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Antibacterial Activity of *Coscinium fenestratum* Against Phytopathogenic Bacteria and Isolation, Characterization of Antibacterial Active Principle

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***Pseudomonas syringae* and *Xanthomonas* pathovars are major phytopathogenic bacteria known to cause severe loss in quality and yield of agriculturally important crops such as paddy, tomato, soybean and sesame. Emergence of drug resistant bacterial strains, toxic effects of synthetic drugs on host crop plants and environment has led to the search for effective, safer and ecofriendly bioactive molecule for the management of plant diseases. Medicinal plant extract is considered as effective, safe, ecofriendly and least toxic to host crop plant. In this study, antibacterial activity of stem extracts of *Coscinium fenestratum* was evaluated against *Pseudomoans syringae* and *Xanthomonas* pathovars *in vitro.* Disc diffusion assay showed that *P. syringae* was more susceptible and *X. oryzae* was less susceptible. MIC of the extract against *P. syringae* was 2.5mg/ml and against *Xanthomonas* pathovars ranged between 1.25mg – 2.5mg/ml. Further, isolation and characterization of antibacterial active principle conducted using TLC-Bioautography-agar over lay method, column chromatography and by spectral analyses (UV, FT-IR, LC-PDA-MS and NMR) revealed that, antibacterial active principle as Berberine, a yellow colored, benzoisoquinoline alkaloid, known to have many bioactive potentialities. In conclusion, the plant extract/ berberine could be utilized for management of bacterial infection/s in crop plants after toxicity and field level evaluations.**

Key words: Medicinal plants, *Coscinium fenestratum*, *Pseudomonas syringae*, *Xanthomonas* pathovars, Berberine, Bacterial disease management.

Pseudomonas syringae (*P. syringae*) and *Xanthomonas* pathovars are the major disease causing plant pathogenic bacteria. Among the top ten list of bacterial pathogens, *P. syringae* stands in the first position and *Xanthomonas* stands in the fourth position[1]. *P. syringae* exists as over 50 pathovars infecting wide range of crop plants, fruits, vegetables, trees and many ornamental plants such as bacterial speck of tomato, bacterial brown of bean, shoot & flower blight, cankers and dieback disease. The average loss incurred reported ranges between 21-30% [2-6]. *Xanthomonas* pathovars viz., *Xanthomonas malvacearum* (*X. malvacearum*), *Xanthomonas vesicatoria* (*X.vesicatoria*), and *Xanthomonas oryzae* (*X. oryzae*) are known to cause blight disease in cotton, bean, crucifers and paddy and the average loss recorded is about 35-50%[7-9].

To manage these plant diseases, copper containing compounds (Bordeaux mixture), systemic bactericide such as streptomycin, oxytetracycline, neomycin, gentamicin and oxolinic acid are being used[10-11]. But there are several limitations to their use including lack of systemic activity, difficulty in timing, incessant and indiscriminate use leading to emergence of resistant bacterial strains, phytotoxicity and

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environmentally hazardous nature. Thus there is an urgent need to search for alternative source of drugs which is natural, stable, eco-friendly and effective even at low concentration. To achieve this, medicinal plants are the suitable bioactive natural resource from which antibacterial active compounds against above mentioned bacterial pathogens could be managed[12]. In this context, *Coscinium fenestratum* (Gaertn.) Colebr. (*C. fenestratum*) – an important medicinal plant belonging to Menispermaceae family was chosen to assess the antibacterial efficacy and to isolate the antibacterial compound. The plant is a dioecious, woody climber reported to occur in Western Ghats of high rain fall and wet evergreen forest at 500- 750MSL. The stem of this plant is used to treat ulcer, jaundice, skin diseases, abdominal disorders, diabetes and general debility. The antiproliferative and antimicrobial activities have been carried out[13-14].

MATERIALS AND METHOD

Plant material and test bacteria

The plant *C. fenestratum* (Family: Menispermaceae) was collected from Subramanya region of South canara district (12° 39' 57"N; 75° 36' 5"E) of Karnataka, India. The identification of plant was done with the help of floras of this region available and confirmed with the help of taxonomist of Department of Studies in Botany, University of Mysore.

Test bacteria

P. syringae (NCIM 1502) and *X. malvacearum* (NCIM 2310) were obtained from National Centre of Industrial Microorganisms (NCIM), National Chemical Laboratory (NCL), Pune and *X. vesicatoria* (MTCC 2286) was obtained from Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh. *X. oryzae* was isolated from diseased paddy seeds. All the test bacteria were maintained on Nutrient agar and periodic sub culturing was carried out.

Solvent extraction

The stem of the plant was washed with running tap water, blot dried, cut into small pieces, shade dried and powdered. Solvent extraction was carried out using Soxhlet apparatus for 48-72h. Pulverized plant material (50g) was initially defatted with 200ml of petroleum spirit followed by methanol. The extract obtained was concentrated and stored at 6°C till further use[15].

Antibacterial activity assay

Inocula preparation

Inoculum preparation was done following the procedures of CLSI (formerly NCCLS) [16]. The inocula density were standardized to a final concentration of 1-2 × 10^8CFU/ml by the direct colony suspension method, where suspending isolated colony from an 18-24h agar plate into Muller Hinton broth to a turbidity matching 0.5 McFarland standard.

Disc diffusion assay

15ml of Muller Hinton agar (HiMedia, Mumbai) was poured into sterile Petri plate and allowed to solidify. 50µl of bacterial inoculum was spread over solidified media using sterile cotton swab. 100µl of test extracts (100mg/ml) was infused onto a 6mm sterile disc (HiMedia, Mumbai). Each plate contained three discs, a disc contained test extract, one disc served as negative control (methanol) and one disc served as positive control (Streptocycline). Inoculated plates were incubated at 28 °C for 24h. Experiment was carried out in triplicates and zone of inhibition in diameters (in mm) were recorded[17]. Results are presented as mean value ± standard error of three replicates.

Determination of Minimum Inhibitory Concentration (MIC)

Sterile 96-well microplates (300µl volume, flat bottom, TARSONS, India) were used for this assay. Antibacterial active test extract of the plant was dissolved in minimal amount of the respective solvent used for extraction. All the wells were filled with 100µl Muller Hinton broth. Test sample (100µl) was added to first well and serial two fold dilution was made of concentration raging from 5mg/ml to 0.15mg/ml. Inocula were standardized as mentioned above and test bacteria (30µl/ well) were inoculated to each well. Plates were sealed and incubated for 24h at 28 °C. MIC was determined based on the absorbance using Microplate reader (LabTech- LT 4000) at 495nm and also by colorimetric estimation by adding 50µl of TTC (0.2mg/ml) (2,3,5-Triphenyltetrazolium chloride, Sigma-Aldrich, India) solution to each well followed by incubation for additional 1-2h. Viable bacteria reduce the colorless dye to pink color. The MIC was determined as the lowest concentration at which less absorbance and no pink color (signifying no

growth) appeared. Tests were performed in triplicate[18].

TLC and bioautrography (Agar overlay method)

It was carried out to determine antibacterial active fraction from the extract.

TLC study

Antibacterial active extract (50µl) was spotted over a precoated silica gel 60GF/UV- 254 (Alugram, Machery- Nagel, Germany) and developed in a solvent system of n- butanol: ethyl acetate: formic acid: water (3:5:1:1 v/v) with developing distance of 80mm, followed by air drying and observed for resolved fraction/s using UV chamber (at 254 & 365 nm) [19].

TLC- Bioautography (Agar over lay method)

The developed chromatogram was encased in a sterile Petri plate and 15ml of Muller Hinton agar containing 0.7% agar, standardized suspension of test bacteria and a reduction dye-TTC was over laid on the chromatogam and allowed to solidify. Plates were initially kept at 4°C for 2h for effective and rapid diffusion of bioactive compound and then incubated at 28°C for 24h. The zone of inhibition, if any was visualized as colorless zone on the chromatogram and the test was performed in triplicate [20].

Isolation of the active principle by Column chromatography

The fraction/ band possessing antibacterial activity was scraped, filtered with HPLC grade methanol and used for further purification using Column chromatography. The pre-activated Silica gel of 60-120 mesh is used, which was packed in the column of size 1 cm diameter and 30cm length with gradient elution system of Chloroform: Methanol (100% to 50%) with a flow rate adjusted to 1ml/min. Column fractions collected was monitored using TLC and based on observation, similar fractions were pooled together and evaporated to dryness.

Structural elucidation of the active principle by various spectral analyses

To elucidate the structure of antibacterial active compound, the fraction obtained in the column chromatography was further subjected to UV, FT- IR, LC-PDA-MS and NMR (both ^{1}H & ^{13}C) spectral analyses[21].

UV spectral analysis

It was conducted for detecting the particular type of unsaturated chromophore. Wavelength of the spectrophotometer was set between 300-600nm. Samples and blank were kept in cuvette. Before analysis cuvettes were washed with sterile distilled water and analysis was performed using Systronics UV- visible spectrophotometer 119 (Systronics, India) assisted with PC control. Absorption spectral measurements were performed using matched quartz cuvettes of 1cm path length. Samples were prepared by mixing one part of the column chromatography fraction and ninety nine parts of HPLC grade methanol.

FT- IR spectral analysis

This was conducted to detect the type of functional group/s present in the antibacterial active compound. The active fraction was subjected to FT- IR spectral analysis using KBr pellet in Perkin Elmer-Spectrum GX FT-IR, v4.07 assisted with PC having 4.31version software progrmme with a scan range from 400-4000 cm^{-1} with resolution of $4cm^{-1}$.

Liquid Chromatography- Photo Diode Array- Mass Spectroscopy (LC-PDA-MS)

Active fraction collected was dissolved in methanol followed by filtration and subjected to LC-PDA-MS analysis. The analysis was performed on an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, Calif) using a Capcell Pak C18 UG120 5mm column (4.6 mm × 150 mm, Shiseido, Tokyo, Japan) followed by a Finnigan LCQ^{DECA} mass spectrometer with electron spray ionization source (Thermo Electron, San Jose, Calif). HPLC conditions were as follows: solvent A, 0.1%TFA/H_2O; solvent B, 50%CH_3 CN/0.1%TFA/ 50%H_2O; linear gradient, initial percentage of B (15%) to 60 minutes (30%); column temperature, 40 °C; flow rate, 0.5 ml/min. Ultraviolet-visible absorption spectra was obtained by a photodiode array detector (PDA) in the range of 250–600nm. MS parameters were as follows: ionization mode, positive; sheath gas, nitrogen; capillary temperature, 320 °C; capillary voltage, 5.0 kV; full scan acquisition, from 50 to 1000 m/z at 2 scan/s.

^{1}H and ^{13}C NMR spectral analysis

It was obtained by dissolving 10mg of the compound dissolved in 500µL MeOD at 298K in a Bruker AMX 400 spectrometer. The coupling constant *J* is given in Hz. The chemical shifts are reported in ppm on scale downfield and signal patterns are indicated as follows: s = singlet, d =

doublet, dd = doublet of doublet, dt = doublet of triplet, t = triplet, q = quartet, qd = quartet of doublet, m = multiplet, br = broad.

RESULTS AND DISCUSSION

Disc diffusion assay

Methanol extract showed antibacterial activity against all the test bacteria. In this study *P. syringae* was highly susceptible and *X. vesicatoria* was moderately susceptible as evidenced by zone of inhibition (Table 1) at the concentration of 10mg/disc.

Determination of Minimum Inhibitory Concentration

MIC of the methanol extract ranged between 1.25mg- 2.5mg/ ml for *Xanthomonas* path ovars and 2.5mg/ml for *P. syringae*.

TLC and bioautrography (Agar over lay method)

The chromatogram illustrated 04 different fractions i.e., Fraction 1, 2, 3 and 4 with *Rf* values 0.2, 0.55, 0.65 and 0.85 respectively. At *Rf* value 0.55, zone of inhibition was observed for *P. syringae*.

Isolation of the active principle by Column chromatography

Fractions were collected in 50 fraction tubes and based on TLC monitoring 4 fractions obtained. Among 4 fractions, fraction possessed bioactivity at*Rf* value of 0.55 was collected, evaporated and further subjected to various spectral analyses.

Structure elucidation of antibacterial active compound by spectroscopic studies

UV spectra (λmax) obtained at 336nm, confirmed the presence of chromatophore belonging to benzoisoquinoline ring, FT- IR spectra obtained shows absorptions between 500-4000 cm^{-1}, where at signal 1035 cm^{-1}, 1354- 1383 cm^{-1}, 1601cm^{-1} and 2849cm^{-1} represents C-O, C-H deformation, C=C & C= N and -CH groups respectively, which confirms that particular functional groups present in the berberine, The LC- PDA- Mass spectra obtained determines the active fraction is having major peak at retention time of 15.58- 17.32 mins, which is under PD analysis determines the λmax at 238nm. Further, mass spectral analysis determines the molecular weight, which is found to be at 336.27 i.e., *m/z* 336.1229 $[M]^+$, major target signals obtained from 1H & 13C NMR of antibacterial active fraction are given in Table 2 & 3.

Thus, the spectral analyses and as per literature, the research work carried on this plant suggests that, the isolated active constituent as a yellow colored benzoisoquinoline alkaloid, known as 'Berberine' having molecular formula $C_{20}H_{18}NO_4^+$.

Table 1. Antibacterial activity of *C. fenestratum* extracts against phytopathogenic bacteria.

Extract and test bacteria	Zone of inhibition (in mm)			
	P. s	*X.m*	*X. v*	*X.o*
Pet ether	-	-	-	-
Methanol	23.66 ± 0.88	17.33 ± 0.33	18.33 ± 0.33	15.66 ± 0.33
Positive control(Streptocycline)	20.66 ± 0.33	21.66 ± 0.33	15.33 ± 0.33	19.33 ± 0.33

The mean diameters of inhibition of triplicate experiments ± standard error. – indicates no growth inhibition
P. s: P. syringae; X.m: X. malvacearum; X.v: *X. vesicatoria; X. o*: *X. oryzae.*

Table 2. Major target signals of the active fractions obtained for ^1H NMR

S. No	[a] Target signal (Proton)	δ_H
1	H-8	9.77 s
2	H-13	8.60 s
3	H-11	8.07 d (9.2)
4	H-12	7.97 d (8.9)
5	H-1	7.57 s
6	H-4	6.93 s
7	-OCH_2O-	6.08 s
8	H-6	4.94 t (6.3)
9	9-OCH_3	4.18 s
10	10- OCH_3	4.07 s
11	H-5	3.25 t (6.3)

J values (Hz) are provided in parentheses. [a]Recorded at 400MHz in MeO

DISCUSSION

The indiscriminate use of antibiotics in plant disease management has resulted in disturbances in the environmental, bacterial resurgence of drug resistant phytopathogenic bacteria and lethal effect to non- target organisms in the agro ecosystem in addition to direct toxicity to users. The medicinal plants acts as a rich source of valuable bioactive compounds and interest have been generated in the development of safer antibacterial agents to control phytopathogenic bacteria [12]. In the present study, *in vitro* antibacterial potency of *C. fenestratum* against major phytopathogenic bacterial pathovars has been demonstrated. Further, the isolation and structural identification of antibacterial active phytoconstituent is reported. Apart from the antimicrobial activity, *C. fenestratum* is reported to possess anti-inflammatory, antioxidant, hypotensive, antiplasmodial, antidiabetic, antiproliferative and antihepatotoxic activities[13]. The major phytochemicals reported from this plant are isoquinoline alkaloid group which includes palmatine, tetrahydropalmatine, crebamine, jatrorhizine, (-)-8- oxotetrahydrothalifendine, (-) - 8- oxoisocorypalmine, (-)-8- oxotheicamine and (-) - 8- oxocanadine[22]. It is rare that specific antibacterial compound have been isolated from medicinal plants following an activity guided assay with phytopathogenic bacteria as test orgainsms. As a natural alkaloid source, this plant extract is demonstrated to inhibit the growth of both gram negative and gram positive human pathogenic bacteria *Staph. aureus*, *E. coli*, *Proteus vulgaris, Salmonella typhimurium, Pseudomonas aeruginasa* and *Bacillus subtilis*[23-24]. None of the earlier workers have evaluated the antibacterial potency against important phytopathogenic bacteria. The antibacterial active compound isolated in the present study is Berberine, an alkaloid that has been used in Ayurvedic and Chinese medicine for the last 3000 years. It is a iminium cation derived from 5,6- dihydrodibenzo (a,g) quniolizinium and demonstrated significant antimicrobial activity against a variety of organisms such as bacteria, viruses, fungi, protozoa and *Chlamydia*[25]. In addition, this alkaloid displays a great variety of biological and pharmacological activities. Also, the fact that berberine and its derivatives exhibit antimicrobial activities as well as hypolipidimic and antineoplastic activities allows considering the berberine as an attractive object for chemical transformations with the purpose of directed amplifying its native biological activity[26]. Presence of plane and multi phenyl cycles, this molecule is reported to intercalate to the DNA/ RNA molecules. The positive electrical charges helps to bind itself to any molecules with negative electrical charges such as DNA, RNA, proteins and others. Thus, antibacterial mechanism of berberine included inhibition of DNA duplication, RNA transcription and protein biosynthesis in bacterial cells, interference with enzyme activity and partially due to the surface morphological changes of bacterial cells [24]. The mode of action of the active principle against test bacteria could be the same as reported by Jin *et al*., (2010)[24]. Present study suggests berberine as a potential phytogenous-bioactive alkaloid, which can be exploited in plant disease management, subject to phytotoxicity studies and field level trails.

Table 3. Major target signals of the active fractions obtained for ^{13}C NMR

S. No.	[a] Target signal (Carbon)	δ_C
1	C1	106.4
2	C2	149.8
3	C3	152.0
4	C4	109.4
5	C4a	132.0
6	C5	28.2
7	C6	57.2
8	C8	146.5
9	C8a	123.2
10	C9	145.7
11	C10	152.1
12	C11	128.0
13	C12	124.6
14	C12a	135.0
15	C13	121.4
16	C13a	139.5
17	C13b	121.8
18	-OCH_2O-	103.6
19	9-OCH_3	62.6
20	10-OCH_3	58.0

[a]Recorded at 400MHz in MeOD.

CONCLUSION

The present study has demonstrated the antibacterial potency of methanol extract of this plant against important phytopathogenic bacteria *P. syringae*; *X. malvacearum*; *X. vesicatoria* and *X. oryzae*, which are known to cause the variety of diseases in many crops. The antibacterial active principle has been identified based on bioautographic studies and isolated in pure form. The spectral analyses suggest that the active principle is a benzoisoquinoline alkaloid with molecular formula $C_{20}H_{18}NO_4^+$, having molecular weight of 336.27, which has identified as Berberine. The study indicates that this plant extract/ metabolite could be exploited for the management of plant diseases caused by *Pseudomonas syringae* and *Xanthomonas* pathovars in a variety of crops subject to standardization of treatment methods.

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