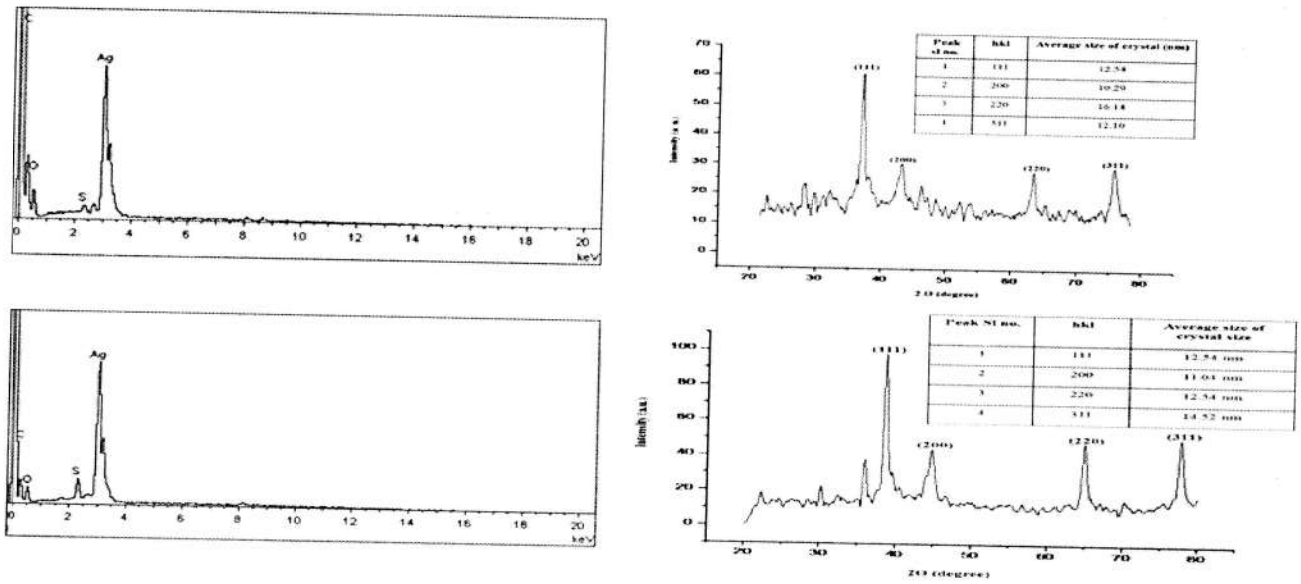


Fig. EDX of the green AgNPs from *S. cumini* (a) and *P. guajava* (b) , X-ray powder diffraction pattern of the green synthesized silver nanoparticles from *S. cumini* (c) and *P. guajava* (d).

Antimicrobial assays and Minimum Inhibitory concentration

Synthesized AgNPs were tested for their antimicrobial activity. AgNPs exhibited significant antibacterial activity against Gram-negative bacteria (*K. pneumoniae*, *P. aeruginosa* and *E. coli*), Gram-positive bacteria (*S. aureus*, *B. subtilis*, *M. smegmatis*) and fungus (*T. rubrum, Aspergillussp*, *F. oxysporum* and *C. albicans*) grown in MHA and PDA medium. The antimicrobial activity expressed in terms of zone of inhibition was quite visible on MHA and PDA plates and corresponding Tables shows the measurement of zone of inhibition of AgNPs, leaf extract and $AgNO_3$ alone.

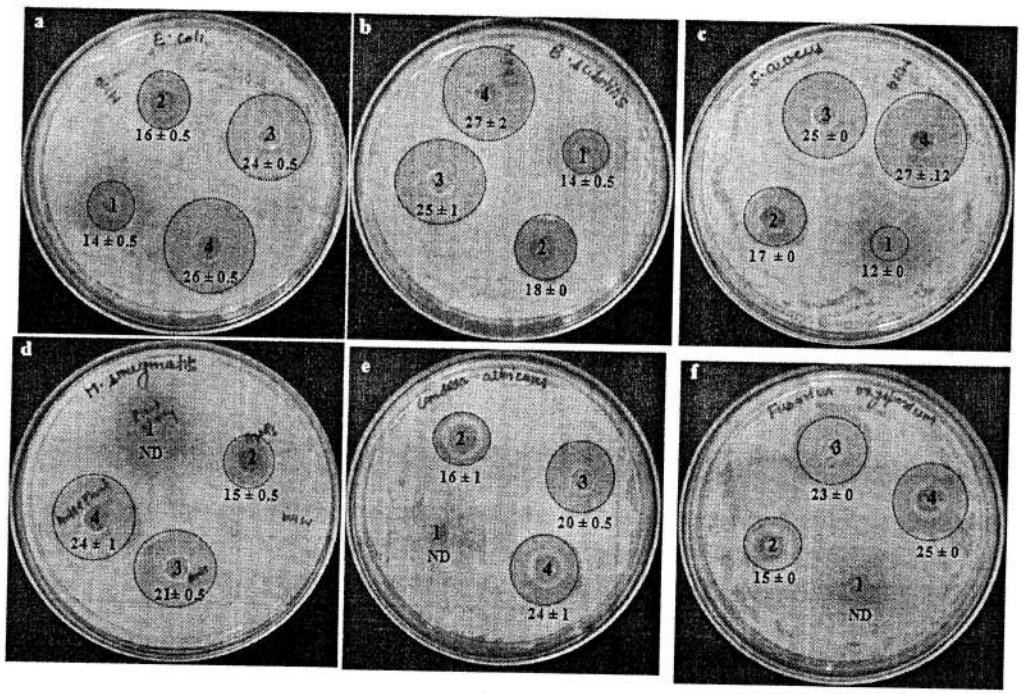


Fig. : Antimicrobial activity of *S. cumini* mediated synthesized AgNPs: Antimicrobial activity of (1) plant extract,(2) silver nitrate, (3)AgNPs and (4) antibiotics against (a)*Escherichiacoli*, (b) *Bacillus subtilis*, (c)*Staphylococcus aureus*, (d)*Mycobacterium smegmatis*, (e) *Candida albicans* and(f) *Fusarium oxysporum.*

Table 1. Anti microbial activity of methanol extract (ME) of *S. cumini*, $AgNO_3$ (1mM), AgNPs (10,000 µg ml^{-1}) and standard antibiotics by agar well diffusion assay

Pathogenic microorganism	Diameter of mean zone of inhibition(mm) *			
	S. cumini	$AgNO_3$	AgNPs	Antibiotics**
Escherichia coli MTCC 40	14 ± 0.5	16 ± 0.5	24 ± 0.5	26 ± 0.5
Pseudomonas aeruginosa MTCC 1688	13 ± 0.5	16 ± 0.5	21 ± 0.5	23 ± .10
Bacillus subtilis MTCC 121	14 ± 0.5	18 ± 0	25 ± 1	27 ± 2
Staphylococcus aureus MTCC 3160	12	17	25	27 ± .12
Mycobacterium smegmatis MTCC 14468	NA	15 ± 0.5	21 ± 0.5	24 ± 1
Candida albicans MTCC 183	NA	16 ± 1	20 ± 0.5	21 ± 1
Fusarium oxysporium MTCC 284	NA	15	23	25
Trichophyton rubrum (sk)	NA	NA	17 ± 1	22 ± 0.5
Aspergillus niger(1)	NA	NA	18 ± 0.5	22 ± 0.5
Aspergillus versicolor(2)	NA	NA	10 ± 0.5	22 ± 0.5

(NA): Not active. Values are given as mean ± SEM (n =3). *The diameter of well is 6 mm
**Gentamicin is used for bacterial strain, Nystatin and Clotrimazole is used for fungal strain as control.

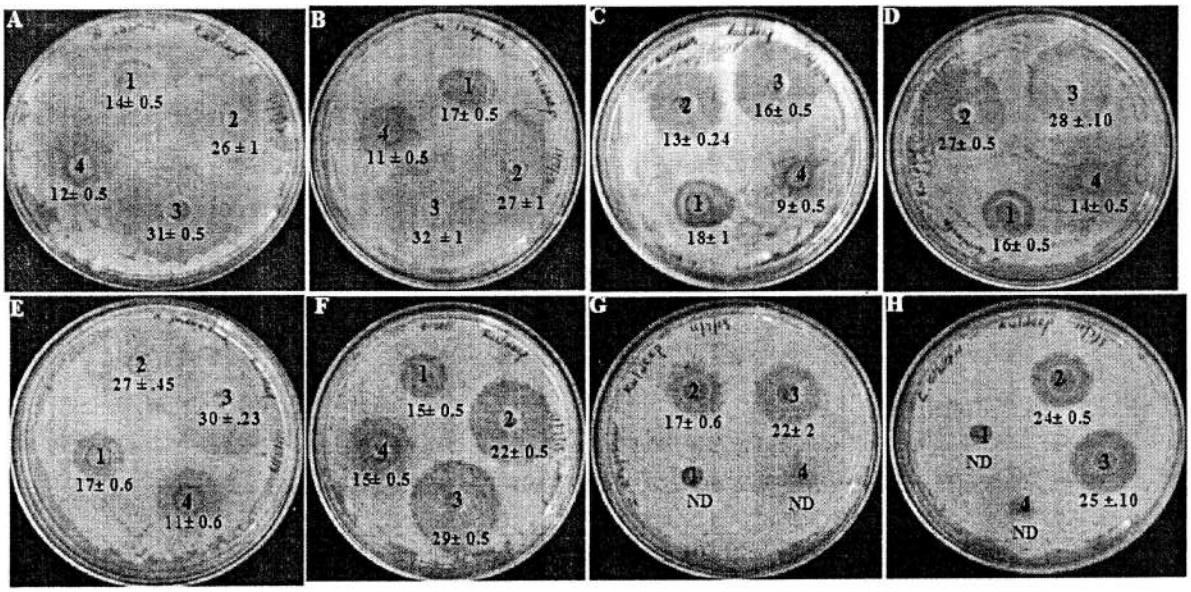


Figure: Antimicrobial activity of *P. guajava* mediated synthesized AgNPs: Antimicrobial activity of (1) silver nitrate (2), AgNPs (3) antibiotics and (4) *P. guajava* extract against (A) *Bacillus subtilis*, (B) *Mycobacterium smegmatis*, (C) Staphylococcus aureus, (D) *P. diminuta*, (E) K*lebsiella pneumonia*, (F) *Escherichia coli*, (G) *Fusarium oxysporum* and (H) *Candida albicans*.

Table 1. Anti microbial activity of AgNO3, plant extract (ME) and silver nanoparticle (AgNPs) by agar well diffusion assay

Pathogenic microorganism	Diameter of mean zone of inhibition(mm)			
	AgNO3	*P.guajava*	AgNPs	Antibiotics
Escherichia coli MTCC 40	15± 0.5	15± 0.5	22± 0.5	29± 0.5
Pseudomonas diminuta MTCC 1688	16± 0.5	14± 0.5	27± 0.5	28 ± .10
Bacillus subtilis MTCC 121	14± 0.5	12± 0	26± 1	31± .23
Staphylococcus aureus MTCC 3160	18	-	23	26± .12
Mycobacterium smegmatis MTCC 14468	17± 0.5	11± 0.5	27± 0.5	28 ± .23
Klebsiella pneumonae MTCC 618	16± 0.5	11± 0.5	27 ± .45	30 ± .23
Candida albicans MTCC 183	-		24± 0.5	25 ±.10
Fusarium oxysporum MTCC 284	-	15± 1	27± 0.6	30± 2

(–): Not active. Values are given as mean ± SEM (n =3). *The diameter of well is 6 mm
**Amp is used for gram (+) microrganism, Gentamicin is used for gram(-) microorganism, Nystatin and Clotrimazoleis used for fungal strain as control

In vitro time dependent killing assay

From the antimicrobial assay it is clear that AgNPs have prominent activity against microorganisms. Again AgNPs showed significant inhibitory activity toward *S. aureus*, *E. coli*, *B. subtilis*, *P. aeruginosa*, *M. smegmatis C. albicans* and *F. oxysporrum* in a time dependent manner. After 30 min of exposure to AgNPs more than 75% of the microbes were killed, whereas after 2 h of incubation more than 95% killing was observed, relative to the control.

Fig 4

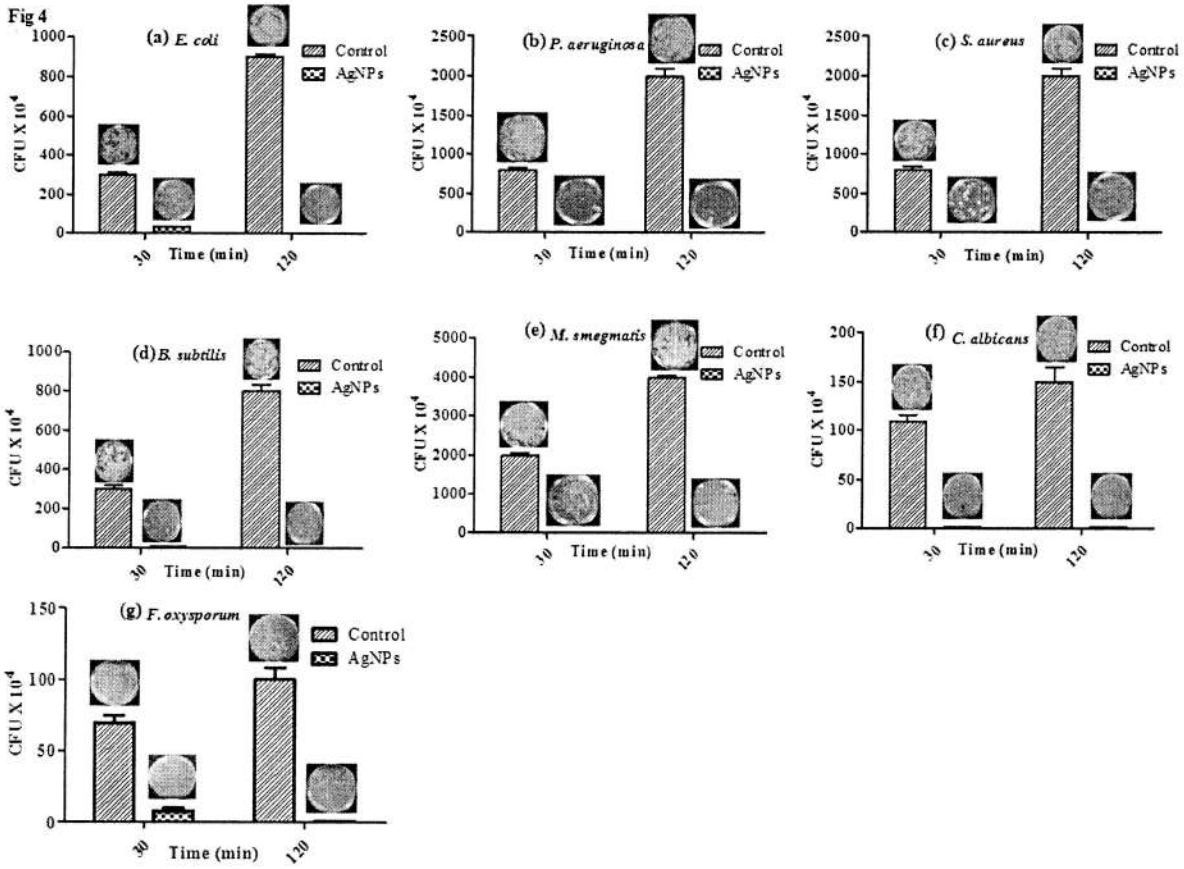


Fig.Time dependent Effect of AgNPs on microbial survivability:effect of AgNPs (10 mg mL^{-1}) on the microbial survival using CFU assay. (a) *Escherichiacoli*, (b) *Pseudomonas aeruginosa*, (c) *Staphylococcus aureus*, (d) *Bacillus subtilis*, (e) *Mycobacterium smegmatis*, (f) *Candida albicans* and (g) *Fusarium oxysporum*

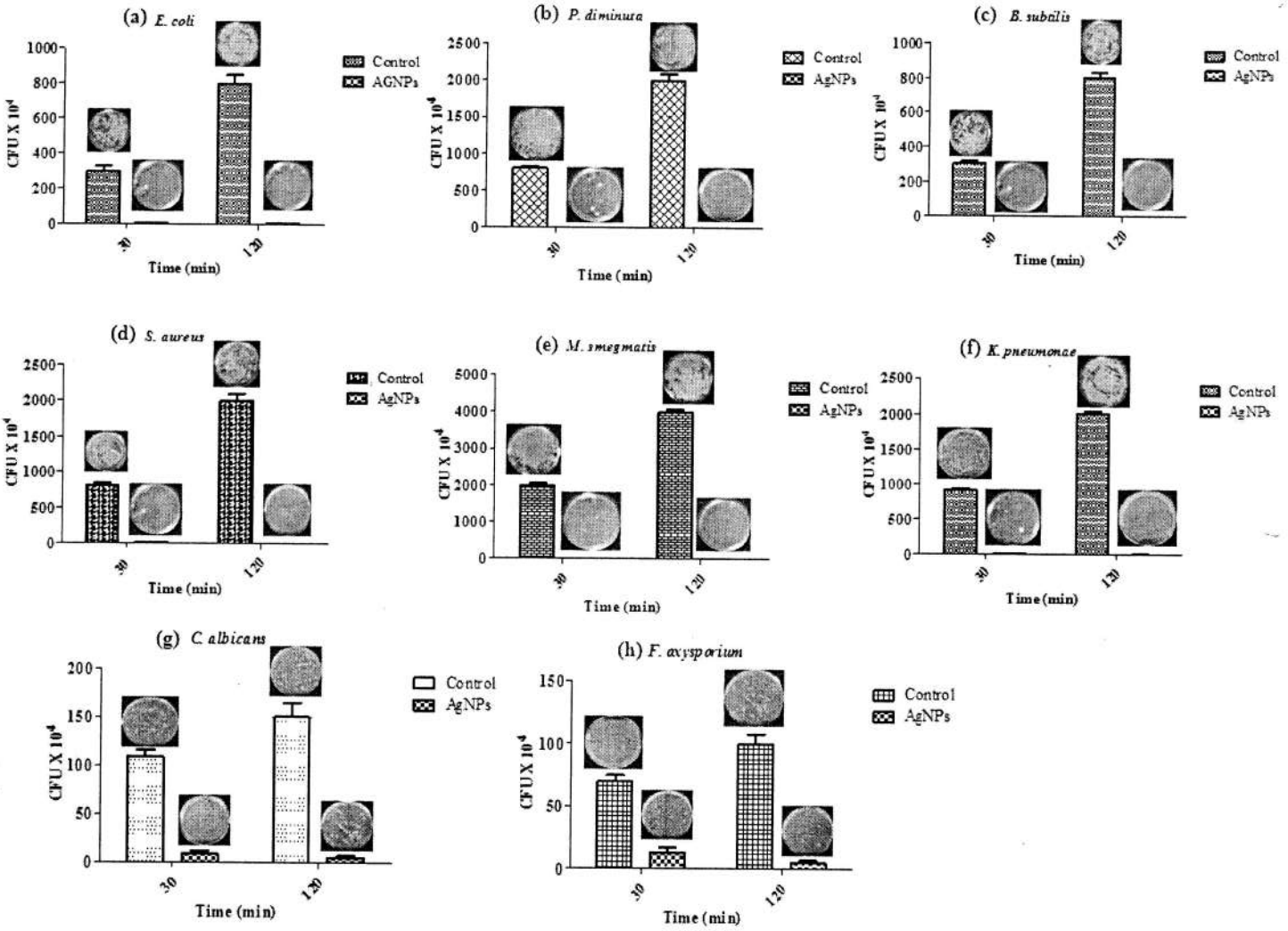


Figure.Time dependent Effect of AgNPs on microbial survivability:effect of AgNPs on the microbial survival using CFU assay. (a) *Escherichia coli*, (b) *Pseudomonas diminuta*, (c) *Bacillus subtilis*, (d) *Staphylococcus aureus*, (e) *Mycobacterium smegmatis*, (f) *Klebsiella pneumonae (g) Candida albicans* and (h) *Fusarium oxysporum*

Change in cellular morphology after AgNPs treatment

SEM images of three representative microbial strains (*E. coli, S. aureus* and *C. albicans*) were taken to observe changes in the surface morphology of the microbial cells after treatments with AgNPs for 30 min. Native *E. coli, S. aureus and C. albicans* cells showed normal morphologies without any treatment (control) and the cell wall of the treated microorganisms is showing some abnormalities (AgNPs).Thus, membrane damage may be one mechanism for inactivation of microorganism treated with AgNPs.

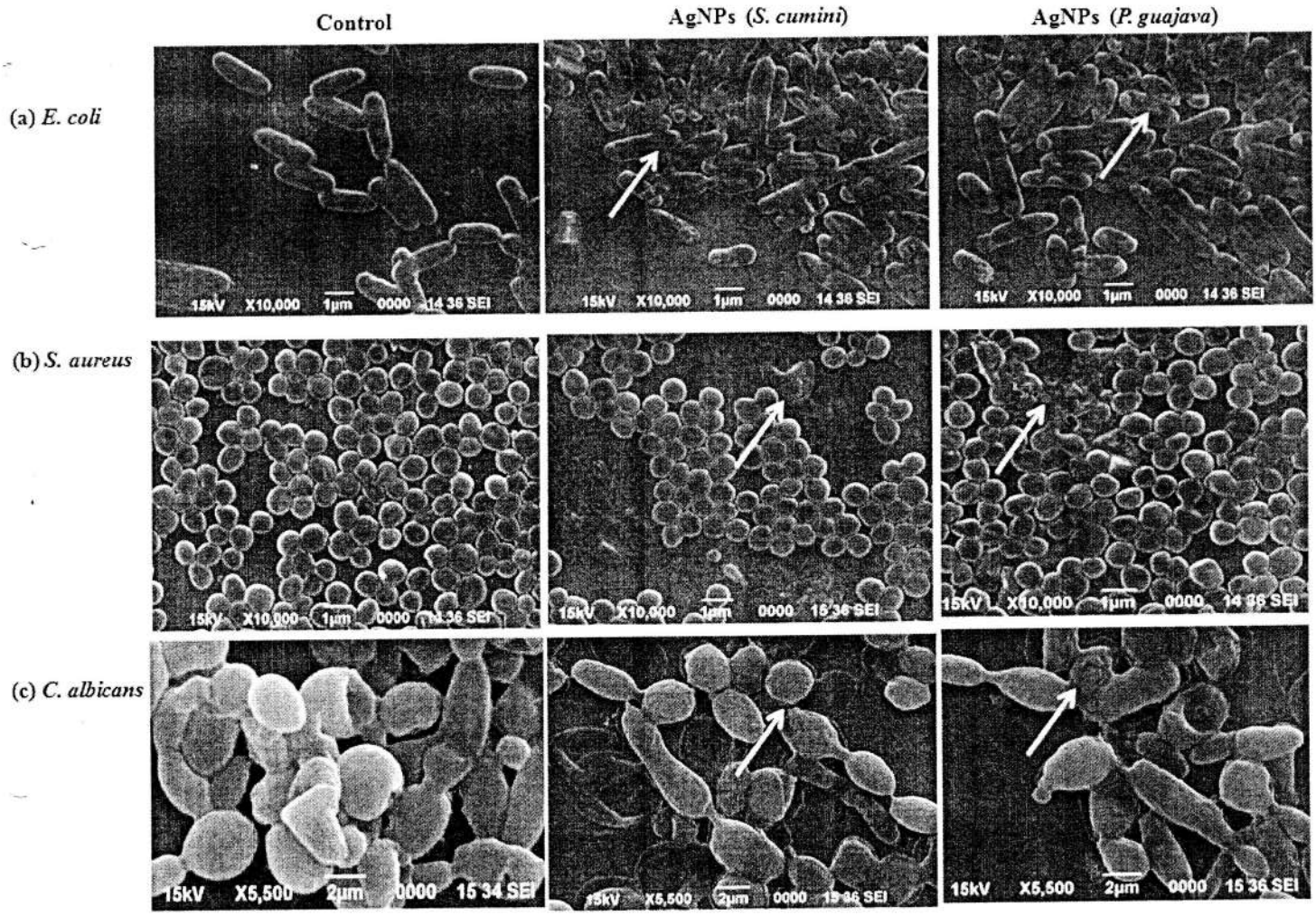


Fig. Scanning electron microscopy images of microbial cells upon treatment with AgNPs: SEM micrograph of microbial cells (a) *Escherichia coli*(b) *Staphylococcus aureus* and (c) *Candida albicans* without treatment of AgNPs (control) and after treatment (AgNPs) Solid arrows show morphological deformation of treated cells.

Action of AgNPs on biofilm formation

Among the microbes tested here, *E.coli P. aeruginosa*, *S. aureus* and *C. albicans* have been studied in detail with respect to their ability to form biofilms. In this study, we tested the effect of AgNPs on biofilm formation under *in vitro* conditions by monitoring the binding of the crystal violet to adherent cells, which directly reflects the effective ability of the biofilm inhibition. Treatment of AgNPs for 24 h resulted in a decrease of biofilm formation with increasing AgNPs concentration. More than 90% biofilm inhibition was observed at higher concentrations. In addition the controls DMSO and water did not have significant biofilm inhibition activity, whereas 1mM $AgNO_3$ has the biofilm inhibition activity which may be due its antimicrobial activity. These data indicate that AgNPs impede biofilm formation of *E. coli*, *P. aeruginosa, S. aureus* and *C. albicans.*

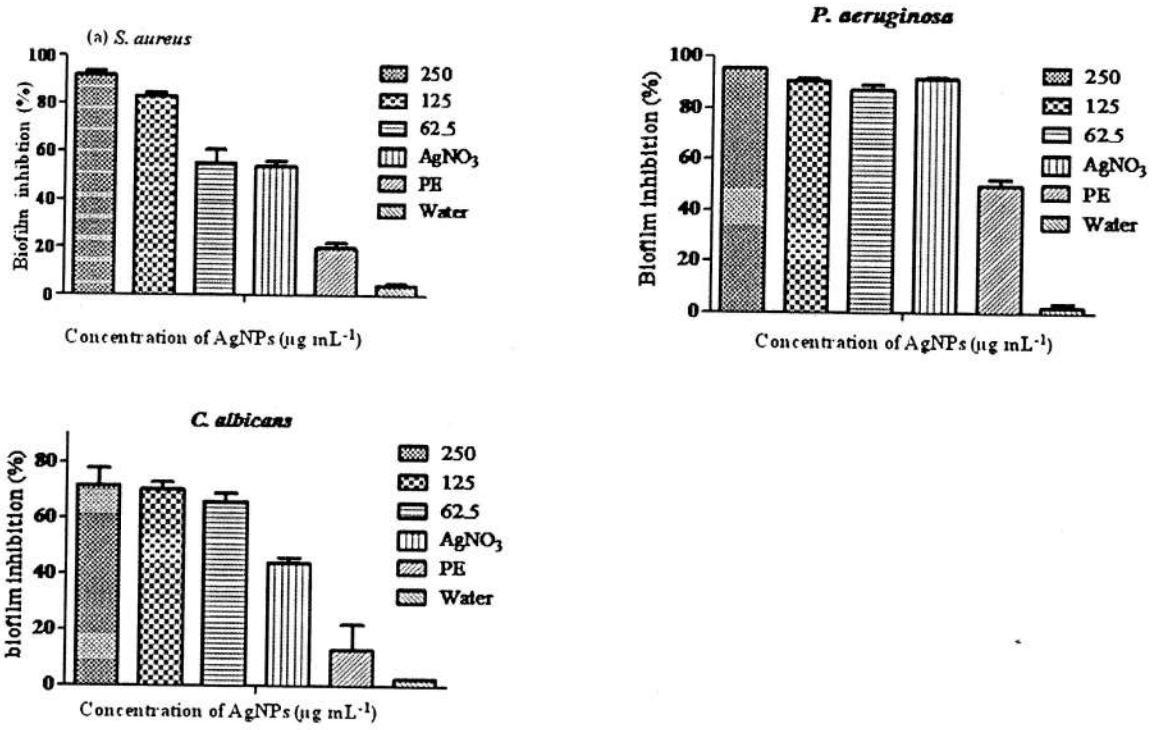


Fig. Inhibition of biofilm formation of (a) *Staphylococcus aureus,* (b) *Pseudomonas aeruginosa* and(c) *Candida albicans* by *S.cumini* mediated synthesized AgNPs.

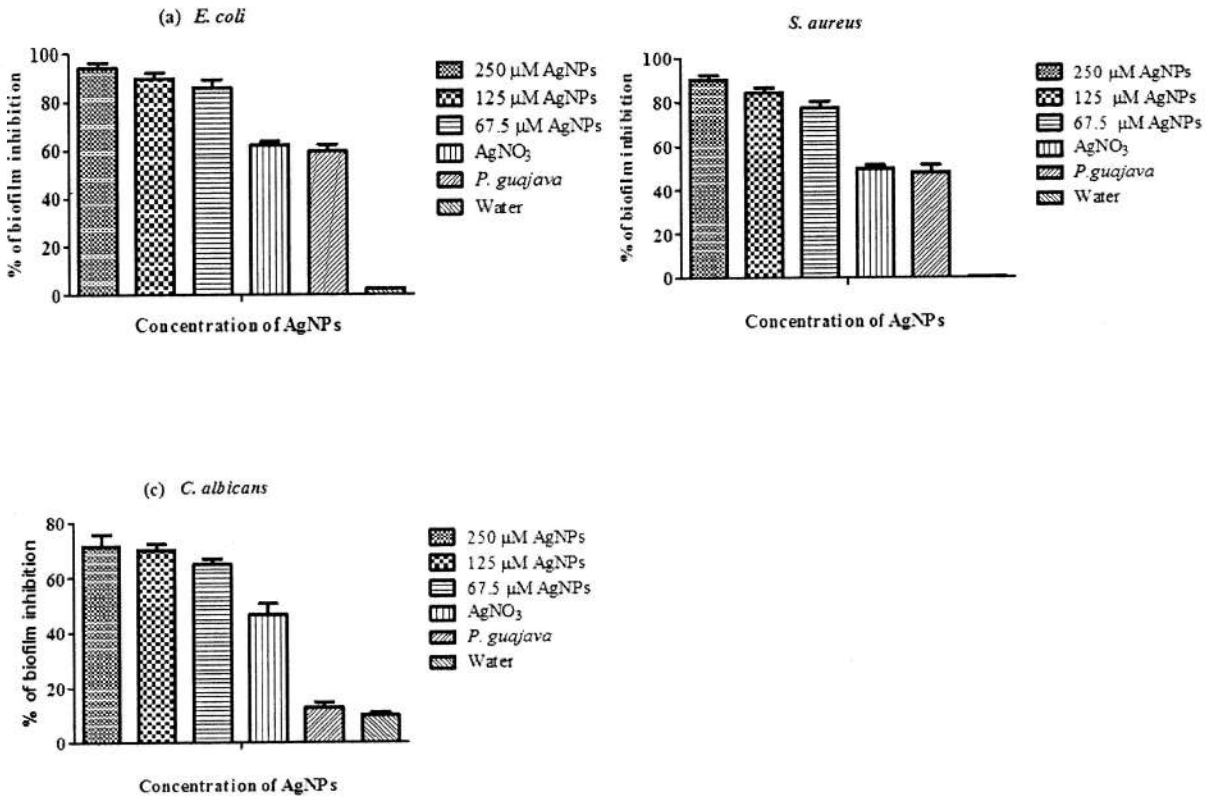


Fig. Inhibition of biofilm formation of (a) *E.coli,* (b) *S. aureus* (c) *Candida albicans* by *P. guajava* mediated synthesized AgNPs.

10. Detailed analysis of results indicating contributions made towards increasing the state of knowledge in the subject.

Natural products are potent fighters against the new emerging infections and also the resistance arising in the microbes. The plant extract could be a promising tool for compromising the biofilm associated infection. Studies related to natural products have been shown to inhibit the biofilm formation and also to inhibit virulence gene expression, related to quorum sensing. The success of plant extracts in inhibiting biofilm formation and quorum sensing mediated virulence factors in this study is a promising tool for reducing microbial colonization and inhibiting quorum sensing associated virulence factors. The anti-bacterial activity by well diffusion assay and growth curve analysis showed that EA did not have direct effect on the growth of *S. aureus* and *P. aeruginosa*. Accordingly, we evaluated the ability of EA for assessing its anti-biofilm activity in-vitro. When the microbes (*S. aureus* and *P. aeruginosa*) were grown in the presence of EA, it shows reduction in biofilm formation in *S. aureus* and *P. aeruginosa* as evident from the crystal violet staining assay. The Scanning Electron Microscopy images

also showed the inhibition of biofilm formation in both the micro organisms. Swarming motility, which is a quorum sensing mediated phenomenon, leads to bacterial translocation and colonization of the cells in a nutrient rich environment . Therefore, any interference with the swarming motility will affect the biofilm formation . It is evident from the results that the swarming motility of the *P. aeruginosa* was remarkably reduced (as compared to the control) by the treatment with different concentrations of EA. In addition to swarming motility, flagella-mediated swimming motility is also associated with virulence and biofilm formation by instigating the cell-to-surface attachment. Therefore, any interruption in swimming motility will also affect the biofilm formation. It is clear that the swimming and swarming motility of *P. aeruginosa* was comparatively poor with that of control when treated with EA, and hence, EA appeared to reduce the biofilm formation by interfering with its ability to reach the substratum.

S. aureus causes a wide spectrum of clinically acquired and community-acquired infections, including skin and soft-tissue infections and life threatening systemic infection. . Staphyloxanthin in *S. aureus* has been associated with enhancing bacterial survival in harsh environments and during infections. To investigate the biological activities of EA on *S. aureus* pigment production, staphyloxanthin was extracted from EA treated *S. aureus* and it was found that the treated *S. aureus* cells exhibited less pigment production and this may offer a potential target for treatment of complicated *S. aureus* infections.

GC–MS of EA revealed a large number of phytochemicals comprising many groups. Further, the molecular docking simulation carried out against the bacterial enzyme (PDB ID: 2B4Q, 2W38 and 3JPU) comes to a conclusion that Heptacosanoic acid (compound 9), 3-N-Hexylthiane s, s-Dioxide (compound 2) and 3-Methyl 2-(2-Oxopropyl) Furan (Compound 11) is most likely to form molecular interaction with the active site of the enzyme (2B4Q, 2W38 and 3JPU) as evidenced from the MolDock Score and Rerank Score Table 1 (a), 1 (b) and 1 (c). The MolDock Score employed in the present investigation is derived from the PLP scoring functions with new hydrogen bonding term and new charge schemes. It is clear that Heptacosanoic acid formed molecular interaction with Gly98 of PDB ID: 2B4Q and Gln136 of PDB ID: 2W38. 3-N-Hexylthiane s, s-Dioxide formed molecular interaction

with Gly156, Gly163 of PDB ID: 2B4Q; Ty243 of PDB ID: 2W38 and Tyr93 of PDB ID: 3JPU .While 3-Methyl 2-(2-Oxopropyl) formed molecular interactions with Tyr75, Trp88, Tyr93, Thr115 and Ser129 of PDB ID: 3JPU. This molecular interaction comes to a conclusion that these compounds will form a strong interaction with the bacterial proteins.

RhlG/NADP active-site complex (PDB ID: 2B4Q) is essential for rhamnolipid formation which regulate the swarming motility of *P. aeruginosa* and LasR is also related in quorum sensing of the bacteria. Studies also showed that mutation in the gene encoding sialidase (Pseudaminidase) resulted in the reduction of biofilm. Overall the molecular docking analysis revealed that Heptacosanoic acid, 3-N-Hexylthiane s, s-Dioxide and 3-Methyl 2-(2-Oxopropyl) is likely to involve in inhibition of bacterial biofilm and quorum sensing.

Biofabricated metal nanoparticles are biocompatible, inexpensive and eco-friendly. They find immense utility in the domain of biomedical and material science. We successfully developed an inexpensive, rapid and a single-step technique for greener synthesis of silver nanoparticles using leaf biomass of medicinal plants *P. guajava* and *S. cumini*. The results of UV-VIS absorbance, XRD, FTIR, EDX, and TEM have confirmed an *in situ* bio-reduction of Ag^{+} ions. AgNPs showed an effective with broad-spectrum antimicrobial behavior, which caused nearly 100% mortality within two hours against the microbial strains used in this study. Further synthesized AgNPs have the potential to inhibit biofilm formation in a variety of micro-organisms

Thus the prepared AgNPs could be of promising application in biomedical field as an efficient antimicrobial and antibiofilm agents.

List of Research publications

S No.	Authors	Title of paper	Name of the Journal	Volume	Pages	Year
1	Kuldeep Gupta et al.	Green silver nanoparticles: Enhanced antimicrobial and antibiofilm activity with effects on DNA replication and cell cytotoxicity	RSC Advances	4 (2014)	52845-52855	2014
2	Kuldeep Gupta et al.	One step green synthesis and anti-microbial and anti-biofilm properties of Psidium guajava L. leaf extract-mediated silver nanoparticles	Materials Letters	125 (2014)	67– 70	2014

i. Manpower trained on the project

a) Research Scientists or Research Associates: **Nil**

b) No. of Ph.D. produced: **Nil**

c) Other Technical Personnel trained: **Nil**

ii. Patents taken, if any: **Nil**

11. Financial Position:

No	Financial Position/ Budget Head	Funds Sanctioned	Expenditure	% of Total cost
I	Salaries/ Manpower costs			
II	Equipment			
III	Supplies & Materials			
IV	Contingencies			
V	Travel			
VI	Overhead Expenses			
VII	Others, if any			
	Total	Rs. 5,50,000	Rs. 5,19,058	94.37

12. Procurement/ Usage of Equipment: **Nil**

b) Plans for utilising the equipment facilities in future: **NA**

Name and Signature with Date

a. _____

(Principal Investigator)

Annexure-III

UTILISATION CERTIFICATE (2 COPIES)
17 November 2013- 31 March 2014

1. Title of the Project/ Scheme: **Study of microbial biofilm inhibition by traditionally used medicinal plants of Assam and Arunachal Pradesh and their role on extracellular polymeric substances (EPS) secretion**

2. Name of the Institution: **Tezpur University**

3. Principal Investigator: **Dr. Manabendra Mandal**

4. Science & Engineering Research Board (SERB) Sanction order No & date sanctioning the project (First financial sanction order): **SB/EMEQ-139/2013, 19.11.13**

5. Head of account as given in the original sanction order: **General head**

6. Amount brought forward from the previous Financial year quoting SERB letter no and date in which the authority to carry forward the said amount was given
   i. Amount: **NA**
   ii. Letter No
   iii. Date

7. Amount received during the financial year (Please give SERB Sanction order no and date)
   i. Amount :**5,50,000.00**
   ii. Order No: **SB/EMEQ-139/2013**
   iii. Date : **19.11.13**

8. Total amount that was available for expenditure (excluding commitments) during the financial year (Sr. No. 6+7) Rs. **5,50,000.00**

9. Actual Expenditure (excluding commitments) Incurred during the financial year (upto 31st March) Rs. 0.00

10. Balance amount available at the end of the financial year: Rs. **5,50,000.00**

11. Unspent balance refunded, if any (please give details of cheque no etc.): **NA**

12. Amount to be carried forward to the next financial year (if applicable): Rs. **5,50,000.00**

UTILISATION CERTIFICATE

Certified that out of Rs. **5,50,000.00** of grants-in-aid sanctioned during the year 2013-2014 in favor of **Dr. Manabendra Mandal** vide SERB order No **SB/EMEQ-139/2013** dated **19.11.13** and Rs. **0.00** on account of unspent balance of the previous year, a sum of **Rs 0.00** has been utilized for the purpose of **NA** for which it was sanctioned and that the balance of Rs. **5,50,000.00** remaining unutilized at the end of the year and will be adjusted towards the grants-in-aid payable during the next year i.e.**2014-2015**.

Signature of PI

Date 7.10.15

Signature of Registrar/ Head of the Institute

Date

Registrar
Tezpur University

Accounts Officer of the Institute

Date 7.11.15

Finance Officer
Tezpur University

(Countersigned in SERB))

Signature:_____

Designation:_____

Date:_____

Annexure-III

UTILISATION CERTIFICATE (2 COPIES)
1 April 2014 – 31 March 2015

1. Title of the Project/ Scheme: **Study of microbial biofilm inhibition by traditionally used medicinal plants of Assam and Arunachal Pradesh and their role on extracellular polymeric substances (EPS) secretion**

2. Name of the Institution: **Tezpur University**

3. Principal Investigator: **Dr. Manabendra Mandal**

4. Science & Engineering Research Board (SERB) Sanction order No & date sanctioning the project (First financial sanction order): **SB/EMEQ-139/2013, 19.11.13**

5. Head of account as given in the original sanction order: **General head**

6. Amount brought forward from the previous Financial year quoting SERB letter no and date in which the authority to carry forward the said amount was given
   i. Amount: **NA**
   ii. Letter No
   iii. Date

7. Amount received during the financial year (Please give SERB Sanction order no and date)
   i. Amount :**5,50,000.00**
   ii. Order No: **SB/EMEQ-139/2013**
   iii. Date : **19.11.13**

8. Total amount that was available for expenditure (excluding commitments) during the financial year (Sr. No. 6+7)
   Rs. **5,50,000.00**

9. Actual Expenditure (excluding commitments) Incurred during the financial year (upto 31st March)
   Rs **Rs. 5,19,058.00**

10. Balance amount available at the end of the financial year: Rs. **Rs. 30,942.00**

11. Unspent balance refunded, if any (please give details of cheque no etc.): **NA**

12. Amount to be carried forward to the next financial year (if applicable): Nil

UTILISATION CERTIFICATE

Certified that out of Rs. **0.00** of grants-in-aid sanctioned during the year 2014-2015 in favour of **Dr. Manabendra Mandal** vide SERB order No **SB/EMEQ-139/2013** dated **19.11.13** and Rs. **5,50,000.00** on account of unspent balance of the previous year, a sum of **Rs. 5,19,058.00** has been utilised for the purpose of **general items and overhead** for which it was sanctioned and that the balance of Rs. **30,942.00** remaining unutilised at the end of the year and has been refunded/returned to SERB (vide DD/Cheque No_____ dated _____).

Signature of PI	Signature of Registrar/ Head of the Institute	Accounts Officer of the Institute
Date 7.10.15	Date	Date 7.11.15
	Registrar Tezpur University	Finance Officer Tezpur University

(Countersigned in SERB))

Signature:_____
Designation:_____
Date:_____

Annexure-II

REQUEST FOR ANNUAL INSTALMENT WITH UP-TO-DATE STATEMENT OF EXPENDITURE

1. Sanction Order No and date: **SB/EMEQ-139/2013, 19.11.13**

2. Name of the PI**: Dr. Manabendra Mandal**

3. Total Project Cost: **Rs. 6,00,000.00**

4. Revised Project Cost: **NA**
   (if applicable)
5. Date of Commencement: **19.11.13**

6. Statement of Expenditure:
   (month wise expenditure incurred during current financial year)

Month & year	Expenditure incurred/ committed
November 2013	Nil
December 2013	Nil
January 2014	Nil
February 2014	Nil
March 2014	Nil
April 2014	Nil
May 2014	Nil
June 2014	Rs. 62,500.00
July 2014	Nil
August 2014	Nil
September 2014	Nil
October 2014	Nil
November 2014	Rs. 2,00,426.00
December 2014	Rs. 38,500.00
January 2015	Nil
February 2015	Nil
March 2015	Rs. 2,17,632.00
April 2015	Nil

1. Grant received in each year:
   a. 1st Year : **Rs. 5,50,000.00**
   b. 2nd Year :**Nil**
   c. 3rd Year : **Nil**
   d. Interest, if any : **Nil**
   e. Total (a+b+c+d): **Rs. 5,50,000.00**

Statement of Expenditure
(19-11-2013 to 31st March 2014, 01-04-2014 till 31.03.2015)

Sr No (I)	Sanctioned Heads (II)	Total Funds Allocated (indicate sanctioned or revised) (III)	Expenditure Incurred			Total Expenditure till.. (VII = IV + V + VI)	Balance as on (date) (VIII = III – VII)	Requirement of Funds upto 31st March next year	Remarks (if any)
			1st Year (17-11-2013 to 31st March 2014) (IV)	2nd Year (1st April 2014 to 31st March 2015) (V)	3rd Year & so on (1st April 2015 to 31st March 2016) (VI)				
1.	Recurring Items(General)	Rs. 4,50,000.00	0.00	Rs. 4,22,268.00	Nil	Rs. 4,22,268.00	Rs. 27,732.00		
2.	Overhead expenses	Rs. 1,00,000.00	0.00	Rs. 96,790.00	Nil	Rs. 96,790.00	Rs. 3210.00		
3.	Total	Rs.5,50,000.00	0.00	Rs. 5,19,058.00	Nil	Rs. 5,19,058.00	Rs. 30,942.00		

Name and Signature of Principal Investigator:

7.11.15

Signature of Competent financial authority: _____
(with seal) Date:_____

Finance Officer
Tezpur University

*** DOS – Date of Start of project**

Note :

1. Expenditure under the sanctioned heads, at any point of time, should not exceed funds allocated under that head, without prior approval of DST i.e. Figures in Column (VIII) should not exceed corresponding figures in Column (III)
2. Utilisation Certificate (Annexure III) for each financial year ending 31st March has to be enclosed along with request for carry-forward permission to the next financial year.