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In Vitro Antimicrobial Activity Screening of Bacteria Endophytic to Ethnomedicinal Plant *Rauvolfia serpentina* (L.) Benth. ex. Kurz

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Abstract

Introduction: Microbial endophytes colonizing internal tissues of living plants provide benefits to their host by promoting plant growth and protection against microbial infectious through the production of wide ranges of metabolites. Members of such endophytic community harbouring medicinally important plants are known to synthesize several antimicrobial compounds. The present study aims to explore bacterial endophytes of ethnomedicinal plant *Rauvolfia serpentina* (Apocynaceae) for producing novel antimicrobial metabolites of pharmaceutical and biotechnological importance.

Materials and Methods: The culturable bacterial endophytic diversity of *R. serpentina* (L.) Benth. ex. Kurz. has been screened for producing antimicrobial compounds following cross-streak and agar well diffusion assay methods against several test microbial strains. The bioactive compound was isolated and partially purified from the cell-free culture filtrate following chromatographic methods.

Results: The endophytes revealed low colonization frequency and isolation rates in the root and stem respectively. *In vitro* antimicrobial screening of 12 phenotypically distinguishable endophytes resulted in the selection of a potent antimicrobial isolate RAU 305 identified as *Pseudomonas aeruginosa* RAU 305 (Genbank accession number KR816098). Cell-free culture filtrate of RAU 305 showed broad spectrum of antimicrobial activity by inhibiting *Paenibacillus, Micrococcus, Arthrobacter, Rhodobacter, Mycobacterium, Bacillus, Escherichia, Staphylococcus, Klebsiella, Aspergillus, Colletotrichum* and *Pythium*. The antimicrobially active component extracted in butanol and chloroform was partially purified by column chromatography followed by a preparative thin layer chromatography and the homogeneity of the compound was confirmed using different solvent systems.

Conclusions: More detailed characterization and identification of the active component is essential to explore the metabolic potential of this endophytic bacterium in future.

Keywords: Bacterial Endophytes, *Rauvolfia serpentina,* Antimicrobial Activity, *Pseudomonas aeruginosa,* Solvent Extraction, Chromatographic Separation

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Introduction

The ever increasing development of antibiotic resistance amongst the microbial pathogens has directed the attention towards the discovery as well as synthesis of new drugs to combat and control the resistant microorganisms and the disease incidence. Studies on plant microbe interactions have encouraged scientists throughout the world towards fostering valuable pharmaceutical resources particularly through phytobiome and endophytic microbiome research.[1-3] The endophytic microbiome, that occur ubiquitously within every plant on earth and form symbiotic, mutualistic and/or trophobiotic relationship with the host[4] has received much attention in recent times as an un-trapped hidden reservoir of wide array secondary metabolites inside plants. The beneficial roles of endophytes in promoting endurance of the host against phytopathogenic attack and as a promising source of novel functional metabolites have been unfolded with great success. It has been established that endophytes protect their hosts from infectious agents and adverse conditions by secreting a plethora of bioactive secondary metabolites[5,6] and also promote plant growth.[7,8] Several of these endophytic bacterial and fungal strains have been explored and exploited as sustainable bioresources for the discovery of novel therapeutic compounds effective against drug-resistant microorganisms.[8,9]

Medicinal plants having traditional ethnobotanical uses have been found to harbour novel endophytic populations and the secondary metabolites elaborated by those endophytes have shown antimicrobial, antiviral, antitumour, antioxidant, cytotoxic or antioxidant properties.[8,10] Members of the genus *Rauvolfia* (Apocynaceae) are well known endemic ethnomedicinal herbs used extensively in traditional

pharmacological as well as ayurvedic preparations as sources of alkaloids, flavonoids, tannins, phenolic compounds, vitamins, etc.[11] Though the pharmacological importance of native Indian species of *Rauvolfia* has been extensively reported, studies elucidating the endophytic diversity of this medicinal plant are rare. Endophytic fungal assemblage of *R. serpentina* have revealed the presence of different species of *Trichoderma, Curvularia, Nigrospora, Chaetomium, Penicillium, Cladosporium*, and *Aspergillus* in their bark, stem and root. Crude extracts of many of these fungal endophytes have the potential to act as the natural sources of hypocholesterolemic, antimicrobial, antioxidant and anti-phytopathogenic compounds.[12,13] However, reports on the occurrence of endophytic bacteria associated with *Rauvolfia* spp. and their potential for production of functional metabolites are lacking. Endophytic *Streptomyces* sp. reported from *R. densiflora* showed glucose uptake by porcine hemidiaphragm indicating its potential as a source of antidiabetic agent.[14] In the present study, the occurrence of bacterial endophytes in the root, stem and leaf tissues of *R. serpentina* (L.) Benth. ex. Kurz. has been reported. Also, their antimicrobial potentials have been enumerated under laboratory conditions with a view to exploit their pharmaceutical and biotechnological applications in the future.

Materials and Methods

Collection of Plant and Isolation of Bacterial Endophytes

Healthy *Rauvolfia serpentina* (L.) Benth. ex. Kurz. plants were collected from the medicinal plant garden, Serampore College, West Bengal, India in sterile zip-lock polythene bags and brought immediately to the laboratory for isolation of bacterial endophytes following the method described by Sun et al.[10] Root, stem and leaf samples were washed thoroughly under running tap water and then surface sterilized aseptically with 0.5 % sodium hypochlorite (2 minutes) followed by 70% ethanol (twice, 30 seconds each). Samples were washed thrice in sterile distilled water, aseptically blotted dry on sterile tissue paper, cut into small segments using sterile scalpel and plated on the surface of nutrient agar, glycerol asparagine agar and tryptic soy agar plates. The plates were incubated at 28-30°C for 2–7 days and were observed for the growth of morphologically distinguishable bacterial colonies surrounding the plant segments. The washings of the sterile plant samples were also streaked on the same media and observed for any microbial growth to confirm successful surface sterilization. The bacterial isolates were further purified by dilution-streaking on the respective medium and the pure cultures were maintained on slopes of tryptic soy agar, by regular sub-culturing at monthly interval.

Colonization frequency or endophytic incidence (EI) was calculated as the percentage of total number of plant segments colonized by bacteria amongst the total number of segments incubated. Isolation rate was determined as the ratio of the number bacterial isolates obtained to the total number of segments incubated. The Shanon Weaver diversity index H' was calculated as: $H' = -\Sigma\ Pi\ X\ \ln Pi$, where, Pi is the proportion of individuals that species "i" contributes to the total (15).

Identification of Endophytic Bacteria

Bacterial endophytes were characterized and identified following micro-morphological and physio-biochemical analysis according to standard protocols.[16,17] The 16S rRNA gene sequence of the potent bacterial strain was determined by direct sequencing of PCR-amplified 16S rRNA. Cells were lysed by boiling for 15 minutes in 5% Chelex suspension (Chelex 100 resin, Bio-Rad) and centrifuged at 13 000 g for 10 minutes. An aliquot of the supernatant (3 µL) containing extracted DNA was PCR amplified using 16S rDNA primers 1522R and 27F. The PCR products were purified using a NucleoSpin purification column (Macherey-Nagel, Germany). The sequencing reactions were performed with an ABI PRISM Dye Terminator cycle-sequencing ready reaction kit (Applied Biosystems) according to the manufacturer's instructions. The sequencing products were purified and electrophoresed on polyacrylamide sequencing gel using an Applied Biosystems ABI 3730xl cycle sequencer. The obtained 16S rRNA gene sequence was aligned using the multiple sequence alignment software Multi Alin and compared with the sequence data available in GenBank using the BLAST program. The 16S rRNA gene similarity values were calculated by pairwise comparison of the sequences within the alignment using MEGA 7.0.

Antibiotic Sensitivity Assay

Antibiotic sensitivity of the endophytic isolate was performed following the Kirby Baur disc-diffusion method using antibiotic impregnated discs (6 mm diameter) from Himedia (HiMedia Laboratories Pvt. Limited, Mumbai, India). Based on the diameter of inhibition zone recorded to nearest mm, the organism was categorized as resistant, intermediate and sensitive following DIFCO Manual 10th edition.[18]

Screening of Endophytes for Antibacterial Activity

Bacterial endophytes were primarily screened for their antimicrobial activity following cross-streak and agar well diffusion methods against the test bacterial strains *Bacillus subtilis, Staphylococcus aureus*, *Escherichia coli, Pseudomonas cepacia* and *Klebsiella pneumoniae*. In cross-streak assay, antagonism was scored by the length of inhibition zone formed by the test bacterial strains used. For agar well-diffusion assay, the endophytic isolates were grown individually in tryptic soy broth (50 mL per 250 mL flask) under continuous shaking (120 rpm) for 48 to 96 hours. Biomass was separated aseptically by centrifugation (12 000 g, 10 minutes, 4°C) and cell-free supernatant was passed through bacteriological filter (Millipore filter, pore size 0.22 µm diameter). About 100 µL of filter sterilized cell-free supernatant was added to previously prepared wells (8 mm diameter) in the Muller-Hinton agar (Himedia, India) plates seeded with test bacterial strains. The plates were incubated overnight at 32°C and antimicrobial activity was determined by measuring the diameter of inhibition zone formed surrounding each well.

Extraction and Partial Purification of Antibacterial Compound

The active compound present in the crude cell-free culture filtrate was extracted in different solvents (2:1 v/v) following

agitation for 45 minutes in a rotary shaker and separation in separating funnel. The solvent fraction was concentrated to a minimum volume in a rotary evaporator under reduced pressure. The crude concentrate was then loaded onto the silica gel (60-120 mesh) column and eluted with the chloroform : methanol (2:1) mixture. The active fractions showing antibacterial activity were pooled, concentrated and further subjected to thin layer chromatography using a number of different solvent systems. The TLC plates (Merck, Germany) were exposed to iodine vapour or UV light for detection of spots. The silica gel portion containing the individual spots were scraped from the plate, eluted in the same solvent, evaporated to dryness, dissolved in sterile distilled water and used for antibacterial assay by the usual agar well diffusion assay.

Results

Isolation of Endophytic Bacteria

A total of 127 surface sterilized segments of leaf (33), stem (39) and root (55) of *Rauvolfia serpentina* were incubated at 28-30°C for 2–7 days on tryptic soy agar, nutrient agar and glycerol asparagine agar media and only 67 of them showed associated bacterial growth within 96 hours (Figure 1). Although, colonization frequency was reported substantially low (29.1%) in the root segments as compared to leaf (72.7%) and stem (71.8%) segments, the isolation rate was found to be almost uniform for leaf (0.12) and root (0.11) but very low in stem (0.051) segments. By avoiding the repetitive strains, a total of 12 phenotypically distinct bacterial endophytes were isolated in pure form following dilution-streaking method. Out of these 12 bacterial isolates, only 4 were obtained from leaf segments, while 2 and 6 isolates were derived from sterilized stem and root segments respectively (Table 1). Shannon Weaver diversity index indicated highest endophytic diversity in root tissues of the host plant. Among the 12 bacterial isolates obtained, only four were Gram-positive while, the rest were Gram-negative. The majority of the isolates were rod-shaped, however, no filamentous forms were reported from any parts of the host plant. Most of the bacteria (7 out of 12) were motile and only two isolates produced diffusible pigments on solid media (Table 2).

Figure 1. Development of Bacterial Endophytes From Root Samples of *R. serpentina* Showing Different Colony Morphologies on Nutrient Agar Plate. [Surface sterilized root segments were plated on nutrient agar and incubated at 30°C for 48 to 72 h.]

Table 1. Occurrence of Endophytic Bacteria in Root, Stem and Leaf Tissues of *R. serpentina*

Parameters	Plant Parts			
	Leaf	Stem	Root	Total
Number of samples[a]	33	39	55	127
Samples yielding isolates	24	28	15	67
Number of isolates	04	02	06	12
Colonization frequency, %	72.7	71.8	29.1	52.76
Isolation rate	0.12	0.051	0.11	0.09
Shannon Weaver index	0.91	1.13	1.69	1.23

[a] For isolation of endophytic bacteria plant samples were surface sterilized and plated on nutrient agar, tryptic soy agar and glycerol asparagine agar plates and incubated in 30ºC for 7 days.

Screening of Endophytes for Production of Antibacterial Substances

The bacterial endophytes were primarily screened for antimicrobial activity following cross-streak method on Mueller-Hinton agar plates against five pathogenic test strains, which include *B. subtilis, S. aureus, P. cepacia, E. coli* and *K. pneumoniae*. With the exception of isolates RAU 104 and RAU 303, all showed inhibitory activity against at least one of the five tested strains (Table 3). While the majority of the endophytes (9 out of 12) were active against *E. coli*, isolate RAU 305, a root endophyte alone demonstrated antibacterial activity against four of the five test strains (Table 3).

Isolates (10) which showed antibacterial activity in cross-streak method were subjected to secondary screening. The isolates were grown in tryptic soy broth under shake-flask conditions and the filter sterilized cell-free culture filtrates were used in agar well diffusion assay against test bacterial strains. Antibacterial activity was scored by measuring the diameter of inhibition zone formed surrounding each well after overnight incubation at 32°C. Cell-free culture filtrate of only five endophytic isolates showed distinct zones of inhibition against selected test bacterial strains (Table 4). The isolate RAU 305 alone demonstrated significant antibacterial activity against *S. aureus* (Figure 2) followed by *B. subtilis, E. coli* and *K. pneumoniae* while *P. cepacia* was not inhibited. Bacterial isolate RAU 305 was therefore selected as the most potent strain for production of antimicrobial substance.

Characterization and Identification of the Potent Isolate

The potent endophytic bacterial isolate RAU 305 was a Gram-negative, short rod-shaped, non-endospore forming motile bacterium producing several enzymes like catalase, gelatinase, oxidase, caseinase, amylase, lysine decarboxylase and nitrate reductase. It produced siderophore and grows well on King's A and King's B medium but is unable to produce polyhydroxyalkanoates (Table 5). As a sole source of carbon, it utilizes glucose, sucrose, galactose, maltose, glycerol, arabinose, xylose and saccharose and ferments malonate and citrate along with adonitol, rhamnose, cellobiose, melibiose, raffinose, trehalose, and lactose. Based on the analysis of 16S rDNA sequence homology, the bacterium was found to possess 99.9% similarity with *Pseudomonas aeruginosa* ATCC 10145 and identified as *P. aeruginosa* RAU 305. The 16S rDNA

Table 2. Morphological and Physio-biochemical Characterization of Bacterial Endophytes Isolated From *R. serpentina*

Characters	Bacterial Endophytes From Plant Tissue											
	Leaf			Stem				Root				
	RAU 101	**RAU 102**	**RAU 103**	**RAU 104**	**RAU 201**	**RAU 202**	**RAU 301**	**RAU 302**	**RAU 303**	**RAU 304**	**RAU 305**	**RAU 306**
Cell morphology	Rod	Coccus	Short rod	Rod	Rod	Short rod	Short rod	Short rod	Rod	Coccus	Short rod	Short rod
Gram nature	G(+)	G(+)	G(-)	G(-)	G(-)	G(-)	G(+)	G (-)	G (-)	G (+)	G (-)	-
Motility	-	+	-	+	+	-	-	+	+	+	+	-
Endospore	+	-	-	-	-	-	+	-	+	-	-	-
Diffusable pigments	-	-	Yellow	-	-	-	-	-	-	-	Green	-
Production of enzymes												
Catalase	+	+	+	+	+	+	+	-	+	+	+	+
Amylase	+	+	+	+	+	+	+	+	+	+	+	+
Gelatinase	+	-	+	+	-	+	+	-	-	+	+	+
Lipase	+	-	-	+	+	-	+	-	-	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+
Caseinase	+	-	-	-	+	+	+	-	-	-	+	-
Fermentation of sugars												
Glucose	+	+	+	+	+	+	+	+	+	-	+	+
Fructose	+	-	+	+	+	+	-	-	+	-	-	+
Sucrose	+	+	+	+	+	+	-	+	+	-	+	+
Galactose	+	-	-	+	+	-	-	+	+	-	+	+
Maltose	+	-	+	-	-	+	-	-	+	-	+	+
Lactose	+	-	-	-	+	+	-	+	+	-	-	+

"+" indicates positive response; "-" indicates negative response; "G(+)" indicates Gram-positive; "G(-)" indicates Gram-negative

Table 3. Primary Screening of Bacterial Endophytes Isolated From *R. serpentina* for Antimicrobial Activity Following Cross-Streak Method

Plant Part	Isolate no.	Length of Inhibition Zone, mm				
		Test Organisms				
		Bacillus subtilis	*Staphylococcus aureus*	*Pseudomonas cepacia*	*Escherichia coli*	*Klebsiella pneumoniae*
Leaf	RAU 101	2.5 ± 0.1	3.5 ± 0.2	NI	5.0 ± 0.1	2.0 ± 0.0
	RAU 102	NI	NI	NI	5.5 ± 0.0	1.0
	RAU 103	NI	NI	NI	3.0 ± 0.0	NI
	RAU 104	NI	NI	NI	NI	NI
Stem	RAU 201	NI	NI	3.5 ± 0.1	NI	NI
	RAU 202	NI	2.0 ± 0.1	NI	7.0 ± 0.1	NI
Root	RAU 301	NI	NI	7.5 ± 0.1	11.0 ± 0.1	12.0 ± 0.1
	RAU 302	NI	NI	NI	2.0 ± 0.1	NI
	RAU303	NI	NI	NI	NI	NI
	RAU 304	NI	NI	3.5 ± 0.1	3.0 ± 0.1	NI
	RAU 305	21.0 ± 0.7	22.5 ± 0.9	NI	17.0 ± 0.7	15.0 ± 1.0
	RAU 306	5.0 ± 0.6	NI	NI	6.0 ± 0.0	NI

'NI' = no inhibition.
Results represents mean of triplicate experiments ± standard error.

Table 4. Secondary Screening of Bacterial Endophytes Isolated From *R. serpentina* for Production of Antimicrobial Substances Following Agar Well Diffusion Assay

Plant Part	Isolate no.	Diameter of Inhibition Zone, mm				
		Test Organisms				
		Bacillus subtilis	*Staphylococcus aureus*	*Pseudomonas cepacia*	*Escherichia coli*	*Klebsiella pneumoniae*
Leaf	RAU 101	NI	NI	NI	NI	NI
	RAU 102	NI	NI	NI	8.5 ± 0.5	NI
	RAU 103	NI	NI	NI	NI	NI
	RAU 201	NI	NI	NI	NI	NI
Stem	RAU 202	NI	NI	NI	8.5 ± 0.5	NI
	RAU 301	NI	NI	NI	9.0	10.0
Root	RAU 302	NI	NI	NI	NI	NI
	RAU 304	NI	NI	NI	NI	NI
	RAU 305	27.0 ± 0.9	28 ± 0.7	NI	25.0 ± 1.1	26.0 ± 1.1
	RAU 306	8.5 ± 0.6	NI	NI	8.5 ± 0.7	NI
	RAU 305	21.0 ± 0.7	22.5 ± 0.9	NI	17.0 ± 0.7	15.0 ± 1.0
	RAU 306	5.0 ± 0.6	NI	NI	6.0 ± 0.0	NI

'NI' = no inhibition.
Results represents mean of triplicate experiments ± standard error.

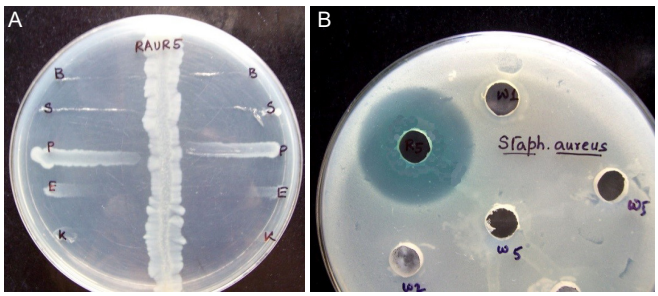

Figure 2. Demonstration of Antimicrobial Activity of Root Endophyte Pseudomonas RAU 305 by Cross-Streak Method (A) Against Test Organisms (B = *Bacillus subtilis*, S = *Staphylococcus aureus*, P = *Pseudomonas cepacia*, E = *Escherichia coli*, K = *Klebsiella pneumoniae*) and Agar Well Diffusion Assay (B) Against *Staphylococcus aureus* in Muller Hinton Agar Plates.

sequence of RAU 305 has been deposited in the GenBank under the accession number KR816098. Evolutionary relationship of *P. aeruginosa* RAU 305 was depicted from the dendrogram that showed clear rooted evolution (Figure 3).

Growth Associated Production of Antibacterial Compound

Time course of growth and antibiotic production by *Pseudomonas* RAU 305 was monitored in tryptic soy broth as well as Lindenbein synthetic medium and antibacterial activity of the cell-free culture filtrates was assayed at regular time intervals following agar well diffusion assay using *S. aureus* as the test organism (Figure 4). It was revealed that the production of antimicrobial substance was initiated on the onset of stationary phase of growth (24 hours) and maximum

Table 5. Morphological, Physiological and Biochemical Characterization of Endophytic Bacterial Isolate RAU 305

Characters	Response
Colony morphology	Round, elevated, translucent, creamish, smooth margin
Cell morphology	Short rod, mostly solitary
Gram nature	Gram –ve
Optimal growth temperature	32ºC
Optimal growth pH	7.2
NaCl tolerance range	0.5 – 1.3%
Growth on Mac Conkey agar	-
Growth on King's A medium	+
Growth on King's B medium	+
Reduction of nitrate	+
Indole production	+
Decarboxylation of lysine and ornithine	+
ONPG test	-
Urease production	-
H_2S production	+
Siderophore production	+
Phenylalanine deamination	+
Polyhydroxyalkanoates production	-
Esculine hydrolysis	-
Methyl Red and Voges Proskauer test	-
Utilization of glucose, sucrose, galactose, maltose, glycerol, arabinose, xylose, saccharose	+
Malonate and citrate fermentation	+
Fermentation of adonitol, rhamnose, cellobiose, melibiose, raffinose, trehalose	-
Antibiotic sensitivity profile	Am^r, Amp^r, B^r, E^r, M^r, Nv^r, P^r, Pb^r, R^r, VA^r, Cf^s, G^s, Nx^s, S^s, TE^s

'r', resistant; 's', sensitive; Am, Amoxycillin; Amp, Ampicillin; B, Bacitracin; E, Erythromycin; M, Methicillin; Nv, Novobiocin; P, Penicilin G; Pb, Polymyxin B; R, Rifampicin; VA, Vancomycin; Cf, Ciprofloxacin; G, Gentamycin; Nx, Nalidixic acid; S, Streptomycin; TE, Tetracycline.
+ sign indicates positive response and - sign indicates negative response.

production was achieved after 72 hours in complex medium (Figure 4A), whereas in synthetic medium, growth was much delayed and antimicrobial activity was initiated during mid-log phase (60 hours) that reached maximum after 102 hours of growth (Figure 4B).

Antimicrobial Spectrum

Antimicrobial spectrum of the active compound elaborated by *Pseudomonas* RAU 305 was established through agar well diffusion assay against different pathogenic test bacterial and fungal strains. Results enumerated in Table 6 demonstrated that the active compound of the cell-free culture filtrate was potentially antibacterial being effective against *Bacillus cereus, B. amyloliquefaciens, Paenibacillus polymyxa, P. amylolyticus, Micrococcus luteus, Arthrobacter citreus, Rhodobacter sphaeroides* and *Mycobacterium smegmatis*. Antifungal activity of the compound was restricted only against *Aspergillus niger, Colletotrichum* sp. however, *Pythium* sp., *Alternaria* and *Penicillium* spp. remained unaffected.

Isolation and Partial Purification of Antimicrobial Compound

In an attempt towards isolation and purification of the active

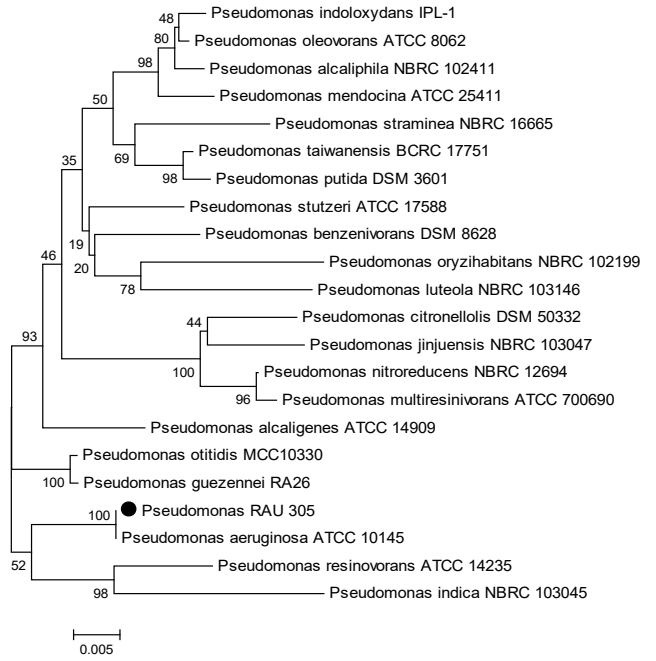

Figure 3. Phylogenetic Relationship of *Pseudomonas* RAU 305 With Closely Allied NCBI Library Strains Based on 16S rDNA Sequence Analysis. Evolutionary relationships were computed using Neighbour-Joining method and analysis was conducted in MEGA 7.0. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site.

component, *Pseudomonas* RAU 305 was grown in tryptic soy broth for 72 hours under continuous shaking (120 rpm) and the antimicrobial compound was extracted from the cell-free culture filtrate with different solvents (2:1 v/v) such as benzene, hexane, diethyl ether, petroleum ether, chloroform, n-butanol and ethyl acetate. The individual solvent fractions were evaporated to dryness, dissolved in distilled water, filter sterilized and assayed for antibacterial activity against *S. aureus* by the usual agar well diffusion method. Maximum antibacterial activity was observed in the butanol fraction followed by chloroform. However, hexane as a solvent was not inferior (Table 7). The butanol and chloroform fractions were further purified following silica gel column chromatography.

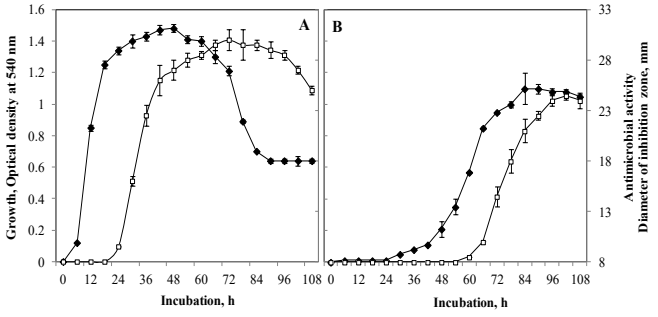

Figure 4. Time Course of Growth (-◆-) and Production of Antimicrobial Substances (-□-) in Tryptic soy broth (A) and Lindenbien Synthetic Medium (B) by *P. aeruginosa* RAU 305. Diameter of inhibition zone was measured in mm against the test organism *S. aureus*.

Table 6. Antimicrobial Spectrum of Bacterial Endophyte *P. aeruginosa* RAU 305 Against Test Bacterial and Fungal Strains

Test Organism	Diameter of Inhibition Zone, mm	
	48 h	96 h
Bacteria		
Arthrobacter citreus	9.5 ± 0.1	20.0 ±0.1
Acinetobacter baumanii	12.0 ± 0.5	19.0 ± 1.0
Bacillus cereus	15.0 ± 1.0	25.0 ± 1.0
Bacillus amyloliquefaciens	12.5 ± 0.5	25.0 ± 0.5
Micrococcus luteus	11.5 ± 0.5	25.5 ± 0.1
Mycobacterium smegmatis	12.0 ± 0.1	23.0 ± 0.3
Paenibacillus polymyxa	10.0 ± 0.2	24.5 ± 0.2
Paenibacillus amylolyticus	9.0 ± 0.2	19.5 ± 0.1
Rhodobacter sphaeroides	12.0 ± 0.4	24.0 ±0.3
Fungi		
Aspergillus niger	12.0 ± 0.1	26.0 ± 0.5
Colletotrichum sp.	11.5 ± 0.1	23.5 ± 0.6
Pythium sp.	8.2 ± 0.1	9.5 ± 0.1
Alternaria alternata	NI	NI
Alternaria solani	NI	NI
Penicillum sp.	NI	NI

NI= No inhibition.
Antimicrobial activity was demonstrated following agar well diffusion assay.
Results represents mean of triplicate experiments ± standard error.

Table 7. Extraction of Antimicrobial Substance From the Cell-Free Culture Filtrate of *P. aeruginosa* RAU 305 Using Different Solvents

Extraction Solvents	Agar Well Diffusion Assay	
	Diameter of Inhibition Zone, mm	
	Bacillus subtilis	***Staphylococcus aureus***
Benzene	21 ± 1.0	16 ± 0.5
Hexane	23 ± 0.8	21 ± 0.6
Diethyl ether	15 ± 0.8	15 ± 0.5
Petroleum ether	17 ± 0.5	14 ± 0.5
Chloroform	26 ± 0.5	26 ± 1.0
Butanol	26 ± 1.0	29 ± 1.0
Ethyl acetate	10 ± 0.5	12 ± 0.1

NI = No Inhibition.
Antimicrobial activity was demonstrated following agar well diffusion assay.
Results represents mean of triplicate experiments ± standard error.

A total of 15 fractions (20 mL each) were collected using chloroform : methanol (2:1) mixture as the elutant. The active fractions (No. 11-12) were pooled, evaporated to dryness and further separated by thin layer chromatography using different solvent systems. Homogeneity of the active compound was indicated by a single spot with Rf values ranging from 0.16–0.8 and 0.12–0.72 for butanol and chloroform fractions respectively (Table 8). Each of these spots after elution in the same solvent, exhibited inhibitory activity against both *S. aureus* and *B. subtilis*.

Table 8. Thin layer chromatographic separation and Rf values of antimicrobial compound produced by *P. aeruginosa* RAU 305

Solvent system	Rf	
	Butanol extract	Chloroform extract
Chloroform : Methanol (1:1)	0.80	0.71
Butanol : Acetic Acid : Water (3:1:1)	0.69	0.72
Isopropanol : Ammonia : Water (8:1:1)	0.75	0.55
Ethyl acetate : Chloroform (9:1)	0.70	0.51
Chloroform : Acetone (9:1)	0.22	0.2
Toluene : Acetone (4:1)	0.16	0.12

The spots were detected in iodine vapour chamber as well as exposure under UV light.

Discussion

The ethnomedicinal plant *R. serpentina* (L.) Benth. ex. Kurz (Indian serpent wood) has been exploited to a large extent for several bioactive metabolites which are used in treatment of insanity, snake bite, gastrointestinal disorders like diarrhoea, dysentery, cholera, etc.[19,20] Although several studies report the occurrence of multiple natural products with medicinal properties, the endophytic biology of this plant is relatively underexplored. This study aims towards exploration of endophytic bacterial resources associated with root, leaf and stem of *R. serpentina* for production of antimicrobial compounds.

Microbiological analysis of endophytic bacterial population in root, stem and leaf tissues of *R. serpentina* clearly revealed that the colonization frequency of endophytes varied widely in different tissues of the plant (Table 1) which might be due to variation in tissue texture, age of the organ, physiology, biochemistry and microenvironmental parameters of the plant.[21,22] Moreover, the efficacy of isolation of endophytes solely depends on the degree of effectiveness of surface sterilization processes.[23] The standard surface sterilization[10] followed by incubation of plant segments in media of different composition resulted in the isolation of only 12 phenotypically distinguishable bacterial strains by avoiding the repetitive ones (Table 2). More detailed morphological, physiological, biochemical and genetic characterisation of all these isolates leading to determination of their taxonomic identity, however, have not been undertaken during the course of this research.

While screening for antimicrobial activity, each of the endophytic bacterial isolates were subjected to preliminary screening by cross-streak method followed by secondary screening which assessed the inhibitory potential of the cell-free culture filtrate by agar well diffusion method.[24] The majority of the isolated endophytic bacteria were selectively inhibitory to only *E. coli* as against the potent isolate RAU 305 (Figure 3) which displayed comparatively broader antibacterial activity in both primary and secondary screening (Tables 3 and 4). The isolate RAU 305, identified as *P. aeruginosa* RAU 305 based on morphological, physio-biochemical and 16S rDNA sequence analysis (GenBank accession no. KR816098) appeared to be a close relative (99.9% similarity) of *P. aeruginosa* ATCC 10145 (Figure 3).

Scientific evidences proving the predominance of pseudomonads as endomicrobiome have been lately reported[25,26] and have been extensively exploited for their secondary metabolites towards improving plant growth and as biocontrol agents.[27] The present strain *P. aeruginosa* RAU 305 also showed broad spectrum antibacterial activity along

with selective antifungal activity against *Aspergillus* and *Colletotrichum* spp. (Table 6). Endophytic fungal assemblages of *R. serpentina* have also been exploited as an alternative source of natural hypocholesterolemic, antimicrobial, antioxidant and anti-phytopathogenic compounds.[12,13] A novel family of peptide antimycotics, termed ecomycins, have been described from plant associated *Pseudomonas viridiflava* showing inhibitory response against a wide number of human and plant pathogenic fungi.[6]

Contrary to the Lindenbein synthetic broth, growth associated production of antimicrobial compound during batch culture of *Pseudomonas* RAU 305 was promoted by the complex organic constituents of the tryptic soy broth (Figure 4). Production of pyrrolnitrin and chloropyrrolnitrin antibiotics by *P. cepacia* isolated from apple leaf was found to be higher in cultures grown in minimal salt's broth as compared to nutrient broth or King's B medium.[28] However, the production of antimicrobials by *P. fluorescens* was stimulated in diluted nutrient broth-yeast extract medium amended with glucose or glycerol.[29] It has also been reported that constituents of the medium including presence of minerals exerted control over the concentration of phenazine antimicrobial compounds produced by different strains of *P. aeruginosa*.[30,31]

The active compound elaborated by *Pseudomonas* RAU 305 was extracted effectively and efficiently from the cell-free culture supernatant with butanol and chloroform (Table 7) which supported the earlier findings of Atta and Radwan[32] and Rosales et al.[31] The active compound was further purified by silica gel column chromatography and the homogeneity of the compound was established through TLC separation using different solvent systems[31,33,34] and a single spot showing the presence of active component was detected under UV light or iodine vapour (Table 8). The TLC analysis substantiates the findings of several research[31,32,35,36] who observed an Rf value ranging from 0.5 to 0.81 for extracted antimicrobial compounds including phenazines from several strains of *P. aeruginosa* using different solvent systems.

Conclusions

This research, as far as we are aware is the first report on the occurrence of bacterial endophytes in root, stem and leaf tissues of *R. serpentina* (L.) Benth. ex. Kurz. and enumeration of their antimicrobial potentials under *in vitro* conditions. *P. aeruginosa* RAU 305, the root endophyte showed a broad spectrum of antimicrobial activity being both antibacterial as well as antifungal. The active component has been isolated from the cell-free culture filtrate and partially purified by chromatographic methods. However, a more detailed characterisation of the antimicrobial compound is essential not only to establish its chemical nature but also for future exploitation.

Authors' Contributions

Both authors contributed equally to this research.

Conflict of Interest Disclosures

The authors declare they have no conflicts of interest.

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References

1. Berg G, Grube M, Schloter M, Smalla K. The plant microbiome and its importance for plant and human health. Front Microbiol. 2014;5:491. doi:10.3389/fmicb.2014.00491.
2. Busby PE, Soman C, Wagner MR, et al. Research priorities for harnessing plant microbiomes in sustainable agriculture. PLoS Biol. 2017;15(3):e2001793. doi:10.1371/journal.pbio.2001793.
3. Cardoso Filho CA. Production and application of novel bio-active compounds by endophytic microbes. In: Akhtar M, Swamy M, Sinniah U, eds. Natural Bio-active Compounds. Singapore: Springer; 2019. p. 1-40. doi:10.1007/978-981-13-7154-7_1.
4. Reinhold-Hurek B, Hurek T. Living inside plants: bacterial endophytes. Curr Opin Plant Biol. 2011;14(4):435-443. doi:10.1016/j.pbi.2011.04.004.
5. Hardoim PR, van Overbeek LS, Berg G, et al. The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. Microbiol Mol Biol Rev. 2015;79(3):293-320. doi:10.1128/mmbr.00050-14.
6. Strobel GA. Endophytes as sources of bioactive products. Microbes Infect. 2003;5(6):535-544. doi:10.1016/s1286-4579(03)00073-x.
7. Miller CM, Miller RV, Garton-Kenny D, et al. Ecomycins, unique antimycotics from *Pseudomonas viridiflava*. J Appl Microbiol. 1998;84(6):937-944. doi:10.1046/j.1365-2672.1998.00415.x.
8. Strobel G, Daisy B, Castillo U, Harper J. Natural products from endophytic microorganisms. J Nat Prod. 2004;67(2):257-268. doi:10.1021/np030397v.
9. Ryan RP, Germaine K, Franks A, Ryan DJ, Dowling DN. Bacterial endophytes: recent developments and applications. FEMS Microbiol Lett. 2008;278(1):1-9. doi:10.1111/j.1574-6968.2007.00918.x.
10. Sun J, Guo L, Zang W, Ping W, Chi D. Diversity and ecological distribution of endophytic fungi associated with medicinal plants. Sci China C Life Sci. 2008;51(8):751-759. doi:10.1007/s11427-008-0091-z.
11. Craig WJ. Health-promoting properties of common herbs. Am J Clin Nutr. 1999;70(3 Suppl):491S-499S. doi:10.1093/ajcn/70.3.491s.
12. Doley P, Jha D. Entophytic fungal assemblages from ethnomedicinal plant *Rauvolfia serpentina*. J Mycol Plant Pathol. 2010;40(1):44-48.
13. Nath A, Chattopadhyay A, Joshi SR. Biological activity of endophytic fungi of *Rauwolfia serpentina* Benth: an ethnomedicinal plant used in folk medicines in Northeast India. Proc Natl Acad Sci India Sect B Biol Sci. 2015;85(1):233-240. doi:10.1007/s40011-013-0184-8.
14. Akshatha VJ, Nalini MS, D'Souza C, Prakash HS. Streptomycete endophytes from anti-diabetic medicinal plants of the Western Ghats inhibit alpha-amylase and promote glucose uptake. Lett Appl Microbiol. 2014;58(5):433-439. doi:10.1111/lam.12209.
15. Pielou E. Ecological Diversity. New York: John Wiley & Sons; 1975.
16. Bergey DH, Hendricks D, Holt JG, Sneath PH. Bergey's Manual of Systematic Bacteriology. Williams & Wilkins; 1984. Vol. 2
17. Smibert RM, Krieg NR. Phenotypic characterization. In: Gerhardt P, Murray RGE, Wood WA, Krieg NR, eds. Methods for General and Molecular Bacteriology. Washington, DC: American Sociiety for Microbiology; 1994. p. 607-654.
18. Difco Manual (Tenth Edition): Dehydrated Culture Media and Reagents for Microbiology. 10th ed. Difco Laboratories; 1984.
19. Dey A, De JN. Ethnobotanical aspects of *Rauvolfia serpentina* (L). Benth. ex Kurz. in India, Nepal and Bangladesh. J Med Plants Res. 2011;5(2):144-150. doi:10.5897/jmpr.9000585.
20. Qureshi SA, Nawaz A, Udani SK, Azmi B. Hypoglycaemic and hypolipidemic activities of *Rauwolfia serpentina* in alloxan-induced diabetic rats. Int J Pharmacol. 2009;5(5):323-326. doi:10.3923/ijp.2009.323.326.
21. Arnold AE, Maynard Z, Gilbert GS. Fungal endophytes in

dicotyledonous neotropical trees: patterns of abundance and diversity. Mycol Res. 2001;105(12):1502-1507. doi:10.1017/s0953756201004956.
22. Liu H, Carvalhais LC, Crawford M, et al. Inner plant values: diversity, colonization and benefits from endophytic bacteria. Front Microbiol. 2017;8:2552. doi:10.3389/fmicb.2017.02552.
23. Yang CJ, Zhang XG, Shi GY, et al. Isolation and identification of endophytic bacterium W4 against tomato *Botrytis cinerea* and antagonistic activity stability. Afr J Microbiol Res. 2011;5(2):131-136.
24. Balouiri M, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial activity: a review. J Pharm Anal. 2016;6(2):71-79. doi:10.1016/j.jpha.2015.11.005.
25. Oteino N, Lally RD, Kiwanuka S, et al. Plant growth promotion induced by phosphate solubilizing endophytic *Pseudomonas* isolates. Front Microbiol. 2015;6:745. doi:10.3389/fmicb.2015.00745.
26. Pratiwi RH, Hidayat I, Hanafi M, Mangunwardoyo W. Antibacterial compound produced by *Pseudomonas aeruginosa* strain UICC B-40, an endophytic bacterium isolated from *Neesia altissima*. J Microbiol. 2017;55(4):289-295. doi:10.1007/s12275-017-6311-0.
27. Bajpai A, Johri BN. Endophytic *Pseudomonads* and their metabolites. In: Jha S, ed. Endophytes and Secondary Metabolites. Reference Series in Phytochemistry. Cham: Springer; 2019. p. 33-59. doi:10.1007/978-3-319-90484-9_8.
28. Roitman JN, Mahoney NE, Janisiewicz WJ. Production and composition of phenylpyrrole metabolites prodcued by *Pseudomonas cepacia*. Applied Microbiol Biotechnol. 1990; 34(3):381-386. doi:10.1007/bf00170064.
29. Duffy BK, Défago G. Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescens* biocontrol strains. Appl Environ Microbiol. 1999;65(6):2429-2438. doi:10.1128/aem.65.6.2429-2438.1999.
30. El-Shouny WA, Al-Baidani AR, Hamza WT. Antimicrobial activity of pyocyanin produced by *Pseudomonas aeruginosa* isolated from surgical wound-infections. Int J Pharm Med Sci. 2011;1(1):1-7.
31. Rosales AM, Thomashow L, Cook RJ, Mew TW. Isolation and identification of antifungal metabolites produced by rice-associated antagonistic *Pseudomonas* spp. Phytopathology. 1995; 85(9):1028-1032. doi:10.1094/Phyto-85-1028.
32. Atta HM, Radwan HG. Biochemical studies on the production of Sparsomycin antibiotic by *Pseudomonas aeurginosa*, AZ-SH-B8 using plastic wastes as fermented substrate. J Saudi Chem Soc. 2012;16(1):35-44. doi:10.1016/j.jscs.2010.10.019.
33. Atta HM, Abul-Hamd AT, Radwan HG. Production of destomycin-A antibiotic by *Streptomyces* sp. using rice straw as fermented substrate. Commun Agric Appl Biol Sci. 2009;74(3):879-897.
34. Zhang Z, Xie J, Zhang F, Linhardt RJ. Thin-layer chromatography for the analysis of glycosaminoglycan oligosaccharides. Anal Biochem. 2007;371(1):118-120. doi:10.1016/j.ab.2007.07.003.
35. Kavitha K, Mathiyazhagan S, Sendhilvel V, Nakkeeran S, Chandrasekar G, Dilantha Fernando WG. Broad spectrum action of phenazine against active and dormant structures of fungal pathogens and root knot nematode. Arch Phytopath Plant Prot. 2005;38(1):69-76. doi:10.1080/03235400400008408.
36. Ohfuji K, Sato N, Hamada-Sato N, et al. Construction of a glucose sensor based on a screen-printed electrode and a novel mediator pyocyanin from *Pseudomonas aeruginosa*. Biosens Bioelectron. 2004;19(10):1237-1244. doi:10.1016/j.bios.2003.11.010.