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ANTI-BIOFILM POTENTIALITY OF *ALTERNANTHERA PHILOXEROIDES*: A STUDY WITH METHANOLIC PLANT EXTRACT.

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ABSTRACT

Alternanthera philoxeroides is a well-known ethnomedicinal plant of Tripura. Ethnic communities of the state use several parts including aerial parts and leaves of this plant for the treatment of several ailments like stomach infection, diarrhoea, appetiser etc. With this background we have proposed to perform chemical characterisation, investigation of antimicrobial and antibiofilm potentiality of methanolic extract of aerial parts of *Alternanthera philoxeroides* (**APME**) against biofilm causing model microorganism *Pseudomonas aeruginosa*. Biofilms are clustered form of microorganisms attached to a substratum. Biofilm causing microorganisms (e.g. *Pseudomonas aeruginosa*) have been found to cause chronic inflammatory, infectious diseases and can become resistant to antibiotics. In the present study, **APME** has shown the presence of UV active phytochemicals as evident from UV-Vis spectroscopy and biochemical colour tests. **APME** has executed maximum 65.23% antimicrobial activity against *Pseudomonas aeruginosa* at 100 µg/ml dose. At sub-inhibitory dose (10 µg/ml), **APME** has exerted maximum 32.57% antibiofilm activity as analysed by safranin staining, protein extraction and EPS quantification assay. Effect of **APME** on attenuation of bacterial biofilm was validated by observation under fluorescent microscope. The extract at 10 µg/ml concentration also executes maximum attenuation in bacterial swarming motility. All these findings suggest that **APME** is a potential phyto-therapeutic extract which may be employed to formulate preventive strategies against biofilm associated infections caused by *P. aeruginosa*.

KEYWORDS: *Alternanthera philoxeroides*, *Pseudomonas areuginosa*, Antimicrobial, Antibiofilm, Motility.

INTRODUCTION

Biofilm represents a cluster of microorganisms embedded by self secretary extracellular polymeric substances (EPS). Several bacteria develop biofilm as an alternative survival strategy for their growth and development. *Pseudomonas aeruginosa*, an efficient biofilm forming gram negative bacterium often form biofilm during virulence exhibition and disease progression.[1] It has been observed that *Pseudomonas aeruginosa* causes several serious pathogenic manifestations on human host like urinary tract infection, skin infection, lung infection etc which may even be life threatening.[2,3] Antibiotics are routinely used to control gram positive as well as gram negative pathogenic microbial growth and survival. But recently, it has been observed that biofilm forming bacterial strains develop antibiotic resistance very fast.[4] Thus, treatment of microbial biofilm with conventional antibiotics becomes troublesome. Therefore, the uses of new antibacterial and antibiofilm compounds have drawn more attention in order to prevent microbial growth and biofilm formation.[4,5] In this context, it has been reported that the plant derived extracts often exhibit high antibacterial and antibiofilm properties against a range of microorganisms.[6] The north eastern region of India including Tripura is rich in higher biodiversity which is enriched with diverse medicinal plants including some rare and endangered taxa. The north eastern region represents about 50% of the total plant species of India.[7] Thus, the potentialities of ethno medicinal plants provide a strong platform to explore efficient phytochemical bioactive compounds from them.

The north eastern part of India is one of the ethnobotanical biodiversity hotspot in the world. The region is a unique bio-geographic province encompassing major biomes recognised in the world. More than 200 tribes of different groups with different cultural entities inhabits in the region. Some of the ethnic group use this plant extract or crude plant for the treatment of different diseases and ailment. Young shoot paste with black pepper is prescribed to cure acute cough.[7] Leaves with a pinch of salt are orally administered to cure intestinal worms by the south Odisa tribes.[8] While some of the ethnic communities of Bangladesh use the plant for hazy vision, night

blindness, malaria, post-natal complains, dysentery and perperual fever.[9] The Manipuri inhabitants use the shoot extract of this plant along with little common salt to cure from dysentery.[10] The ethnic community and rural villagers of Tripura use this plant as a vegetable and also to recover from anaemia.[11] In Kerala and West Bengal it is also sold in the market as vegetable and used as a medicine.[12] Surprisingly, the basic phytochemistry has not yet been discovered, only sulphated polysaccharides are isolated from this plant which has showed significant *in vitro* anti-HIV activity.[13] On this background we have screened the antibacterial and antibiofilm activity of the methanolic plant extract of *Alternanthera philoxeroides*.

MATERIALS AND METHODS

Chemicals and plant material

All chemicals used in the present study were purchased from Himedia, India. All solvents used in the extraction process were purchased from Merck, India.

Whole plant in flowering season, were collected from Kakrabon of Gomati district, Tripura in the month of September, 2015. The plant was independently identified by Prof. B.K. Datta, Dept of Botany, Tripura University (A Central University). A voucher specimen (TU/H/1450) has been deposited at the Herbarium of Plant Taxonomy and Biodiversity Laboratory, Department of Botany, Tripura University, Agartala, India.

EXTRACTION AND ISOLATION

Fresh air dried semi-powdered aerial parts (1 kg) were extracted with MeOH (1L×2). The concentrated semisolid extract (42.5 g) was suspended in H_2O (ca. 100 mL), defatted with hexane and then successively partitioned with $CHCl_3$, EtOAc and *n*-butanol. The n-butanol extract (20.8 g) was subjected to CC over Diaion HP-20; the column was eluted with H_2O, H_2O-MeOH (4:1 and 1:5) and MeOH, (500 mL each). Fractions (50 ml each) were collected and concentrated under reduced pressure. Fractions of H_2O-MeOH (1:4) exhibiting identical TLC pattern were mixed and concentrated to an oily mass (5.2 g). This oily mass was subjected to CC over silica gel eluted with petroleum ether-EtOAc (1:1 and 1:9) and EtOAc with a volume of 500 ml for each eluent. The fractions from petroleum ether-EtOAc (1:6 and 1:7) were almost in similar composition on TLC. These were mixed and subjected to repeated CC over silica gel (60-120 mesh) to obtain **APME** (12 mg) as white amorphous solid. Other known compounds were also isolated from different sub-fraction during isolation of **APME**.[14]

Ultra Violet (UV) spectrophotometric analysis

APME (0.1 mg/ ml) was dissolved in dimethyl sulphoxide (DMSO) and wavelength scan was done from 200 nm to 800 nm (Parkin-Elmer). Absorbances were plotted against wavelength to find out absorbance peaks corresponding to specific compounds.[14]

Qualitative phytochemical screening of the plant extract

Identification of flavonoids by alkaline reagent test

To test presence of flavonoid, in a test tube 1 ml of extract was taken and to that a few drops of dilute sodium hydroxide was added. An intense yellow colour was produced in the plant extract which becomes colourless on addition of few drops of dilute hydrochloric acid which indicates the presence of flavonoid.[15]

Identification of Alkaloid by Mayer's Test and Wagner's Test

To the extract solution, 2 ml of mayer's reagent [Dissolve 2.72 gm mercuric chloride in 120 mL distilled water, separately dissolve 10 gm potassium iodide in 40 mL distilled water. Mix the two solutions and make up to 200 ml with distilled water] was added, a dull white precipitate revealed the presence of alkaloids.[16]

To the 1 ml of extract, 2 mL of wagner's reagent (Dissolve 2 g of iodine and 6 g of KI in 100ml of water) was added, the formation of a reddish brown precipitate indicates the presence of alkaloids.[16]

Identification of Tannin by Ferric chloride Test

Few drops of diluted solution of $FeCl_3$ was added to the test tube containing 0.5 mL of extract solution, production of a greenish-black colour that changes to olive green as more ferric chloride was added indicates the presence of tannins.[17]

Identification of sterol by Libermann-Burchard Test and Salkowski Test

To 1 mL of extract solution, 1 mL of glacial acetic acid and 1 mL of acetic anhydride and two drops of concentrated sulphuric acid were added. The solution becomes red, then blue and finally bluish green, indicates the presence of steroids.[16]

1 mL of extract was treated with few drops of concentrated sulfuric acid in 0.5 mL of chloroform, shake well and allow to stand for 10 min, red colour appears at the lower layer indicates the presence of steroids.[15]

Identification of anthraquinone by Borntrager's Test

1 mL of extract was taken in a test tube, to that 1 mL of chloroform was added and shaken for 5 min. The mixture was filtered and the filtrate shaken with equal volume of 10% ammonia solution. A ink colour in the aqueous layer after shaking indicates the presence of free anthraquinone.[17]

Test for Phenol

In a test tube 1 mL of extract and 2 mL of distilled water were added followed by few drops of 10% ferric chloride ($FeCl_3$). Appearance of green colour indicates presence of phenols.[18]

Test for Terpenoid

In a test tube 1 mL of each extract was mixed with 0.5 mL of chloroform. 0.5 mL of concentrated sulphuric acid (H_2SO_4) was then added to form a layer. A brown colouration at the interface formed indicated the presence of terpenoids. [18]

Test for Anthocyanin

To the 1 mL of extract, 0.5 mL of 10% sodium hydroxide was added; blue color shows the presence of anthocyanins. [16]

Identification of Reducing Sugar by Fehling's solution test and Benedict's test

1 mL of extract solution was mixed with 1 mL of Fehling's solution [Fehling's A: Copper sulfate in distilled water, Fehling's B: Potassium tartarate and Sodium hydroxide in distilled water] and boiled for 5 minutes. Formation of brick red precipitate of cuprous oxide demonstrated the positive test for reducing sugar.[15]

To 0.5 mL of the extract solution, 2 mL of Benedict's solution was added in a test tube and boiled for few minutes. Brick red precipitate was developed confirm the presence of carbohydrates. [16]

Test for Quinone

To 1 ml of extract, 1 ml of concentrated sulphuric acid was added. Formation of red color indicated the presence of quinones. [18]

Identification of Saponin by Froth Formation Test

1 ml of extract solution was diluted with distilled water to 5 mL and shaken in a graduated cylinder for 15 minutes. Development of stable foam suggested the presence of saponin.[18]

Microbial strain

In the present study, *Pseudomonas aeruginosa* MTCC 2488 was used as test organism. Tryptic soy broth (TSB) media was used for the cultivation of *P. aeruginosa*. Initially bacteria were streaked from a −80°C glycerol stock onto respective agar plate, a fresh single colony was inoculated into 25 ml liquid media and incubated at 37°C for 24 hrs. From there, 10^6 CFU/mL bacterial cell suspensions were taken for all subsequent experiments.

Antibacterial activity assay

Antibacterial activity of **APME** against *P. aeruginosa* was determined by using standard broth micro dilution assay.[19] Briefly, 250.0 µl of 10^6 CFU/mL bacterial cell suspensions were taken in microplate wells and to that **APME** of different concentrations were added to attain final concentrations 7.5 to 400 µg/ml. The microplate was then incubated at 37^0C for 48 hrs. After this incubation period, bacterial population density was measured by taking absorbance (at 600 nm) by a microplate reader (Synergy H1 hybrid reader, Biotek).

Evaluation of biofilm forming capability of *P. aeruginosa*

Prior to antibiofilm assay, *P. aeruginosa* were tested for its ability to form biofilm. To perform the experiment, bacteria was grown in 96 wel micrtitre plate containing TSB at 37^0C for 48 hrs. After the incubation, tubes were then washed three times with sterile phosphate buffer saline (PBS) and stained with 0.1% (v/v) Safranin for 10 min. The excess stain was removed by washing with sterile PBS. Tubes were then dried for overnight at 37°C. Safranin-stained adherent *P. aeruginosa* bacteria were re-dissolved in 30% (v/v) glacial acetic acid. Thereafter, absorbance was measured at 492 nm for Safranin stained glacial acetic acid suspension.[19] Each assay was performed in triplicate.

Antibiofilm activity assay

Interference of biofilm formation upon **APME** treatment was performed using the protocol mentioned above for examining the biofilm forming ability of bacteria. Wels of microtitre plate was filled with 1 ml of diluted bacterial suspension. Sub-inhibitory concentrations of **APME** (50 µg/ml, 40 µg/ml, 30 µg/ml, 20 µg/ml, 10 µg/ml, 5 µg/ml) and sub-MIC dose (5 µg/ml, 1 µg/ml) of standard antibiotic gentamicin were directly added to the bacterial suspension at time zero and incubated at 37°C for 48 hours. All test tubes (**APME** treated and untreated) were then washed and stained with safranin as per the biofilm detection assay procedure described previously.[4] Absorbances of all tubes were measured at 492 nm. To find out percentage of biofilm inhibition in all treated tubes with respect to **APME** untreated control tube, the following formula was used:

Biofilm Inhibition (in %) = {(OD of **APME** untreated control) − (OD of **APME** treated sample) / (OD of **APME** untreated control)} X 100.

Estimation of total extractable protein concentration from biofilm

The microbial population density in biofilm is directly proportional to the amount of extractable protein concentration.[4,20] Therefore, to estimate the total extractable protein from tube surface, *P. aeruginosa* was inoculated into sterile test tubes containing TSB and incubated them with and without sub- inhibitory doses of **APME** (50 µg/ml, 40 µg/ml, 30 µg/ml, 20 µg/ml, 10 µg/ml, 5 µg/ml) and gentamicin (5 µg/ml, 1 µg/ml) at 37^0C for 48 hrs. After the incubation, planktonic cells were removed following which the adhered biofilm cells were washed gently with sterile PBS and boiled for 30 min in 5 ml of 0.5 N NaOH (Himedia, India) to extract the protein. After that, the suspension was centrifuged at 10,000 rpm for 5 min and the resulting clear supernatant was collected. From the supernatant, protein concentration was determined according to the Lowry's method.[21] Each experiment was performed in triplicate.

Extraction and measurement of exopolysaccharide (EPS)

EPS were measured as described previously by Das et al. 2016. In brief, biofilm was allowed to grow on glass surface after treatment with sub- inhibitory doses of **APME** and gentamicin. Subsequently, formed biofilm was extracted in sterile water and the suspension was centrifuged at 3500 g for 20 min at 4°C. The supernatant was collected and pellet was treated with 10 mmol/l EDTA, vortexed for 15 min and re-centrifuged to extract cell-bound EPS. The supernatant was collected and mixed with the previous supernatant. Pooled supernatant was then mixed with 2.2 volume of chilled absolute ethanol, incubated at -20°C for 1 h and centrifuged at 3500 g for 20 min at 4°C. The pellet containing EPS was dissolved in sterile water and measured by phenol–sulphuric acid method.[22]

Observation of biofilm formed on glass surface by fluorescence microscope

To examine the antibiofilm potentiality of selected sub-inhibitory doses of **APME** against *P. aeruginosa* biofilm, *P. aeruginosa* was separately grown in media containing varying concentration of **APME** and gentamicin with respect to untreated control. Sterile glass cover slips were added to each growth media to allow the development of microbial biofilm over it and incubated them at 37^0C for 48 hours. After the incubation, cover slips were recovered from all the conditioned media, stained with acridine orange (4 µgml−1), air-dried and observed under a fluorescence microscope (Leica DM 4000B, Germany).[4,20]

Motility analysis of *P. aeruginosa*

Swarming motility of *P. aeruginosa* was investigated in small 35 X 10 mm polystyrene plates containing swarming motility media [nutrient agar (8g/l) (Himedia, India) supplemented with glucose (5.0 g/l) (Himedia, India)]. An aliquot of 2 µl of overnight culture either treated or untreated with sub-inhibitory doses of **APME** and gentamicin with respect to untreated control was point inoculated in the centre of the plates and subsequently dried the spot for 20 min at room temperature and plates were incubated for 48 hrs at 37^0C. Diameter of circular bacterial growth from the point of inoculation was used to measure swarming motility of bacteria.[4,20]

STATISTICAL ANALYSIS

All experiments were performed in triplicate. Presented data are value of mean ± Standard deviation. Statistical significance was determined through one way ANOVA and level of significance are mentioned as *P* value < 0.01 (noted with *), *P* value <0.001 (noted with **) and *P* value <0.0001 (noted with ***). Statistical analyses were performed using Graph Pad Prism 6.0 software.

RESULT AND DISCUSSION

Methanolic plant extract is a crude extract which may contain all probable phytoconstituents as secondary metabolite of that plant. There have been several ethnobotanical reports of *Alternanthera philoxeroides*, which reveals that the plant has antimicrobial activity.[23] In this direction to further explore the effect of all active constituents of the plant in combination we have prepared methanolic extract from the aerial part of the plant. Though methanolic plant extract as a crude extract may contain several phytoconstituents, but to further confirm the type of these constituents we have examined the extract through UV-Vis spectrophotometric analysis. Spectroscopic data shows peaks in both UV (203 nm, 219 nm, 233 nm, 327 nm and 333 nm) and visible region (660 nm) (**Figure 1**). All secondary metabolites and any other phytoconstituents usually show absorbance at UV region.[14,15] But all colour pigments present in the aerial part always will show peak in visible region. To further know the type of phytochemicals present in the crude extract qualitative biochemical colour reaction tests were performed. Observed results confirm that the methanolic extract (**APME**) contain flavonoid, alkaloid, anthraquinone and phenolic groups (**Table 1**). These depicts that **APME** contains flavonoid, alkaloid, anthraquinone and phenolic groups for which absorption peaks were found in UV region.

There are several flavonoids like vitexin, baicalein, chrysin etc., alkaloid like chelerithrime and anthraquinone like soranjidiol, rubiadin has been reported to execute antibacterial activity.[4,24,25] On this background we have assayed the antibacterial activity of **APME** against *P. aeruginosa* MTCC 2488. The organism *P. aeruginosa* is a model biofilm forming bacteria which can form biofilm over both biotic as well as abiotic surfaces.[4,20] *P. aeruginosa* can produce severe pathologic manifestation like severe lung infection, nosocomial infection, cystic fibrosis etc. Thus we have selected this organism to find whether **APME** has any effect against its biofilm formation. Before antibiofilm assay we have estimated the antibacterial effect of **APME**. Result shows that at 100 µg/ml dose **APME** has maximum **65.23%** growth inhibitory effect against *P. aeruginosa*. Thus for antibiofilm study we have selected several sub-inhibitory doses starting from ½ to $1/20^{th}$ fraction of inhibitory concentration (100 µg/ml). For this particular study gentamicin was selected as standard drug against which activity of all other treatments were measured. Minimum inhibitory concentration (MIC) of gentamicin was taken as10 µg/ml.[4]

Results of the antibiofilm activity study reveal that **APME** has maximum 32.57% biofilm inhibition at 10 µg/ml dose whereas activity was gradually reduced with reduction in concentration (**Figure 2A**). Biofilm is a bacterial community where cells adhere with each other through formation of EPS. In this process EPS helps biofilm to develop further through accumulation of more cells together. After formation of biofilm they attain characteristics to adhere with surfaces of the culture vessel or in case of *in vivo* condition adhere with tissue or blood vessel surface. With the development of

biofilm, bacterial total protein also increases gradually. Thus estimation of biofilm total protein becomes an indirect parameter through which biofilm attenuation can be measured. In the present study we have observed 30.16% and 53.39% inhibition in biofilm total protein by **APME** (10 µg/ml) and gentamicin (5 µg/ml) respectively (**Figure 2B**). Further bacterial EPS were also quantified as a measure of extent of biofilm with or without treatment of **APME**. This has been reported that *P. aeruginosa* produce significant quantity of EPS which helps cells to adhere within this EPS network. Thus with the growth of EPS network biofilm quantity and thickness also increases proportionately. On this background, quantification of EPS may be taken as a very significant measure to quantify the extent of biofilm inhibition with respect to untreated control. EPS quantification result shows 15.23% (50 µg/ml of **APME**), 29.69% (10 µg/ml of **APME**), 29.39% (5 µg/ml of **APME**) and 52.57% (5 µg/ml of **Gen**) inhibition with respect to untreated control (**Figure 3A**). In addition, above mentioned results were further validated by comparing ratio of bacterial existing EPS and total protein in all treatments. Results show that, in 10 µg/ml of **APME** ratio was 1.007 whereas in 5 µg/ml of **Gentamicin** the ratio was 1.018 (**Figure 3B**). This infers that with **APME** treatment EPS percentage was more inhibited than gentamicin in comparison. Furthermore, *P. aeruginosa* biofilm attenuation by **APME** was observed under fluorescence microscope. Observed images also confirms that 10 µg/ml of **APME** shows highest biofilm inhibition whereas within gentamicin 5 µg/ml concentration shows highest attenuation of *P. aeruginosa* biofilm (**Figure 4**).

Swarming motility is a typical colonisation property of *P. aeruginosa* where colonisation was usually done through motility of the bacteria over agar surface. Such kind of colonisation helps bacteria to spread from the point of inoculation in the agar medium. This kind of motility helps bacteria to re-localise in various other tissues of the host body to execute pathologic change. In the present study, we have estimated the effect of sub-inhibitory concentrations of **APME** on swarming motility of *P. aeruginosa*. We have observed very significant attenuation of swarming motility by 5 µg/ml dose of gentamicin whereas among **APME** 10 µg/ml dose (**Figure 5**) shows maximum reduction in diameter (**Table 2**) of bacterial motility zone. From above all observation this can be concluded that **APME** at 10 µg/ml dose has significant antibiofilm activity against biofilm forming model organism *P. aeruginosa*. This particular dose of **APME** is also able to minimize bacterial swarming motility and virulence property as well.

Figure Legend

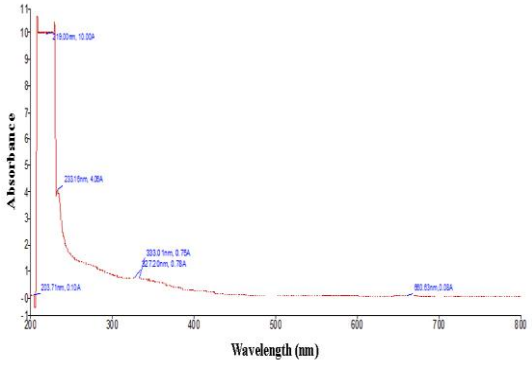

Figure 1: Ultra-Violet spectrophotometric absorption curve of APME in the range of 200 nm – 800 nm.

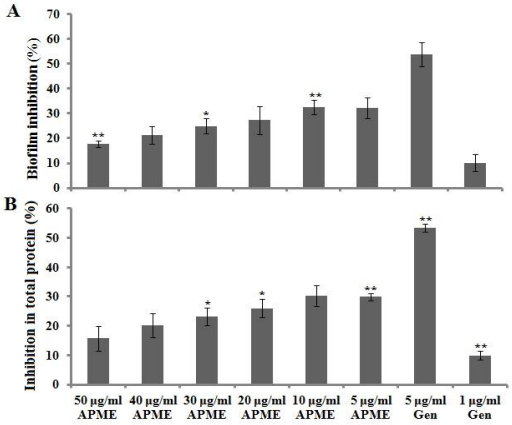

Figure 2: Antibiofilm effect of sub-inhibitory doses of APME from 5 µg/mL to 50 µg/mL in comparison with sub-inhibitory doses (5 µg/mL and 1 µg/mL) of standard antibiotic gentamicin [A]. Inhibition of *P. aeruginosa* biofilm total protein at sub-inhibitory doses of APME with respect to sub-inhibitory doses of gentamicin [B].

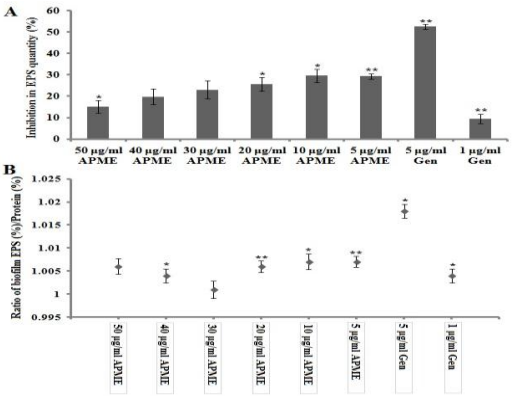

Figure 3: Inhibition of *P. aeruginosa* exo-polysaccharide quantity by sub-inhibitory doses of APME in comparison with sub-inhibitory doses of gentamicin [A]. Ration of *P. aeruginosa* biofilm EPS quantity with biofilm total protein quantity after treatment with sub-inhibitory doses of APME and gentamicin [B].

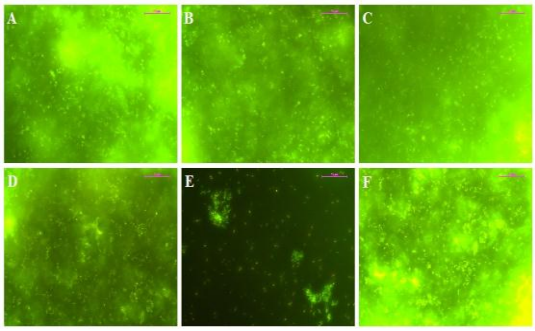

Figure 4: Observation of *P. aeruginosa* biofilm under fluorescent microscope after treatment with sub-inhibitory doses of APME and gentamicin with respect to untreated control. Images are represented as untreated control [A], 20 µg/mL APME [B], 10 µg/mL APME [C], 5 µg/mL APME [D], 5 µg/mL gentamicin [E] and 1 µg/mL gentamicin [F].

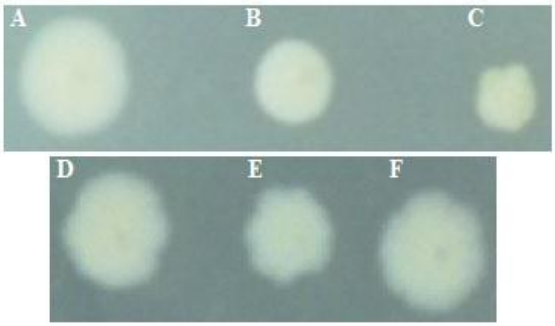

Figure 5: Inhibition of *P. aeruginosa* swarming motility after treatment with sub-inhibitory doses of APME and gentamicin with respect to untreated control. Motility zones are represented as untreated control [A], 10 µg/mL APME [B], 5 µg/mL gentamicin [C], 20 µg/mL APME [D], 5 µg/mL APME [E] and1 µg/mL gentamicin [F].

Table 1: Qualitative estimation of presence of phytochemicals in APME.

Phytochemicals	Results
Flavonoids	+
Alkaloids	+
Tannin	-
Sterol	-
Anthraquinone	-
Phenol	+
Terpenoid	_
Anthocyanin	_
Reducing sugar	_
Quinone	_
Saponin	_

N.B. + indicates present; - indicates absent.

Table 2: Effect of sub-inhibitory concentrations of APME and gentamicin on reduction of *Pseudomonas aeruginosa* swarming motility with respect to untreated control.

	Untreated control	**APME** 50 µg/ml	**APME** 10 µg/ml	**APME** 5 µg/ml	Gen 5 µg/ml	Gen 1 µg/ml
Distance of migration from centre (mm)	23±0.5	20±0.84	14±0.3**	16±0.03**	10±0.14**	21±0.87

Values are expressed as mean ± SD of 3 independent experiments. * *P* value < 0.05 and ** *P* value <0.005 (vs. untreated control).

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