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ANTIBACTERIAL ACTIVITIES OF THE PLANT EXTRACTS AND BIOCHEMICAL CHARACTERIZATION OF ASSOCIATED MICROBES OF *ANGIOPTERIS EVECTA* (G. FORST.) HOFFM. AND *TECTARIA COADUNATA* (J. SM.) C. CHR.

MAITRI SAHA AND JNAN BIKASH BHANDARI*

Pteridology and Palaeobotany Laboratory, Department of Botany, University of North Bengal, Raja Rammohunpur, P.O.- N.B.U., Dist.- Darjeeling, Pin-734013, West Bengal, India

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ABSTRACT

Plants are important source of medicine. From time immemorial fern and fern allies are used as medicine in different cultural communities. Different plant parts such as leaf or rhizome are used for curing diseases has antimicrobial activity which is experimentally proved from time to time by different Pteridologists. Besides this pteridophytes also maintain the soil environment by their rhizome associated microorganisms which play important role in growth and establishment of plants in environment. Microorganisms produce various types of secondary metabolites and different types of enzymes which are commercially and industrially important .In the present study it is observed that leaves may have antibacterial activity and its associated rhizobacteria produces some enzymes which can be exploited further commercially.

Key Words : *Angiopteris evecta, Tectaria coadunata,* Antibacterial, Rhizospheric, Biochemical

INTRODUCTION

Since the time immemorial humans are dependent upon the plants as an important source of medicine. Even today, many tribal communities and rural population are dependent heavily upon the natural resources obtained from the surrounding forest regions for treatment of various ailments and diseases. The Indian traditional medicine is based on different systems such as Ayurveda, Siddha and Unani used by various tribal communities. Though, lot of studies were already done focusing on the medicinal properties of plants, especially angiosperms, unfortunately in Pteridophytes limited number of studies have been done to explore the medicinal potentialities.

Pteridophytes form an important part of the biodiversity of this blue green planet. Besides showing economic values large number of pteridophytes are used by different communities as herbal medicine and folk medicines. The tribal people of Barak Valley area of Southern Assam, are using different species of pteridophytes to cure various diseases (Louis *et al.*, 2016). The medicinal values of ferns have been known to many for more than 2000 years. Different Pteridologist mentioned the uses of medicinal ferns in their books.

Antimicrobial activity of ferns was tested by several workers, who reported that the different fern extracts are effective against both Gram positive and Gram-negative bacteria (Guha *et al.*, 2004; Ghosh *et al.*, 2005). Glands of superficial hairs on leaves and rhizome

* Corresponding Author - E-mail : jnanbikashbhandari@nbu.ac.in

contain chemicals that are found to have antimicrobial activity. About 600 plant derived alkaloids have been analyzed by different workers for their bio medicinal properties and many of them have become important drug leads or actual drugs in pharmaceutical industry (Parihar and Bohra, 2002).

The leaf extracts of *Tectaria coadunata* are used in asthma, bronchitis; stings of honeybee, dysentery and disease of blood ulcer and the rhizome extract are used for preventing diarrhoea and colitisin the treatment of leprosy and other skin disease.

The rhizosphere represents a highly dynamic front for interactions between roots and pathogenic and beneficial soil microbes. Microorganisms are ubiquitous in nature and play a very important role in growth and establishment of plant in its natural environment. The rhizosphere microflora affects the health of plants in many ways (Odunfa, 1979). Therefore, it becomes necessary to record the adequate information of the rhizosphere and non-rhizosphere microflora of these ferns.

However, the soil microbial community is often ignored in studies examining the effects of plant species and functional group losses on ecosystem functioning (Marshall, McLaren & Turkington 2011). Soil microbes play key roles in ecosystems and mediate many ecological processes that are central to ecosystem functioning, including nutrient cycling (Zeller *et al.* 2008), decomposition processes (Subke *et al.* 2004; Carney *et al.* 2007) and the regulation and maintenance of plant biodiversity (Zak *et al.* 2003).

MATERIALS AND METHODS

2.1. Collection of Plant Materials

Fresh plant materials were collected from Sukna and Rongtong of Darjeeling district. The plants were identified and preserved as herbarium for further reference in the departmental Herbaria (PLATE I, Figure 1).The necessary work in laboratory was done from fresh and oven dried material.

2.2 Plants

(a) *Angiopteris evecta* (G. Forst.) Hoffm. and (b) *Tectaria coadunata* (Wall. ex Haines).

2.3. Bacterial Strains

The bacterial strains were collected. These includes *Bacillus subtilis* (MTCC NO. 1610), *Escherichia coli* (MTCC NO. 736).

2.4. Controls

(a) Positive control – Streptomycin (10 mcg)
(b) Negative control – DMSO (Dimethyl Sulfoxide)

2.5. Preparation of Plant Extract

Fresh plant materials were surface sterilized with 70% alcohol to remove air

microbial contamination and dried in hot air oven, grinded to powder. 5 grams of each plant powder was dipped in 20 ml of four different solvents i.e., methanol (50% and 80%) and ethanol (50% and 80%) for 72 hrs. After 72 hours the extract was filtered and left in the room temperature so that the solvent could evaporate. Then DMSO was mixed with dried plant extract and stored the sample.

2.6 The Inoculum

The inoculum was prepared by inoculation of freshly grown bacterial culture in nutrient broth and incubated for 24 hrs at 37°C in shaker.

2.7. *In Vitro* Antibacterial Assay

In Nutrient Agar medium, pores were made by sterile cork borer (diameter 3 mm). Then bacterial strains were seeded with sterile cotton swab, first horizontally then vertically to ensure the equal distribution of the bacteria in petri plates and allowed to dry. Then leaf extracts were poured into it and plates were incubated at 37°C for 24 hrs. After that inhibition zone was formed it was measured in mm. as routine procedure Manhas *et al.* (2018).

2.8. Bacterial Isolates from Soil and Rhizome

Soil samples of these ferns were collected and processed through serial dilution up to (10^-9) dilution. Then 1 ml of highly diluted solutions were poured into agar medium and kept it into the incubator for 24 hrs at 37°C. Whereas collected rhizomes were washed with tap water, sterilized with 70% alcohol and cut into small pieces. The prepared nutrient agar medium, poured into the petri plates on which rhizome pieces were inoculated after solidifications.The whole set up was kept in incubator for 24 hrs at 37°C temp.

TABLE 1 : The Result Showing Antibacterial Activity of Leaves against Two Bacteria.

Plants	Solvents		Diameter of hollow zone in mm	
			Bacillus subtilis	*Escherichia coli*
Angiopteris evecta	Methanol	50%	10	9
		80%	11	6
	Ethanol	50%	5	5
		80%	6	10
Tectaria coadunata	Methanol	50%	12	10
		80%	10	9
	Ethanol	50%	6	5
		80%	7	6

2.9. Morphological Characterization

The isolated bacterial colony were differentiated from one to another on the basis of their shape, colour, texture and Gram staining.

2.10. Biochemical Characterization

The isolated bacterial colonies were further characterized on the basis of their biochemical characters such as amylase, caseinase, cellulase, catalase, urease etc. enzymes present or not. All the biochemical characterization was performed by the procedures of Aneja K R (2005).

2.10.1. Amylase production - In SAM (Starch agar medium) the isolates were streaked and allowed to incubate at 37°C for 24 hrs. After 1 day, the plates was flooded with iodine solution for 30 sec and pour off the excess iodine solution. After sometimes the plates showed the hollow zone around the growth line of organism if they produced amylase otherwise no hollow zone were produced.

2.10.2 Caseinase production - In SMA (Skim milk agar) medium isolates was inoculated by isolates and incubated for 24 hrs at 37°C in an inverted position. Positive result showed clearing growth around streak line.

2.10.3 Fermentation of Carbohydrate - A fermentation medium (sucrose) was prepared and 5 ml sucrose solution was taken in a test tube and then Durham tubes were filled up by sucrose and placed as inverted position in each test tube. Each isolated bacterial strain was inoculated in each test tubes and allowed to incubate for 24 hrs at 37°C. Gas formation and colour changes will be observed if positive results come.

2.10.4 Citrase test - Simmon's citrate agar (SCA) media (pH – 6.9) was prepared and the isolated bacterial strains were inoculated. Then the plates were allowed to incubate at 37°C for 24-48 hrs. Positive result showed blue coloration around the streak line area and negative result showed no change of the medium colour which remains green.

2.10.5 Catalase test - Tryptone soya agar (TSA)/broth medium was prepared, inoculated with the organism from different isolates which allowed incubating at 35°C for 24-48 hrs. After 1-2 days, added 3-4 drops of hydrogen peroxide to show whether the gas bubbles were formed or not. Positive result showed gas bubbles on the upper surface of the inoculated medium in test tube.

2.10.6 Cellulase test - CMC (Carboxymethyl cellulose) medium (pH – 7.0) was prepared and bacterial isolates were seeded into medium which allowed to incubate at 37°C for 2-5 days. Incubated plates were then flooded with congo red and stands for 15 mins and then 1M NaCl solution was added. Positive results showed hollow zone around the streak line.

2.10.7 Gelatinase test - Gelatin agar media (GAM) (pH – 7.0) was prepared and inoculated with the bacteria. Plates were kept to incubate at 37°C for 4-7 days. After

PLATE I



Figure 1 : Herbarium of ferns. **(A)** *Angiopteris evecta* **(B)** *Tectaria coadunata*

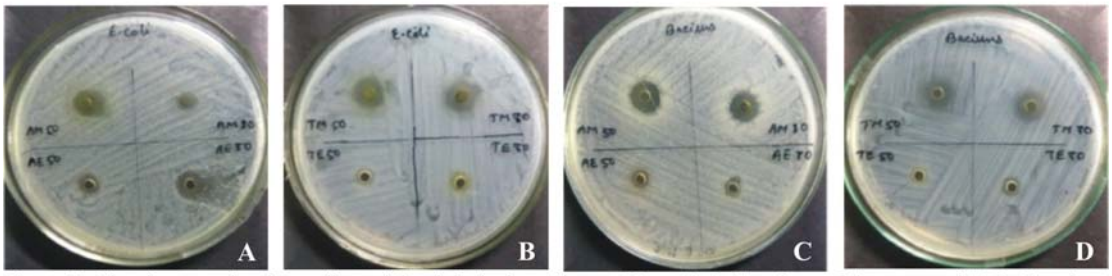


Figure 2 : The plates are showing antibacterial activity in four solvents. **Here, (A & C) :** plates of *A. evecta* and **(B & D) :** plates of *T. coadunata* against *E. coli* and *Bacillus subtilis*

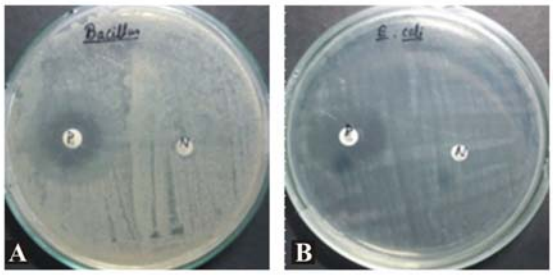


Figure 3 : Zone of inhibition produced by positive control used streptomycin (denoted as P) and no inhibition zone was produced by negative control used DMSO (denoted as N) by **A)** *B. subtilis*; **B)** *E. coli*

PLATE II

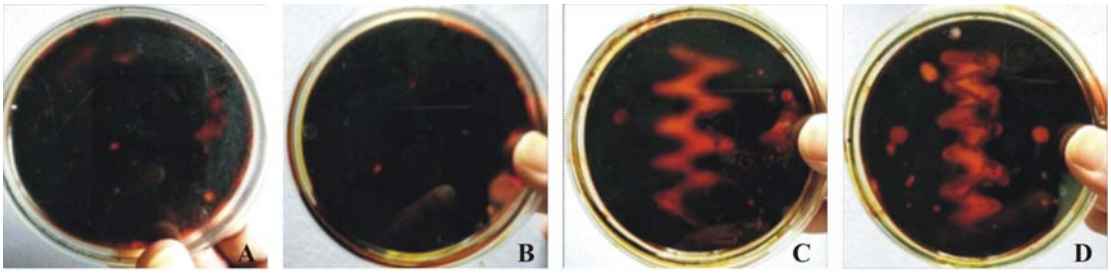


Figure 4 : Starch hydrolysis test. **A & B :** Isolates of *A. evecta*, **C & D :** Isolates of *T. coadunata*



Figure 5 : Casein hydrolysis test. **A & B :** Isolates of *A. evecta*, **C & D :** Isolates of *T. coadunata*

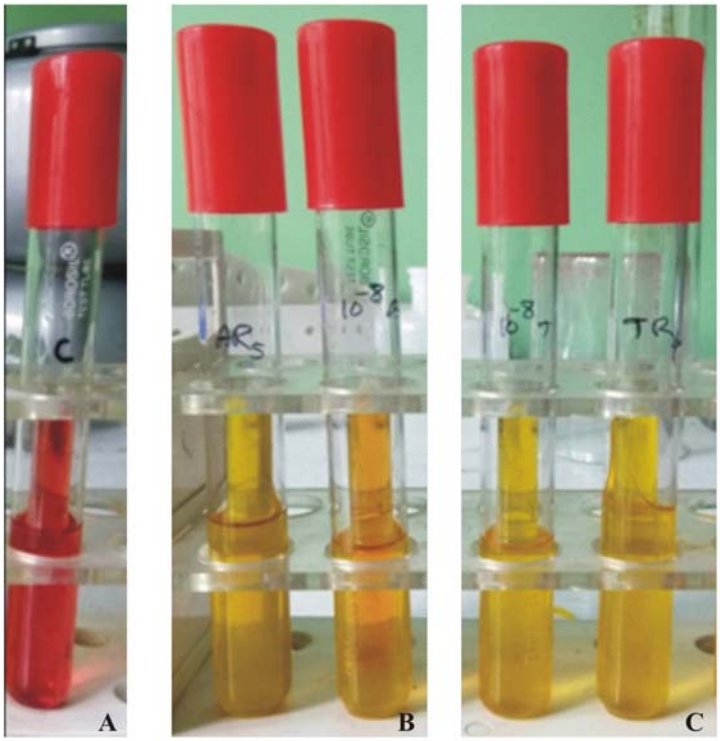


Figure 6 : Carbohydrate fermentation. Here, **A :** Control, **B :** *A. evecta*, **C :** *T. coadunata*

PLATE III

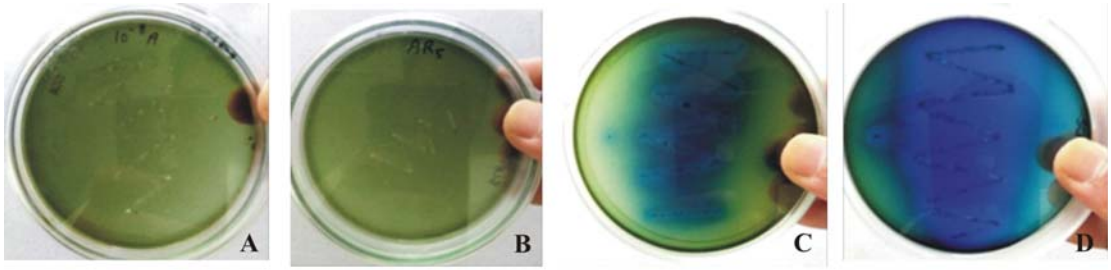


Figure 7 : Citrate utilization test. Here, **A & B :** Isolates of *A. evecta,* **C & D :** Isolates of *T. coadunata*

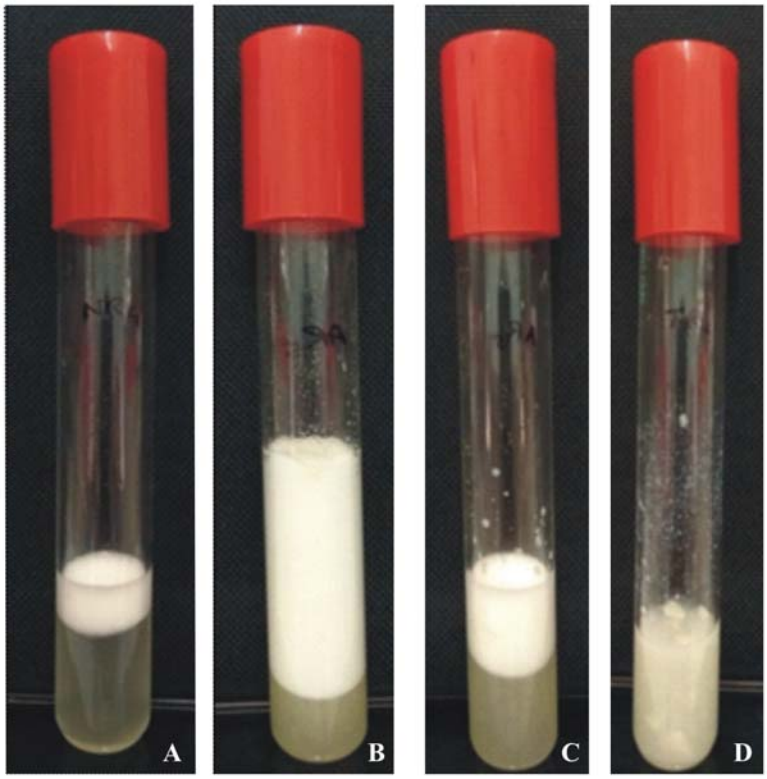


Figure 8 : Catalase test. Here, **A & C :** Isolates of *A. evecta* soil & rhizome respectively, **B & D :** Isolates of *T. coadunata* rhizome & soil respectively

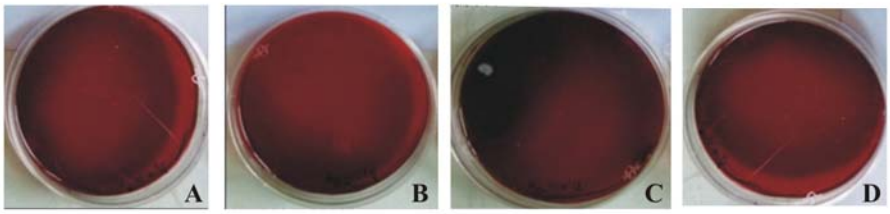


Figure 9 : Cellulose test. Here, **A & B :** Isolates of *A. evecta*, **C & D :** Isolates of *T. coadunata*

PLATE IV

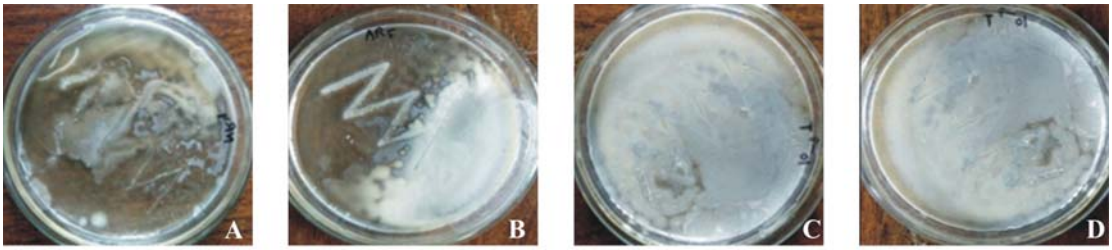


Figure 10 : Hydrolysis of Gelatin test. Here, **A&B :** Isolates of *A. evectasoil* & rhizome respectively, **C&D :** Isolates of *T. coadunata* rhizome and soil respectively

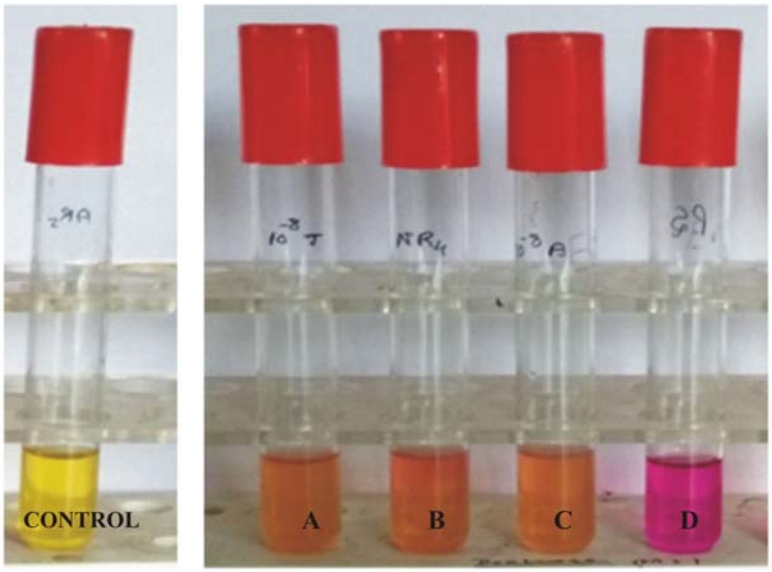


Figure 11 : Urease test. Here, **B & D :** Isolates of *A. evecta* rhizome & soil respectively. **A & C :** Isolates of *T. coadunata* rhizome and soil respectively

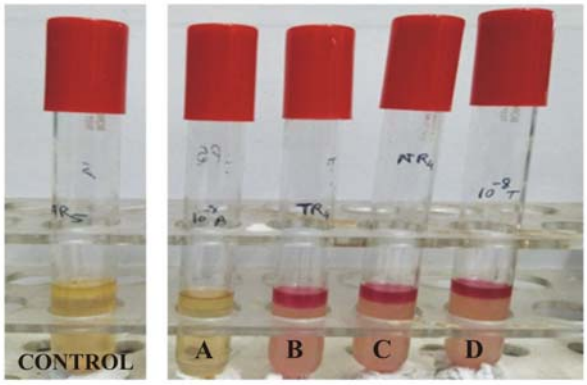


Figure 12 : Indole production test. Here, **B & D :** Isolates of *A. evecta* rhizome & soil respectively, **A & C :** Isolates of *T. coadunata* rhizome and soil respectively

incubation the plates were placed into a refrigerator at 4°C for 15 minutes. Then plates were flooded with mercuric chloride solution and allowed to see whether the medium was solid or liquid and the flooded plates for clearing, around the line of growth. Positive result showed the liquefied gelatin medium and negative results showed solid medium.

2.10.8 Urease test - Urea broth (pH 6.8) was prepared and bacteria inoculated from different isolates and incubate for 24-48 hrs at 37°C. After incubation, the phenol red changes from a yellow colour to a red or deep pink colour. Failure of the development of a deep pink color is negative response.

2.10.9 Tryptophanase test - Tryptone (1%) broth was prepared and isolated bacteria were inoculated and allowed to incubate for 24-48 hrs at 37°C. After 48 hrs of incubation, 1 ml of Kovacs reagent was added to each test tube. Shake the tubes gently after intervals for 10-15 minutes and allowed the tubes to stand to permit the reagent to come to the top.

OBSERVATION

3.1 Antimicrobial Activity

We observed the results of antibacterial activity of leaf extracts of two selected ferns in different solvent and measured the diameter of inhibition zone of all plates. The result, it was seen that the maximum inhibition zone was formed in *T. coadunata*fern. The diameter of large inhibition zone & small inhibition zone is 12 mm and 5 mm respectively. The highest inhibition zone was found in 50% methanolic extract against *Bacillus subtilis*, the Gram-positive bacteria. (PLATE I Figure 2)

3.2. Morphological Characterization

Out of four isolates, three isolates showed gram negative and one isolate showed gram positive results. In *A. evecta* the isolates which were isolated from the soil & rhizome showed gram negative & gram-positive results respectively. In *T. coadunata* the isolates which were isolated from soil and rhizome showed gram negative result.

TABLE 2 : The Result Showing Morphological Characteristics of The Selected Isolates.

Name of the sample	Colony colour	Colony shape and texture	Gram +ve/ -ve
D1	Off white & light yellow	Shiny, smooth, oval	-ve
D2	Light yellowish	Shiny, smooth, round	-ve
D3	White	Rough, faint round	+ve
D4	Bright white	Oval and smooth	-ve

Note : D1 : *Angiopteris* soil D2 : *Tectaria* rhizome D3 : *Angiopteris* rhizome D4 : *Tectaria* soil

TABLE 3 : Biochemical Characterization of Isolates

Biochemical Characterization	D1	D2	D3	D4
Starch Hydrolysis	-ve	+ve	-ve	+ve
Casein Hydrolysis	-ve	-ve	+ve	+ve
Carbohydrate Fermentation	+ve	+ve	+ve	+ve
Citrate Test	-ve	+ve	-ve	+ve
Catalase Test	+ve	+ve	+ve	-ve
Cellulose Test	-ve	-ve	-ve	-ve
Gelatin Hydrolysis	+ve	+ve	-ve	-ve
Urease Test	-ve	-ve	+ve	-ve
Indole Production Test	+ve	-ve	+ve	+ve

Note : +ve means positive response, -ve means negative response towards the reaction

3.3. Biochemical Characterization

3.3.1 Amylase production - In *A. evecta* the isolates gave negative response. Whereas, in *T. coadunata* the isolates gave positive response (PLATE II, Figure 4).

3.3.2 Caseinase production - In *A. evecta* the isolates from soil & rhizome showed both negative and positive response respectively and the isolates from *T. coadunata* soil & rhizome also showed positive and negative response respectively (PLATE II, Figure 5).

3.3.3 Fermentation of carbohydrates - Here, all the isolates which were isolated from *A. evecta* and *T. coadunata,* showed positive results comparing to control (PLATE II, Figure 6).

3.3.5 Citrase test - In inoculated plate, the media colour turns from green into blue showed positive response while other plates no change in the colour of the media showed negative response. Here, in *A. evecta* the isolates gave negative response. But in *T. coadunata* the isolates gave positive response (PLATE III, Figure 7).

3.3.6 Catalase test - In this test positive culture showed the bubbles of oxygen at the upper surface of the broth culture after addition of hydrogen peroxide. In *A. evecta* the isolates which were isolated from soil & rhizome showed positive results as well as negative results respectively. In *T. coadunata* isolates from soil & rhizome gave positive & negative response respectively (PLATE III, Figure 8).

3.3.7 Cellulase test - A clear zone around the streaking line showed positive results and in negative results there was no growth of bacteria. Here, all the isolates which were isolated from *A. evecta* and *T. coadunata* showed negative results (PLATE III, Figure 9).

3.3.6 Gelatinase test - After incubation, liquifaction of the media showed positive results and solid media demonstrated the negative results. In *A. evecta* the isolates from

soil & rhizome gave positive & negative response respectively. In *T. coadunata* the isolates from soil & rhizome also gave negative & positive response respectively (PLATE IV, Figure 10).

3.3.8 Urease test - The culture tube showed deep pink colouration of the medium showing positive reaction while the other culture tubes did not show any change in the colouration of the medium which showing negative response. In *A. evecta* isolates from soil & rhizome showed negative & positive results respectively. Whereas, in *T. coadunata* the isolates gave negative response (PLATE IV, Figure 11).

3.3.9 Tryptophanase test - Development of a cherry (deep) red colour in the top layer of the culture tube showed the positive result & absence of red colouration showed negative result. Here, in *A. evecta* isolates showed positive results. In *T. coadunata* the isolates from rhizome & soil gave negative & positive response respectively (PLATE IV, Figure12).

DISCUSSION

In the extracts of 114 species of pteridophytes (27 families, 61 genera) has been surveyed due to antimicrobial activity and most of them showed antibiotically active. The plants were extracted in four different solvents like water, 80% methanol, 80% ethanol, 80% acetone and ether and assayed against 3 gram positive, 1 acid-fast and 5 gram-negative bacteria and 3 fungal plant pathogens. The active substances were in most cases antibacterial and only 3 possessed antifungal activity (Banerjee & Sen, 1980).

In the present study it was found that methanolic extracts have more antibacterial activity than ethanolic extracts. 50% methanolic extract of *T. coadunata* leaves showed 12 mm inhibition zone against *Bacillus subtilis* whereas the positive control showed the inhibition zone around 18 mm (PLATE I, Figure 3). 80% methanolic leaf extract of *Davallia griffithiana* showed around 10 mm of inhibition zone (Saha *et al.,* 2019). 80% ethanolic extract of *A. evecta* leaves and 50% methanolic extract of *T.coadunata*leaves showed highest inhibition zone against *E. coli* and it was around 10 mm whereas positive control showed 21mm inhibition zone. This type of inhibition zone was also found in *Blechnum orientale* leaf extracts against *E. coli* (Saha *et al.,* 2019).

It is possible that several non-analyzed ferns and fern allies could contain effective antibiotic substances. So, the more antibiotic activities of the Pteridophytes are analyzed, the more natural antibiotics could be developed (Lee *et al.*, 2011).

Aqueous and alcoholic extracts of plant parts of *Athyrium pectinatum* (Wall.) Presl were tested against the growth of some human and plant pathogenic bacteria like, *Agrobacterium tumefaciens, Escherichia coli, Salmonella arizonae, Salmonella typhi* and *Staphylococcus aureus*. Nearly all the extracts were found effective against these bacteria. It was found that extracts when mixed in equal proportion with the antibiotic (Tetracycline) were more effective against bacteria than the antibiotic alone .

Biochemical Characterization of Bacteria

1. Amylase production - When microbes produce amylase then it degrades into amylose and amylopectin. Iodine solution reacts with amylase and form a dark-blue colouration and a yellow zone around a colony in blue medium indicates amylolytic activity.

Angiopteris isolates do not produced amylase like *D. griffithiana, B.orientale* and *P. cuspidatus* isolates (Saha *et al.,* 2020) whereas *Tectaria* isolates produced amylase.

2. Caseinase production - The enzyme caseinase breaks the peptide bond and liberating smaller chains of amino acids by extracellular or intracellular peptidases. The medium will be clear around the growth of bacteria due to casein hydrolysis whereas it will remain opaque due to casein colloidal suspension.

In this case, D3 and D4 showed positive result like *B. orientale* and *P. cuspidatus* isolates and D1 and D2 showed negative result like one of the *D. griffithiana* isolates (Saha *et al.,* 2020).

3. Fermentation of Carbohydrate - Fermentation of carbohydrates by microbes is carried out in a fermentation tube which contains a Durham tube for the detection of gas production. The fermentation broth contains carbohydrate and a pH indicator phenol red, which is red at pH 7 and becomes yellow at <pH of 6.8 due to acid.

Here, all the isolates showed positive result like *D. griffithiana, B. orientale* and *P. cuspidatus* isolates (Saha *et al.,* 2020).

4. Citrase test - The enzyme citrase break citrate and produced oxaloacetic acid and acetic acid. This acetic acid further divided into pyruvic acid and CO_2. Bromothymol Blue is used which is an indication of pH of medium. When citrate form CO_2 then it reacts and form an alkaline product which change the colour of the medium from green (pH 6.8 and below) to blue (pH 7.6 and higher).

Only *T. coadunata* isolates showed positive response and *Angiopteris* isolates showed negative response like *D. griffithiana, B. orientale* and *P. cuspidatus* isolates (Saha *et al.,* 2020).

5. Catalase test - In aerobic respiration, microorganisms produce H_2O_2 which is lethal to cell. H_2O_2 is breaks down by catalase and produced H_2O and O_2.

All isolates released oxygen except *Tectaria* soil isolates similar to *D. griffithiana* isolates (Saha *et al.,* 2020).

6. Cellulase test - Cellulose is the most abundant biomolecule in the Earth. This cellulose is degraded by enzyme cellulase which is made up of endoglucanase, exoglucanase and β-glucosidase.

All isolates did not show hollow zone after addition of congo red and 1M NaCl solution similar to *D. griffithiana, B. orientale* and *P. cuspidatus* isolates (Saha *et al.*, 2020).

7. Gelatinase test - Gelatin is hydrolysed into amino acids by gelatinase which is a proteolytic enzyme.

D1 and D2 isolates showed positive response like *B. orientale* isolates (Saha *et al.,* 2020).

8. Urease test - Urea is the major waste product of vertebrates and some microorganisms produced urease which breaks the amide bond, form ammonia. In urea broth pH indicator phenol red (yellow) (pH 6.8) is used. When pH increases, yellow colour changes to red or deep pink colour.

Only D3 isolate showed positive result like *D. griffithiana, B. orientale* and *P. cuspidatus* isolates (Saha *et al.,* 2020).

9. Tryptophanase test - An essential amino acid tryptophan is oxidized by tryptophanase. In this test, indole produced during reaction which is detected by Kovac's reagent by changing a cherry red reagent layer.

Only D2 gave negative response like *D. griffithiana, B. orientale* and *P. cuspidatus* isolates (Saha *et al.,* 2020).

CONCLUSION

From the above experiments we concluded that the selected ferns show a great antibacterial activity against *Bacillus subtilis* & *Escherichia coli*. Plant extracts have great potential as antimicrobial compounds against microorganisms. Thus, they can be used in the treatment of infectious diseases caused by resistant microbes. The synergistic effect from the association of antibiotic with plant extracts against resistant bacteria leads to new choices for the treatment of infectious diseases. This effect enables the use of the respective antibiotic when it is no longer effective by itself during therapeutic treatment.

Moreover, several biochemical tests were performed with the bacterial isolates obtained from the respective rhizosphere of the ferns which shows a great variety of positive as well as negative results. Some of the microbes produce enzymes and chemicals that are highly useful for human. These soil microorganisms may be an important source of producing chemicals having biochemical and pharmacological importance. Although many useful chemicals have been discovered as microbial metabolites, there might be many more products yet to be discovered from soil microorganisms.

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