Bioprospecting of medicinal plants for antibiofilm activity against MDR-UTI pathogens

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Abstract
Biofilms produced by urinary tract pathogens play vital role in the development of infections. These biofilms make UTI treatment more complex, therefore it is the need of time to develop a strategy that effectively controls the biofilm formation in these pathogens. Bioactive compounds from medicinal plants have been known to cure number of diseases since ancient times. In the present study, two medicinal plants E. milii, Des Moul and C. thevetia, (L.) H. Lippold were selected for the evaluation of antibiofilm potential. Soxhlet apparatus was used with methanol as a solvent for extraction purpose. Different conc. of plant extracts (2mg/ml, 4mg/ml, 6mg/ml, 8mg/ml and 10mg/ml) in 3% DMSO were used for evaluation of antibiofilm activity against three potent MDR-UTI pathogens. The selected pathogens were successfully identified by MALDI-TOF as E. coli, P. aeruginosa and S. equorum. The MDR potential of these pathogens was confirmed by using different antibiotics (Ampicillin 10µg, Cephoxitin 30 µg, Ceftazidime 30µg, etc.).

Crystal violet assay was used for antibiofilm activity evaluation. E. milii, Des Moul extract showed maximum biofilm inhibition potential upto 55.65% against P. aeruginosa. C. thevetia, (L.) H. Lippold showed maximum biofilm inhibition potential upto 86.72% against S. equorum. E.milii, Des Moul and C. thevetia, (L.) H. Lippold extract showed minimum antibiofilm activity against P. aeruginosa (7.98%) and (7.25%) respectively. The result provides evidence that plant investigated in the present study might be used as potential candidate for development of antibiofilm agent for UTI infections in future.

Keywords: *E. coli*, *P. aeruginosa*, *S. equorum*, *E. milii*, Des Moul, *C. thevetia*, (L.) H. Lippold, Soxhlet extraction, Crystal violet assay, MALDI-TOF MS.

Introduction

A Dutch researcher, Antonie van Leeuwenhoek observed 'animalcule' on surfaces of tooth by using a simple microscope and this was considered as the microbial biofilm discovery[7]. A biofilm constitutes of many microbial cells associated with surface, present in an extracellular polymeric substance matrix. Biofilms are defined as microbial derived sessile communities characterized by the cells that are irreversibly attached to a substratum or to each other. They are embedded in a matrix of extracellular polymeric substances they have produced and exhibit an altered phenotype with respect to growth rate and gene transcription[12]. Within a biofilm, bacteria communicate with each other by production of chemotactic substances.

Biofilms are present on different surfaces like biological tissues, medical equipment, pipes of water systems etc[15]. Biofilms are responsible for a large number of microbial diseases in humans. Infections due to biofilm mainly include urinary tract infections (UTI), middle-ear infections, catheter infections, dental plaque, coating contact lenses, gingivitis, endocarditic, cystic fibrosis and infections of persistent indwelling devices such as heart valves and joint prostheses[13]. Many UTI and bloodstream infections are caused due to biofilm associated in dwelling medical devices[14]. Several protozoa, bacterial and fungal biofilms are related to 60% urinary tract infections[32]. Common bacteria isolated from indwelling medical devices are *E. faecalis, S. aureus, S. epidermidis, S. viridians, E. coli*, *K. pneumoniae*, *P. mirabilis* and *P. aeruginosa*[41].

In the last decade, medicinal plants have raised the attention of researchers because of their promising potential as a source of antibiofilm compounds. Medicinal plants and their extracts are used in traditional treatments of various diseases [23]. Extracts and oils obtained from plants have been used for a great variety of objectives for centuries[24]. The bioactive compounds from plants were mainly identified as terpenes, steroids, carotenoids, phenols, furanones, alkaloids, peptides and lactones[44].

The family Euphorbiaceae consists of 2000 species. The genus *Euphorbia* is the largest genus of medical plant. *E. milii*, Des Moul is commonly known as "crown of thrown"[37]. It is a succulent shrub growing to 1.8 m (5 ft 11 in) tall with densely spiny stems, the straight, slender spines up to 3 cm long which help it scramble over other plants. The leaves are found mainly on new growth and are obovate up to 3.5 cm long and 1.5 cm broad. The flowers are small subtended by a pair of conspicuous petal-like bracts, variably red, pink or white, up to 12 mm broad. It plays a role in folk medicine. It acts as a cure for cancer and it can cure warts[36].

C. thevetia, (L.) H. Lippold (*Thevetia peruviana*) belongs to the family Apocynaceae and is commonly known as Yellow oleander and Lucky nut. *C. thevetia*, (L.) H. Lippold is an evergreen shrub or small tree usually about 3-8m tall. The

leaves are spirally arranged, linear and about 13-15cm in length. Flowers are bright yellow and funnel shaped with spirally twisted. The fruits are somewhat globular, slightly fleshy and have a diameter of 4-5cm. The fruits, which are green in color, become black on ripening. Each fruit contains a nut, which is longitudinally and transversely divided. All parts of the plant contain the milky juice.

Different parts of *C. thevetia*, (L.) H. Lippold are known to possess various medicinal properties. *C. thevetia*, (L.) H. Lippold is used traditionally in treatment of amenorrhea, malarial fever, jaundice, hemorrhoids, constipation, headaches, skin disorders etc. The main medically active compounds found in the plant are a range of cardiac glycosides. The latex is applied to decayed teeth to relieve toothache and is used to treat chronic sores and ulcers[1].

The main objective of the present study was to investigate the antibiofilm property of these Indian medicinal plants such as *E. milii*, Des Moul and *C. thevetia*, (L.) H. Lippold against MDR-UTI pathogens.

Material and Methods

Collection of clinical samples: Various midstream urine samples from UTI patients were collected from Silver jubilee sub district hospital (Baramati), Giriraj hospital (Baramati) and Swami Samarth hospital (Pune). The samples were collected in sterile capped containers and transported to the laboratory for microbial isolation and stored in the refrigeration condition until use[28].

Enrichment, isolation and screening of UTI pathogens

Enrichment: Four urine samples collected from UTI patients were centrifuged at 2000 rpm for 10 mins. A loopful of the pellet was inoculated in a flask of nutrient broth for enrichment purpose and incubated at 37^{o}C for 24 hrs[29].

Isolation: For isolation of UTI pathogens, 0.1 ml of enriched urine sample was spread onto the nutrient agar and MacConkey's agar plates and incubated at 37^{o}C for 24 hrs. After incubation, isolated colonies were selected for screening purpose[33].

Screening: Isolated pathogens were screened on the basis of their multi-drug resistant potential[31]. Antibiotic susceptibility pattern of every isolate was determined on MH agar by using disc diffusion method according to the modified Kirby-Bauer technique[43]. Hi-media antibiotic polydiscs having antibiotics such as amikacin (Ak), Ampicillin (A), Cephoxitin (Cn), Ceftazidime (Ca), Ceftriaxone (Ci), Chloramphenicol (C), Gentamicin (G) and Piperacillin (Pc) were used in present study[10].

Characterizations of the selected isolates

Morphological characters: All the three screened isolates were successfully characterized by using morphological character. Colony characters of the isolates were recorded such as size, shape, color, margin, elevation, opacity and consistency etc. The Gram character of the isolates was also determined by using standard Gram staining procedure. Motility of the isolates was determined. The ability of isolates to produce capsule and spore was also checked successfully[6].

Biochemical tests: All the three selected isolates were further characterized by using biochemical tests on the basis of Bergey's Manual for Determinative Bacteriology[5]. For biochemical characterization, catalase test, oxidase test, sugar fermentation test, IMViC and nitrate reduction tests were performed.

Molecular test: Finally, the results were confirmed and a selected bacterium was identified upto species level by MALDI- TOF technique.

Bioprospecting of plant material: Plants are the rich source of various antibiofilm compounds, so in the present study we have screened two medicinal plants to check their antibiofilm activity against selected pathogens.

Selection of plants: Medicinally important plants were screened and selected for extraction purpose. The selected plants were *E. milii*, Des Moul and *C. thevetia*, (L.) H. Lippold.

Collection of plant material: The healthy plant part samples like leaves were collected from the different sites from Baramati, Pune (Maharashtra). The collected samples were transferred to laboratory and stored until use.

Processing and extraction of collected samples

Cleaning, drying and grinding of plant materials: Collected samples were cleaned thoroughly with fresh water to remove adhering debris and associated biota. After cleaning, leaves were dried in shade at room temp. for three weeks[19]. Grinding of the dried leaves was carried out by mixer grinder to reduce the size of the leaves which makes solvent extraction more efficient[9].

Soxhlet extraction: Soxhlet extractor setup consists of a round bottom flask, siphon tube, distillation path, expansion adapter, condenser, cooling water inlet, cooling water outlet, heat source and thimble. In this study, methanol was selected as a solvent. Total 150 ml of the methanol was added in the round bottom flask followed by assembling the apparatus. The extraction process was carried out at 75^{o}C temp. 15g of powder sample of the plant was placed in the thimble. The extraction was carried out for 24 hrs to complete 15 cycles. The extract collected was subjected to evaporation and dried extract was stored at low temp. until use[2].

Evaluation of antibiofilm potential of plant extract: Five different concentrations (2mg/ml, 4mg/ml, 6mg/ml, 8mg/ml and 10mg/ml) of plant extracts were prepared by dissolving in 3% DMSO. Freshly prepared 24 hrs old culture of the selected MDR isolates in LB (Luria Bertani) broth was used

for assay purpose. The cultures were serially diluted to 1:100 in fresh medium (Turbidity adjusted to 0.5 McFarland standards). The different concentrations of plant extracts were added in clean and sterile test tubes. One ml of 24 hrs old bacterial culture was added in each test tube. A positive control tube was treated with one ml of bacterial culture without plant extract while a negative control (Blank) tube was treated with one ml sterile broth without bacterial culture and plant extract.

All the tubes were incubated for 24 hrs at 37ºC. After incubation, the unbound cells were removed by washing the tubes thrice with distilled water. The bound cells were then stained with 0.1% crystal violet solution for 20 min. Excess stain was rinsed off by thorough washing with distilled water and tubes were kept for air drying[22].

For the quantification of antibiofilm activity of plant extract, the adherent bacteria associated with crystal violet were solubilized with 30% acetic acid and the absorbance was recorded at 600nm using UV-Vis spectrophotometer. From the absorbance, the percentage of biofilm inhibition was calculated by the formula:

$$\% \text{ of inhibition} = \frac{\text{OD of control} - \text{OD of test}}{\text{OD of control}} \times 100$$

All the experiments were carried out in triplicate. Standard error was determined and shown by error bars.

Results and Discussion

Collection of samples: Various urine samples from different hospitals of Baramati and Pune from the UTI patients were collected.

Ouno et. al[29] also used the urine samples from UTI patients and they have used these samples for successful isolation of the UTI pathogens. According to Ouno et. al[29] the urine samples of the UTI patients show acidic pH, fruity/aromatic/ammonical smell, color change, clear/turbid appearance etc. and they generally show presence of *S. aureus, E.coli, E. faecalis, P. aeruginosa* and *K. pneumonia* pathogens.

Enrichment, isolation and screening of UTI pathogens

Enrichment: The selected UTI samples showed sufficient amount of growth in the broth after enrichment. Similar approach was used by Ouno et al[29] for the enrichment of the pathogens from urine samples. They used centrifugation technique in which 0.5 ml pellets from the centrifuge were inoculated into the thioglycolate broth. In the present study instead of using thioglycolate broth we used nutrient broth for the enrichment purpose.

Isolation: After incubation, first urine sample showed the maximum colonies as compared to second, third and fourth. Priyadharsini et al[33] also used same medium for isolation of pathogens from urine sample. The total six pathogens were isolated by them and the result obtained is in agreement with the findings.

Screening: Effective screening process is very necessary for the selection of potent pathogens. In the present study, we used the multi-drug resistant criteria for primary screening of the isolate. In this approach we use the polydiscs of Hi-media (OD025) for checking the MDR potential of the pathogen. We have shown the result in table 1.

Out of six isolated pathogens, three were screened and selected on the basis of their ability to resist the antibiotic. Out of selected pathogens, isolate 1 shows resistance against Ampicillin (10µg), Cephoxitin (30µg), Ceftazidime (30µg) and Piperacillin (100µg). Isolate 3 and isolate 6 show resistant to Ampicillin (10µg), Cephoxitin (30µg), Ceftazidime (30µg) and Ceftriaxone (30µg).

Table 1
Antimicrobial susceptibility testing of isolated pathogens against different antibiotics

S.N.	Antibiotic	Sym	Conc. (µg)	Isolates					
				1	2	3	4	5	6
1	Amikacin	Ak	30	-	-	-	-	-	-
2	Ampicillin	A	10	+	+	+	+	-	+
3	Cephoxitin	Cn	30	+	+	+	+	+	+
4	Ceftazidime	Ca	30	+	+	+	-	-	+
5	Ceftriaxone	Ci	30	-	-	+	-	-	+
6	Chloramphenicol	C	30	-	-	-	-	-	-
7	Gentamicin	G	10	-	-	-	-	-	-
8	Piperacillin	Pc	100	+	-	-	-	-	-

+ = Resistant; - = Sensitive

Dash et al[10] also used multi drug resistant approach for selection of biofilm forming organism. They found that UTI pathogens can grow in presence of antibiotics like Penicillin, Ampicillin, Gentamicin, Erythromycin, Kanamycin, Oxacillin, Ciprofloxin, Norfloxacillin etc. Our results are in agreement with these findings.

Identification of pathogens: After isolation and screening, the selected three potent pathogens were identified by morphological, biochemical and molecular methods.

Morphological characters: In morphological characterization process, the colony morphology of the isolates such as size, shape, color, margin, elevation, opacity, consistency, Gram character, motility etc. were determined and the results obtained were shown in table 2.

The results of morphological characterization were used for selection of biochemical test with the help of Bergey's Manual for Determinative Bacteriology[5].

Biochemical tests: By using Bergey's Manual for Determinative Bacteriology, following biochemical tests were performed for all the three isolates. Standard protocols were used and results obtained were shown in table 3. On the basis of the results of biochemical tests, all the three isolates were successfully identified upto genus level as *Escherichia* spp., *Pseudomonas* spp. and *Staphylococcus* spp.

Fig. 1: MDR activity of isolate One

Fig. 2: MDR activity of isolate two

Fig. 3: MDR activity of isolate three

Table 2
Morphological characters of isolates

Organism	Colony characters								
	Size	**Shape**	**Color**	**Margin**	**Elevation**	**Opacity**	**Consistency**	**Gram character**	**Motility**
Isolate 1	1mm	Circular	White	Entire	Slightly raised	Opaque	Smooth	Gram negative rod shaped	Motile
Isolate 2	2mm	Circular	Bluish green	Serrated	Flat	Opaque	Smooth	Gram negative rod shaped	Motile
Isolate 3	1mm	Circular	White	Entire	Convex	Opaque	Smooth	Gram positive cocci	Non motile

Table 3
Biochemical tests of isolates

Biochemical tests	Isolates			Biochemical tests	Isolates		
	Isolate 1	**Isolate 2**	**Isolate 3**		**Isolate 1**	**Isolate 2**	**Isolate 3**
Catalase	+	+	+	Indole	+	-	-
Oxidase	-	+	-	MR	+	+	-
Glucose	A	A	A	VP	-	-	-
Lactose	AG	A	-	Citrate	-	+	-
Xylose	A	-	A	Nitrate	+	+	+
Mannitol	AG	-	A	Urease	-	-	+
Maltose	-	-	A	H2S	-	-	-
Sucrose	-	-	A	Gelatinase	+	+	-

+ = Positive; - = Negative; A = Acid; AG = Acid + Gas

Priyadharsini et al[33] used similar biochemical tests for identification of isolates. They found three Gram positive isolates viz. *S. aureus, S. epidermidis* and *Streptococcus* spp. and three Gram negative isolates like *E.coli, K. pneumoniae* and *P. aeruginosa*. Generally maximum reported UTI infections are caused by these three pathogens world-wide.

MALDI-TOF MS identification: For the identification of organisms at species level, MALDI-TOF approach was used. The technique is very sensitive and accurate to find out the organism up to species level.

MALDI-TOF MS detects many different biomolecules such as nucleic acids, peptides, proteins, sugars and small molecules. This technology identifies microorganisms via the generation of fingerprints of highly abundant proteins followed by correlation to reference spectra in a database. The basic principle of all mass spectrometric methods is the ionization of neutral molecules and the subsequent accurate determination of the resulting primary ions and their decay products in high vacuum. A typical mass spectrometer is composed of three components: an ion source, a mass analyzer and the detector.

Mechanism of microbial identification using MALDI-TOF MS is quite simple. In brief, it requires mixing of microbial growth from a pure colony with the matrix solution followed by smear preparation on the target plate, air drying, loading of plate inside instrument and finally exposing the samples to source of ionization. After ionization, ionized peptides and proteins travel towards detector in a vacuum tube and they get separated based on their mass to charge ratio (m/z). A mass spectrum of the strain under study is generated which was then compared with that of the other strains present in the reference database.

The database includes biomarkers detected in MALDI spectra of intracellular proteins primarily in the range of 2 to 20 kDa[17,35]. Most of the biomarkers detected in MALDI-TOF spectra of intact bacterial cells have a molecular mass below 15 kDa[8,38,42]. Lysis of organisms with organic solvents in acidic conditions favors extraction of basic cytoplasmic proteins.

By the MALDI-TOF we got the results as shown in table 4. MALDI-TOF spectrum identified isolate 1, 2 and 3 as *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus equorum* respectively. Spectrum shows best score for *E. coli, P. aeruginosa* and *S. equorum* as 2.002, 2.081 and 2.346 respectively.

Ferreira et al[18] used MALDI-TOF for identification of the pathogens upto species level. They found that it is very accurate and sensitive technique to identify the organism. According to them, there is 93.1% consistency between MALDI-TOF MS identification and conventional methods. They identified UTI pathogens as *E. coli, P. aeruginosa* and *E. faecalis*[18].

Table 4
Comparison with the Bruker taxonomy database using Biotyper 3.1 software the test strain

S.N.	Sample	Organism (best match)	Score	Organism (second match)	Score
1	S1	*Escherichia coli* DSM 682 DSM	2.002	*Escherichia coli* ATCC 25922 THL	1.952
2	S2	*Pseudomonas aeruginosa* DSM 50071T HAM	2.081	*Pseudomonas aeruginosa* 8147_2 CHB	2.038
3	S3	*Staphylococcus equorum* spp. *equorum* DSM 20675 DSM	2.346	*Staphylococcus xylosus* FI FLR	1.653

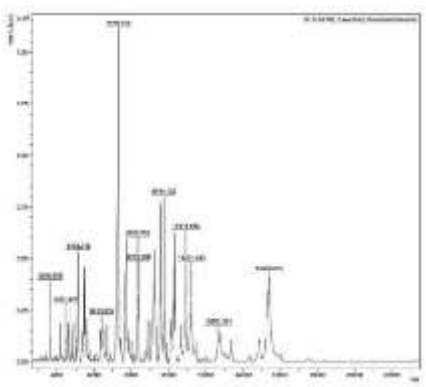

Fig. 4: MALDI-TOF analysis of isolate one

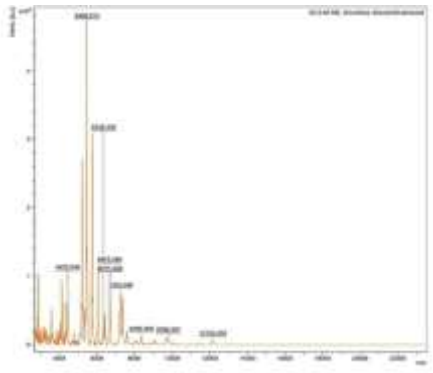

Fig. 5: MALDI-TOF analysis of isolate two

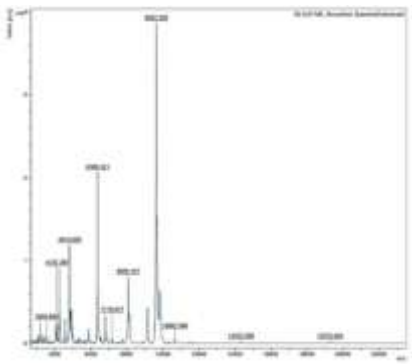

Fig. 6: MALDI-TOF analysis of isolate three

Kanwar et al[25] also used MALDI-TOF in for identification of the pathogens up to species level. According to them, the technique show high rate of accuracy for the identification of Gram negative bacteria and Gram positive bacteria in urine sample without any major error.

Bioprospecting of plant materials

Selection of plants: In India, a medicinal plant forms the backbone of several indigenous traditional systems of medicine. Pharmacological studies have acknowledged the value of medicinal plants as potential source of bioactive compounds[34], antimicrobial compounds and several researchers throughout the world are investigating the antimicrobial activity of medicinal plants which are utilized in the traditional or alternative healthcare systems[21].

In the present study, two important plant varieties were used namely *E. milii,* Des Moul *and C. thevetia,* (L.) H. Lippold. We have selected these plant varieties because they are easily found in large no. in the region of Baramati, Pune and Maharashtra. They are easy to grow and show less generation time, so the sample can be easily collected from these plants throughout the year.

Gapuz et al[19] checked antioxidant activity of leaves of *E. milii,* Des Moul plant extract. In previous study, plants with flavonoids have been reported to have antioxidant activity and are known to have health promoting effect like anti-cancer, anti-microbial, anti-inflammation and anti-viral properties and they found that *E. milii,* Des Moul had the highest total flavonoids per gram of dried material.

Kumar et al[26] checked the antimicrobial, antifungal, anti-oxidant, anti-tumor and anti-inflammatory activity of leaves of *C. thevetia,* (L.) H. Lippold plant extract and they have got notable results.

Fig. 7: *E. milii,* Des Moul

Fig. 8: *C. thevetia,* (L.) H. Lippold

Collection of plant materials: Healthy plant leaves from *E. milii,* Des Moul and *C. thevetia,* (L.) H. Lippold was collected. According to previous report, the leaves of these medicinally important plants are the main source of bioactive compounds[19,26].

Processing and extraction of collected samples

Cleaning, drying and grinding of collected samples: Collected samples were thoroughly cleaned by distilled water to remove impurities and biota because water is universal solvent and is having ability to remove many impurities and contaminants from the plant sample, so we select water for cleaning purpose.

According to Bandiola[3], the cleaning with distilled water using the hands leads to better results and less damage on the plant and wiping the samples with clean and dry cloth enhances the drying process. After cleaning the samples were dried under shade for three weeks. According to previous report, drying of leaves sample in sun may affect the bioactive compound quality because heat denatures the lead compound present in nature.

According to Gopalasatheeskumar,[20] overheat can lose the volatile substances from plant materials and some of the light sensitive constituents may lose in light condition, so air dry process under shade in the dark room gives better results and it is a convenient process.

In our study we used mixer grinder for size reduction, as grinding reduces the particle size which increases surface area for extraction. So, grinding directly affects the Soxhlet extraction process. According to Namasivayam et al,[27] grinding in mortal and pestle does not provide sufficient results as compared to mixer grinding. Figures 9 to 14 show different stages of processing of collected leaves samples from both the plants.

Soxhlet extraction: There are various methods used by the researchers for the extraction of the bioactive compound from the environmental samples. These methods are broadly classified into two types such as hot extraction and cold extraction. The temp. sensitive compound if present in the sample, then the cold extraction approach is used by researcher but after analysis of phytochemical present in the two selected plant samples, we used hot extraction method (Soxhlet extraction).

It is extremely effective, accurate and time saving approach for extraction of the compound from the natural source. The method can be effectively used to extract compound with more efficiency[11]. After 15 cycles of the Soxhlet extraction, the solvent is removed from the round bottom flask and evaporated successfully to obtain a pure dried extract.

According to Sahne et al,[39] Soxhlet extraction is one of the foremost and most common extraction techniques where in long extraction time at high temperature facilitates the

extraction of target compound; moreover, the repeated contact of solvent with extract can enhance the extraction yield.

Evaluation of antibiofilm potential of plant extract: The antibiofilm potential of the extract against the selected three isolates was successfully determined by using crystal violet assay. Five different concentrations (2, 4, 6, 8 and 10 mg/ml) of extract were used for this purpose. After washing the quantitative estimation of antibiofilm activity was done by the spectrophotometric assay (600nm). % inhibition of biofilm was determined at each concentration by using standard formula.

Evaluation of antibiofilm activity of *E. milii,* Des Moul: Table 5 showed % inhibition of biofilm produced by these three organisms at five different concentrations. Control was kept without extract to compare with test.

Fig. 9: Leaves of *E. milii,* Des Moul before drying

Fig. 10: Leaves of *E. milii,* Des Moul after drying

Fig. 11: Leaves of *C. thevetia* (L.) H. Lippold before drying

Fig. 12: Leaves of *C. thevetia* (L.) H. Lippold after drying

Fig. 13: Sample of *E. milii,* Des Moul after grinding

Fig. 14: Sample of *C. thevetia* (L.) H. Lippold after grinding

Fig. 15: Soxhlet extraction assembly

Table 5
Percent inhibition of antibiofilm activity of the *E. milii,* Des Moul against three isolates by crystal violet assay

Organisms	% Inhibition				
	2 mg/ml	**4 mg/ml**	**6 mg/ml**	**8 mg/ml**	**10 mg/ml**
E. coli	25.42±4.96	48.78±5.74	53.32±4.24	35.72±5.82	48.01±6.24
P. aeruginosa	7.98±6.01	44.03±5.73	51.87±4.59	34.24±4.01	55.65±7.65
S. equorum	30.72±4.05	38.94±5.13	20.2±4.53	34.78±7.24	32.34±6.89

*Mean values ± Standard error of means

The different conc. of the extract tested for antibiofilm activity found that *E. coli* showed highest sensitivity to *E. milii*, Des Moul extract at 6 mg/ml (53.32%) followed by 4 mg/ml (48.78%), 10 mg/ml (48.01%) and 8 mg/ml (35.72%). The lowest antibiofilm activity was observed at 2 mg/ml (25.42%).

P. aeruginosa also showed sensitivity to the different conc. of the extract but it was found that maximum activity was observed at 10 mg/ml (55.65%) followed by 6 mg/ml (51.87%), 4 mg/ml (44.03%), 8 mg/ml (34.24%) and 2 mg/ml (7.98%). Further increase or decrease in conc. did not show significant effect on antibiofilm potential of the isolates.

By using % inhibition formula, the value obtained showed that *S. equorum* has highest sensitivity to 4 mg/ml (38.94%) conc. of the extract followed by 8 mg/ml (34.78%), 10 mg/ml (32.34%), 2 mg/ml (30.72%) and 6 mg/ml (20.2%). These results showed that there was no linear relationship that means increase or decrease in conc. did not show significant effect on antibiofilm potential of the isolates.

Evaluation of antibiofilm activity of *C. thevetia,* (L.) H. Lippold: Table 6 showed % inhibition of biofilm produce by these three organisms at five different concentrations. Control was kept without extract to compare with test. The different conc. of the extract tested for antibiofilm activity found that *E. coli* showed highest sensitivity to *C. thevetia* (L.) H. Lippold extract at 10 mg/ml (59.76%) followed by 4 mg/ml (54.19%), 6 mg/ml (53.58%) and 2 mg/ml (52.95%). The lowest antibiofilm activity was observed at 8 mg/ml (30.91%).

Fig. 16: Effect of different conc. of *E. milii,* Des Moul against *E. coli*

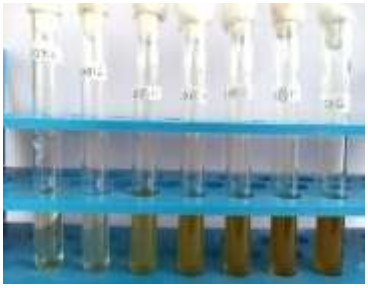

Fig. 17: Effect of different conc. of *E. milii,* Des Moul against *P. aeruginosa*

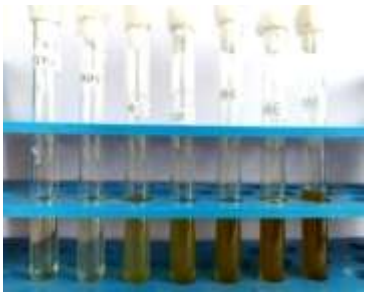

Fig. 18: Effect of different conc. of *E. milii,* Des Moul against *S. equorum*

Fig. 19: Antibiofilm activity of *E. milii,* Des Moul against *E. coli* by crystal violet assay

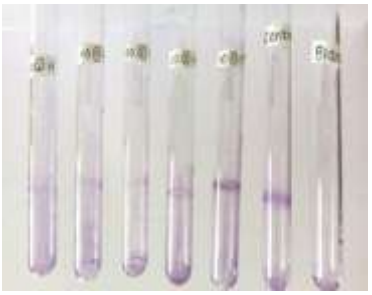

Fig. 20: Antibiofilm activity of *E. milii,* Des Moul against *P. aeruginosa* by crystal violet assay

Fig. 21: Antibiofilm activity of *E. milii,* Des Moul against *S. equorum* by crystal violet assay

Fig. 22: Effect of different conc. of *C. thevetia,* (L.) H. Lippold against *E. coli*

Fig. 23: Effect of different conc. of *C. thevetia,* (L.) H. Lippold against *P. aeruginosa*

Fig. 24: Effect of different conc. of *C. thevetia,* (L.) H. Lippold against *S. equorum*

Fig. 25: Antibiofilm activity of *C. thevetia,* (L.) H. Lippold against *E. coli* by crystal violet assay

Fig. 26: Antibiofilm activity of *C. thevetia,* (L.) H. Lippold against *P. aeruginosa* by crystal violet assay

Fig. 27: Antibiofilm activity of *C. thevetia,* (L.) H. Lippold against *S. equorum* by crystal violet assay

Table 6
Percent inhibition of antibiofilm activity of the *C. thevetia,* (L.) H. Lippold against three isolates by crystal violet assay

Organisms	% Inhibition				
	2 mg/ml	4 mg/ml	6 mg/ml	8 mg/ml	10 mg/ml
E. coli	52.95±5.05	54.19±8.08	53.58±4.17	30.91±4.06	59.76±7.61
P. aeruginosa	17.66±5.30	7.25±4.91	67.11±5.09	54.15±6.93	51.08±7.50
S. equorum	67.81±5.44	73.73±4.44	70.39±4.08	86.72±4.58	62.33±7.06

*Mean values ± Standard error of means

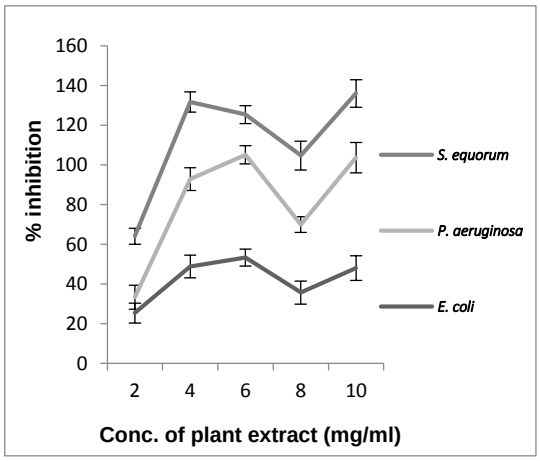

Fig. 28: Antibiofilm activity of *E. milii,* Des Moul plant extract against isolates

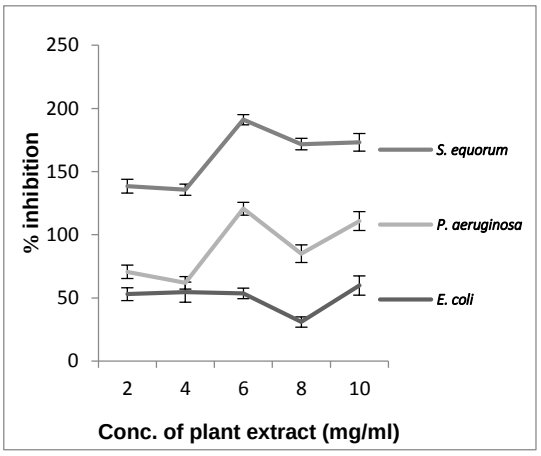

Fig. 29: Antibiofilm activity of *C. thevetia,* (L.) H. Lippold plant extract against isolates

P. aeruginosa also showed sensitivity to the different conc. of the extract but it was found that maximum activity was observed at 6 mg/ml (67.11%) followed by 8 mg/ml (54.15%), 10 mg/ml (51.08%), 2 mg/ml (17.66%) and 4 mg/ml (7.25%). Further increase or decrease in conc. did not show significant effect on antibiofilm potential of the isolates.

By using % inhibition formula, the value obtained showed that *S. equorum* has highest sensitivity to 8 mg/ml (86.72%) conc. of the extract followed by 4 mg/ml (73.73%), 6 mg/ml (70.39%), 2 mg/ml (67.81%) and 10 mg/ml (62.33%). The results suggest that there was no linear relationship between conc. of the extract and antibiofilm activity % inhibition.

All the experiments were carried out in triplicate. Standard error was successfully calculated and shown by error bars in the figures 28 and 29. According to Namashiwayam et al[27] *Azadirachta indica* is the significant bioactive compound which shows the good antibiofilm inhibition up to 59% against *E. coli*.

According to the Bazargani et al,[4] essential oils of coriander, hexane extract of Anise and ethanol extract of Peppermint could inhibit the bacterial cell attachment of *Staphylococcus* spp. completely (100% inhibition activity) while the other extracts and essential oils generally displayed percentage inhibition in range of 23- 96%. The tested plant extract and essential oils show strong anti-adhesion activity with an inhibition value of 98.4%.

According to Packiavathy et al,[30] *Curcuma longa* plant extract has the antibiofilm, antifungal, antibacterial and antiviral activity. According to them, the plant extract of *Curcuma longa* shows 81% and 68% maximum antibiofilm activity against *E. coli* and *P. aeruginosa* respectively. Our

finding showed that plant extract of *E. milii*, Des Moul and *C. thevetia*, (L.) H. Lippold showed maximum antibiofilm activity 53.32% and 59.76% against *E. coli* and against *P. aeruginosa.* This plant extract shows maximum antibiofilm activity upto 55.65% and 67.11% respectively.

Sridevi et al[40] used *Chlorella vulgaris* plant extract which is green microalgae. They found that this plant extract has presence of flavoids, steroids and phenolic compounds. According to them, the plant extract of *Chlorella vulgaris* shows 88% maximum antibiofilm activity against *Staphylococcus* spp.

El-Bashiti et al[16] used *Melissa officinalis* plant extract to check antibiofilm activity against *P. aeruginosa* and *Staphylococcus* spp. In the study, they found maximum antibiofilm activity up to 84% and 77% against *P. aeruginosa* and *Staphylococcus* spp. respectively.

Conclusion

The study found that UTI pathogens from different patients showed potent ability to resist action of many effective antibiotics which will be a matter of concern in the treatment of infections caused by them. As this pathogens use biofilms as a important tool in pathogenesis, so antibiofilm compounds can be effective in treatment of infections caused by them.

MALDI-TOF is a relatively modern approach for characterization of microbes, as it is a quick, time saving, cost effective and reliable method. In the present study, all the three selected pathogens were identified up to species level by this technique.

Biofilm inhibition studies carried out using bioactive compounds of plant extracts of *E. milii*, Des Moul and *C. thevetia,* (L.) H. Lippold proved potential of this plant in treatment of UTI infections. All the tested conc. of plant extracts showed inhibition of biofilm formation in *E. coli, P. aeruginosa* and *S. equorum.* The plant extracts from the selected plants inhibit the biofilm formation in dose-dependent manner.

The study concludes that bioactive compounds from *E. milii*, Des Moul and *C. thevetia* (L.) H. Lippold can be used as potent antibiofilm agent against MDR pathogens in future to reduce UTI infection. More detailed analysis of the plant extracts and characterization of the bioactive compounds present in them are necessary.

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