ELUCIDATION OF ANTIBACTERIAL PRINCIPLE FROM *ACROSTICHUM AUREUM* L.

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INTRODUCTION

Acrostichum aureum L., belongs to the family Pteridaceae, a large terrestrial plant observed in flooded areas during rainy seasons and at high tides, association with mangroves and its status is common in kerala (Easa, 2003) Whole plant is used as an antihelmintic and styptic, also used as a worm remedy and as an astringent in haemorrhage (Chopra ,1992). Widespread use of antibiotics caused significant increase in antibiotic resistance of bacteria. Flavonoid of *Drynaria fortunei* against renal failure (Long et al., 2005). Reports of *Drynaria quercifolia* against Neisseria gonorrhoeae (Shokeen et al., 2005). Friedelin, epifriedelirol, betaamyrin, beta sitosterol, 3-beta-D-glucopyraroside, and naringin were isolated from dried rhizome of *Drynaria quercifolia.* The methanol extract showed braoad & concentration dependent antimicrobial activity (Ramesh et al., 2001). Cytotoxic & antioxidant effects by *Drynaria fortunei* (Liu et al., 2001). Gleichenia linearis show antibacterial properties (Vasudeva, 1999).

Over the past few years strains of many highly pathogenic species resistant to all widely available antibiotics have emerged and proliferated at rates that can never be envisaged (Tomasz, 1994). It is commonly assumed that infections caused by multiple resistant strains occur throughout the developing world (Gibbons, 1992). In other words, a generalized sense of alarm regarding the problem of multidrug resistant microorganism is arising simultaneously in a number of places in the world (Cohen, 1992). Resistant pathogens are increasing in prevalence, for example: multidrug resistant *Acinetobacter baumannii*, methicillin resistant *Staphylococcus aureus*, vancomycin resistant beta-lactamase producing *Enterococcus* species etc. have been observed in hospital acquired pathogen (Kaatz et al., 1990).

Currently, these multi-drug resistant bacteria have been emerging as one of the most important hospital and community pathogens worldwide. The emergence of these resistant bacteria has caused a major concern and thus the urgent need for new antibacterial agents. Present study is an attempt to evaluate antibacterial potential of the plant in various extracts of increasing polarity and to understand the phytochemial background of the

extracts. The extracts were tested towards pathogenic bacteria involved in various diseases in human beings.

METHODOLOGY

Preparation of plant extract

The air-dried fronds (Leaves) of the plant material (100g) was ground and utilised for preparing extracts. Soxhlet extracts of petroleum ether, acetone, ethanol and water were made successively with yield of 0.58%, 4.7%, 5.4%, and 0.9% respectively (Reichardt, 1998).

Microorganisms Used

The test organisms were collected from the culture collection of the institute of Microbial Technology (IMTECH), Chandigarh. These include *Staphylococcus aureus* (MTCC 96), *Escherichia coli* (MTCC 443), *Pseudomomas aeruginosa* (MTCC 741), *Micrococcus luteus* (MTCC 6164) and *Serratia marcescens* (MTCC 6164). antibacterial activity were evaluated against thirteen strains of pathgenic bacteria, extracts were prepared in different solvents of increasing polarity.

In vitro Antibacterial Assay

The disc diffusion method as illustrated by Bauer et al., 1966 was used to determine the growth inhibition of bacteria by plant extracts. Sterile liquid Mueller Hinton Agar media (pH 7.4 ± 2) was poured into sterile petridish and after solidification; the bacteria (1 ml broth of approximately 10^5 CFU) were swabbed with a sterile needle under aseptic conditions. Sterile discs prepared using Whatman No. 4 Filter Paper, of 5-mm diameter were used in the study. The original solvent in which the extract prepared was used as a control. Test materials were dissolved in the respective solvent to obtain a stock solution of concentration of 100 mg/ml. 20 µL of the solution was loaded per disc to attain a concentration of 1 mg/disc. The discs (including control) were used after drying them in an incubator at 40°C to remove any trace of solvent. Discs were introduced onto the surface of the medium. The plates were incubated at 37^{o}C for 24 hours to obtain inhibition zones. Experiments were conducted in more than three replicates and average inhibitory zone diameter was determined along with standard deviation.

Minimum inhibitory Concentration (MIC)

The MIC of the extracts was performed by incorporating various amounts (128–0.125 mg/ml) of the extract into sets of test tubes with the culture media. 50 µl of the bacterial broth culture was added into each of the test tubes. The bacterial cultures containing the plant extracts were incubated at 37^{o} C for 24 hours. Test tube containing only the growth medium and each of the organisms was also incubated under the same conditions as positive controls. The minimum inhibitory concentration was expressed as the lowest concentration of the extracts that did not permit any visible growth when compared to that of the control tubes

Minimum bactericidal concentration (MBC)

Samples from the tubes used in the MIC assays, which did not show any visible growth after a period of incubation were subcultured onto a freshly prepared nutrient medium The minimum bactericidal concentration was taken as the lowest concentration of the extract that did not yield a single colony on the nutrient agar plate after 24 hours incubation period (Ratimi et al., 1998)

Preliminary detection of phytochemicals

Alkaloids were detected by Iodine Potassium iodide reagent, Dragendroff reagent and Marguis reagent (Harbone, 1973). Presence of flavonoids was predicted by 25% aqueous solution of basic lead acetate spot fluorescence in long wave U.V light and 1% aluminium chloride solution in ethanol yielded yellow fluorescence in long wave U.V light. Phenols were detected by spraying TLC plates with saturated aqueous silver nitrate solution; light pink to deep green spots were yielded after spraying and Folin ciocalteu reagent yielded dark brown spots. Stannic chloride for titerpenes, sterols and steroids, phenol and poly phenols (Scheidegger and Cherbuliez, 1955)

Separation of the crude extract using Thin Layer Chromatography (TLC)

This involves the differential adsorption of substances as they pass through a uniform layer of inert adsorbent, which has been uniformly spread over a suitable supporting plate such as glass. TLC is an excellent qualitative and quantitative method for separating the components of a mixture. In TLC a thin layer of the stationary phase is formed on a suitable flat-surface such as glass, foil or plastic plate. Since the layer is thin, the movement of the mobile phase across the layer generally by simple capillary action, is

rapid, there being little resistance to flow. As the mobile phase moves across the layer from one edge to the opposite, it transfers any analytes placed on the layer at a rate determined by their distribution coefficients between the stationary and mobile phases. The movement of the analyte is expressed by its retardation factor.

$$R_f = \frac{\text{Distance moved by analyte from origin}}{\text{Distance moved by solvent from origin}}$$

The crude acetone extract (5 μL) was loaded with using micropipettes with a suitable solvent system i.e Chloroform-ethyl acetate (1:1) v/v. Separations were carried out at room temperature

Selection of suitable solvent system for fractionating Acetone Extract

Different combinations of solvents with different polarities were tried to find out the maximum differential separation. It was found that Ethyl acetae: Acetone (1:1) provided good separation of compounds and therefore different gradations of Ethyl acetate: Acetone starting from 9 Ethyl acetate and 1 Acetone to 1 Ethyl acetate : 9 acetone could be used to separate the extract into components. The second set of chromatographic separation was conducted by solvent system Ethyl acetate: chloroform in the gradation of increase in polarity.

Visualisation of substances on chromatogram

15% Con. H_2SO_4 in ethanol was taken as the visualising agent in this experiment. The reagent was applied as a fine spray in order to distribute it uniformly over the whole layer. Optimum colour development was attained by heating the plate after spraying 15% Con. H_2SO_4 ethanol in hot air oven. Presence of alkaloid, flavonoids, phenols and sterols were also evaluated by procedure described earlier.

Column chromatographic separation

Column chromatography is conducted by applying the material to be tested to one end of a column in which the adsorptive material (Silica gel mesh size 60 to 120) is packed as uniformly as possible and as the material moves, the various constituents adhere to the surface of the solid particles at different distances from the starting point in accordance with their chemical characteristics.

The crude ethanol extract (1 mL) was transferred to a column of silica gel (70g). The column was eluted successively with Ethyl acetate and Acetone (increasing polarity).

Volume of 25 mL was collected and concentrated. The collected volumes were monitored by TLC and divided into different groups The active-fraction contains two spots. This was separated again by using solvent system, Ethyl acetate: chloroform (in the gradation of increasing polarity) and finally obtained a single active flavonoid Fraction.

Preparation of the stock solution of Active Principle

Dissolved 10 mg of pure 'X' in 10 ml of a solution containing 9.0 mL sterile water and 1.0 mL dimethyl sulphoxide (DMSO). Therefore the concentration of the stock solution was 1mg mL^{-1}. (Jones et al ; 1985)

Experimental procedure

Arrange five empty tubes labelled 1 to 10 and the stock solution was serially diluted with Nutrient broth (1g peptone, 500mg NaCl dissolved in 100ml distilled water) to obtain solutions having concentration ranging from 500µgmL^{-1} to 1.96µgmL^{-1}. Pipette out 1ml of nutrient broth to each of the test tubes, plugged and sterilised. With a sterile micropipette pipette out 1 ml of the stock solution to 1ml of nutrient broth in test tube and shake well. Transfer 1ml into the tube 2 and subsequently in rest of the tubes except the last one that did not contain any stock solution. Inoculate each tube with a loop full inoculum of bacteria and incubate them at 37^{o}C for 48 hrs to observe any visible growth. A tube with 10%DMSO was also set up as control. The test was done in duplicate.

RESULTS AND DISCUSSION

Ethanolic extract of *Acrostichum aureum* showed maximum activity against *Pseudomonas aeruginosa*, gram-negative bacteria. While the acetone and methanolic extracts of *Acrostichum* showed moderate level of inhibition towards *Escherichia coli*, gram-negative bacteria. The plant showed lower level of inhibition towards *Serratia marcescens* when compared to the other bacterial strains. None of the water extracts showed any antibacterial activity. *Pseudomonas aeruginosa* and *staphylococcus aureus* are the most sensitive organisms. The plants did not show any antibacterial activity against M*icrococcus lutens*. No control discs exhibited antibacterial activity.

Table 1: Antibacterial activities of *Acrostichum aureum*

Name of plant Extract used		Zone diameter (in millimetre)				
		Pseudomonas aeruginosa (MTCC-741)	*Staphylococcus aureus* (MTCC-96)	*Escherichia coli* (MTCC-443)	*Micrococcus luteus* (MTCC-6164)	*Serratia marcesens* (MTCC-97)
Acrostichum aureum	Petroleum ether	-	-	-	-	-
	Acetone	-	-	10 ± 0.12	-	7.6 ± 0.58
	Ethanol	12.3 ± 0.23	9.7 ± 0.48	10.6 ± 0.14	-	7 ± 0.32
	Water	-	-		-	-

Value = Mean ± Standard deviation; No inhibition; control dics no inhibition

Table 2: Results of Phytochemical evaluation of *Acrostichum aureum*

Name of plant	Plant extracts	Test for Flavanoids	Test For Alkaloids	Test for Phenols	Test for Sterols, steroid, phenol and poly phenol
Acrostichum aureum	Petroleum ether	+	-	+	+
	Acetone	+	-	+	+
	Ethanol	+	-	+	-
	Water	+	-	+	-

Value = '+' : Present '–' : Absent

Table 3: Antibacterial activities of standard antibiotics

Name of Antibiotic (Con. 25µg/Disc)	Zone diameter (in millimetre)		
	Micrococcus luteus (MTCC-6164)	*Staphylococcus aureus* (MTCC-96)	*Pseudomonas aeruginosa* (MTCC-741)
Streptomycin	25 ± 0.23	18 ± 0.56	19 ± 0.42
Amoxylin	35 ± 0.48	31 ± 0.52	-
Chloramphenicol	-	25 ± 0.49	-

The phytochemical evaluation of *Acrostichum* is shown in the Table 2. The presence of Flavanoids and phenols observed as general feature the plant extracts. Test for sterols,

steroids, phenol and poly phenols exhibited positive result in all cases except the methanolic and water extracts of *Acrostichum aureum*. The plant extracts showed negative results with alkaloids. Flavonoid and phenol content observed in methanol extract of the plant; it might be one of the reasons for its antibacterial activity. Table 3 shows the results of antibacterial assays of pathogenic organisms towards standard antibiotics. Ethanolic extract *Acrostichum aureum* showed stronger inhibition towards *Pseudomonas aeruginosa* when compared to standard antibiotics like amoxylin and chloramphenicol. Antibacterial activity of ethanol extract towards *Pseudomonas aeruginosa* is comparable with that of streptomycin.

Table 4: Antibacterial activity of gradient extracts

Name of the plant and Family	Part used	Extract used	Disc diffusion method **Bacterial species**												
			S.al	S.au	M.lu	S.ma	S.ty	S.pt	C.fr	K.pn	S.so	S.dy	E.co	P.ae	B.sub
Acrostichum aureum Pteridaceae	Stem & Fronds	PE	-	-	-	-	-	-	-	-	-	-	-	-	-
		AE	-	++	-	-	-	+	-	-	+	-	-	+	+
		EtOH	-	+	+	-	-	-	+	-	-	-	-	-	-
		WA	-	-	-	-	-	-	-	-	-	-	-	-	-

Value= no obvious growth inhibition (-); zone of inhibition with diameter 7mm-10.99mm (+); 11mm-14.99mm as ++

Abbreviations: PE - Petroleum ether Extract; AE - Acetone Extract; EtOH - Ethanol Extract WE - Water Extract.

S.al - *Staphylococcus albus*; S.au - *Staphylococcus aureus*; M.lu – *Micrococcus luteus*; S.ma - *Serratia marcescens*; S.ty - *Salmonella typhi*; S.pt - *Salmonella paratyphi*; C.fe - *Citrobacter freundii*; K.pn - *Klebsiella pneumoniae*; S.so - *Shigella sonnei*; S.dy - *Shigella dysentriae*; E. co - *Escherichia coli*; P.ae - *Pseudomonas aeruginosa*; B.sub - *Bacillus subtilis*

The present investigation clarified the antibacterial property of the leaves towards one of the clinically important multi-drug resistant stains, *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* is often encountered in nosocomial infections and its infection is common in patients receiving treatment of severe burns or other traumatic skin damage and in people suffering from cystic fibrosis. This pathogen colonises the lungs of patients and increasing mortality rate of individuals with the disease (Madigan et al., 2000) . The present antibacterial analysis of the plant confirms the ethnobotanical importance of and *Acrostichum aureum* by Chopra et al., (1992) The study proves that *Acrostichum aureum*

possesses antibacterial principles, soluble in methanol, which hinder the growth and multiplication of some multidrug resistant bacterial strains. In view of the analysis, the leaves can be recommended as source for isolating and characterizing new antibacterial drugs for modern medicine. Further investigations are necessary to isolate and purify antibacterial principles from active acetone extract of the plant and may be later used as a potential phytomedicine instead of synthetic antibiotics especially towards multidrug resistant pathogenic bacterial species.

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