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Bioactive *Streptomyces* sp. S5 Associated with *Marsilea minuta* Linn.; Purification and Characterization of Antimicrobial Substance

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Abstract: Endophytic actinomycetes are the hidden treasure of future pharmaceutical research. Plants with ethno medicinal value are most important resource of endophytic microorganisms possessing bioactivity. *Marsilea minuta* Linn., a very common pteridophyta and is well known for being used as folk medicine. In this investigation we have isolated 7 actinomycetes strains form suface sterilized petioles of this plant. Antimicrobial property evaluation of these isolates revealed one strain, S5 with both antibacterial and antifungal property. The strain, S5 was identified as *Streptomyces* sp. S5 through polyphasic approach. Active antimicrobial substance was isolated from S5 fermented cell free liquor and purified by activity guided TLC and analytical HPLC study. Purified substance was found to inhibit bacterial pathogen; *Aeremonas caviae*, *Bacillus cereus* and plant pathogenic fungi *Fusarium solani*, *Sclerotinia sp*. UV and FTIR spectrum of purified substance indicated presence of phenolics with poly-ene aliphatics as active principal. However further characterizations are necessary for structural information of the antimicrobial substance.

Key words: *Marsilea minuta*, Endophyte, *Streptomyces*, identification, phenoilics.

Introduction

Ferns belong to a leading division of the Plant Kingdom, also known as Pteridophyta. *Marsilea* spp. is common species of sub-division pteridophyta and family Marsileaceae. It is an amphibious and aquatic plant with roots ingrained in the soil, mud or in shallow pools and is extensively found all over India. Roots of these plants are produced both at the nodes and internodes of the rhizome. The glabrous (sometimes pubescent) slender petioles are usually 5.5-17 cm long [24]. One of the most crucial factors for the evolutionary success is that the pteridophytes are not vitiated by any microbial pathogens. It is a notable fact that they survived for more than 350 million years [22]. As per folk medicine, the ayurvedic systems of medicine recommended the therapeutic benefits of the ferns and showed its bioactivity properties such as antimicrobial, anti-inflammatory, antitussive, antitumor, etc [22].

A prospective observational study on *Marsilea minuta* has reported that it contains steroidal/ triterpenoid, sapogenols, phenolic compounds, including flavonoids viz., quercetin-3-O-glucoside, kaempferol-3-O-glucoside, quercetin-3-O-galactoside, chalcone-3-O-glucoside, quercetin-3-rutinoside, and naringenin-7-O-glucoside [6] etc. Besides these marsilin (1-triacontanol-cerotate), 3-hydoxy-triacontan- 11-one, hentriacontan-6-ol, methylamine, beta-sitosterol, marsileagenin A, flavonol-Omono- and-diglycoside, C-lucoyl-flavones and C-glucosylxanthones[13] were also isolated from another species, *M. quadrifolia* [6].

Ayurveda being the world's ancient holistic healing system has recommended this plant for the treatment of psycopathy, diarrhea, cough,

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bronchitis, and skin diseases [31] which indicates some sort of anti-infective property of *Marsilea* spp. However, several recent studies also exhibited the wide range of beneficial properties as anti-amnesic [2], anxiolytic, antidepressant, hypocholesterolemic, antifertility activity [30], tranquilizing activity and hepato-protective property of the extracts of this plant. Leaves of these plants have various cures for many ailments like diuretic, febrifuge, snakebite and abscesses [34]. Since, there have been very few studies conducted regarding the antimicrobial properties of *Marsilea minuta* so; there is no well reported description of any compound having antibacterial or antifungal properties.

There are a number of infectious agents that are getting more resistant to commercial anti-microbial compounds. Because of the impenetrable wall of Gram-negative bacteria, they are more resistant against antibiotics compared to Gram-positive bacteria [5]. So, there is a need to develop new drugs through modern scientific scrutiny, with varied strategies or some new source of active compounds. Various medicinal values have been evaluated from *Marsilea minuta* which are dwelled in some chemical substances that harvest a definite physiological action on the human body reflecting it's unique and diversified uses to mankind. Since the revelation of penicillin in 1942, as a source of antimicrobial compounds microorganisms like various bacteria and fungi were also screened. To minimize the dereplication of natural products that are higher during studies of microorganisms from soils or water bodies, endophytic micro-organism of plant tissues are profusely screened new anti-microbial substances which are comparatively less scrutinized biotope. Residing inside the plant's internal tissue, these microorganisms live asymptomatically and plays propitious role in many respect including protection from pathogens by producing secondary metabolites [28]. Because of the feasibility for genetic recombination with host during coevolution, these groups of microbes furnish better information in drug discovery through symbiotic association[8]. This phenomenon again attracts importance towards the production of active natural products by plants with ethno botanical properties which are more potent sources of endophytes when compared with other plants [27]. There are abundant endophtyic actinomycetes which are already isolated as endophyte to various medicinal plants cultivating significant bioactive compound and some of them having new chemical structures [7,15,16]. Present study emphasizes the isolation and identification of a *Streptomyces* sp. from surface sterilized petiole of *Marsilea minuta* Linn. The endophytic strain was found antagonist against some pathogenic bacteria and fungi. Active component were purified from fermented broth of this endophytic strain. For structural knowledge of antimicrobial substance the spectral analyses were also carried out.

Materials and methods

Plant sample preparation and endophytic actinomycetes isolation

Healthy *Marsilea minuta* Linn. were collected from various shallow pools of Paschim Medinipur, West Bengal, India. All the plants were washed under tap water and placed in sterile plastic container after soaking in tissue papers and brought to laboratory for isolation of actinomycetes. Petioles were cut in 1cm in length. These samples were surface sterilized with ethanol and sodium hypochlorite (3-4 % free chlorine) [35]. After proper surface sterilization, efficiency of technique was examined by washing the samples in double distilled autoclaved water and spreading that water on nutrient agar (NA) media. No colony formation indicates positive surface sterilization. Surface sterilized samples were taken and barks were removed with sterile scalpel. Inner tissues were sectioned and placed on ISP2 (International Streptomyces Project) media supplemented with cycloheximide and streptomycin (50 µg/ml). Media containing plant samples were incubated at 30°C with 8 h illuminated condition.

Pure culture and preservation of isolates

Actinomycetes strains emerging from plant tissues were immediately subculture in same media and pure cultures were maintained on slant at 4°C and in liquid culture with 30 % (v/v) glycerol at -20°C.

Antimicrobial screening of isolates

Isolated strains were screened for antimicrobial activity by dual culture method. Antimicrobial study was conducted against four bacterial pathogens; *Bacillus cereus* (ATCC 14579), *Shigella flexnerii* (ATCC 12022), *Proteus vulgaris* (ATCC 12453), *Aeromonas caviae* (ATCC 15468) and two fungal pathogens *Fusarium solani* (NCPF 580016), *Sclerotinia* sp. The mycelial plugs (5 mm diameter) of fungal pathogens and isolated actinomycetes were placed on the same Sabouraud's dextrose agar media 4 cm apart from each other with control plate containing fungal pathogens only. Isolated actinomycetes strains were placed 5 days earlier than fungal pathogens due to their slow growth. For antibacterial evaluation, 5 mm agar plugs containing actinomycetes colony were placed on ISP2 agar media and grown for 5 days. A loopful of bacteria was then streaked (3 cm long) up to 1 cm away from the actinomycetes colony. Percentage of growth inhibition was calculated from following formula;

PGI (for fungal pathogen) (%) = D*c*- D*t*/D*c* x 100

where D*c* is distance between point of inoculation and margin of fungal colony on control plate and D*t* is distance between point of inoculation and fungal colony margin to the actinomycetes phase on treated plate.

PGI (for bacterial pathogen) (%) = 30 – L*s*/30 x 100 where 30 = 3 cm *i.e.* length of streaked bacterial line and L*s* is length of streaked bacterial colony appeared.

Characterization of endophytic strain S5

Due to broad spectrum antimicrobial activity the endophytic actinomycetes strain S5 was characterized from polyphasic approach. Colony morphology and soluble pigmentation was checked on various media [23,32]. Spore morphology was determined after scanning electron microscopy. Various sugar utilization pattern of S5 was also carried out [10].

Genomic DNA of strain S5 was extracted by Li *et al.*, [14]. Specific amplification of 16s rRNA gene was done with 27 F and 1492 R primers [21]. Amplified products were purified with Hi-PurA™ PCR product purification spin kit (Himedia Laboratories, India) and sequenced using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequences of 1440 bp of rRNA gene were generated and carried out for BLAST in NCBI database. Based on maximum identity score sequences were selected and aligned using multiple alignment software program, Clustal W. Distance matrix was generated using RDP (Ribosomal Database Project) database and the phylogenetic tree was constructed using MEGA 6 [29]. The evolutionary history was inferred using the Neighbor- Joining method [20]. The evolutionary distances were computed using the Kimura 2-parameter method [12].

Fermentation and extraction of antimicrobial compound

For production of antimicrobial substance in larger amount, 20 Erlenmeyer flasks (250 ml) were taken containing 50 ml production media (ISP2). The strain S5 was grown in liquid ISP2 media for 2 days and a 2 % (v/v) inoculum was transferred to final production media. Fermentation was carried in batch fashion at 30°C for 12 days. After fermentation biomass was separated by filtration and centrifugation at 10,000 rpm for 12 m. Filtrate was extracted twice with equal volume of ethyl-acetate. Organic phase was taken and made water free with anhydrous Na_2SO_4. It was then evaporated to dryness under reduced pressure in a rotary evaporator (HS-2005S, HAHNSHIN, Korea) at 40°C. The dried residual was dissolved in 1 ml methanol.

Activity guided purification and analytical HPLC

The methnolic extract was checked for bio-activity against *B. cereus* and *Fusarium* sp. by disk diffusion assay (disk content 25 µl). Active ingredient in extracted mixture was separated by thin layer chromatography. Silica coated alumina TLC plates were taken of 6 cm length and 2 cm breadth (TLC aluminium sheets; Silica gel 60 F_{254}). A 25 µl methanolic extract was loaded as thin band on TLC plates and compounds were separated with various combinations of chloroform and methanol (1:0, 10:1, 5:1, 4:1 and 1:1). Separation of components was visualized after UV

exposition of TLC plates. Active component on TLC plate was located by bioautogram analysis. MHA plate was seeded with *B. cereus* and TLC plate was placed inverted on the media for 2 hr to diffuse separated components from TLC plates to MHA media. TLC plate was removed and bacteria were allowed to grow for 24h at 37°C. Bacterial growth inhibition was located on spraying of methylthiazoletetrazolium (MTT-5 mg mL-1) of MHA surface. After TLC separation and detection of active compound by bioautogram the active component was eluted from TLC plates and was checked for antimicrobial activity against a Gram positive bacteria; *B. cereus*, Gram negative; *A. caviae* as well as two fungi *F. solani* and *Sclerotinia* sp. by agar well diffusion method [1].

Anlytical HPLC and structural characterization of active substance

Active component was scrapped from TLC plate and dissolved in methanol. It was then centrifuged at 10,000 rpm for 5 m and methanol was taken in another centrifuge tube. A 10 µl of such sample was analyzed in reverse phase high performance liquid chromatography (Agilnet, 1260, USA) under the following conditions: Flow rate 1.0 mL min-1; stationary phase Delta Pak C18 column (Zorbax high purity porous silica microsphere); 23°C; mobile phase 80 % methanol in chloroform; detector wavelength 260 nm. Numbers of peaks with their retention time were recorded.

A 500 µl methanolic solution of active substance was analyzed for UV- Vis absorption by UV-1800, Shimadzu and 100 µl methanolic solution of active band was taken in zinc selenide cuvette and analyzed for IR spectrum by FT-IR spectrophotometer (Spectrum T, Perkin Elmer).

Results

Several endophytic actionmycetes were obtained on ISP2 media from surface sterilized petioles of *Marsilea minuta* Linn.after incubation of 1-3 weeks of incubation. Hyphael emergences were detected for endophytic actinomycetes and immediately subcultured. A total of seven actinomycetes strains were isolated as endophyte in this study. Within these isolates strain S5 was found with broad spectrum antimicrobial activity against all bacterial and fungal pathogens selected in this study, except for *Shigella flexnerii.* Table 1 represents all the isolated strain and their ability to inhibit pathogen growth.

Endophytic strain S5 produced white dry colony on ISP2 agar media that turns to offwhite in color within 5 days with strong earthy smell. Light

Table 1. Isolated actinomycets strains form *Marsilea* and their antagonism profile

Plant Name	Code of isolates	*Antagonism (growth reduction %)					
		Antibacterial				Antifungal	
		BC	SF	PV	AC	FS	SC#
	S1	++	-	-	+	-	+
Marsilea minuta Linn.	S2	-	-	-	-	-	-
	S3	-	-	-	-	-	-
(Petioles)	S5	++	-	+	++	+	++
	S6	+	-	-	-	-	-
	S7	+	+	-	-	-	-
	S8	-	-	-	-	-	-

* For antagonism profiling: - indicates no pathogen inhibition
+ indicates pathogen inhibition of 30 % or less
++ indicates pathogen inhibition of 50 % or more
# BC: *Bacillus cereus:* SF: *Shigella flexnerii*
PV: *Proteus vulgaris:* AC: *Aeromonas caviae*
FS: *Fusarium solani:* SC: *Sclerotinia* sp

brownish pigmentation was found on ISP2 media surrounding the colony of this endophyte. Scanning electron microscopy of strain S5 revealed long mycelia with very less branched and possessing 3-5 cylindrical spore an the tip of hyphae (Fig. 1). It grows well aerobically. The strain could utilize dextrose, maltose and lactose most efficiently than other sugar tested in this experiment. Characterizations data those were taken for S5 were summarized in Table 2. Sequencing of 16s rDNA gene produced 1440 nucleotides and phylogeny constructed for this strain showed its close similarity (98 %) with various species of *Streptomyces* (Fig. 2).

Thin layer chromatography of methanolic extract of strain S5 produced four distinct bands (A-D) upon exposing on UV rays and bioautogram analysis indicated compound B (Rf- 0.262)

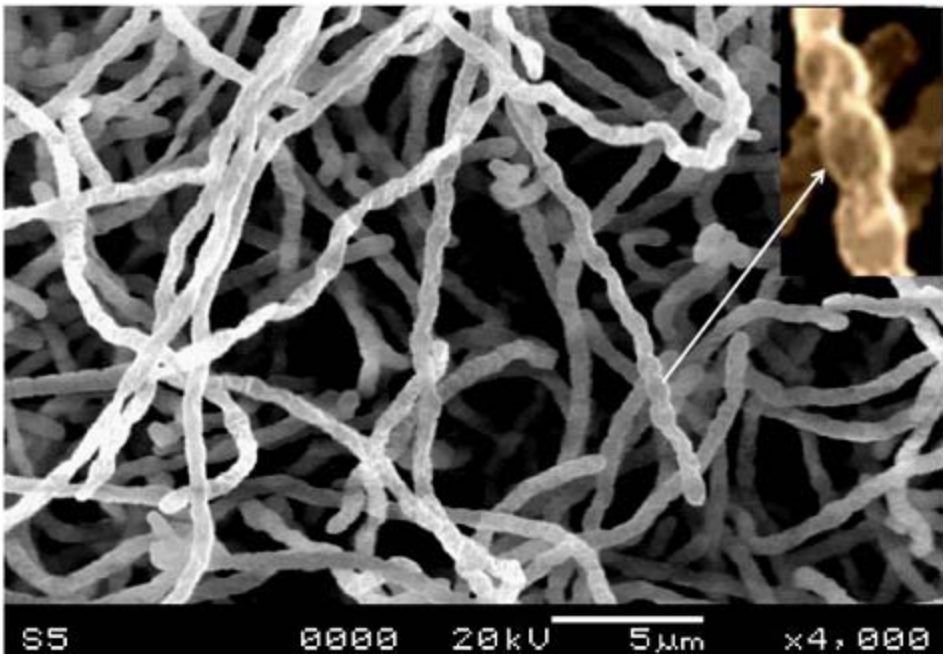

Fig. 1. Scanning electron microscopy of endophytic strain *Streptomyces* S5; (inset, arrow indicates spore nature)

Table 2. Cultural and physiological characteristics of endophytic*Streptomyces*sp. Strain S5

Colony morphology	**Flat, rough surface, dry, white colony**
Growth on ISP2	Good
Substrate mycelia	Brownish
Aerial mycelia	White
Soluble pigment	Light bownish
Growth on ISP4	Moderate
Substrate mycelia	Gray
Aerial mycelia	White
Soluble pigment	None
Growth of ISP5	Very less
Substrate mycelia	Cream
Aerial mycelia	No
Soluble pigment	No

table 2. (continued).

Colony morphology	**Flat, rough surface, dry, white colony**
Spore morphology	3- 5 spores at tip of the filaments Cylindrical, rough surface About 1 to1.2 µm in length and 0.8 µm diameter
Cell morphology	Very less branched filamentous Gram positive
Extra cellular enzymes	Cellulase, amylase, protease (+)
Carbon source utilization	Dextrose, fructose, galactose, lactose, maltose, sucrose, starch (+), mannitol, xylose, inositol, rhamnose (-)
Growth temperatue	
20°C	+
28°C	++ (optimum*)
35°C	+
40°C	-
Growth pH	
5	-
6	+
7	++ (optimum*)
8	+
9	-
10	-

(*Optimum has been determined as mean of triplicate study)

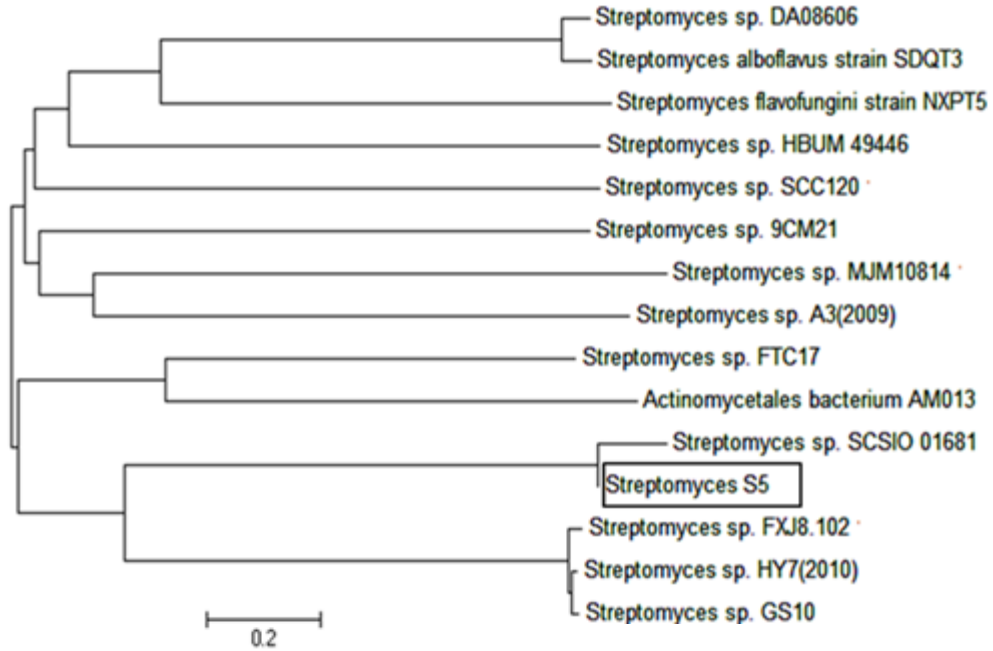

Fig. 2. Phylogenetic tree based on 16s ribosomal RNA gene sequences showing relationship between S5 and members of the genus *Streptomyces*

as active antimicrobial ingredient produced by endophytic S5. The HPLC analysis of TLC purified active compound with 80 % methanol in chloroform showed a major peak (B) of retention time 2.983 m with three other minor peaks A, C, D of retention time 2.527, 3.162 and 3.264 respectively (Fig. 3). Antimicrobial activity of compound B isolated form TLC plates was determined against selected pathogens and was found to possess both anti bacterial and antifungal property. Pathogen inhibition of purified compound produced by strain S5 is represented in table 3. It showed potential inhibitory activity against both *A. caviae* and *B. cereus* whereas among fungi, *Sclerotinia* sp. was inhibited most (Fig. 4).

The purified antimicrobial substance strongly absorbs 213, 457 and 667 strongly at UV-Vis zone. However FTIR analysis indicates peaks at 1764 cm^{-1}, 1696-1730 cm^{-1},1395-1510 cm^{-1}, 1225 cm^{-1} and 750-800 cm^{-1} which are indicative for various

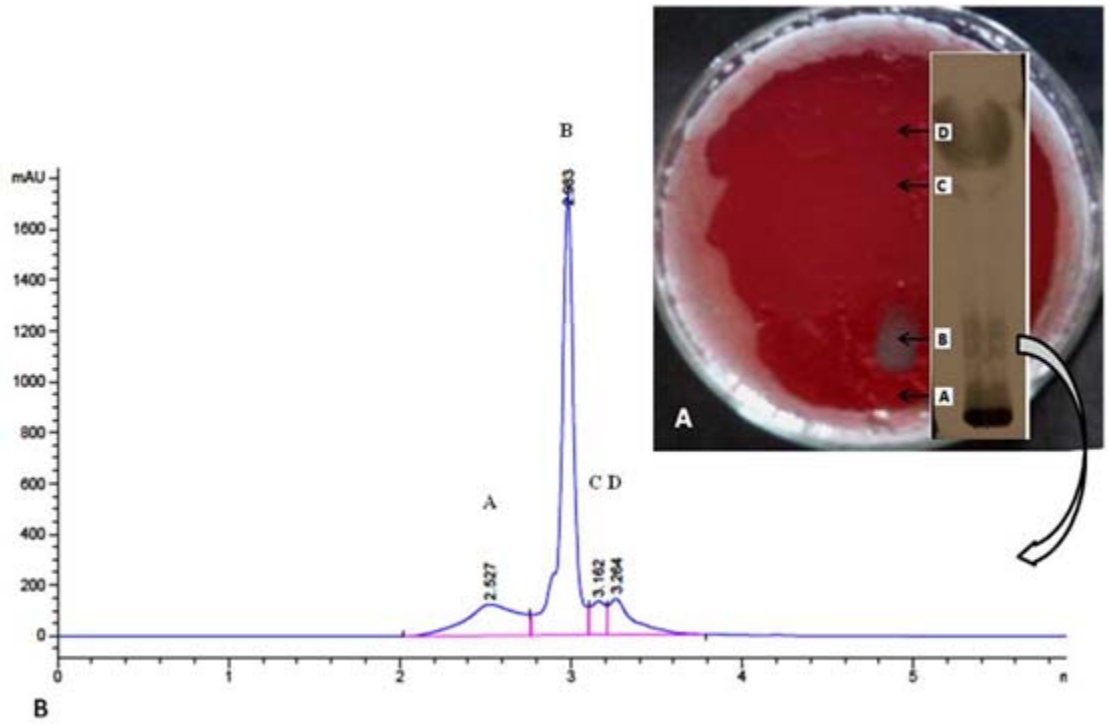

Fig. 3. Bioautogram (A) of TLC plate showing compound 'B' as active compound where as HPLC analysis (B) of compound B indicates a major peak at retention time 2.983

Table 3. antimicrobial activity of TLC purified methanolic active substance produced by *Streptomyces* sp. S5

Endophyte	*Antagonism			
	Antibacterial		Antifungal	
	Zone of inhibition (mm)		Growth reduction (%)	
	BC	AC	FS	SC #
Streptomyces sp. S5 TLC purified methanolic substance (15 µl)	16.6	25.3	57.14	75

*values were represented as mean of triplicate study
#BC: *Bacillus cereus:* AC: *Aeromonas caviae*
FS: *Fusarium solani:* SC: *Sclerotinia* sp.

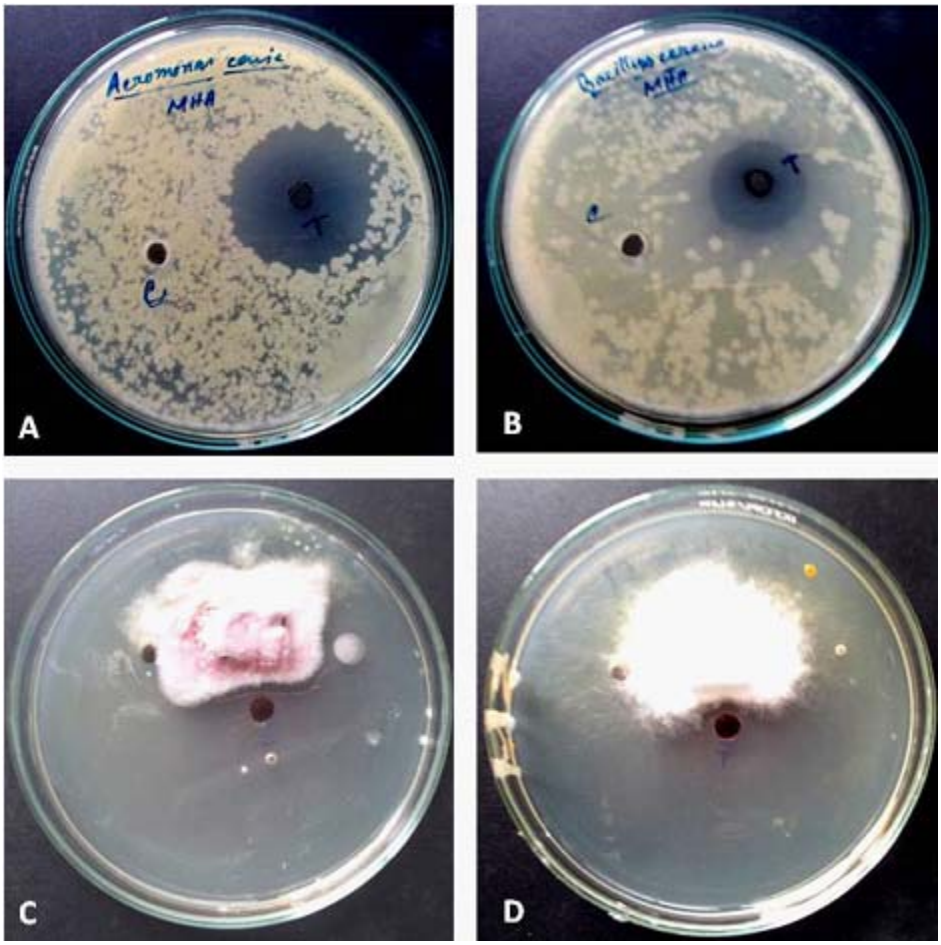

Fig. 4. Antimicrobial activity of TLC purified substance produced by Streptomyces sp. S5; A: activity against *Aeremonas caviae*, B: activity against *Bacillus cereus*, C: activity against *Fusarium solani* and D: activity against *Sclerotinia sp.*

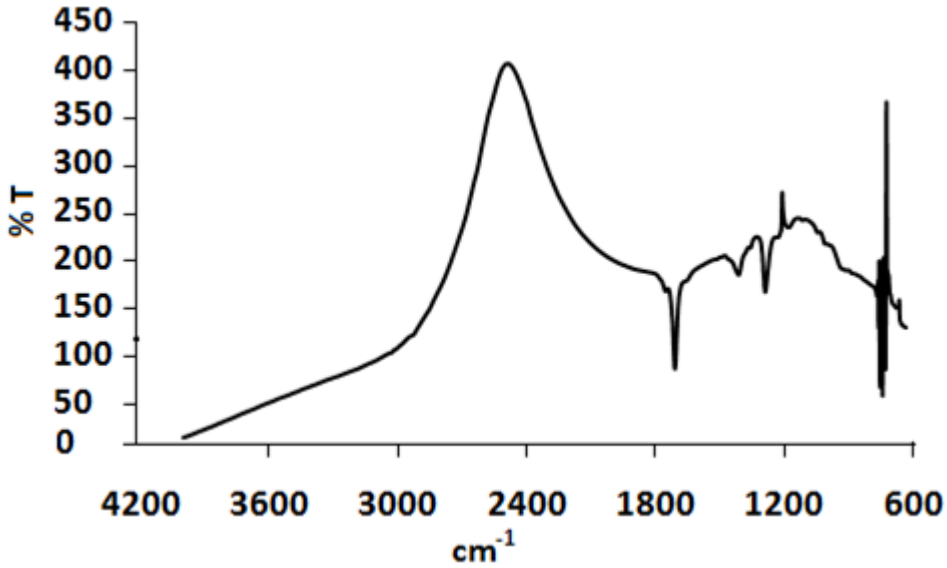

Fig. 5. FTIR spectrum of active antimicrobial substance produced by endophytic strain S5

functional groups present in the purified substance (Fig. 5).

Discussion

The plant, *Marsilea minuta* Linn. is a very popular herb and eminently used as folk medicine. Current study was to isolate endophytic actinomycetes associated with this plant and the strain *Streptomyces* S5 was isolated as endophyte which emulates potential antimicrobial activity against most of the test pathogens. It will effective against both pathogenic bacteria and plant pathogenic fungi. The endophytic strain has been identified as *Streptomyces* sp. S5 from polyphasic approach. It reflected the typical *Streptomyces* properties like oval swollen spore at the tip of mycelia which are less branched and other colony characteristics presented in table 2. *Streptomyces* are famous for their antimicrobial activities due to retaining cluster for secondary metabolite genes [3]. Earlier experiments also indicate presence of *Streptomyces rochei* CH1 from *Cinnamomum* sp. with broad spectrum antibacterial activity and many bioactive endophytic actinomycetes from various plant species [18,19].

The strong absorbance of UV at 213 nm by purified antimicrobial compound produced by this strain indicates presence of conjugated di-ene system in the active compound. Other absoption perhaps was due to the nature of the color. Recommendation of the presence of various functional groups such as orthodisubstituted benzene for 750-800 cm^{-1}, carboxylic acid anhydride for peak at 1764 cm-1, C=O functional group was indicated at 1696-1730 cm^{-1}, secondary alcohol at 1395-1510 cm^{-1} and presence of aromatic ether at 1225 cm^{-1} was certainly given by the FTIR data. The active compound is assumed to be some phenolic derivatives bearing carboxylic anhydrides and aliphatic chains with single bond double bond characters. Phenolics are eventually reported in a phytochemical study *Marsilea minuta* Linn. [9] but their structural information is unavailable yet. Nevertheless, this green method is eco-friendly, non-toxic and cheap for isolation of pharmaceutically active compound; unlike the chemical synthesis or purified plant products.

Medicinal plants, an auspicious treasure of nature are an effective source of both traditional and modern medicines, which have endless usefulness for primary health care. It has been advocated by the World Health Organization that traditional medicine are safe remedies for ailments of microbial and non-microbial origin. Plants have a mystical power to synthesize aromatic substances, especially secondary metabolites for defending from invaders like fungi, bacteria, viruses, nematodes etc. People of all sections of India from generation to generation kept track of using many plants widely for pharmaceutical preparation of modern medicine either directly as ethnic remedies or indirectly. The open awareness over "Green Medicine" has paved the way to acknowledge it to be safe, more accessible and more affordable when compared with the limitation associated with the synthetic pharmaceutical products. But recent study related to endophytic microorganism has not only enlighten a new direction but also offered a better pattern in the field of pharmaceutical research. Various studies reported the production of medicinal compounds or other compounds produced by plant are also isolated from inhabiting endophytic microorganisms [33].

Alkaloids like vinblastine and vincristine of *Catharanthus* also had been isolated from endophytic fungus *Fusarium oxysporum* obtained from *Catharanthus roseus* [13] with the help of this hypothesis. Current studies also show that taxol of plant origin, had also been isolated from endophtyic fungi associated with the plant *Taxus brevifolia*. There are enormous endophytic fungi which have been isolated from other Taxus plants such as *Taxus chinensis* [4], *Taxus wallichiana* [26], *Taxus cuspidate* [17] *Taxus baccata* [25] which are also culpable for the production of taxol and related compounds. Since it is economy to purify microbial extracellular product than to purify the same from plant extract, the recent finding no doubt flares up the isolation of medicinal plant-products from their associated endophytic microorganisms. Sacrifices of medicinal plants are even nonobligatory for obtaining pharmaceutical demands.

Conclusion

A total 7 endophytic actinomycetes were

isolated from petioles of the medicinal plant *Marsilea minuta* Linn. in this study. One of the strains was identified as *Streptomyces*sp. S5, and found to produce potential broad spectrum antimicrobial compound. Active compound was found with Rf value 0.262 on silica gel TLC and retention time 2.983 m by HPLC analysiswith methanol-chloroform (4:1). Spectral analysis revealed presence of phenolics derivatized compound with polyene aliphatics as active principal. However more characterization is necessary for structural detail of active compound. This method of production of drugs is in complete synchronization with the environment and when optimized, will lead to surplus production of these drugs, thus reducing their price and making them readily available.

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