Antibacterial Activity of *Bryophyllum pinnatum* Against Pseudomonas Aeruginosa Isolated from UTI

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***Abstract*—Various solvent extract of leaves of *Bryophyllum pinnatum* were analyzed for antibacterial activity against selected species isolated from urine samples. To provide a scientific basis to treat UTIs, the extracts of leaves *Bryophyllum pinnatum* were subjected to antibacterial activity against selected bacterial species by well diffusion assay. MIC values were also determined to find minimum effective concentration. Methanolic extract show good antibacterial activity against highly resistant UTI isolates.**

***Index Terms*—*Bryophyllum pinnatum*, anti-bacterial activity, well diffusion assay, minimum inhibitory concentration, Urinary Tract Infection (UTI).**

I. Introduction

Urinary Tract Infections (UTIs) are the most widespread microbial disease in India as per WHO [1]. The common UTI pathogenic bacteria occurring nosocomial infections. Generally UTIs are caused by a variety of both Gram-positive and Gram-negative bacteria. The Gram-positive bacteria includes *Staphylococcus sp, Enterococcus sp,* and *Streptococcus sp.* Gram-negative bacteria includes huge number of aerobic bacilli such as *Escherichia sp, Pseudomonas sp, Klebsiella sp, Citrobacter sp, Enterobacter sp, Proteus sp, Serratia sp and Salmonella sp* [2]. Invasion of the urinary tract of these pathogenic bacteria which leads to an inflammatory response of the urothelium. Urinary tract infection is caused by the Proliferation of those bacteria in the urinary tract [3]. Many of the antibiotics and also synthetic drugs become sensitive and show many undesirable side effects. So natural alternative must be selected as they are more safe in biological system [4], [5].

Presence of chemical and medicinal contents in natural form, plants and herbal medicines have important position in modern medicine. They contain various secondary metabolites which work together and show wide range of antibacterial activities. Microorganisms may get mutated and become resistant to many antibiotics and so it generates a global health problem. These inspired scientists to search out new natural alternative to treat diseases [6].

Bryophyllum pinnatum (Lam.) Kurz., (Crassulaceae) Synonym: *Kalanchoe pinnata* (Lam.) Oken, *Bryophyllum calycinum* Salisb. It is commonly known as Zakham-e-hyat, Cathedral bells, air or maternity plant, love plant, Life plant, Canterbury bells and also parnabija. It is a widely growing perennial herb and used in medicine with folklore value in, tropical America, tropical Africa India, Australia and China classified as a weed [7]. It is a crassulescent herb of about 1 metre in height, with opposite, glabrous leaves (with 3–5 deeply crenulated, fleshy leaflets [8].This herb is distributed worldwide but growing primarily in the rain forest [9]. The main phytoconstitutes found in Kalanchoe are alkaloids, triterpeness, glycosides flavonoids, steroids, bryophyllin, palmitic acid, oxalic acid etc. [10]. In the present study, *in vitro* antibacterial of aqueous and methanolic extracts leaf *Bryophyllum pinnatum* (Lam.) Kurz. have been evaluated by using MIC and agar well diffusion method.

II. Materials and Methodology

A. Collection of Plant Materials

Plant *Bryophyllum pinnatum* was collected from Santipur, West Bengal, India. Leaves of the plant was washed with running water, dried in shade at room temperature, ground to powder and stored in air tight bag in dry at low temperature.

B. Preparation of Extracts

Leaf powder (50g) of *Bryophyllum pinnatum* (Lam.) Kurz. was macerated separately in 100 ml of methanol and distilled water for 24 hours in mechanical shaker at

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120 rpm. The contents were filtered through Whatman filter paper No. 1 and residues were further macerated thrice using above procedure. The filtrates taken at each step were combined and evaporated separately for leaf in water bath (60±2℃). These extracts were used for *in vitro* antibacterial activities against UTI bacteria.

C. Isolation and Selection of Bacterial Species

Urine samples of the female patient suffering from UTI were collected from JNM medical Hospital, Kalyani, West Bengal, India. Bacteria of UTI were isolated by cetrimide medium. The isolated bacterial species was identified by morphological, physiological and biochemical tests [11]. From the identified causative agent, highly resistant species was selected by performing antibiotic susceptibility test against 5 different antibiotics that are Ampicillin, Chloramphenicol, Ciprofloxacin, Streptomycine and Amoxycilin.

D. Determination of Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of methanolic extracts of leaf of *Bryophyllum pinnatum* (Lam.) Kurz. were determined against selected bacteria separately. Concentration ranging from 300 µg/ml - 1 mg/ml of methanolic extracts of leaf was prepared and 500µl of each dilution was incubated with 5 ml of Mueller Hinton Broth containing 0.1 ml of bacterial suspension at 37℃ for 24 hours. After incubation the tubes were examined for bacterial growth by observing turbidity. The MIC was determined as minimum concentration that showed no visible growth. The experiment was carried out in triplicates.

E. Determination of Antibacterial Activity

Antibacterial activity was carried out using methanolic extracts of leaf of *Bryophyllum pinnatum* (Lam.) Kurz. by agar well diffusion method. Antibacterial activity was determined by measuring the diameter (mm) of zone of inhibition. 200 µl of plant extract (prepared in a particular solvent) was added in each test well by using sterile micropipettes. The plates were incubated at 37℃ for a day. After incubation the diameter of the zone of inhibition was measured in mm.

F. Phytochemical Evaluation

Preliminary phytovhemical analysis was carried out using standard protocol for determination of phytoconstituents: alkloids, tannins, saponin, reducing sugar, anthrocyanine, flavonoids, carbohydrate, protein, terpenoids, cardiac glycoside and phlobatannins as directed by references [12]. The details of the tests are as follows:

- Test for alkaloids

The small portions extract was stirred separately with a few drops of dil. HCl and filtered and then subjected to test for alkaloids.

Dragendorff's test: Extract was treated with dragendorff's reagent. Formation of orange brown precipitation which indicates the presence of alkaloids.

Mayer's test: Plant extract was treated with Mayer's reagent. Formation of cream precipitation is formed. This indicates the presence of alkaloids.

Wagner's test: Plant extract was treated with Wagner's reagent. Formation of reddish brown precipitation indicates the presence of alkaloids.

- Test for tannins

In 2–3 ml of extract, 10% alcoholic ferric chloride solution was added. Dark blue or greenish grey coloration indicate the presence of tannins.

- Test for saponins

Few drops of olive oil was added to plant extract and vigorously shaken. Soluble emulsion is formed that indicates the presence of Saponin.

- Test for reducing sugar

The plant extracts was added with the Fehling's solution (A and B) in a test tube. Colour reaction indicates the presence of reducing sugar.

- Test for anthrocyanine

10% sodium hydroxide is mixed with the plant extracts. Blue colour precipitation indicates the presence of this phytochemicals.

- Test for flavonoids

Alkaline reagent test: Extract was treated with minute amount of sodium hydroxide solution. Change of colour from yellow to colourless on addition of dilutes acid was observed. This indicates presence of flavonoids.

Lead acetate test: Few drops of lead acetate solution were added with the plant extract. Yellow colour precipitation indicates the presence of flavonoids.

- Test of carbohydrate

Extract was dissolved individually in 5 ml distilled water and filtered. The filtrate was used to test for the presence of carbohydrates.

Molisch's test: Filtrate was treated with 2 drops of alcoholic α-naphthol solution in a test tube and 2 ml of concentrated sulphuric acid was added along the sides of the test tube. Appearance of violet ring at the junction indicates the presence of carbohydrates.

Benedict's test: Filtrate of plant extract was treated with Benedict's reagent and heated on water bath. Orange red precipitate indicates the presence of carbohydrate.

- Test for proteins

Few ml of plant extract was added with 1 ml of 40% NaOH and 2 ml of cupric sulphate. Purple of violet colour precipitation indicates the presence of proteins.

- Test for terpenoids

Thionyl chloride was added with the plant extracts .Pink colour appearance is indicating the presence of terpenoids.

- Test for cardiac glycosides

Glacial acetic acid, ferric chloride and concentrated sulphuric acid were added with the plant extracts. Green colour of the mixture indicates the presence of cardiac glycosides.

- Test for phlobatanins

Plant extract was dissolved in distilled water. Then the filtrate is boiled with 2% hydrochloric acid. Red colour precipitation indicates the presence of phlobatannins.

G. Quantitative Phytochemical Analysis

1) Total phenolic content

Modified Folin-Ciocalteu reagent method was used to determine total phenol content in the methanolic extract of the selected plant. 2.5ml of Folin-Ciocalteu reagent (1%) and 2ml of sodium carbonate solution (2%) were added to 1ml of extract. Then the mixture was incubated for half an hour at room temperature. Gallic acid was used as standard (1mg/ml). The absorbance was taken at 765nm. The result was estimated from a standard curve already prepared and was presented as Gallic acid equivalent (mg/g of extracted compound) [13].

2) Total flavonoid content

Aluminum chloride colorimetric method was used with little modifications to determine flavonoid content. One ml of plant extract was added with 3ml of methanol, 0.2ml of $AlCl_3$ (10%), 0.2ml of potassium acetate (1M) and 5.6ml of distilled water and kept at room temperature for half an hour. The absorbance was taken at 420nm. The standard used was quercetin (1mg/ml). A standard curve was prepared to determine the flavonoid content and the result was presented as quercetin equivalent (mg/g of extracted compound) [13].

H. Thin Layer Chromatography

TLC was used for the conformation of the different bioactive compounds on analytical plates. 10µml of the extract were loaded on the analytical plate (2.5cm above from the bottom) and dried on air for thirty minutes. The spotted plates were kept in a previously saturated developing chambers containing mobile phase and allowed to run 3/4th of the height of the prepared plates. Ascending thin layer chromatography was performed using 80:20 proportions of benzene and acetic acid as mobile phase. The plates were air dried and the spots on the plate were located using iodine vapours. Various fractions were pooled based on the Rf values

III. Results

A. Isolation and Selection of Bacterial Species

5 urine samples were collected from laboratories of JNM Hospital, Kalyani, West Bengal, India.Total six bacteria were isolated. Gram negative bacteria, *Pseudomonas aeruginosa* was isolated from urine sample. The bacterial cultures were identified by microscopic, biochemical characters were identified observations as shown in Table I.

TABLE I. Identification of Isolated Microbe Using Biochemical Test

TESTS/CHARACTERS	Bacterial isolates 1	Bacterial isolates 2	Bacterial isolates 3	Bacterial isolates 4	Bacterial isolates 5	Bacterial isolates 6
Cell shape	Rods	Rods	Rods	Rods	Rods	Rods
Gram reaction	-	-	-	-	-	-
Motility	+	+	+	+	+	+
Lipid Hydrolysis	-	-	-	-	-	-
Starch Hydrolysis	+	+	+	+	+	+
Casein Hydrolysis	-	-	-	-	-	-
Catalase Test	+	+	+	+	+	+
Oxidase Test	+	+	+	+	+	+
Urease Test	-	-	-	-	-	-
Growth on TSI	NC	NC	NC	NC	NC	NC
Nitrate Reduction Test	+	+	+	+	+	+
Indole Production Test	-	-	-	-	-	-
Methyl Red Test	+	+	+	+	+	+
Voges-Proskauer Test	-	-	-	-	-	-
Citrate utilization Test	+	+	+	+	+	+
Carbohydrate utilization tests						
Glucose	+	+	+	+	+	+
Fructose	+	+	+	+	+	+
Lactose	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-

- Indicates negative results and + indicates positive results
NC---No change

B. Antimicrobial Sensitivity Test

Based on the results obtained from susceptibility testing it was observed that the bacterium isolated from UTI showed highest degree of MIC value against to Chloramphenicol and Ciprofloxacin (800 µg/ml) which are commonly prescribed drug for UTI treatment (Table II). Lowest MIC value showed against Ampicilin (50 µg/ml).

C. Anti-Bacterial Assay

All the extracts were tested for the antibacterial activity against the selected bacterial species. Zone of inhibition was measured 13 mm. Methanol extract

showed maximum activity against *Pseudomonas aeruginosa* (Fig. 1). The antibacterial activity might be due to presence of bioactive compounds in plant extracts. MIC of the plant extracts was determined by well diffusion method by using different dilutions. The MIC value was shown 500µg/ml by methanolic extract of *B. pinnatum*.

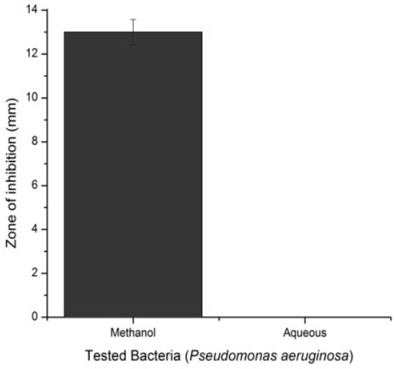

Figure 1. Zone of inhibition of *Bryophyllum pinnatum* aginst tested bacteria

TABLE II. ANTIMICROBIAL SUSCEPTILITY TEST AGAINST ISOLATED PATHOGENS

Antibiotic	MIC (µg/ml)
Ampicillin	1000
Amoxycillin	500
Chloramphenicol	800
Ciprofloxacin	800
Streptomycin	500

D. Phytochemical Evaluation

The phytochemical analysis reveals presence of tannin, flavonoid, carbohydrate, protein, terpenoid, phlobatannins, anthocyanins and cardiac glycoside.

E. Quantitative Phytochemical Analysis

Total phenolic content obtained was 16.3mg/gm and total flavonoid content was found 8.9mg/gm.

F. Thin Layer Chromatography

The TLC plate of leaf of *B. pinnatum* is shown in Fig. 2. Two spots were shown in iodine vapour. The Rf value was observed during the TLC are 0.78 and 0.88.

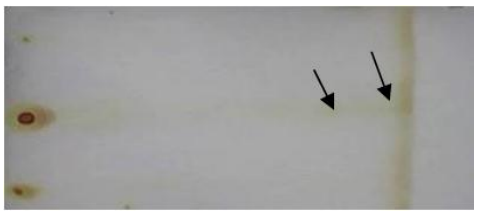

Figure 2. TLC of leaf of *Bryophyllum pinnatum*

IV. DISCUSSION

Antibiotic resistance pathogenic bacteria should be investigate new, cost effective, efficient and safe antibacterial as alternative agents for controlling the infectious diseases [14]. The extent of sensitivity of the test organisms on leaf extracts of B. Pinnatum was measured by measuring the zone of inhibition after 24 hours of inoculation shown in table- The potential for developing antimicrobials from plant materials are found to be promising. Plant based antimicrobials have immense therapeutic potential for the formulation of phytomedicines as they show minimal side effects and not at all. Those are often encountered with synthetic antibiotic [14]. The antibacterial activity might be due to presence of bioactive compounds in plant extracts. According to Dholaria and Desai methanol extract showed maximum activity against all selected isolates except *Pseudomonas aeruginosa* while maximum activity against *Pseudomonas aeruginosa* was shown by ethanol extract [15]. Aqueous extract of leaf of the plant showed no zone of inhibition against tested bacteria. The Rf value indicates the presence of phenolic compounds in the plant extracts which shows antimicrobial activity against UTI pathogens. Presence of components in plant extracts which exhibit medicinal and physiological activities was revealed by phytochemical analysis [16]. The largest and most appearing groups of secondary metabolites of plant is phenolic compounds [17]. These phenolic compounds possess biological properties such as anticarcinogen, antiinflammation, antiapoptosis, antiatherosclerosis, antiaging, improvement of endothelial function, cardiovascular protection and also inhibition of cell proliferation activities and angiogenesis [18]. A large number of studies were made which described the antioxidant properties of medicinal plants are increased in presence of phenolic compounds [19], [20]. Natural antioxidants come mainly from plants in the form of phenolic compounds such as phenolic acids, tocopherols flavonoid, etc. [21]. In case of tannins, it bind to proline rich protein and interact with the synthesis of protein. In response to microbial infection, hydroxylated phenolic substances, flavonoids are released by plants. Flavonoids posses antimicrobial properties against wide variety of microorganisms *in vitro*. Their activity is might be due to their ability to form complex with extracellular and soluble proteins as well as to form complex with cell wall of bacteria [22] (see Table III and Table IV).

TABLE III. MIC OF *BRYOPHYLLUM PINNATUM* EXTRACT AGAINST ISOLATED PATHOGENS

Plant Extracts	MIC Value(µg/ml)
Methanol	500
Aqueous	0

TABLE IV. PHYTOCHEMICAL ANALYSIS OF *BRYOPHYLLUM PINNATUM* LEAVES EXTRACTS

Secondary metabolites	Methanolic leaves extracts
Alkaloids	-
Tannins	+
Steroids	-
cardiac glycoside	+
Flavonoids	+
Terpenoids	+
Proteins	+
Carbohydrates	+
Saponins	-
Reducing Sugars	-
Phlobatannins	+
Anthocyanins	+
Phenolic compounds	+

Indicates nagativeresulta and + indicates positive results

V. Conclusion

The present study showed that the leaf extracts of *Bryophyllum pinnatum* must be present of various ptytoconstituents which might be effective to inhibit microbial growth. These results suggest that solvent extracts of this plant may be a good source of natural treatment of UTI. This study also ascertains the value of Bryophyllum pinnatum used in Unani system of Medicine. This could be considerable interest to the development of new drugs. Further studies on the chemical characteristics of the extract is in progress in order to identify the leads with antimicrobial activity. In the search for new pharmaceuticals, screening of such different natural organic compounds and the proper identification of bio-active agents must be considered as a fruitful approach.

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