

Original Research Article

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Investigation of antibiofilm potential of *Argemone mexicana* and *Calotropis gigantea* against clinical isolates

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ABSTRACT

Keywords

A. mexicana,
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In the current investigation, the anti-biofilm potential of *Argemone mexicana* and *Calotropis gigantea* against 11 clinical isolates viz *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Proteus vulgaris*, *Proteus mirabilis*, *Citrobacter*, *Serratia marcescens* and *Staphylococcus epidermidis* were tested. The phytochemicals extracted in different solvents were analysed for their antibiofilm ability. For detection of biofilm formation, 11 clinical isolates were screened by Tube method (TM), Congo red agar (CRA), Static Glass coupon reactor method and *in vitro* catheter method. Of the 11 isolates, 7(57.8%) displayed a biofilm-positive phenotype under the optimized conditions in the TCP method and strains were further classified as strong 3 (27.27 %) and moderate 4 (36.36 %) while in 4 (36.36 %) isolates weak or no biofilm was detected. Though TM correlated well with the CRA test for 3 (27.3 %) strongly biofilm producing strains, weak producers were difficult to discriminate from biofilm negative isolates. Screening on Static Glass coupon reactor does not correlate well with either of the two methods for detecting biofilm formation in clinical isolates. Methanolic extract of *Argemone mexicana* consistently showed inhibitory effect against the *E. coli*, *P. vulgaris* and *P. aeruginosa*, while acetone extract of *Argemone mexicana* showed inhibitory effect against *S. aureus* and *Enterococcus*. *Calotropis gigantea* however was less efficient when compared to *Argemone mexicana*.

Introduction

Bacteria that adhere to implanted medical devices or damaged tissue can become the cause of persistent infections (Costerton JW *et al.*, 1999) (Costerton JW *et al.*, 2000). These bacteria encase themselves in a hydrated matrix of polysaccharide and protein, forming a slimy layer known as a biofilm.

Direct microscopic examination of colonized surfaces shows dense aggregates of bacteria held together by diffused extracellular polymers. Biofilm formation is important because this mode of growth is associated with the chronic nature of the subsequent infections and with their inherent resistance to antibiotic chemotherapy.

The most important of these characteristics is that bacteria in biofilms evade host defences and withstand antimicrobial chemotherapy. Even in individuals with competent innate and adaptive immune responses, biofilm-based infections are rarely resolved. In fact, tissues adjacent to the biofilm might undergo collateral damage by immune complexes and invading neutrophils. (Høiby N, 1995) Susceptibility tests with in-vitro biofilm models have shown the survival of bacterial biofilms after treatment with antibiotics at concentrations hundreds or even a thousand times the minimum inhibitory concentration of the bacteria measured in a suspension culture. (Ceri H, 1999) In vivo, antibiotics might suppress symptoms of infection by killing free-floating bacteria shed from the attached population, but fail to eradicate those bacterial cells still embedded in the biofilm.

When bacteria are dispersed from a biofilm they usually rapidly become susceptible to antibiotics (Anwar H, 1989),(Williams I, 1997) which suggests that resistance of bacteria in biofilms is not acquired via mutations or mobile genetic elements.

In the present study, microbial contamination of indwelling catheters by biofilms is tested against the phytochemicals. Urinary Tract Infections account for an estimated 25-40% nosocomial infection, out of which 90% are associated with urinary catheter, called Catheter associated urinary tract infection. The microbial populations within CAUTI frequently develop as biofilms. Clinical isolates were collected for following pathogens viz., Methanol, chloroform, water and acetone extracts of leaves of *Argemone mexicana* and *Calotropis gigantea* were prepared. The preliminary phytochemical screening of the plant extracts showed presence of carbohydrates, glycosides, sterols, alkaloids, flavonoids, tannins and saponins. The antimicrobial activity of all the prepared extracts was evaluated on the above

mentioned clinical isolates. Results showed promising antimicrobial activity against *Enterobacter aerogenes* for chloroform extract, *Proteus vulgaris* for methanol extract, *Serratia marcescens* for acetone extract and *Pseudomonas aeruginosa*, *Escherichia coli*, for both acetone and ethanol extracts of *Argemone mexicana*, and only in methanol extract of *Calotropis gigantea* for *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus*, *Enterobacter*, *Staphylococcus aureus*, *Proteus vulgaris*, and *Staphylococcus epidermidis*. Biofilm formation was carried out for all the eleven test organisms by three methods namely Congo red method, Tube method and Static Glass Coupon Reactor method. Then biofilms were formed in catheters and efficiency of extracts upon them was carried out by infecting the catheters with above mentioned organisms which gave positive result for methanol extract and qualitative studies were carried out.

Materials and Methods

Chemicals reagents and media components

Benedict's reagent, Ninhydrin reagent, Mayer's reagent, Wagner's reagent, Dragendorff's reagent, Hager's reagent, Barfoed's reagent, Molish's reagent, Fehling's solution A and B, α -naphthol, lead acetate, sodium hydroxide, potassium iodide, sodium chloride, ferric chloride, benzene, acetone, pyridine, chloroform, acetic anhydride, copper sulphate, copper acetate, anhydrous stannic chloride, vinyl chloride, acetyl chloride, zinc chloride, ammonia solution, bismuth subnitrate, dilute HCl, concentrated HNO₃, concentrated H₂SO₄, glacial acetic acid, methanol and ethanol. All the media components used were of laboratory grade and the details of each is provided as Annexure. All the chemicals including the solvents were of analytical grade and purchased from Sd fine chemicals, Mumbai, India.

Micro-organisms

Clinical samples were collected from Hospitals and micro-organisms were isolated by standard protocols. Reference strains were procured from MTCC, India as mentioned for positive control.

Plant material

The selection of the plant species for the present investigation was mainly based on the traditional and folkloric uses of the plants for the treatment of infection and antibacterial activity. For the current investigation, plants samples namely *Argemone mexicana* and *Calotropis gigantea* were collected from Hessaraghatta, Bangalore with flowers and fruits. It was identified and authenticated from Foundation for Rehabilitation of local Health tradition (FRLHT), Bengaluru. The deposited voucher numbers:120013 and 120170 (FRLH Herbarium) respectively.

Preparation of herbal extract

100g of powdered plant samples were extracted in 200 ml of methanol, acetone, chloroform and water, percentage yield was calculated and stated in table.1. The solvents were evaporated using rotary evaporator and dissolved in DMSO for further use.

Phytochemical screening

Qualitative phytochemical analysis of the crude extracts from each plant extracts were determined. The presence of phytochemical compounds like Tannins, alkaloids, saponins, cardiac glycosides, steroids and flavonoids was tabulated (Table2) (Gopinath, 2012).

Antimicrobial studies

Of the 11 clinical isolates included in the study 9 are gram negative strains viz *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Serratia marcescens*, *Citrobacter* *Enterobacter*,

Klebsiella pneumoniae, *Proteus mirabilis*, *Proteus vulgaris*, and two are gram positive strains viz *Staphylococcus aureus* *Enterococcus*. These strains are obtained from hospitals in Bangalore.

Biofilm Formation Activity

(Mathur T, Singhal, et al., 2006,)

For detection of biofilm formation, the clinical isolates were screened by tissue culture plate (TCP), Tube method (TM) and Congo red agar (CRA) method.

Tube method (TM)

A qualitative evaluation of biofilm formation was determined as described earlier by (Christensen et al, 1982). Microorganism from overnight culture was inoculated into TSBglu (10mL) and incubated at 37 °C for 24 hours. The tubes were decanted and washed with PBS (pH 7.3) and the dried. Dried tubes were stained with crystal violet (0.1%). Surplus stain has been removed and tubes washed with deionised water and dried in inverted position. It was observed for biofilm formation.

Biofilm formation was considered positive if the wall and bottom of the tube were lined with a visible film. The formation of the ring on the liquid interface did not indicate the formation of biofilm. Tubes were examined and the amount of biofilm formation was scored as 0-absent,1-weak,2-moderate or 3-strong in triplicates.

Congo red Agar method (CRA)

(Freeman *et al*, 1989) described an alternative method for screening the formation of biofilms by *Staphylococcus* isolates, cultured in brain heart infusion broth (BHI) supplemented with 5% sucrose and Congo red(0.8g/L) . Autoclaved aqueous solution of congo red was later added to the other media constituents Plates were inoculated and

incubated aerobically for 24 to 48 hours at 37°C. Black colonies with a dry crystalline consistency were shown to have a positive result. Weak slime producers generally remained pink, although sometimes darkening was observed in the centers of the colonies. In the absence of a dry crystalline colonial morphology, the darkening of the colonies indicated an indefinite result. The experiment was conducted in triplicate.

Static Glass Coupon Reactor Method

Overnight cultures of organisms were diluted by pipetting 1 ml of the culture into 9 ml of 1/10 normal strength TSB. The culture was thoroughly mixed by vortexing or by rolling the tube containing the culture between the palms of hands. The filter paper was uniformly moistened with 1 ml of the diluted bacterial culture. A clean 1 X 3 inch microscope slide was sterilized using the forceps and by immersing the slide in a container of alcohol and flaming it. When the flame goes out the slide is sterile. The slide was left to cool for a few seconds and then carefully laid upon the top of the filter paper being careful so that no air bubbles are trapped beneath the slide. After 24-48 hours, the biofilms were harvested by carefully lifting the slides from the filter paper surface with a pair of sterile forceps (flamed). The biofilm appears as a slimy layer coating the under surface of the slide. Lifting too rapidly may disturb the biofilm causing large sections to slough off the slide. (Zelver N,1999)

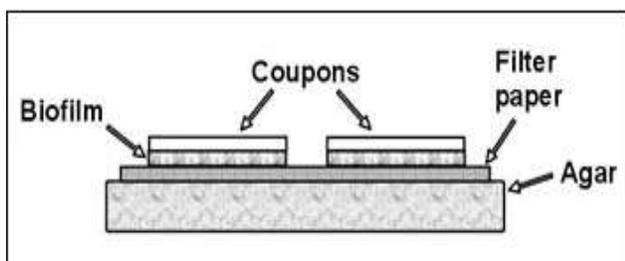


Fig.1 Cross sectional diagram of the construction of a Static Glass Coupon Reactor

In vitro Catheter model

The catheters were infected with six short listed organisms *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus*, *Staphylococcus aureus*, *Proteus vulgaris*, and *Staphylococcus epidermidis* along with the methanolic extract of *A. mexicana* and *C. gigantea* based on antimicrobial activity at a concentration of 0.5mg/mL. After three day of incubation of the catheters at 37°C, the samples were plated on nutrient agar and incubated at 37°C for 24h. After which colony forming units were counted.

Results and Discussion

Extraction yield

Table.1 shows the total yield of the plant extract using different solvents (aqueous, chloroform, methanol and acetone). The methanolic extract of *C. gigantea* gave the highest yield of crude extract (10.3g) while lowest yield was recorded in aqueous extract of *A. mexicana* (3.4g).

Phytochemical screening

Table 2.1 and 2.2 showed the qualitative phytochemical analysis of the *A. mexicana* and *C. gigantea* samples using different solvents (methanol, acetone, water and chloroform). Methanolic extract of *C. gigantea* contained all the phytochemicals followed by chloroform, water and acetone compared to extracts of *A. mexicana*. (Gopinath *et al.*, 2012)

Antimicrobial activity

Screening of antimicrobial activity was evaluated by using disc diffusion method against eleven bacteria given in fig:2. Some of these bacteria are pathogenic to human whereas some are non-pathogenic strains. The zone of inhibition varied significantly

depending upon the solvent and type of bacteria at a concentration of 500µg/mL. Four sample each of methanol, acetone, chloroform and aqueous extract for *A. mexicana* leaf and *C. gigantea* leaf were evaluated for their antimicrobial activity in triplicates. The diameter of zone of inhibition was recorded for evaluating the antibacterial activity. The methanolic extract of *A. mexicana* showed maximum inhibition against *P. vulgaris* followed by *E. coli* and *P. aeruginosa*. Zone of inhibition was maximum in *S. aureus* followed by *P. aeruginosa* and *E. coli* for acetone extract of *A. mexicana* leaves.. Aqueous extract of both the plant species did not show antibacterial activity against the eleven bacteria. Only methanolic extract of *C. gigantea* leaves showed very weak antibacterial activity against the few bacteria while no other extracts of *C. gigantea* showed any antimicrobial activity against the clinical isolates (Ashalatha and Gopinath, 2013).

Biofilm formation

Congo Red method

The cultured petri plates were observed for biofilm formation. Black colonies with a dry crystalline consistency indicated strong biofilm production. Weak biofilm producers usually remained pink, though occasional darkening at the centers of colonies was observed. Biofilm negative strains produced white or very light pink colored colonies. The experiment was performed in triplicates (Fig 3).

Tube Method

The biofilm formation was evaluated using crystal violet stain. The cultured tubes were decanted, washed with PBS (pH 7.3) and stained with 0.1% crystal violet and dried in inverted position after which the adherent biofilm layer was scored visually as either negative or weak, moderate or strong (Table 3).

Static Glass Coupon Reactor method

One side of the biofilm slide was wiped clean with a paper towel. On the remaining biofilm side of the slide, the top and bottom 2 mm of the slide clean were wiped clean. Petroleum jelly was thinly spread on a sheet of paper. Holding a cover slip at right angles to the petroleum jelly, it was scraped in such a way so as to build up a thin ridge along one edge of the cover slip. The process was repeated with the opposite edge of the cover slip. The cover slip was carefully placed onto the biofilm slide preparation petroleum jelly side down so that the jelly was in contact with the 2 mm clean edges. This has the effect of producing a tunnel between the coverslip and the slide. The Gram stain reagents were added at one open end of the slide “tunnel” and drawn through the space under the slide by means of an absorbent paper towel. The reagent sequence was identical to that in the standard Gram stain but the time intervals were longer to compensate for any dilution effect caused by the aqueous phase of the biofilm preparation (Fig 4).

In vitro Catheter model

The catheters were infected with six short listed organisms *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus*, *Staphylococcus aureus*, *Proteus vulgaris*, and *Staphylococcus epidermidis* along with the methanolic extract of *A. mexicana* and *C. gigantea* based on antimicrobial activity at a concentration of 0.5mg/mL. After three day of incubation of the catheters at 37°C , the samples were plated on nutrient agar and incubated at 37°C for 24. After which colony forming units were counted. The percentage reduction of biofilm formation was found to be 25%-30% by methanolic extract of *A. mexicana* against all tested clinical isolates. However *C. gigantea* extract displayed less than 20% of antibiofilm activity (Fig 5)

Table.1 % yield of secondary metabolites from various solvent extraction methods

Sl. No	Methanol extract	Acetone extract	Chloroform extract	Water extract
<i>Argemone mexicana</i>	9.2%	8.1%	5.9%	3.4%
<i>Calotropis gigantea</i>	10.3%	9.6%	9.2%	5.4%

Table.2 Preliminary Phytochemical Screening of *Argemone mexicana* leaves

Phytoconstituents	Methanol extract	Acetone extract	Chloroform extract	Water extract
Carbohydrates	+	+	-	+
Reducing sugar	-	-	+	-
Monosaccharides	-	-	-	-
Pentose sugar	+	+	-	-
Non-reducing polyssacharides	-	-	-	-
Proteins	-	-	-	-
Steroids	+	+	+	-
Glycosides	+	-	+	-
Saponins	-	+	-	+
Alkaloids	+	+	+	-
Flavonoids	+	+	-	+
Tannins and phenolic compounds	-	-	-	-

(+)=positive (-)=negative

Table.2 Preliminary Phytochemical Screening of *Calotropis gigantea* leaves

Phytoconstituents	Methanol extract	Acetone extract	Chloroform extract	Water extract
Carbohydrates	+	+	+	+
Reducing sugar	+	+	-	-
Monosaccharides	-	-	-	-
Pentose sugar	-	+	-	-
Non-reducing polyssacharides	-	-	-	-
Proteins	-	-	-	-
Steroids	+	-	+	+
Glycosides	+	+	+	+
Saponins	+	-	+	+
Alkaloids	+	+	+	+
Flavonoids	+	+	+	+
Tannins and phenolic compounds	+	-	-	-

(+)=positive (-)=negative

Table.3 Biofilm formation in Tube Method

Sl. No.	Name of Bacteria	Biofilm Formation
1.	<i>Escherichia coli</i>	Strong
2.	<i>Pseudomonas aeruginosa</i>	Strong
3.	<i>Staphylococcus aureus</i>	Weak
4.	<i>Staphylococcus epidermidis</i>	Moderate
5.	<i>Serratia marcescens</i>	Weak
6.	<i>Citrobacter</i>	Moderate
7.	<i>Enterococcus</i>	Weak
8.	<i>Proteus vulgaris</i>	Moderate
9.	<i>Proteus mirabilis</i>	weak
10.	<i>Klebsiella pneumoniae</i>	strong
11.	<i>Enterobacter</i>	moderate

Fig.2 Antibacterial effect of *Argemone mexicana* leaf on the test cultures as zone of inhibition

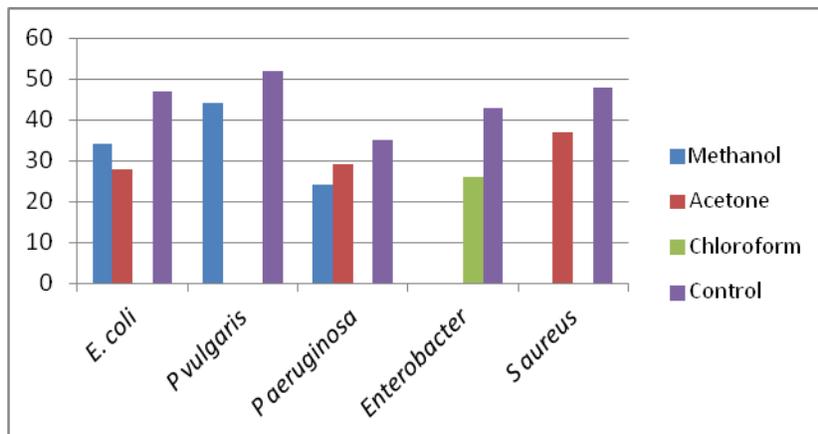


Fig.3 Congo Red Biofilm Analysis Method



Enterobacter aerogenes
Enterobacter –ve for Congo red Test



P aeruginosa showing +ve for Cong red Test

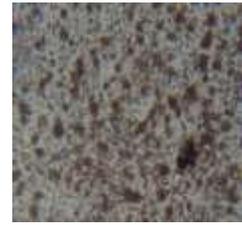
Fig.4 Slide Glass Coupon Reactor method



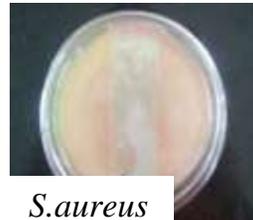
E.coli



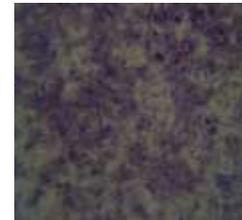
P.aeruginosa



S.marcescens



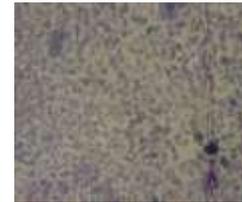
S.aureus



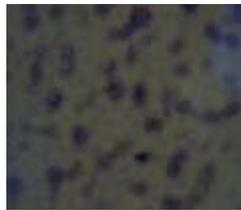
Citrobacter



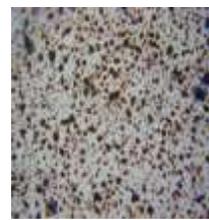
Enterobacter



P.vulgaris



P.mirabilis



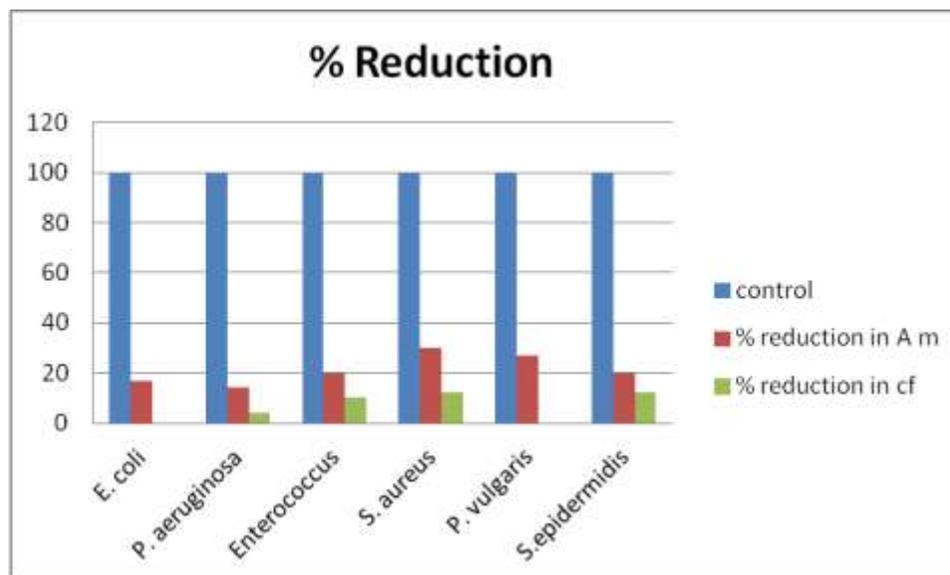
K.pneumoniae



Enterococcus



Fig.5 Percentage Reduction in biofilm formation in catheters.



Effect of herbal extracts on percentage reduction in biofilm forming clinical isolates

Several studies reported *S. aureus* and its manipulation as a primary urinary pathogen. *S. aureus* bacteriuria as an invasive reservoir (Choi *et al.*, 2009; Al-Mathkhur *et al.* 2011). showed the opportunistic gram negative *P. aeruginosa* common urinary catheters colonization and biofilm development on them. Phytochemical analysis showed the presence of carbohydrates, flavonoids, glycosides, alkaloids, steroids saponins and tannins. The methanolic and acetone extract of *Argemone Mexicana* had better results compared to other extracts and only methanolic extract of *Calotropis gigantea* showed antimicrobial activity against very few organisms of the clinical isolates. However the methanol extracts of *Calotropis gigantea* showed better anti-biofilm activity when compared to methanol extract of *Argemone mexicana*. These observations may be attributed to two reasons: firstly, the nature of biological active components whose activity can be enhanced in the presence of methanol and secondly, the stronger extraction capacity of ethanol could have produced greater number of active constituents responsible for antibacterial

activity. The incidence of catheter infection may be as high as 16% by all organisms. We can conclude that certain bioactive agents in methanol extracts of *A mexicana* showed moderately promising results which could quench the biofilm and reduce the catheter infection occurrences.

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