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In Vitro Anti-Biofilm Efficacy Of Achyranthes Aspera (L) Methanolic Extract Against Urinary Tract Infection Causing Escherichia Coli

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Abstract
Urinary track and medical device associated infections caused by biofilm forming *Escherichia coli* (*E. coli*) plays a primary role in the pathogenesis of urinary tract infection (UTI). The biofilm formed by these pathogens are highly resistant to conventional antibiotic therapies and they are also protected by host immune response, hence they are poorly responding to therapeutic agents. Different parts of medicinal plants are largely utilized as potential source of bioactive agents to cure many diseases. Based on this context the present study conducted to determine the anti-biofilm activity of the methanolic extract of folkloric medicinal plant *Achyranthes aspera* (L). The plant extract was subjected to Thin Layer Chromatography (TLC) and column was packed with silica (stationary phase). Totally nine different elusions were collected with the usage of different organic solvents (mobile phase) and TLC was again performed for all fractions. The elusion which shown single band in the Ultra Violet (UV) chamber was selected as a test sample for antibacterial activity.
The Minimum Inhibitory Concentration (MIC) was done for the same, then the least concentration which shown best result was used to check the anti-biofilm activity against UTI causing *E. coli*. The anti-biofilm activity of the elusion has exhibited above 80 %. From this work we have concluded that this plant is highly capable to kill *E. coli* biofilm and which can be used to develop anti-biofilm medicines for UTI.

Keywords: Antibiotics, Elusion, Extract, Infection, Resistant

1. Introduction

Herbal medicines have a strong traditional or conceptual base and the potential to be useful as drugs in terms of safety and effectiveness that leads to treat **(Akhtar MS and Iqubal J, 1991)** different diseases. There has been a tremendous increase in the use of plant based health products in developing as well as developed countries, resulting in an exponential growth of herbal products globally. According to the WHO, more than 80 % of the world population relies on traditional **(Bafna AR and Mishra SH, 2004)** herbal medicine for their primary health care. One of the many plants known for medicinal use is *A. aspera,* this is bitter, sour and pungent in taste. Since ancient times, the tribal **(Alam MT *et al.,* 2009)** and rural people of India commonly use this wild tropical herb for various disorders. The plant extract thus obtained by methanol was applied on different pathogens to determine the antibacterial activity. The phytochemicals present in methanolic extract undoubtedly inhibit the activity of UTI causing *E. Coli* so this plant material was found to be a potent anti-biofilm agent from this study.

2. Materials and Methods

2.1. Collection and Authentication

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Achyranthes aspera (L) fresh plants were collected from the coastal areas of Chennai. Before the extraction process the identification and authentication of the plant was confirmed. After collection, the whole plant sample was surface sterilized with sterile distilled water, shade dried and powdered. The powdered sample was stored in plastic airtight container for extraction.

2.2. Taxonomical Classification

- Kingdom : Plantae
- Subkingdom : Tracheobinota
- Family : *Amaranthaceae*
- Genus : *Achyranthes*
- Species : *Aspera*
- Order : Caryophyllales

2.3. Preparation of Extract

In 500 ml Erlenmeyer flask, about 100 grams of the powdered sample of *A. aspera* was mixed in 300 ml of purified methanol (1:3 ratio) and the flask was kept in a shaker at 150 rpm for 48 hours. On the next day the solvent mixture was extracted **(Dwivedi S *et al.*, 2008)** by using a soxhlet apparatus.

3. Preliminary Analysis for Phytochemicals

3.1. Test for Alkaloids

(i) Hager's Test

About 1 ml of plant extract and 1 ml of Hager's reagent **(Tahiliani P and Kar A, 2000)** were added and mixed. Formation of crystalline yellow precipitates indicated the presence of alkaloids.

(ii) Mayer's Test

About 1 ml of leaf extract and 1 ml of Mayer's reagent were added and mixed. Creamy white precipitate formation indicated the presence of alkaloids.

(iii) Wagner's Test

About 1 ml of leaf extract and 1 ml of Wagner's reagent were added and mixed. Formation of reddish brown precipitate indicated the presence of alkaloids.

3.2. Test for Amino Acids

(i) *Ninhydrin Test*

Two drops of ninhydrin solution were applied to 2 ml of aqueous filtrate (10 mg of ninhydrin in 200 ml of acetone). The appearance of purple colour suggests that amino acids are present.

(ii) *Benedict's Test*

0.5 ml of Benedict's reagent was added to 0.5 ml of the filtrate. The mixture was **(Gokhale AB *et al.*, 2002)** heated for 2 mins in a boiling water pan. A distinctive coloured precipitate shows the presence of sugar.

3.3. Test for Flavonoids

(i) *Shinoda Test*

To 1 ml of the extract, 8 to 10 drops of concentrated HCl was added along with a pinch of magnesium powder or filings. The mixture **(Sutar N *et al.*,2011)** was boiled for 10 to 15 minutes and cooled. A red or orange colouration indicated the presence of flavonoids**.**

(ii) *Ammonia Test*

A small piece of filter paper was dipped to about 1 ml of the extract and exposed to ammonia vapour. Formation of yellow spot on filter paper indicated the presence of flavonoids.

3.4. Test for Cardiac Glycosides

(i) *Keller-Killani Test*

5 ml of the extract was treated with 2 ml of glacial acetic acid, one drop of ferric chloride solution and 1 ml of concentrated sulphuric acid. A brown ring at interface indicates the presence of deoxy sugar characteristic feature of cardenolides. A violet ring may **(Goyal BR *et al.*, 2007)** appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout thin layer.

(ii) *Baljet Test*

To 1 ml of the extract, 2 ml of sodium picrate solution was added. The conversion of yellow to orange colour shows the presence of glycoside.

3.5. Test for Phenol

(i) *Ferric Chloride Test*

In 5 ml of distilled **(Vetrichelvan T and Jagadeesan M, 2003)** water, the extract (50 mg) was dissolved. Few drops of 5 % $FeCl_3$ solution was applied to this. The presence of phenolic compounds indicated by a dark green colour.

(ii) *Gelatin Test*

The extract (50 mg) was dissolved in 5 ml of distilled water and 2 ml of 1 % gelatin containing solution was added to it. The presence of phenolic compounds is indicated by white precipitate.

(iii) *Lead Acetate Test*

The extract (50 mg) was dissolved in distilled water and a 10 % lead acetate solution was added to this 3 ml solution. The presence of phenolic **(Sharma SK *et al.*, 2009)** compounds suggests a bulky white precipitate.

3.6. Test for Saponins

(i) *Froth Test*

About 2 g of the powdered sample was boiled with 20 ml of distilled water in a water bath and filtered. 10 ml of the filtrate was mixed with 5 ml of distilled **(Mishra TN *et al.*,1991)** water and shake vigorously for a stable persistent froth. Formation of 1 cm layer of foam indicates the presence of saponins.

3.7. Test for Steroids

(i) *Libermann Burchard Test*

To 0.5 ml of the extract, 2 ml of acetic anhydride 2 ml of concentrated H_2SO_4 was added along the sides of the tube. The appearance of purple colour which change to blue or green colour indicates the presence of steroids.

3.8. Test for Tannins

(i) *Modified Prussian Blue Test*

To 1 ml of the extract, add 1 ml of 0.008 M potassium ferricyanide and 1 ml of 0.02 M $FeCl_3$ in 0.1 M HCl. Appearance of blue colour denoted **(Sharma V *et al.*, 2013)** the presence of tannins.

3.9. Test for Terpenoids

(i) *Salkowski Test*

To 5 ml of the extract, 2 ml of chloroform and 3 ml of concentrated H_2SO_4 was added. Formation of yellow colour ring at the interface of the two liquids that turns reddish brown after two minutes indicates the presence of terpenoids.

3.10. Test for Xanthoprotein

The plant extract was treated with a few drops of conc. HNO_3. Formation of yellow colour indicated the presence of xanthoprotein.

4. Column Chromatography

A cylinder shaped glass column containing stationary phase (silica gel) is encountered slowly from the top with a liquid solvent (mobile phase - chloroform) that flows down the column. Once the column is ready, the sample is loaded inside **(Nagappa AN and Binu C, 2001)** the top of the column. The mobile solvent is then allowed as follows, ethyl acetate, methanol and water to flow down through the column. The compounds in mixture have different interactions ability with stationary phase (silica gel), and mobile phase, thereby they may flow along the mobile phase at different time intervals. In this way, the separation of compounds from the mixture is achieved. The individual compounds were collected in test tubes as fractions and stored for further analysis.

5. Thin Layer Chromatography (TLC)

The glass slides were evenly coated with silica gel (for TLC) and dried. Glass capillaries were used to spot the sample on TLC plate. The plate was kept in the glass chamber **(Naik *et al.*, 2003)** containing mobile phase which was chloroform: methanol: ethyl acetate (8:1.5:1). Five micro liters (µL) of the sample was spotted on the TLC plate. After the run, plates were dried and kept in the UV radiation to detect the **(Perianayagam JB *et al.*, 2006)** bands on the TLC plates. The movement of the active compound was expressed by its retention factor (Rf) values.

(Rf) = Distance travelled by the solvent / Distance travelled by the solvent front.

6. UV Visible Spectroscopy

The electronic absorption spectra were recorded on a PerkinElmer Lamda 25 UV Visible spectrophotometer **(Raghavendra MP *et al.*, 2006)** controlled by the Win Lab software through computer. The spectra were recorded in the region of 190 - 900 nm using distilled water as solvent using a matched pair of Teflon stoppered quartz cell of path length 1 cm. The concentration of the solution used was 10^{-5} M.

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7. Antibacterial Activity

Antimicrobial activity was performed using the agar-well diffusion bioassay, the methanol extract of *A. aspera* was examined against four different pathogenic bacteria. Control was maintained separately, ampicillin (**Rameswar RD, 2007)** was used as a positive control. Muller Hinton agar medium was prepared and poured in sterile petri dishes. Small wells made by cork borer, 25 µl of the dried elusion obtained from the column, which was already **(Ratra and Misra, 1970)** dissolved in dimethyl sulfoxide (DMSO) loaded into those wells. the plates were sealed, incubated at 37ºC for overnight and the zone of inhibition was calculated.

8. Anti-Biofilm Activity

The effect of fraction from column on biofilm formation was evaluated in 96-well polystyrene flat-bottom plates and the optical density was observed at 600 nm using microplate reader (EPOCH II, Bio Tek, USA). Biofilm forming assay was initially done using pathogens and methanol was used as negative control, an antibiotic ampicillin was used as positive control.

Briefly, 100 μL of inoculated Luria-Bertani broth was aliquoted into each well of microplate and pathogens were cultured. Agar wells containing **(Shankar D and Ved DK, 2003)** test sample were used to compare with controls. Plate was incubated at 37°C for 48 h. After incubation, supernatant was removed and each wells washed thoroughly with sterile distilled water to remove free-floating cells; thereafter plate was air-dried for 30 min and the biofilm formed was stained during 15 min at room temperature with 0.1 % aqueous solution of crystal violet. Following incubation, the excess **(Vijaya Kumar S *et al.* ,2009)** of stain was removed by washing the plate thrice with sterile distilled water. Finally, the dye bound to the cells was solubilized by adding 250 μL of 95 % ethanol to each well and after 15 min of incubation, absorbance was measured using microplate reader at a wavelength of 600 nm. Biofilm determination was made using the formula.

9. Results and Discussion

Screening of Phytochemicals

The qualitative phytochemical screening is an essential step towards discovery of new drug as it provides the information regarding the presence of primary and secondary metabolites in the plant extract. The presence of carbohydrates, phenolic compounds, tannins, etc., were confirmed by the screening tests. The phytochemical screening of (Table. 1) methanolic extract of *A. aspera* are summarized below.

Table 1: Preliminary Phytochemical Analysis of *A. aspera* Methanolic Extract

Phytochemicals	Results
Alkaloids	+
Amino acids	+
Flavonoids	+
Cardiac glycosides	-
Phenols	+
Saponins	-
Steroids	-
Tannins	+
Terpenoids	+
Xanthoproteins	-

Column Chromatography

This research visualized successful application of column-chromatographic technique for the isolation of biologically active secondary metabolites from methanolic extract of *A. aspera*. Totally nine fractions were collected with different (Fig. 1) colours such as, from ethyl acetate (F1, F2 and F3), methanol (F4, F5, F6, F7 and F8) and water (F9). In-order to confirm the purity TLC was done for all those fractions. Among these, fraction six (F-6) shown a clear single band on TLC plate and the same was emitted fluorescence at UV chamber, so F-6 was selected for further studies.

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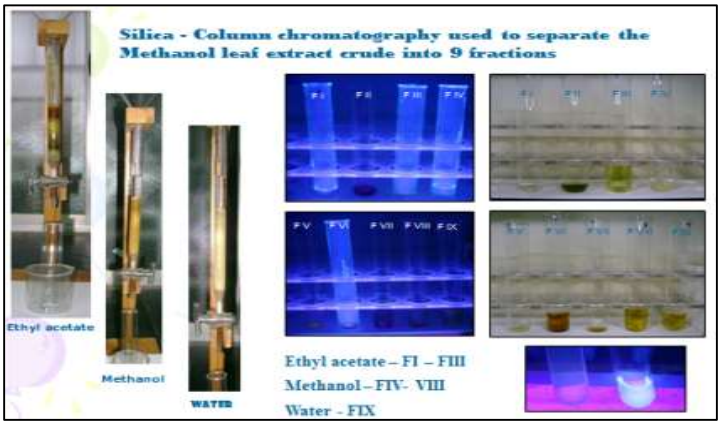

Figure 1: Column Packing of *A. aspera* Methanolic Extract.

Thin Layer Chromatography (TLC)

Fraction six obtained from column was evaporated with the help of fume hood. A pinch of crude obtained was dissolved with 10 µL of methanol and loaded on TLC plate. Then the air dried plate was kept in TLC chamber which containing mobile phase. After the compound (Fig. 2) migrated towards the top of stationary phase, the plate was removed and checked in UV chamber with different nanometers. The compound emitted fluorescence in UV chamber. The plate was exposure to iodine vapours, a single spot observed and the retention factor value calculated as Rf = 0.57 (2.3/4).

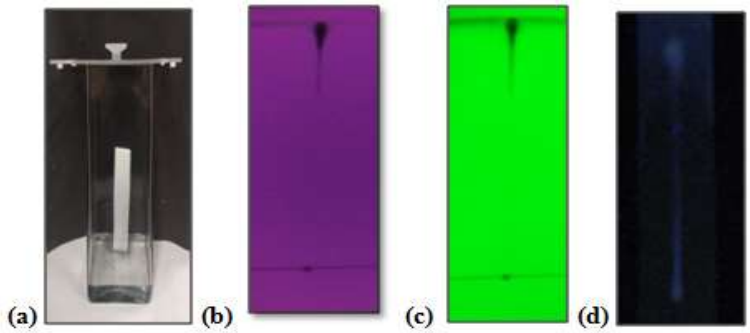

Figure 2: TLC of Fraction-6 from Column (a) Sample in TLC Chamber (b) Single Band at 366 nm (c) Single Band at 254 nm (d) Fluorescence of Compound in UV Chamber.

UV Visible Spectrum

The methanolic extract of *A. aspera* displayed 4 peaks at 470 nm, 536 nm, 670 nm and 688 nm respectively. Four peaks correlated with the four phytochemicals (Fig. 3) found during preliminary phytochemical screening. The appearance of different absorption peaks confirmed the presence of bioactive compounds in the plant extract.

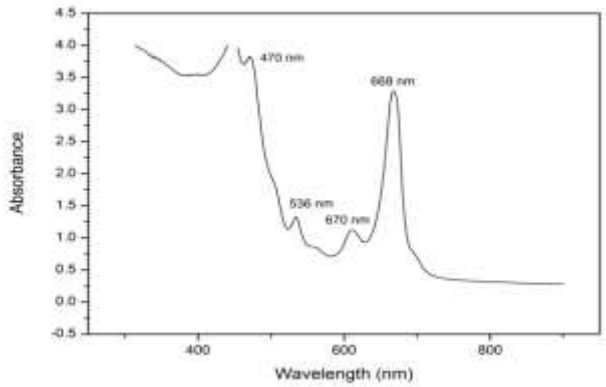

Figure 3: UV Visible Spectrometric Analysis of *A. aspera* Methanolic Extract.

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Antibacterial Activity (Well Diffusion Method)

After the desired incubation period the plates were removed from incubator and the inhibition zone was measured with the help of zone scale. There was no any change around the negative control (DMSO). The test sample shown best activity against all pathogens, which was more or less (Fig.4) equal to the positive control (ampicillin) and the results were (Table.2) included in the table.

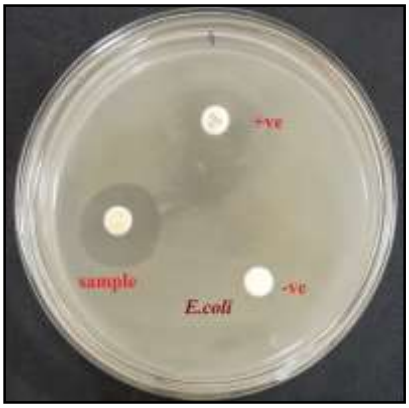

Figure 4: Antibacterial Activity of Evaporated Solvent Elusion (F-6) Obtained from Column.

Table 2: Antibacterial Activity of Crude (F-6) Obtained from Column Chromatography

	Disk Diffusion Method (zone of clearance in mm) conc. 0.1 gm in 500 µl		
Pathogens	Negative control (DMSO)	Positive control (Ampicillin)	Test sample
Staphylococcus aureus	-	9	6
Klebsiella pneumonia	-	8	6.5
Pseudomonas aeruginosa	-	8	8
Escherichia coli	-	5	9

Anti-biofilm Activity (96 Well Plate Method)

The effect of obtained elusion from column on biofilm disruption was evaluated in 96-well microtiter plate. After incubation, supernatant was removed and each wells were washed thoroughly with sterile distilled water to remove free-floating cells; thereafter plates were air-dried for 30 minutes and the biofilm formed was stained at room temperature with 0.1 % crystal violet solution. Following incubation, the excess amount of dye was removed by washing the plate thrice with sterile H_2O, absorbance was (Table 3 and 4) measured using microplate reader (Fig.5) at a wavelength of 600 nm. The percentage of biofilm dispersion was made using the formula B = (X - C) x G, [where X = C - S] C = control (the specific biofilm formation), S = sample (OD at 600 nm of the attached and stained bacteria) and G = 100. The anti-biofilm activity of the elusion has exhibited above 80 %.

Figure 5: Anti-biofilm Activity (a) Biofilm Forming Assay (b) Eradication of Biofilm by Test Sample.

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Table 3: Anti-biofilm Activity of Crude Obtained from Column using Microplate Reader

E.coli	Control	Ampicillin	Sample
A	0.103	0.021	0.020
B	0.106	0.020	0.019
C	0.108	0.019	0.021
Average	0.105	0.019	0.020

Table 4: Biofilm Disrupting Efficacy of Crude Compound Obtained from Column

X = Control - Sample	Dispersal activity (in %) (X / Control x 100)	
	Ampicillin	Sample
0.105 - 0.020 = 0.085	81.90 %	80.95 %

Conclusions

The plant extract contained the phyto constituents such as alkaloid, amino acids, flavonoids, tannins, phenol and terpenoids. The results of this study clearly indicates that the methanolic extract of *A. aspera* capable to produce many phytocompounds. The active metabolites present in this extract have high rate of mortality of clinical pathogens. An antibacterial and anti-biofilm activities of the methanolic extract of *A. aspera* confirmed to the hypothesis of the formulation of herbal drugs against *E. coli*. The results of the present study would be useful in promoting research and development. Thus, *A. aspera* is quite promising as a multipurpose medicinal agent, so further clinical trials should be performed to prove its efficacy in medical field.

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