IJPSR (2012), Vol. 3, Issue 03 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 15 November, 2011; received in revised form 14 December, 2011; accepted 23 February, 2012

ANTIBACTERIAL SCREENING OF A FEW MEDICINAL FERNS AGAINST ANTIBIOTIC RESISTANT PHYTO PATHOGEN

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Keywords:
Biocontrol,
Phytochemical analysis,
Antibacterial activity,
medicinal ferns

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ABSTRACT

Centella asiatica is a medicinal plant. It is severely affected by leaf spot disease caused by a harmful, antibiotic resistant phyto pathogen *Xanthomonas campestris* pv. *centellae* (*X. c.* pv. *c*). Farmers used synthetic pesticides to control the disease. When pesticides are applied to control this pathogen, pesticides may cause environmental hazards and health problems in man. But traditionally plants are used to prevent or control the plant diseases in agriculture. It is an eco- friendly, biocontrol, traditional method. Hence the aim of the present study is to control the pathogen in biocontrol method by screening the phytochemicals and antibacterial activity of petroleum ether, chloroform, benzene, methanol and aqueous extracts of fronds of five ferns (Pteridophytes) *Adiantum caudatum, Angiopteris evecta, Pteris confusa, Pteris argyraea* and *Lygodium microphyllum* against *X. c.* pv. *c*. The methanol extracts of all the ferns gave successful result against the tested bacteria. Phytochemical analysis of all the extracts revealed that antibacterial activity is due to the presence of alkaloids, flavonoids and phenolic compounds. According to the results of MIC (Minimum Inhibitory Concentration) and RPI (Relative Percentage Inhibition) values, *Angiopteris evecta* could be used as potential plant for the management of pathogenic bacteria, *X. c.* pv. *c* which is known to cause diseases on many vegetables and cash crops particularly *Centella asiatica*.

INTRODUCTION: A bacterial leaf spot disease caused by *Xanthomonas campestris* pv. *centellae* (*X. c.* pv. *c*) has been the major problem in cultivation of *Centella asiatica*. To control the disease causing pathogens, number of synthetic pesticides and antibiotics are used by the formers. But pesticides cause environmental pollution and many unwanted effects in man.

Pesticides have made great contribution for quick and effective management of plant diseases and microbial contaminations in several agricultural commodities. Considering the deleterious effects of synthetic pesticides on life supporting system, there is an urgent need for alternative agents for the management of pathogenic microorganisms [1].

Pteridophytes are not infected by microbial pathogens, which may be one of the important factors for the evolutionary success of pteridophytes and the fact that they survived for more than 350 million years [2].

Considering the rich diversity of Indian medicinal plants including Pteridophytes, it is expected that, the screening of plant extract for antibacterial activity may be beneficial for humans and plants diseases. The synergistic interaction among crude extracts or the active compounds may be useful in the preparation of

improved herbal or drug formulations. The extensive survey of antibiotic activity among the ferns conducted and reported about a hundred species having such property [3]. Traditionally people used pteridophytes as medicine and anti bacterial agents. Therefore the aim of the present study is to investigate the phytochemical analysis and *in vitro* antibacterial activity of five solvent extracts of five ferns against gram-negative plant pathogenic bacteria.

MATERIALS AND METHODS:

Preparation of Plant Extracts: Healthy, disease free leaves of five ferns *Adiantum caudatum, Angiopteris evecta, Pteris confusa, Pteris argyraea* and *Lygodium microphyllum* (**Table 1**) were collected from Kothayar and their identification was confirmed with the help of herbarium specimens in XCH (Xavier's College Herbarium), St. Xavier's college, Palayamkottai. These samples were used for the preparation of aqueous and different solvent extracts. Thoroughly washed leaves were shade dried and then powdered with the help of a blender. 25 g of the powder was extracted successively with petroleum ether, benzene, chloroform, methanol and distilled water using a Soxhlet extractor for 48 h. All the extracts were concentrated and preserved in airtight bottle until further use.

TABLE 1: MEDICINAL FERNS SELECTED FOR ANTIBACTERIAL ACTIVITY

Botanical name	Family	Common name	Parts screened	Medicinal uses
Adiantum caudatum L.	Adiantaceae	Walking maiden hair	Fronds	Wound healing
Pteris argyraea T. Moore	Pteridaceae	Silver brake fern	Leaves	Piles
Pteris confusa T.G. Walker	Pteridaceae	Rock brake fern	Leaves	Boils healing
Angiopteris evecta (Forst) Hoff.	Angiopteridaceae	King fern	Rhizomes	leprosy treatment
Lygodium microphyllum (Cav) R.Br.	Lygodiaceae	Snake fern	Leaves	skin diseases

Phytochemical analysis: Phytochemical analysis of all the extracts of the selected ferns was conducted following the standard procedures [4].

Antimicrobial Assay:

Bacterial Strain: The culture of *Xanthomonas campestris* pv. *centellae* isolated from diseased *Centella* plant was maintained in nutrient agar slant at $4^{o}C$.

Antibacterial Activity: The antibacterial activity of five solvent extracts of five medicinal ferns was tested in disc diffusion method [5]. Muller Hinton agar medium was seeded with 100µl of inoculum (1×10^{8} CFU/ml). The impregnated discs containing the test sample (20µg/ml, 40µg/ml and 80µg/ml) were placed on the agar medium seeded with tested microorganisms. Standard antibiotic discs (Kanamycin 30µg/disc, Neomycin 10µg/disc) and blank discs (impregnated with solvent) were used as positive and negative control. The plates were then incubated at 37 ^{0}C for 24 h to allow maximum growth of the microorganisms[5]. The antibacterial activity of the test samples was determined by measuring the diameter of zone of inhibition expressed in millimeter. Experiments were done in triplicate and repeated twice and mean of the three experiments was recorded.

Determination of Minimum Inhibitory Concentration (MIC): The Minimum Inhibitory Concentration (MIC) of the benzene and methanol and benzene extracts of *P. confusa, A. evecta, P. argyraea, A. caudatum* and *L. microphyllum* were determined by using serial dilution technique [6]. 1 mg/ml of the sample solutions of all the extracts were prepared using Dimethyl Sulfoxide (DMSO).

In this technique a large number of test tubes were used and each of the test tubes was filled with 1 ml of sterile nutrient broth media and graded doses of sample solution were added. Then, these test tubes were inoculated with the selected organisms (inoculum contains 1×10^{6} cells/ml) followed by incubation at $37^{o}C$ for 24 hours to allow the growth of the bacteria. The test tubes which showed minimum concentration as well as clear content were selected. This lowest or minimum concentration was considered as Minimum Inhibitory Concentration (MIC). Another three test tubes containing medium, medium and sample, medium and inoculum were used as control. Bacterial growth observed was only in test tubes (solution content was cloudy) containing medium and inoculum and the other two were clear showing no growth [6]. Experiments were done in triplicate and repeated twice.

Sensitivity Tests: Commercially available discs of standard antibiotics (Kanamycin 30µg/disc, Neomycin 10µg/disc, amoxicillin-25µg, chloramphenicol-30µg, and penicillin 5µg) were used to determine antibiotic sensitivity profile of multi antibiotic resistance of *X. c.* pv. *c* by the disc diffusion method. Sensitivity and resistant were evaluated by measuring the inhibition zone diameters.

Relative Percentage Inhibition (RPI): The relative percentage inhibition of the test extracts with respects to positive controls (kanamycin and neomycin) was calculated by using the following formula [7, 8];

Relative percentage inhibition of the test extract =

$$\frac{100\ X\ (x-\ y)}{(z-y)}$$

Where; **x:** total area of inhibition of the test extract, **y:** total area of inhibition of the solvent, **z:** total area of inhibition of the standard drug.

The total area of inhibition was calculated by using area = πr2; where, r = radius of zone of inhibition.

Statistical Analysis: All data were expressed as mean ± SD. Statistical analyses were evaluated by one-way ANOVA followed by Tukey HSD test. Values with P< 0.005 were considered statistically significant. Mean and standard deviation were also calculated using the Microsoft Excel sheet, Office Edition 2007.

RESULTS:

Phytochemical Analysis: The phytochemical analysis revealed the presence of phenolic compounds/tannins, flavonoids, in methanol extracts of the selected two ferns (**Table 2**). Phytochemical analysis of methanol extracts revealed that significant antibacterial activity is due to the presence of phenolic compounds.

TABLE 2: PHYTOCHEMICAL ANALYSIS OF METHANOL EXTRACTS OF SELECTED FERNS

Compounds	*P. confusa*	*A. evecta*	*A. caudatum*	*L. microphyllum*	*P. argyraea*
Steroids	+	+	+	+	+
Triterpinoids	-	-	-	-	-
Reducing sugars	-	+	+	+	+
Sugars	-	+	-	-	-
Alkaloids	+	+	+	-	+
Phenolic compounds	+	+	+	+	-
Flavonoids	+	+	+	+	+
Catechins	-	-	-	-	-
Saponins	-	+	-	-	-
Tannins	-	-	-	-	-
Anthroquinones	+	+	+	-	+
Amino acids	-	-	-	-	-

(+): Present (-): Absent

Antibacterial Activity: To control the isolated harmful phyto pathogen in biocontrol method, petroleum ether, benzene, chloroform, methanol and aqueous extracts of five different medicinal ferns were evaluated for their antibacterial activity against the isolated harmful pathogen in disc diffusion method in three different concentrations (20µg/ml, 40µg/ml and 80µg/ml). All the extracts showed marked inhibitory effects against the isolated bacteria (**Table 3**).

TABLE 3: ANTIBACTERIAL ACTIVITY OF FIVE SOLVENT EXTRACTS OF SELECTED FERNS AGAINST *XANTHOMONAS CAMPESTRIS* PV.CENTELLAE IN THREE CONCENTRATIONS

NS	Petroleum ether (µg/ml)			Benzene (µg/ml)			Chloroform (µg/ml)			Methanol (µg/ml)			Aqueous (µg/ml)		
	20	40	80	20	40	80	20	40	80	20	40	80	20	40	80
Ac	10.6±0.9	13.6±1.2	13.3 ±1.2	13.0±0.8	23.1±1.7	18.0±1.6	10.0±1.2	11.2±1.2	11.0±0.8	21.0±0.8	24.0±0.8	23.0±1.4	5.0 ±1.4	7.0±1.7	6.0±1.7
Pa	13.0±1.4	15.6±0.4	25.0±0.8	26.6±1.2	27.3±1.2	05.6±0.4	18.6±0.4	19.6±1.6	16.0±0.4	15.6±0.4	18.3±1.2	18.0±0.8	7.3±1.7	8.0±0.8	9.3±0.4
Pc	11.3±0.4	15.3±0.4	13.0±1.4	17.6±0.6	18.6±0.4	13.3±0.6	14.6±0.9	16.6±0.4	12.0±1.4	18.0±0.8	23.3±1.2	24.6±0.4	6.0±0.4	7.0±1.7	7.0±0.7
Ae	06.3±0.4	10.0±0.6	07.3±1.4	13.6±1.2	28.0±1.6	26.3±1.8	15.0±0.8	16.6±1.4	10.3±0.2	20.0±1.6	32.6±0.6	29.0±1.2	6.0±0.7	7.0±0.8	7.3±1.6
Lm	12.3±0.4	13.3±1.2	15.0±0.8	20.0±1.6	18.5±0.4	16.6±0.4	08.4±0.4	09.3±0.8	06.9±0.4	15.6±0.4	18.6±1.2	18.4±0.8	4.2±0.7	5.2±0.4	4.3±0.9

Data given are mean of three replicates ± standard error, p < 0.005. NS-Name of the samples, *Ac- Adiantum caudatum* L., *Pa- Pteris argyraea* T. Moore, *Pc- Pteris confusa* T.G. Walker *Ae- Angiopteris evecta* (Forst) Hoff., *Lm- Lygodium microphyllum* (Cav) R.Br.

Among these, the benzene and methanol extracts of *P. argyraea, P. confusa, A. caudatum, L. microphyllum* and *A. evecta* showed significant inhibitory effect compared with other solvent extracts in 40µg/ml and 80µg/ml concentration. The test pathogen was more susceptible to the methanol extract of each plant material than the other four extracts. The highest susceptibility was recorded with the methanol and benzene extract of *A. evecta*, followed by the extracts of *P. argyraea A. caudatum* and *P. confusa* and the least, being recorded with the aqueous extracts of *L. microphyllum.* The petroleum ether and chloroform extracts of all the samples showed marked inhibitory effects. The aqueous extracts of all the selected samples also exhibit some inhibitory effects on the isolated bacteria.

The ANOVA analysis of the data revealed that among the five samples methanol extracts. *A. evecta* ($p<0.005$) showed highly significant activity against the tested pathogen (Table 3). Tukey HSD analysis of the data revealed that *X. c.* pv. *c* was highly susceptible to methanol extracts compared with other extracts. Antibacterial activity of methanol and benzene extract of *A. evecta* was highly significant ($p<0.005$) compared to Kanamycin and Neomycin (**Table 4**).

TABLE 4: ZONE OF INHIBITION IN POSITIVE AND NEGATIVE CONTROLS

Antibiotics	Type of control	Inhibition zone
Kanamycin(30µg/ml)	Positive	15.70±0.85
Neomycin (10µg/ml)	Positive	16.23±0.47
Petroleum ether(Blank)	Negative	0.00±0.00
Benzene (Blank)	Negative	0.00±0.00
Chloroform (Blank)	Negative	0.00±0.00
Methanol (Blank)	Negative	0.00±0.00
Aqueous (Blank)	Negative	0.00±0.00

Minimum Inhibitory Concentration (MIC): The MIC value of methanol and benzene extracts *A. evecta* was 8µg/ml and 16µg/ml against *X. c.* pv. *c* respectively. Similarly the MIC value of methanol and benzene extracts of *P. confusa,* and *P. argyraea* was 64µg/ml, 128µg/ml against *X. c.* pv. *c* respectively. The highest MIC value was found in the benzene extracts of *A. caudatum, L. microphyllum* i.e. 128µg/ml, 256µg/ml, and 256µg/ml respectively. Hence it is concluded that the methanol extracts of *A. evecta* showed inhibition of bacterial growth even at low concentrations (**Table 5**).

Among these five samples, the MIC value of *A. evecta* is the lowest against *X. c.* pv. *c*. Hence *A. evecta* shows significant ($p<0.005$) bactericidal activity compared to other samples. According to the results of antibacterial assay, the methanol extracts of *A. evecta* might be used as antibacterial agent against *X. c.* pv. *c* which severely affect plants.

TABLE 5: MINIMUM INHIBITORY CONCENTRATION OF THE FERNS EXTRACT AGAINST *X. C.* PV. *C*

Name of the samples	Methanol (µg/ml)	Benzene(µg/ml)
A. evecta	8.00±0.00	16.00±0.00
P. confusa	64.00±0.00	128.00±0.00
P. argyraea	64.00±0.00	128.00±0.00
A. caudatum	128.00±0.00	256.00±0.00
L. microphyllum	128.00±0.00	256.00±0.00

Relative Percentage Inhibition (RPI): The results of antimicrobial activity of methanol and benzene extracts of ferns (40µg/ml) were compared with the positive control (kanamycin and neomycin) for evaluating their relative percentage inhibition (**Table 6**). For kanamycin the methanol extract of *A. evecta* exhibits maximum relative percentage inhibition against the test inoculum (207.64%) followed by *P. argyraea* (167.51%) and *A. caudatum* (152.86%) respectively. For neomycin the methanol extract of *A. evecta* exhibits maximum relative percentage inhibition against the test inoculum (200.86%) followed by *P. argyraea* (162.04%) and *A. caudatum* (147.87%) respectively.

TABLE 6: RELATIVE PERCENTAGE INHIBITION OF METHANOL AND BENZENE EXTRACTS OF FERNS COMPARED TO STANDARD ANTIBIOTIC - KANAMYCIN AND NEOMYCIN

Samples	Kanamycin		Neomycin	
	Methanol	Benzene	Methanol	Benzene
A. caudatum	152.86	147.13	147.87	142.32
P. argyraea	167.51	173.88	162.04	168.20
P. confusa	148.40	118.47	143.56	114.60
A. evecta	207.64	178.34	200.86	172.52
L. microphyllum	118.47	117.83	114.60	113.98

Sensitivity of the Bacteria Standard Antibiotics: Table 7 reveals the antibacterial effectiveness of the plant materials as was compared with the choicest commercially prepared antibiotics for *Xanthomonas* infection.

TABLE 7: SENSITIVITY OF *X.C.PV.C* TO STANDARD ANTIBIOTICS

Antibiotics	Sensitivity	Inhibition zone (mm)
Kanamycin 30µg/disc,	Susceptible	15.70±0.85
Neomycin 10µg/disc	Susceptible	16.23±0.47
Amoxicillin-25µg/disc	Resistant	-
Chloramphenicol-30µg/disc	Resistant	-
Penicillin 5µg/disc	Resistant	-

DISCUSSION: Very less work has been done on the antimicrobial activity of pteridophytes, yet ethanobotanical importance of these plants have been investigated and studied by various authors. They have been found for their biological activity [9]. The phytochemical composition of *Adiantum radiata* has been studied and found that the isolated phytochmicals were effective against the growth of microorganisms [10]. Antibiotic activity of pteridophytes has been studied by [3], while the antiviral activity of crude extracts of some pteridophytes have also been analyzed by [11]. The antibacterial activity of *Adiantum capillus-veneris* was also been studied and found that nearly all the extracts were effective against the selected microorganisms [12, 13].

In general, gram-negative bacteria were more resistant to antibiotics than gram- positive bacteria [14, 15]. The resistance is due to the differences in their cell wall composition. In gram-negative bacteria the outer membrane acts as a barrier to many environmental substances including antibiotics [16]. Presence of thick murine layer in the cell wall prevents the entry of the inhibitors [17]. But in the present study gram-negative bacteria were more susceptible to the crude extracts than gram-positive bacteria. It may be due to the presence of broad spectrum of antibiotic compounds present in the selected ferns.

An important characteristic of plant extracts and their components is their hydrophobicity, which enable them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable. Extensive leakage from bacterial cells or the exit of critical molecules and ions will lead to death [18]. Hence, the active compounds which are present in ferns inhibit the tested antibiotic resistant gram negative bacteria by using the above mechanism. The results of the MIC showed that the plant materials are very potent against the test pathogen even at very low concentration.

The result of RPI also indicates the selected ferns act as significant antimicrobial agents compared with standard antibiotics like Kanamycin and Neomycin.

CONCLUSIONS: This study has confirmed the antibacterial potentials of ferns, thus supporting their application as a biocontrol herbal remedy for *Xanthomonas* infection in plants. With these, there is need for the preparation of different formulations towards ensuring acceptable dosing to field trials. It is hoped that this study would lead to the establishment of some compounds that could be used to formulate new and more potent antimicrobial agents of natural origin for the treatment of *Xanthomonas* infection in plants particularly causing leaf spot disease in *Centella asiatica.*

ACKNOWLEDGEMENT: The authors are grateful to the Council of Scientific and Industrial Research (CSIR), New Delhi for financial support (Ref. No: 38(1260)/10/EMR-II 17/05/2010).

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