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Research Article

Biological Control of Leaf Spot Disease by a Few South Indian Medicinal Ferns

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Abstract: **Background:** Leaf spot is a common disease in agriculture which results in severe yield loss. This harmful disease is caused by a phyto-pathogenic bacteria namely *Xanthomonas campestris* which is a multi-antibiotic resistant bacterium. Many vegetable and cash crops are severely affected by the leaf spot disease which is caused by *X. campestris*. These bacteria have acquired resistance to many synthetic pesticides. Pathovars of *Xanthomonas* are reported to have developed resistance to kanamycin, ampicillin, penicillin and streptomycin. Considering the resistant potency of *X. campestris*, there is an urgent need for alternative agents for the management of this pathogenic microorganism. Pteridophytes (ferns) are one of the oldest land plant groups on earth and constitute a vast group of vascular cryptograms. Ferns are also show medicinal utility and many of them have been used medicinally from ancient time. The rich diversity of Indian medicinal ferns has been evaluated for their antimicrobial properties, and this may prove beneficial for mankind. All the parts including rhizome, stem, fronds, pinnae and spores contain antimicrobial and medicinal potency. Hence, in the current study we report the antibacterial activity of ten south Indian medicinal ferns towards *X. campestris.* **Methods:** *X. campestris* was isolated from the infected plant leaves of agricultural fields in Tirunelveli, Tamil Nadu, India. The isolation was performed by serial dilution and plating technique on mTBM medium. The isolate was identified on the basis of its morphology and biochemical properties. The isolate was checked for the drug susceptibility by disc diffusion method. **Results:** *X. campestris* was found sensitive toward kanamycin (15.70 ± 0.85) and neomycin (16.23 ± 0.47) but resistant to amoxicillin, chloramphenicol, and penicillin. The antibacterial activity of five solvent extracts of ten medicinal ferns collected from the Western Ghats of south India was checked by agar disc diffusion method on MH agar medium. The methanol extracts of all the ferns displayed antibacterial activity against the tested bacteria. Phytochemical analysis of all the extracts revealed that the antibacterial activity may be due to the presence of alkaloids, flavonoids and phenolic compounds. **Conclusions:** According to the results of MIC (Minimum Inhibitory Concentration) and RPI (Relative Percentage Inhibition) values, ferns extracts could be used as bio control agents for the management of pathogenic bacteria *X. campestris*.

KEYWORDS: Leaf spot disease, *Xanthomonas campestris*, biocontrol, medicinal ferns.

INTRODUCTION

Xanthomonas campestris is a very important kind of phytopathogenic bacteria which causes plant diseases all around the world. Pathovars of *Xanthomonas* are known to cause diseases on several vegetable and cash crops. Among the Pathovars *Xanthomonas campestris* is particularly serious. The hosts of this genus include at least 124 monocotyledonous and 268 dicotyledonous plants, among which the rice bacterial blight, cabbage black rot disease, and citrus blight disease are the most serious diseases, causing a big economic impact on agricultural production every year. Many vegetable and cash crops are severely affected by the leaf spot disease which is caused by *X. campestris.* These bacteria have acquired resistance to synthetic pesticides. Pathovars of *Xanthomonas* are reported to have developed resistance to kanamycin, ampicillin, penicillin and streptomycin.[1] To control the disease causing pathogens, a number of synthetic pesticides and antibiotics are used by the farmers. Pesticides have made great contribution for

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quick and effective management of plant diseases and microbial contaminations in several agricultural commodities. However, pesticides cause environmental pollution and many unwanted effects in man. Incessant and extensive use of these synthetic pesticides are posing serious problem to the life supporting systems due to their residual toxicity.[2] 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.[3]

A green plant represents a reservoir of effective chem other apeutants and can provide valuable sources of natural pesticides.[4, 5, 6] Pteridophytes are one of the oldest land plant groups on earth and constitute a vast group of vascular cryptograms. Pteridophytes have also shown medicinal utility and many of them have been and continue to be used medicinally from ancient time.[7] Traditionally people used pteridophytes as medicine and anti-bacterial agents. Tribal communities throughout the world utilize plant parts such as the rhizome, stem, fronds, pinnae and spores in various ways for the treatment of various ailments since ancient time. Phytotherapy studies about Pteridophytes have been published from time to time, although not enough attention has been paid towards their medicinally useful aspects.[8] 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 individual active compounds may be useful in the preparation of improved herbal or drug formulations. Very little work has been published on the phytochemical and antimicrobial activity of pteridophytes.[9] More recently, modern biological and pharmaceutical studies were carried out on pteridophytes by different workers.[10, 11, 12, 13, 14]

Hence, the present study was carried out to investigate the phytochemical analysis and *in vitro* antibacterial activity of five solvent extracts of ten south Indian medicinal ferns against *X. campestris* which was isolated from diseased plants.

MATERIALS AND METHODS

Isolation of *Xanthomonas campestris*

Sample collection

The infected leaves showing the typical symptoms of bacterial spot were collected from farmer's fields in Tirunelveli and Tuticorin districts in Tamilnadu. The collected samples were transported to Plant Molecular Biology Research Unit (PMBRU) in fresh condition in plastic bags and stored at 4°C for 24 h for further analysis. All the leaves showed typical external symptoms like yellowish brown spots, decolouration, minute water soaked lesions, cankers lesions and irregular yellow and brown patches.

Bioassay and collection of fern plant materials

Healthy, disease free leaves of ten ferns *Adiantum lunulatum, Adiantum capillus – veneris Pteris otaria, Pteris aspericaulis, Pteris kleiniana, Pteris confusa, Pteris multiaurita, Pteris vittata, Asplenium polyodon* and *Hypodematium crenatum* 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.

Methods – isolation of *Xanthomonas campestris*

The bacterium was isolated by extracting the ooze in sterile distilled water in test tubes, followed by dilution plate technique on nutrient agar and on two semi- selective media, mTBM and mMD5A. The isolated colonies were purified on YDCA medium.

Identification of causal organism

The morphological characteristics such as cell shape, Gram reaction, capsule and spore staining characters of the isolate was studied as described by Society of American Bacteriologists.[15, 16] Pathogenicity tests were also carried out to identify the bacteria.

Preparation of solvent extracts

25 g of shade dried, powdered plant materials were extracted successively with 150 ml each of petroleum ether, benzene, chloroform, methanol and distilled water for 48 h using a Soxhlet extractor. All the extracts were concentrated using a rotary flash evaporator. After complete solvent evaporation, each of these solvent extracts was weighed and preserved at 4°C in airtight bottles until further use.

Phytochemical analysis

Phytochemical analysis of methanol extracts of the selected plants was conducted following the standard procedure.[17]

Antimicrobial assay

Bacterial strain

The culture of *Xanthomonas campestris* isolated from diseased plant was maintained in nutrient agar slant at 4°C.

Antibacterial activity

The antibacterial activity of five solvent extracts of the selected fern was tested in disc diffusion method.[18]

Sensitivity tests

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

Determination of Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) of the benzene, chloroform and methanol extracts of *P. vittata* was determined by using serial dilution technique.[19]

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 standard formula.[20, 21]

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

Isolation of *Xanthomonas campestris*

Yellow, slimy, glistening and round colonies were isolated on nutrient agar and the two selective media, mTBM and mMD5A. In purification, the bacterial colonies on YDCA medium were deep yellow, slimy, highly viscous and irregular to round in shape.

Identification of causal organism

The results of the various morphological, physiological and biochemical tests are given in Table 1. The isolated bacterium was compared with the original culture of *X. campestris* (reference strain MTCC 2286) by studying the type of staining morphology of the colonies and biochemical characters and was found to be consistent with the reference strain. Pathogenicity testing also gave positive results.

Phytochemical analysis

The preliminary phytochemical analysis of the methanol extracts of the ferns showed the presence of steroids, triterpinoids, reducing sugars, sugars, alkaloids, phenolic compounds, flavonoids and tannins (Table 2).

Table 1: The morphological and biochemical characteristics of isolated *Xanthomonas campestris* compared with the reference strain (MTCC 2286)

Characters	Isolated strain	Reference strain
I. Morphology:		
a. shape	Small rods	Small rods
b. Occurrence	In single	In single
c. Flagellation	Monotrichous	Monotrichous
II. staining		
a. Gram reaction	G $^{-}$ve	G $^{-}$ve
b. Capsule staining	+	+
c. Spore staining	–	–
d. Acid fast	–	–
III. Biochemical characters		
a. Utilization of glucose, sucrose, fructose for acid production	+	+
b. Utilization of Asparagine as sole source of carbon and nitrogen	–	–
c. Catalase reaction	+	+
d. Gelatin liquefaction	+	+
e. Casein hydrolysis	+	+
f. Methyl red reaction	–	–
g. Reduction of nitrate to nitrite	–	–
h. Urease reaction	–	–
i. Indole production	–	–
j. Hydrogen sulphide production	+	+
k. Oxidase reaction	–	–
l. Starch hydrolysis	+	+

Antibacterial activity

The petroleum ether, benzene, chloroform, methanol and aqueous extracts of ten different medicinal ferns were evaluated for their antibacterial activity against the isolated harmful pathogen in disc diffusion method at three different concentrations (20 µg/ml, 40 µg/ml and 80 µg/ml). All the extracts showed marked inhibitory effects against the isolated bacteria (Table 3). Among these, the benzene and methanol extracts of all the selected ferns showed the most significant inhibitory effect (compared with other solvent extracts) at 40 µg/ml and 80 µg/ml concentrations. The test pathogen was more susceptible to the methanol and benzene extract of each plant material than all other extracts. The highest susceptibility was recorded with the methanol and benzene extract of *P. vittata,* followed by the extracts of *A. lunulatum, A. capillus – veneris, P. confusa* and the least, being recorded with the aqueous extracts of *P. otaria.* The petroleum ether and chloroform extracts of all the species also showed marked inhibitory effects. The aqueous extracts

Table 2: Preliminary phytochemical tests for methanol extracts of selected medicinal ferns

Compounds	1	2	3	4	5	6	7	8	9	10
Steroids	+	+	–	+	+	+	+	+	+	+
Triterpinoids	+	–	–	–	–	–	–	+	–	–
Reducing sugars	–	–	+	–	–	–	–	–	–	–
Sugars	–	–	–	–	–	–	–	–	–	–
Alkaloids	+	+	–	+	+	+	+	+	+	+
Phenolic compounds	+	+	+	+	+	–	+	+	+	+
Flavonoids	+	+	–	+	+	+	+	+	+	+
Catechins	–	–	–	–	–	–	–	–	–	–
Saponins	+	+	–	–	–	+	–	+	–	–
Tannins	+	–	–	–	–	–	–	+	–	–
Anthroquinones	–	–	–	–	–	–	–	–	–	–
Amino acids	–	–	–	–	–	–	–	+	–	–

1. A. lunulatum 2. A. capillus–veneris 3. P. otaria, 4. P. aspericaulis, 5. P. kleiniana, 6. P. confusa, 7. P. multiaurita, 8. P. vittata, 9. A. polyodon 10. H. crenatum.

of all the selected samples also exhibit some inhibitory effects on the isolated bacteria, albeit with lower efficacy

The ANOVA analysis of the data revealed that among the ten samples, the methanol extracts of *P. vittata* ($p<0.05$) showed highly significant activity against the tested pathogen (Table 3). Tukey HSD analysis of the data revealed that *X. campestris* was highly susceptible to methanol extracts compared with other extracts. Antibacterial activity of methanol and benzene extract of *P. vittata* was highly significant ($p<0.05$) compared to Kanamycin and Neomycin (Table 4).

Minimum Inhibitory Concentration (MIC)

The MIC value of methanol and benzene extracts of *P. vittata* was 8 µg/ml and 16 µg/ml against *X. campestris* respectively. Likewise the MIC value of methanol and benzene extracts of *A. lunulatum* was 16 µg/ml and 32µg/ml. Similarly the MIC value of methanol and benzene extracts of *A. capillus – veneris* and *P. confusa* was 64 µg/ml, 128 µg/ml against *X. campestris* respectively. The highest MIC value was found in the benzene extracts of *P. otaria* i.e. 256 µg/ml. Hence it is concluded that the methanol extracts of *P. vittata* showed inhibition of bacterial growth even at low concentrations (Table 5). Among these five samples, the MIC value of *P. vittata* is the lowest against *X. campestris*. Hence *P. vittata* shows significant ($p<0.05$) bactericidal activity compared to other samples. According to the results of antibacterial assay, the methanol extracts of *P. vittata* might be used as antibacterial agent against *X. campestris*.

Relative Percentage Inhibition (RPI)

The results of antimicrobial activity of methanol and benzene extracts of ferns (40 µg/ml) were compared

Table 3: Antibacterial activity of five solvent extracts of selected ferns against *Xanthomonas campestris* 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
Al	10.3±0.4	11.3±0.7	10.0±0.8	13.6±1.2	25.0±1.2	15.3±0.4	15.0±1.4	15.3±0.4	10.6±0.9	23.3±1.2	26.3±0.4	28.3±0.4	8.0±1.4	9.6±0.9	4.0±0.7
Av	11.3±0.9	15.6±0.4	12.3±0.8	13.6±1.2	18.6±0.4	16.0±0.4	07.6±0.9	16.3±1.8	14.0±0.8	15.6±0.4	18.3±1.2	18.0±0.8	4.3±1.2	6.3±0.9	5.3±0.4
Pc	06.3±0.4	08.3±0.4	07.0±0.8	05.3±0.6	13.0±0.8	07.3±1.2	10.6±0.8	12.3±1.8	11.3±0.8	14.0±0.4	16.3±1.2	15.3±0.4	5.6±1.7	8.0±0.8	6.0±0.4
Po	05.0±1.4	06.0±1.7	07.0±1.7	04.3±1.2	06.3±0.9	05.3±0.4	05.6±1.7	08.0±0.8	06.0±0.4	07.3±1.7	08.0±0.8	09.3±0.4	6.0±0.4	7.0±1.7	7.3±0.7
Ps	07.3±0.4	08.0±0.8	07.4±1.2	12.0±0.7	17.3±1.6	13.6±1.2	05.6±0.4	10.0±0.8	07.3±1.2	07.3±1.2	08.4±0.8	09.3±0.4	8.0±0.4	9.0±0.8	7.0±1.7
Pk	05.3±0.4	06.2±0.4	05.6±0.8	11.3±1.2	18.3±0.4	15.0±0.8	16.0±1.4	10.0±0.2	08.0±0.8	09.0±0.4	12.0±0.3	13.3±0.9	4.3±0.4	5.6±1.4	5.0±0.8
Pm	08.3±0.4	07.0±0.8	06.0±0.4	15.0±0.8	19.0±0.4	18.3±0.4	10.0±0.4	12.3±0.8	08.0±0.4	07.3±0.8	08.3±0.9	06.0±0.4	6.3±0.9	5.3±0.4	4.3±1.2
Pv	14.3±1.7	16.0±0.4	14.0±1.2	23.3±1.2	27.6±0.8	26.0±0.8	13.6±1.2	18.6±0.4	16.0±0.4	25.3±0.8	31.3±0.8	26.6±0.8	5.6±1.7	8.0±0.8	6.0±0.4
Ap	07.3±0.4	08.0±0.8	07.4±1.2	12.0±0.7	17.3±1.6	13.6±1.2	05.6±0.4	09.3±0.4	07.3±1.2	07.3±1.2	10.0±0.8	08.4±0.8	8.0±0.4	9.0±0.8	7.0±1.7
Hc	11.3±0.4	12.0±0.4	11.0±0.8	10.3±0.4	11.3±0.7	10.0±0.8	08.3±0.4	09.0±0.8	06.0±0.4	15.0±0.8	16.0±1.4	10.0±0.2	3.0±0.4	5.0±0.7	7.3±1.7

1. ***Al****-Adiantum lunulatum* Burm. *2.* ***Av****- Adiantum capillus - veneris* L. *3.* ***Pc****- Pteris confusa* T.G. Walker *4.* ***Po*** *– Pteris otaria* Beddome *5.* ***Ps*** *– Pteris aspericaulis* Wall. Ex.Ag. *6.* ***Pk*** *– Pteris kleiniana* Christ. *7.* ***Pm****- Pteris multiaurita* Ag. *8.* ***Pv****-Pteris vittata* L. *9.* ***Ap*** *– Asplenium polydon* G. Foster *10.* ***Hc****-Hypodematium crenatum* (Forssk) Kuhn.

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

Table 5: Minimum Inhibitory Concentration of the ferns extract against *X. campestris*

Name of the samples	Methanol (µg/ml)	Benzene (µg/ml)
P. vittata	8.00±0.00	16.00±0.00
P. confusa	64.00±0.00	128.00±0.00
P. otaria	128.00±0.00	256.00±0.00
A. lunulatum	16.00±0.00	32.00±0.00
A. capillus-veneris	64.00±0.00	128.00±0.00

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
P. vittata	199.36	175.79	192.85	170.05
A. c. veneris	116.56	118.47	112.75	114.60
P. confusa	103.82	82.80	100.43	80.09
A. lunulatum	167.51	159.23	162.04	154.03
P. otaria	63.69	110.19	61.61	106.59

with the positive control (kanamycin and neomycin) for evaluating their relative percentage inhibition (Table 6). For kanamycin the methanol extract of *P. vittata* exhibits maximum relative percentage inhibition against the test inoculum (199.36 %) followed by *A. lunulatum* (167.51 %) and *A. capillus – veneris* (116.56 %). For neomycin the methanol extract of *P. vittata* exhibits maximum relative percentage inhibition against the test inoculum (192.85 %) followed by *A. lunulatum* (162.04 %) and *A. capillus – veneris* (112.75 %) respectively.

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.

DISCUSSION

The rich diversity of Indian medicinal ferns has been evaluated for their antimicrobial properties, and this may have proved beneficial for mankind.[22–24] The ferns which have ethno-medicinal importance were found and are used by the local and tribal people. Ferns show various economic values towards food and fodder indicators, biofertilizers, insect repellents, medicine and folk medicines.[25] Very little work has been done on the antimicrobial activity of pteridophytes, yet the ethanobotanical importance of these plants have been investigated and studied by various authors. They have been studied for their biological activity. The phytochemical composition of *Adiantum radiata* has been studied and found that the isolated phytochemicals were effective against the growth of microorganisms.[26]

Table 7: Sensitivity of *X. campestris* 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	–

In general, Gram-negative bacteria were more resistant to antibiotics than Gram-positive bacteria.[27, 28] 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.[29] Presence of thick murine layer in the cell wall prevents the entry of the inhibitors.[30] In the present study Gram-negative bacteria were more susceptible to the crude extracts than Gram-positive bacteria. This 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.[31]

Hence the active compounds which are present in selected ferns inhibit the tested antibiotic resistant Gram-negative bacteria by using this mechanism. The results of the MIC showed that the plant materials are very potent against the test pathogen, even at very low concentrations.

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 cash crops.

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