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Antibacterial and antioxidant potentials, detection of host origin compounds, and metabolic profiling of endophytic Bacillus spp. isolated from *Rauvolfia serpentina* (L.) Benth. ex Kurz

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The research highlights the importance of exploring endophytic microbiomes of medicinal plants to uncover their potential for secondary metabolite production and their role in the biosynthesis of host-derived compounds. This study was aimed to isolate leaf endophytic bacteria of *Rauvolfia serpentina*, investigate their antibacterial, antioxidant potentials and detect host-origin compound reserpine using Reverse Phase High-Performance Liquid Chromatography (RPHPLC). Untargeted analysis via Ultra High-Performance Liquid Chromatography-High-Resolution Mass Spectrometry (UHPLC-HRMS/MS) was conducted for profiling main phytochemicals in the leaves and to explore potential bioactive compounds in bacterial extracts. Nine bacterial isolates were obtained from *R. serpentina* leaves. These isolates exhibited positive results in various biochemical tests including indole production, methyl red, Voges-Proskauer, citrate utilization, catalase and oxidase production, nitrate reduction, oxidative fermentation, and citrate reduction tests. Endophytic isolates RSLB3 and RSLB18 exhibited most potential antibacterial activity against tested human pathogenic bacteria and were identified as *Bacillus* sp. The extract of RSLB3 and RSLB18 also showed significant antioxidant activity compared to leaf extract. The total phenol content was similar in both these isolates while flavonoids content and DPPH scavenging activity was higher in isolate RSLB3. RPHPLC analysis confirmed the presence of reserpine in bacterial metabolites when compared to a standard reference. UHPLC-HRMS profiling unveiled a diverse range of host-derived compounds and reaction intermediates with known and unknown bioactive properties in leaf extract, RSLB3, and RSLB18. To our knowledge, this is the first study to achieve a comprehensive profiling.

Keywords Alkaloid, *Bacillus*, Endophytes, Reserpine, Secondary metabolites

Rauvolfia serpentina (L.) Benth. ex Kurz. is an evergreen, woody, glabrous and perennial shrub with a maximum height of 60 cm. The plant belongs to the family Apocynaceae and occurs in habitats of tropical and subtropical regions. The family is distributed worldwide in the region of the Himalayas, Indian peninsula, Myanmar, Indonesia and Sri Lanka and is native to India, Bangladesh and other regions of Asia[1] (Fig. 1). In Ayurvedic literature, the powdered root of this plant has been used for the treatment of snake bites, feverish illnesses and mental illness from ancient time. The available reports suggest for its antimicrobial, anti-inflammatory, antioxidant, anti-proliferative, anticancerous, antidiuretic, antifibrillar, antiarrhythmic, anticholinergic, anti-dysentery, antidiarrhoeal, anti-hypotensive, anti-contractile, sympathomimetic and tranquilizing activity[1,2].

The plant contains more than 70 distinct alkaloids belonging to the monoterpene indole alkaloid (MIA) family. These alkaloids are extensively located in the roots. The major indole alkaloids with biological and therapeutic potential reported from various *Rauvolfia* species are reserpine, serpentine, ajmaline, deserpidine,

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Fig. 1. *R. serpentina* plants in their natural habitat and distribution: Ref: WFO (2024): *Rauvolfia serpentina* (L.) Benth. ex Kurz.

indobine, reserpiline, rescinnamine, and yohimbine[3]. Alkaloids have therapeutic significance in treatment of cardiovascular diseases, hypertension, breast cancer, and human promyelocytic leukemia[4], psychotic disorders like schizophrenia, anxiety, epilepsy, insomnia, insanity, and furthermore, utilized as a sedative, a hypnotic drug[5]. *Rauvolfia*has also been studied widely in research as a treatment for autistic children between the ages of 3.5 and 9 years[6].

Endophytic microbes have been studied in majority of plant species, existing in either obligate or facultative relationships without causing harm to their host. Their interaction represents a highly researched domain aimed at deciphering their roles in various aspects[7]. Host plants offers nutrients and a conducive environment for endophytes, which in turn aid in plant growth, disease management, phytoremediation, sustainable crop production, and the secondary metabolites production that aid the plants in resisting biotic and abiotic stresses. They are well-known for their ability to produce a wide range of pharmacologically important compounds (Taxol, Swainsonine, Huperzine, Rohutikine, Kaempferol, Maytansine, etc.,) with enormous therapeutic potentials that are similar or even identical to those of their hosts, especially medicinal plants[8]. These bioactive compounds have been identified as antiviral, antifungal, antibacterial, antitumor and anticancer agents[9]. Many antimicrobial compounds produced by endophytes belong to diverse structural classes such as peptides, alkaloids, steroids, quinones, terpenoids, phenols, and flavonoids[10].

Our goal was to isolate and characterize endophytic bacteria from *R. serpentina* leaves, evaluate their antimicrobial potential,and detect host-derived compounds using RP-HPLC, as well as perform untargeted metabolic profiling via UHPLC-HRMS. Thus the study aims to explore the untapped potential of bacterial endophytes in producing both host-origin and novel bioactive metabolites of microbial origin. The overexploitation of *R. serpentina* for medicinal value especially for reserpine has threatened plant in an endangered stage. The metabolic profiling of *Rauvolfia* endophytes will offer a significant potential for discovering host-origin compounds and a hope to control the exploitation of medicinal plant. The bacterial endophytes can be easily optimized under a specific culture condition for fermentation and elucidating secondary metabolite pathways.

Materials and methods

Collection of plant samples

Healthy plant samples of *R. serpentina* were randomly collected during the month of April-May 2018 from five sites around the Botanical garden of Department of Botany, Banaras Hindu University, Varanasi, India.

Isolation and biochemical characterization of endophytic bacteria

The endophytic bacteria were isolated from leaves of *R. serpentina* plants[11]. The plant parts were thoroughly washed with running tap water to remove soil particles and attached microbes. Leaves were individually surface sterilized. by serial washing in 70% ethanol (1 min), 2% sodium hypochlorite solution (2% available Cl^{-}) for 3–4 min and 70% ethanol (60 s) followed by rinsing three times with sterile distilled water. Isolation was performed using the direct plating method on nutrient agar (NA) medium supplemented with 2% sodium chloride (NaCl). The sterilized leaves were cut into 0.5 cm x 0.5 cm sections. A minimum of five sections were placed on NA plates and incubated at 35 ± 2 °C for one week. The plates were checked every 24 h for bacterial colony emergence. The emerging bacterial colonies were selected, sub-cultured, purified, and used for further studies. The bacterial isolates were characterized after 48 h for the following traits: color, form, elevation, margin, surface texture, and opacity. Biochemical tests such as indole production, methyl red and Vogues-Proskauer tests, citrate utilization, catalase and oxidase production tests, nitrate reduction, and oxidative fermentation tests were performed for the endophytic isolates[12–14]. Amylase, protease, lipase and cellulase tests were also performed for the endophytic isolates following standard protocols[15,16].

Extraction of secondary metabolites of endophytic bacterial isolates

Previously isolated leaf endophytic bacterial strains were subjected to secondary metabolite extraction. A suspension of 10^7 CFU.mL^{-1} of endophytic bacteria was inoculated into 1 L of nutrient broth (NB) and incubated at 35 ± 2 °C for 4–5 days in a BOD shaker incubator at 150 rpm (Supertech, New Delhi). After incubation, the

broth was centrifuged at 8000 × g for 20 min at room temperature. Secondary metabolites were extracted from the supernatant using a solvent extraction method. Equal volumes of ethyl acetate and culture filtrate were combined in a separatory funnel and shaken vigorously for 10 min. The ethyl acetate layer was then separated and concentrated using a Rotary Evaporator (Ika, Germany).

Antibacterial activity of metabolites against human pathogenic bacteria

The disc diffusion assay was conducted on Mueller-Hinton agar (MHA) plates against eight human pathogenic bacteria to identify the most potent crude secondary metabolites produced by the endophytic bacteria[17]. Secondary metabolites showing significant zones of inhibition against the majority of pathogens were selected for determination of minimum inhibitory concentration (MIC) values. MIC values for two standard antibiotics and secondary metabolites were determined by broth dilution well plate assay in 96-well microtitre plates following established protocols[18]. In broth dilution method crude extract were prepared and subjected to serial dilution (0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, 35.6 mg.mL^{-1}) using Mueller–Hinton broth and dimethyl sulfoxide (DMSO) in a 1:1 ratio. Tetracycline, nalidixic acid, and streptomycin served as standard positive controls (0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, 35.6 µg.mL^{-1}, while a 1:1 ratio of DMSO and Mueller–Hinton broth was utilized as the negative control. Each well of the microplate was inoculated with 75 µl of a 24-hour cultured broth of human pathogenic bacteria containing 10^7 CFU.mL^{-1}, followed by addition of blank Mueller–Hinton broth. Antibiotic solutions and test metabolites (75 µL) were added to separate wells, resulting in a final assay volume of 150 µL. Absorbance readings were measured at 630 nm using a microplate reader (Agilent BioTek Synergy HTX Multimode Reader) immediately after inoculation to establish the baseline (*T*0). Plates were then incubated at 35 ± 2 °C without shaking until the stationary phase was reached. Absorbance was measured for *TF* (Final Time i.e. 48 h). The inhibition percentage was calculated as:

$$\%\ \boldsymbol{Inhibition} = 1 - \left(\frac{\boldsymbol{TFsample} - \boldsymbol{T0sample}}{\boldsymbol{TFsample}}\right)\ \boldsymbol{X}\ 100$$

The *T*0 sample and *TF* sample represent the absorbance at 630 nm of bacterial growth in the presence of test metabolites before and after incubation, respectively. Inhibition values below 10% were deemed insignificant. Bacterial pathogens from wells were reinoculated onto MHA plates to observe colony appearance and determine the MIC of antibiotics and test metabolites.

Molecular identification of endophytic bacterial isolates and their phylogenetic characterization

Genomic DNA of isolates showing antibacterial activity was extracted following Wilson[19]. The isolated genomic DNA strains were analysed on 0.8% agarose gel (Himedia) and amplification of the 16S rRNA (~ 1500 bp) gene was carried in PCR using universal primers [Forward primer (27 F) AGAGTTTGATCCTGGCTCAG; Reverse primer (1492R), CGGTTACCTTGTTACGACTT (Eurofin Genomics, India)]. PCR was performed with 25 µl reaction mixture composed of PCR buffer 2.5 µl; $MgCl_2$ 1.5 µl; 0.2 mm dNTPs 2 µl; Primers each 1.25 µl; Taq DNA polymerase 0.25 µl (Thermo Fischer Scientific); template DNA 2 µl; PCR water 14.75 µl. The PCR conditions were as follows: initial denaturation 94 °C for 4 min, followed by 30 cycles of 30 s denaturation at 94 °C, 1 min annealing at 55 °C, 1 min extension at 72 °C and a 7-min final extension at 72 °C. The PCR products were purified before they were sent for sequencing.

The PCR products of the endophytes were sequenced by BIOKART INDIA Pvt. Ltd (Bengaluru, India) by Sanger's dideoxy nucleotide sequencing method. All obtained sequences were compared with those in the GenBank database by using the BLASTN search program. Similar sequences were further aligned by CLUSTALW. Phylogenetic analysis was conducted by the neighbour-joining method. Bootstrap analysis was performed with 1000 replications to determine the support for each clade with grouping by the neighbour-joining method. A phylogenetic tree was constructed based on evolutionary distance data by using MEGA 11. The 16S rRNA nucleotide sequences acquired have been submitted to the National Center for Biotechnology Information (NCBI) GenBank to secure accession numbers. The unique accession numbers assigned to the bacterial isolates were MW7415340 and MW7415343.

Biochemical characterization of metabolites

Determination of total phenolic and total flavonoid content

The total phenolic content in both the leaf extract and bacterial secondary metabolites was assessed using the Folin-Ciocalteu method[20], with Gallic acid as the standard. Phenolic compound levels were expressed as milligrams of Gallic acid equivalents (mg GAE.mg^{-1} dw). Total flavonoid content was quantified using the $AlCl_3$ method[21], expressed as mg Quercetin equivalents (mg QE.mg^{-1} dw) of extract.

Determination of total antioxidant capacity (TAC), FRAP assay, and DPPH radical scavenging activity

The total antioxidant capacity of the test metabolites was evaluated according to the method described by Prieto et al. 1999[22]. The absorbance of the aqueous solution of each sample was measured at 695 nm against blank. The antioxidant capacity was estimated using the following formula:

$$\textbf{Total antioxidant capacity}\ \% = \left(\frac{\boldsymbol{As} - \boldsymbol{Ac}}{\boldsymbol{As}}\right)\ \boldsymbol{X}\ 100$$

Where, *Ac* is absorbance of negative control; *As* is absorbance of sample at time (t) = 20 min.

A concentration range (0.25, 0.5, 1, 2, 4, 8, 16, 32 mg.mL^{-1}) of the leaf extracts and crude secondary metabolites of RSLB3 and RSLB18 was prepared for determining the EC_{50} (Effective concentration). Total free radical scavenging capacity of the *R. serpentina* leaf extract and secondary metabolite of endophytic bacteria were estimated following Blois et al. (1958)[23] with slight modification using the stable DPPH radical, which has an absorption maximum at 515 nm. A concentration range (0.25, 0.5, 1, 2, 4, 8, 16, 32 mg.mL^{-1}) of the leaf extracts and crude secondary metabolites of RSLB3 and RSLB18 was prepared for determining the IC_{50}. A calibration curve was plotted with % DPPH scavenged versus concentration of standard antioxidant (Ascorbic acid and Gallic acid). DPPH scavenging activity was calculated by using the following equation:

$$\%\ \textbf{Radical scavenging activity} = 1 - \left(\frac{\boldsymbol{As} - \boldsymbol{Ac}}{\boldsymbol{As}}\right) \boldsymbol{X}\ 100$$

Where, *Ac* is absorbance of control; *As* is absorbance of sample at time (t) = 20 min. The ferric reducing antioxidant power (FRAP) assay for the leaf extract and bacterial secondary metabolites was conducted following Benzie and Strain, 1999[24] as a measure of antioxidant power. FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), a solution of 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3$ at 10:1:1 (v/v/v). The reagent (150 µL) and sample solutions (50 µL) were added to each well and mixed thoroughly. The absorbance was taken at 593 nm at 4 min. A potential antioxidant will reduce the ferric ion (Fe^{3+}) to the ferrous ion (Fe^{2+}); the latter forms a blue complex (Fe^{2+}/TPTZ), which increases the absorption at 593 nm. Standard curve was prepared using different concentrations of $FeSO_4$. The results were expressed as mg Fe^{2+} mg^{-1} dw. All analyses were performed in triplicate on each extract and absorbance readings were measured using a microplate reader (Agilent BioTek Synergy HTX Multimode Reader).

HPLC detection of host origin compound reserpine in test metabolites of endophytic bacteria

RP-HPLC analysis was conducted to detect the presence of the host-derived compound reserpine in the secondary metabolites produced by endophytic bacteria, compared to a standard curve of reserpine (Himedia). The HPLC instrument used was a Shimadzu Prominence model, equipped with an LC-20 AD pump and an SPD 20 A detector. The mobile phase comprised 0.1 M phosphate buffer (A) with 0.1% formic acid and methanol (B), both of which were filtered through a Millipore PTFE 0.45 µm membrane. Separations were carried out using a linear gradient, starting at 85% B for 0.01 min, maintaining at 85% B for 9 min, transitioning to 75% B at 9.01 min, maintaining at 75% B until 10 min, further transitioning to 70% B at 10.01 min, maintaining at 70% B until 12.0 min, transitioning to 65% B at 12.01 min, and maintaining at 65% B until 30 min. The flow rate of the mobile phase was set at 1.0 mL.min^{-1}, with an injection volume of 20 µL. Chromatographic runs were conducted at 40°C, and UV detection was performed at 268 nm.

Untargeted metabolite profiling of R. serpentina leaves and test metabolites of endophytic bacteria through UHPLC-HRMS

R. serpentina fresh leaves were treated with liquid nitrogen and crushed in 80% methanol and suspended in 10 mL solvent for 10 min in a 55 kHz ultrasonic bath. Samples were filtered through a 0.25 µm PTFE membrane and stored at −20 °C until analysis. The resulting samples were filtered through a 0.25 µm PTFE membrane and stored at −20 °C until analysis. The hydro-alcoholic leaf extract and ethyl acetate extract containing secondary metabolites from endophytic bacteria were analyzed using Ultra-high performance liquid chromatography-high resolution electrospray ionization mass spectrometry (UHPLC-HRMS/MS) (Model: Q Exactive Plus; Make: Thermo Fischer Scientific; for small molecules UHPLC: Dionex Ultimate 3000 RS Series). The full scan MS was set at: resolution 70,000 (at m/z 100), AGC target 1e6, max IT 100 ms, scan range 100 to 1000 m/z. The MS2 conditions were: resolution 17,500 (at m/z 200), AGC target 1e5, max IT 50 ms, mass range m/z 200 to 2000, isolation window 4.0 m/z and (N)CE 30, 45, and 60. UHPLC separations were achieved using a Hypersil GOLD™ C18 Selectivity HPLC Column (Particle size 1.9 µm, Diameter 2.1 mm, Length 100 mm) with a column temperature set at 40 °C and a flow rate of 300 µL.min-1. The mobile phase consisted of a tertiary gradient of water (A), acetonitrile (B), and methanol (C), all containing 0.1% formic acid. The solvent composition gradient was programmed as follows: 0 min, 0% C; 0–7 min, 0–5% C; 7–15 min, 5–30% C; 15–20 min, 30–60% C; 20–25 min, 30–90% C; 25–28 min, 90 – 5% C; 28–30 min, 5 – 0% C; with a post-run of 8 min at 0.6 mL.min-1. Mass spectrometry, equipped with an ESI source, was operated in negative (NI) and positive (PI) ionization modes.

Statistical analyses

All samples underwent triplicate analysis, and the data were reported as the mean value accompanied by the standard deviation for each data point. Data processing and visualization software options include GraphPad Prism (version 10.1.0), Molecular Evolutionary Genetics Analysis (MEGA version 11.0.13). Data analysis from the UHPLC-HRMS for compound annotation were performed via Thermo Compound Discoverer 3.3.2.31 by using default settings and online databases.

Results

Isolation of bacterial endophytes and biochemical characterization

A total of 9 endophytic strains (Table 1) were isolated from the leaves of *R. serpentina*. The strains were designated as RSLB 1, 3, 4, 5, 6, 9, 12, 14, and 18. All the isolates were indole and oxidase negative with five being catalase positive. Most of the isolates exhibited positive result for Voges-Proskauer test, and oxidative fermentation, with less than half isolates showing positive result for nitrate reduction test citrate utilization. Protease and amylase activity was exhibited by all the isolates. All isolates except RSLB14 and RSLB18 had shown positive result for presence of cellulase activity. Four out of 9 isolates were positive for lipase activity (Table 2).

ISOLATE	Colony characteristics				
	Color	Form	Margin	Surface texture	Gram character
RSLB 1	Beige	Circular	Irregular	Butyrous	Gram-positive
RSLB 3	Cream colored	Floral colony with off-white base	Irregularly lobed ridged margin	Butyrous	Gram-positive
RSLB 4	Cream colored	Small compact	Irregular	Slimy	Gram-positive
RSLB 5	Off-white	Compact circular	Entire	Rough	Gram-negative
RSLB 6	Yellow	Compact circular colorless base	Entire	Rough	Gram-negative
RSLB 9	Yellow	Circular	Irregular	Slimy	Gram-negative
RSLB 12	White	Compact circular colony	Entire	Slimy	Gram-negative
RSLB 14	Cream colored/ yellow	Off-white	Entire	Slimy	Gram -positive
RSLB 18	Off-white	Small dotted	Irregular	Slimy	Gram-positive

Table 1. Microscopic study of bacterial isolates of *R. serpentina*. *RSLB: *R. serpentina* Leaf bacteria.

Antibacterial activity

Antibacterial activity against human pathogenic bacteria was exhibited by majority of crude secondary metabolite extracts of endophytic bacteria when compared with standard antibiotics such as a tetracycline, streptomycin and nalidixic acid that showed inhibitory action against all pathogens. 22.22% of the isolates showed antibacterial activity in the form of zone of inhibition (Fig. 2a) against majority of pathogen. The Table 3 highlights the endophytic bacterial isolates RSLB3 and RSLB18 as the most effective isolates in inhibiting the growth of the majority of the tested pathogens, with the largest zones of inhibitions. Zone of inhibition of secondary metabolite of RSLB3 was maximum, ranging between 20 mm and 25 mm, subsequently zone of inhibition of RSLB18 ranged between 7 mm and 26 mm (Fig. 2b). RSLB3 appears to be more effective against *Shigella boydii* and MRSA, while RSLB18 is more potent against *Enterococcus faecalis*. *R. serpentina* leaf extract also showed significant inhibitory activity (against 8 pathogens), with zone of inhibitions ranging from 7 mm to 12 mm. Endophtyic bacterial isolates RSLB9, RSLB14, RSLB4 and RSLB12 were active against 8, 4 and 3 pathogens respectively while isolates RSLB1 and RSLB6 inhibited growth of 1 pathogen each. The endophytic bacterial isolates, RSLB5 exhibited limited or no antibacterial activity, as indicated by the absence of zone of inhibition.

MIC values of secondary metabolites of two endophytic bacteria (RSLB3 and RSLB18) against clinical isolates of human pathogenic bacteria was determined by broth dilution method. The MIC values obtained from broth dilution plate assay ranged from <0.1 mg.mL^{-1} to 0.2 mg.mL^{-1} for RSLB3; maximum MIC value (0.2 mg.mL^{-1}) being for *M. morganii* and minimum (<0.05 mg.L.mL^{-1}) for MRSA). RSLB18 had a MIC range of <0.1 mg.mL^{-1} to 3.2 mg.mL^{-1} (<0.1 mg.mL^{-1} for *E. faecalis* and 3.2 mg.mL^{-1} for MRSA). For standard antibiotics tetracycline and streptomycin MIC values ranged from 1 µg.mL^{-1} to 128 µg.mL^{-1} and 16 µg.mL^{-1} to 128 µg.mL^{-1}, respectively (Table 4). This suggests that the crude secondary metabolites from RSLB3 and RSLB18 could be a promising source of novel antibacterial agents.

Identification of bacterial endophytes

The isolates exhibited 96–100% similarity to sequences in the National Center for Biotechnology Information (NCBI) database. The phylogenetic analysis grouped the two identified endophytic bacteria into the Bacillus group. The analysis is represented by order Bacillales. The 16S rRNA sequences were deposited in the GenBank with accession numbers MW680956–MW680964 (Table 5). The resulting phylogenetic tree of bacterial isolates revealed that isolates and reference sequences were clustered according to established taxonomic orders, with high bootstrap support (Fig. 3).

Biochemical characterization of metabolites

The results presented in Table 6 provides insight into the total phenolic content, total flavonoid content, total antioxidant capacity, DPPH radical scavenging activity, and ferric reducing antioxidant power (FRAP) of the *R. serpentina* leaf extract and the secondary metabolites of the endophytic bacterial isolates RSLB3 and RSLB18. Total polyphenol and flavonoid content were measured and expressed as mg gallic acid equivalents (GAE) and mg Quercetin equivalents (QE) respectively. The total phenolic content of the *R. serpentina* leaf extract was determined to be 0.103 ± 0.003 mg GAE.mg^{-1} dw indicating the presence of a significant number of phenolic compounds in the plant material. In comparison, the secondary metabolites of the endophytic isolates RSLB3 and RSLB18 exhibited total phenolic contents of 0.027 ± 0.001 and 0.027 ± 0.001 mg GAE.mg^{-1} dw, respectively. The total flavonoid content followed a similar trend, with the leaf extract showing 0.016 ± 0.0008 mg QE.mg^{-1} dw, while the secondary metabolites of RSLB3 and RSLB18 contained 0.042 ± 0.0004 and 0.022 ± 0.001 mg QE.mg^{-1} dw, respectively (Fig. 4).

The total antioxidant capacity of extracts measured by phosphomolybdenum blue method was calculated as EC_{50} was 1.2 ± 0.05 mg.mL^{-1} for leaf extract, 3.21 ± 0.05 mg.mL^{-1} for RSLB3 and 0.66 ± 0.1 mg.mL^{-1} for RSLB18 compared to the EC_{50} of 0.197 ± 0.05 mg.mL^{-1} for ascorbic acid. The DPPH assay further corroborated the antioxidant properties of the samples. The leaf extract had an IC_{50} value of 0.82 ± 0.003 mg.mL^{-1}, while the secondary metabolites of RSLB3 and RSLB18 IC_{50} values of 0.53 ± 0.05 mg.mL^{-1} and 0.33 ± 0.04 mg.mL^{-1}, respectively when compared to IC_{50} of ascorbic acid (0.0129 ± 0.03 mg.mL^{-1}) and gallic acid (0.005 ± 0.0001 mg.mL^{-1}). The ferric reducing antioxidant power (FRAP) assay showed that the *R. serpentina* leaf extract had

Isolates	Indole production	Motility	H_2S production	Catalase	Oxidase	Methyl Red Test	Voges-Proskauer Test	Citrate Utillisation	Oxidative Fermentation		NO^-_3 reductase	Protease	Amylase	Cellulase	Lipase
									with oil	without oil					
RSLB 1	-	+	-	+	-	-	+	-	-	+	-	+++	++	+++	-
RSLB 3	-	+	-	-		-	+	+	-	+	-	+++	+++	++	+
RSLB 4	-	+	-	-	-	-	+	-	++	++	-	+	+	+	-
RSLB 5	-	+	-	+	-	+	-	-	+++	+++	-	++	+++	++	+
RSLB 6	-	+	-	+	-	-	+	-	-	+	+	++	++	+	-
RSLB 9	-	+	-	+	-	+	-	-	-	+	+	+	+++	+	-
RSLB 12	-	+	-	-	-	-	+	-	-	+	+	+	+++	+	+
RSLB 14	-	+	-	-	-	-	+	-	+++	+	-	+	+++	-	-
RSLB 18	-	-	-	+	-	-	+	-	-	+		+++	++	-	+

Table 2. Biochemical characterization of endophytic bacteria. (+) = presence of activity; (-) = absence of activity.

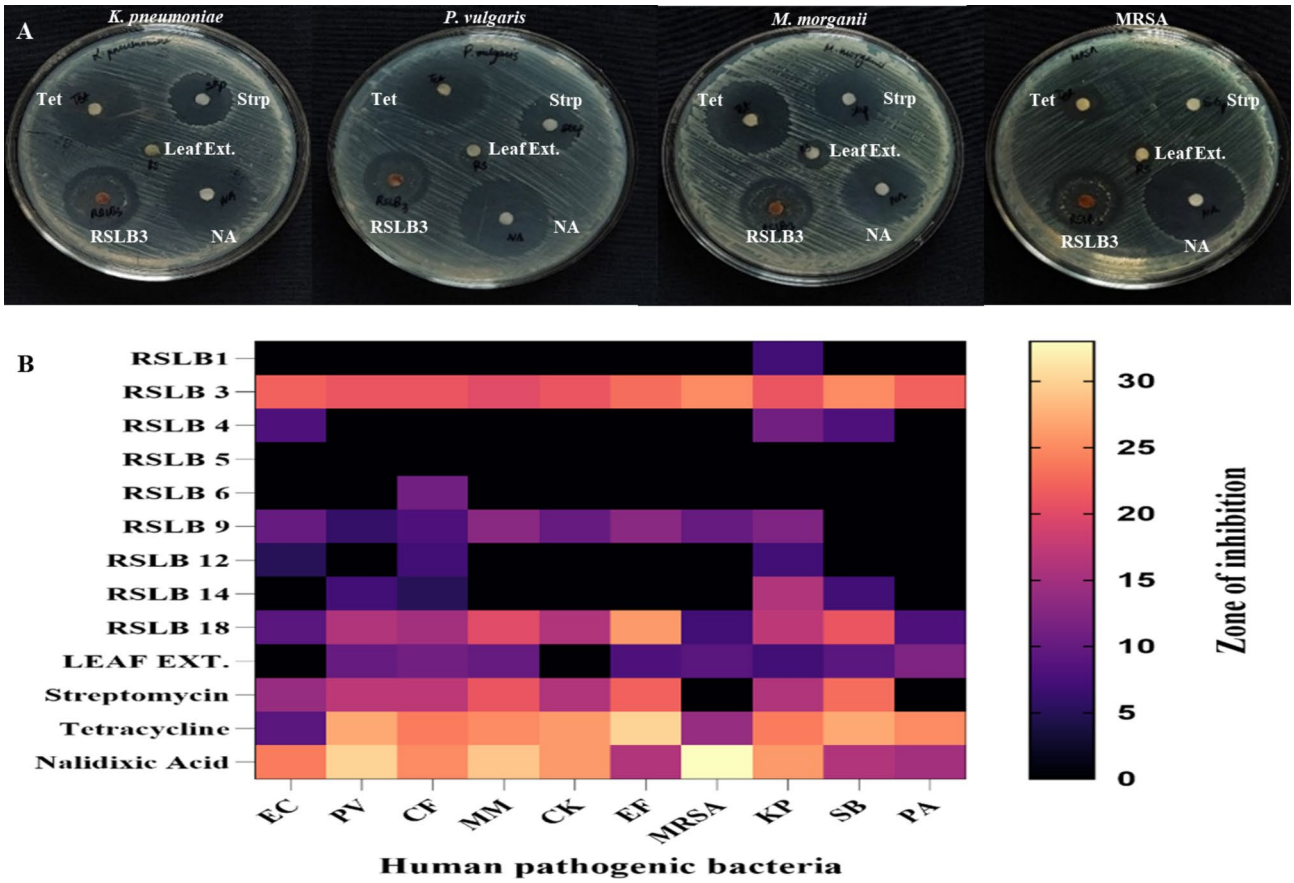

Fig. 2. **(A)** Antibacterial activity of metabolites against some human pathogenic bacteria (RSLB3 and RSLB18). **(B)** Heatmap for antibacterial activity of secondary metabolites of bacterial endophytes was prepared in terms of color intensity by normalization of mean values of the results. EC: *Escherichia coli;* PV: *Proteus vulgaris*; CF: *Citrobacter freundii*; MM: *Morganella morganii*; CK: *Citrobacter koserii*; EF: *Enterococcus faecalis*; MRSA: Methicillin resistant *Staphylococcus aureus*; KP: *Klebsiella pneumoniae*; SB: *Shigella boydii*; PA: *Pseudomonas aeruginosa*.

	Zone of Inhibition in mm									
Isolates	**EC**	**PV**	**CF**	**MM**	**CK**	**EF**	**MRSA**	**KP**	**SB**	**PA**
RSLB1	0	0	0	0	0	0	0	6.33 ± 0.5	0	0
RSLB 3	**22.0 ± 0.8**	**21.0 ± 0.8**	0	**19.7 ± 0.5**	**21.3 ± 0.5**	**21.7 ± 0.9**	**25.3 ± 1.2**	**20.3 ± 0.9**	**24.3 ± 0.9**	**21.3 ± 0.9**
RSLB 4	8.3 ± 0.47	0	0	0	0	0	0	11 ± 0.81	8.0 ± 0.81	0
RSLB 5	0	0	0	0	0	0	0	0	0	0
RSLB 6	0	0	11.5 ± 0.40	0	0	0	0	0	0	0
RSLB 9	**10.67 ± 0.94**	**6.33 ± 0.47**	**8.33 ± 0.47**	**13.00 ± 0.82**	**9.00 ± 0.82**	**13.00 ± 0.00**	**9.67 ± 0.47**	**12.33 ± 0.47**	0	0
RSLB 12	5.33 ± 0.47	-	7.67 ± 0.47	0	0	0	0	6.67 ± 0.47	0	0
RSLB 14	0	7.67 ± 0.47	6.00 ± 0.82	0	0	0	0	15.67 ± 1.25	6.67 ± 0.47	0
RSLB 18	**8.3 ± 0.9**	**14.7 ± 0.9**	**14.7 ± 1.2**	**18.7 ± 0.9**	**14.7 ± 0.5**	**24.3 ± 1.2**	**7.0 ± 0.8**	**16.3 ± 7.0**	**20.7 ± 0.5**	**7.7 ± 0.5**
Leaf extract	0	**9.3 ± 0.9**	**11.0 ± 0.0**	**10.0 ± 0.0**	0	**8.0 ± 0.0**	**8.3 ± 0.5**	**7.0 ± 0.0**	**8.0 ± 1.4**	**11.7 ± 0.5**
Streptomycin	14.0 ± 0.8	16.3 ± 0.5	16.3 ± 0.5	20.3 ± 0.9	15.7 ± 1.2	21.7 ± 1.2	0	15.7 ± 0.5	22.0 ± 0.8	0
Tetracycline	8.7 ± 0.5	25.7 ± 0.9	24.3 ± 0.9	24.3 ± 0.9	26.0 ± 0.0	30.3 ± 0.5	13.3 ± 0.9	23.3 ± 0.9	26.3 ± 1.7	25.3 ± 0.5
Nalidixic Acid	24.0 ± 0.82	29.33 ± 0.47	25.33 ± 0.47	29.33 ± 0.47	25.33 ± 0.94	15.67 ± 0.47	32.0 ± 1.41	26.33 ± 0.47	15.67 ± 0.47	14.33 ± 0.94

Table 3. Antibacterial activity of extracellular crude secondary metabolites of *R. serpentina* leaf endophytic bacterial isolates. EC: *Escherichia coli;* PV: *Proteus vulgaris;* CF: *Citrobacter freundii;* MM: *Morganella morganii;* CK: *Citrobacter koserii;* EF: *Enterococcus faecalis;* MRSA: Methicillin resistant *Staphylococcus aureus;* KP: *Klebsiella pneumoniae;* SB: *Shigella boydii*; PA: *Pseudomonas aeruginosa*. Concentration of extracts: 50 mg.mL^{-1}; Concentration of antibiotics: 10 mg.mL^{-1}; Volume loaded per disk: 5 µL.

Isolates	MIC AGAINST PATHOGENIC BACTERIA									
	EC	PV	CF	MM	CK	EF	MRSA	KP	SB	PA
RSB 3	0.1	0.1	0.1	0.2	0.1	0.1	< 0.1	0.1	< 0.1	0.1
RSB 18	0.8	0.4	0.8	0.1	0.2	< 0.1	3.2	0.2	0.1	1.6
Streptomycin	< 1.6	6.4	> 12.8	> 6.4	< 1.6	1.6	> 12.8	> 6.4	3.2	> 12.8
Tetracycline	< 0.8	3.2	0.8	6.4	< 0.8	0.4	> 12.8	> 6.4	1	16

Table 4. MIC determination by broth dilution assay of secondary metabolites of endophytic bacterial isolates. EC: *Escherichia coli;* PV: *Proteus vulgaris;* CF: *Citrobacter freundii;* MM: *Morganella morganii;* CK: *Citrobacter koserii;* EF: *Enterococcus faecalis;* MRSA: Methicillin resistant *Staphylococcus aureus;* KP: *Klebsiella pneumoniae;* SB: *Shigella boydii*; PA: *Pseudomonas aeruginosa*. Concentration of extracts in $mg.mL^{-1}$; Concentration of standard antibiotics: $\mu g.mL^{-1}$.

Endophytic Bacterial isolates	Accession number	Identified as	Accession number of closest match	Partial Sequence size	% Similarity
RSLB 3	MW7415340	*Bacillus mojavensis*	NR_118290.1	1221 bp	98.54%
RSLB 18	MW7415343	*Bacillus wiedmannii* strain FSL W8-0169	NR_152692.1	1232 bp	99.76%

Table 5. Taxonomic identification and sequence homology of bacterial endophytes (showing antimicrobial activity) with Genus or species affiliation and corresponding identity percentage in the NCBI database.

an antioxidant capacity of 0.146 ± 0.0004 mg Fe^{2+} mg^{-1} dw, while the RSLB3 and RSLB18 secondary metabolites demonstrated FRAP values of 0.043 ± 0.001 and 0.041 ± 0.001 mg Fe^{2+} mg^{-1} dw, respectively (Fig. 4).

HPLC Analysis

HPLC analysis was conducted to detect reserpine in the crude extracts of secondary metabolites from endophytic bacterial isolates RSLB3 and RSLB18, as well as in leaf extract. The distinct peak of the reserpine standard served as a reference for comparison. The concentrations found were 61.91 ppm in leaf extract, 53.51 ppm in RSLB3, and 63.02 ppm in RSLB18 (Fig. 5).

Metabolite profiling of *R. Serpentina* Leaf extracts and secondary metabolites of endophytic isolates RSLB3 and RSLB18

Untargeted analysis by Ultra high-performance liquid chromatography-high resolution electrospray ionization mass spectrometry (UHPLC-HRMS/MS) was performed to determine the profile of the main phytochemicals and bioactive metabolites present in the leaves of *R. serpentina* and endophytic bacterial isolates RSLB3 and RSLB18 respectively (Supplementary material S1). Based on the observed mass spectra (Figs. 6, 7 and 8), the compounds were identified by matching to different libraries, and bioactivities were reported by comparing them to literature. The list of some of the identified metabolites for leaf extract, RSLB3 and RSLB18 are reported in Tables 7, 8 and 9 respectively, and detailed HRMS, MS/MS data are reported in the Supplementary materials (S1). In the hydro-alcoholic leaf extract of *R. serpentina*, we observed the presence of a diverse array of plant metabolites, including alkaloids (e.g. gelsemine, ajmaline, yohimbine), indole alkaloids (e.g. catharanthine, vindoline, vincristine), iridoid glycosides (loganin, sarpagine), coumarins (fraxetin, rescinnamine), and flavonoids (kaempferol, kaempferitrin) (Table 7, Fig. 7:Supplementary file S2).

The ethyl acetate extract of the leaf endophytic bacteria *B. mojavensis* (RSLB3) exhibited a diverse array of compounds, both plant and microbial origin (Table 8; Fig. 8: Supplementary file S2). Among the identified compounds, the naphthoquinone plumbagin has been reported to exhibited anticancer, antimicrobial, and anti-inflammatory properties[46]. The extract also contained compounds of microbial origin, including nucleoside antibiotics like sangivamycin produced by various microorganisms. The bacterium *B. wiedmannii* (RSLB18) produces a diverse array of secondary metabolites with various biological activities (Table 9, Supplementary file S2). Notably, this strain synthesizes muscimol, a mycotoxin acting as a GABA receptor agonist with neuroactive properties and also generates mycocyclosin, an antibiotic with significant antibacterial activity against *Mycobacterium tuberculosis*[73]. Additionally, maculosin, a phytotoxin serving as a virulence factor, is another product of RSLB18 as well as Diacetylphloroglucinol, a compound known for its antimicrobial properties against plant pathogens[74]. Scopoletin acetate, a coumarin, demonstrates antimicrobial, anti-inflammatory, and antioxidant properties. The strain also produced harmine, a β-carboline alkaloid with antimicrobial, antiparasitic, and psychoactive effects. Harmaline, another β-carboline alkaloid, shares similar properties with harmine.

Several important intermediates of the alkaloid biosynthetic pathways in *R. serpentina* were also detected in the crude secondary metabolites of bacterial endophytes (Supplementary file S2). The identified key metabolites were utilized to construct a simplified metabolic pathway (Supplementary file S2) illustrates the synthesis of reserpine and indole alkaloids through the MIA pathway.

Discussion

The isolation and biochemical characterization of the nine endophytic bacterial isolates (RSLB1, RSLB3, RSLB4, RSLB5, RSLB6, RSLB9, RSLB12, RSLB14, and RSLB18) from the leaves of *R. serpentina* revealed metabolically

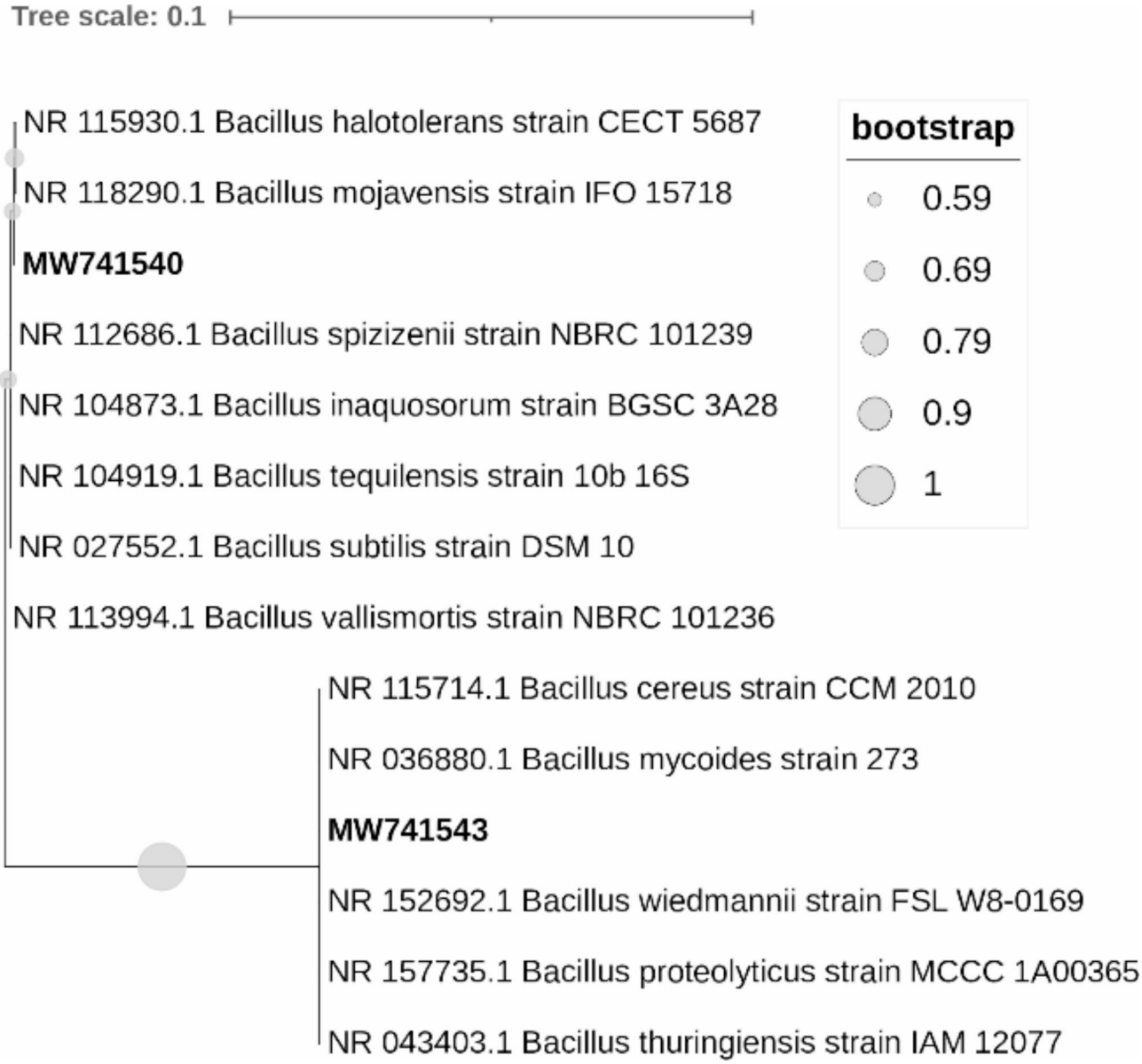

Fig. 3. Neighbor-joining phylogenetic tree based on the partial sequence of the 16S rRNA gene of bacterial isolates from *R. serpentina* leaf and their related type strains. The evolutionary distances were computed using the p-distance. Bootstrap values are given at branch nodes and are based on 1000 replicates.

active strains. The positive results obtained in various biochemical tests, including indole production, methyl red, Voges-Proskauer, citrate utilization, catalase and oxidase production, nitrate reduction, oxidative fermentation, and citrate reduction, indicate the metabolic versatility of these endophytic bacteria and their potential to produce a wide range of secondary metabolites.

The assessment of antibacterial activity of the crude secondary metabolites from the endophytic bacteria demonstrated significant inhibitory effects against a panel of clinically relevant human pathogenic bacteria. Isolate RSLB3 identified as *B. mojavensis* has been known for its ability to biosurfactant lipopeptides surfactins A, B, and C, pumilacidin, esperin, lichenysin, fengycin, iturin, Bacillomycin, etc[110]. RSLB18, identified as *B. wiedmannii*, has been reported for production of hemolysin BL[111] and, bacteriocins (Bawcin) having cytotoxic activity[112]. The MIC values of two isolates (RSLB3 and RSLB18) were found to be in the range of 0.05 $mg.mL^{-1}$ to 1.6 $mg.mL^{-1}$. This suggests that the crude secondary metabolites from RSLB3 and RSLB18 could be a promising source of novel antibacterial agents.

The endophytic bacteria were capable of producing phenolic compounds, potentially through the biotransformation of host plant-derived precursors or de novo synthesis. The higher flavonoid content in the endophytic bacterial extracts indicates their potential to produce a diverse array of flavonoid-based compounds, which are known for their antioxidant, antimicrobial, and anti-inflammatory properties. The lower IC_{50} value of RSLB3 and RSLB18 indicate a higher free radical scavenging ability of the endophytic bacterial extracts compared

Total phenolic content [a]			Total flavonoid content [b]			Total antioxidant capacity (EC_{50} mg.mL^{-1})			DPPH ASSAY (IC_{50} mg.mL^{-1})			FRAP assay [c]		
LEAF	RSLB3	RSLB18	LEAF	RSLB3	RSLB18	LEAF	RSLB3	RSLB18	LEAF	RSLB3	RSLB18	LEAF	RSLB3	RSLB18
0.103 ± 0.003	0.027 ± 0.001	0.027 ± 0.001	0.016 ± 0.0008	0.042 ± 0.0004	0.022 ± 0.001	1.2 ± 0.05	3.21 ± 0.05	0.66 ± 0.1	0.82 ± 0.03	0.59 ± 0.05	0.33 ± 0.04	0.146 ± 0.0004	0.043 ± 0.001	0.041 ± 0.001

Table 6. Total phenol and flavonoid estimation of *R. serpentina* leaf extract and secondary metabolites of leaf endophytic bacteria RSLB3 AND RSLB18. [a]Total phenolic content expressed as mg Gallic acid equivalent mg^{-1}dw;[b]Total flavonoid expressed as mg Quercetin equivalent mg^{-1}dw;[c]Total Frap value expressed as mg Fe^{2+}mg^{-1}dw.

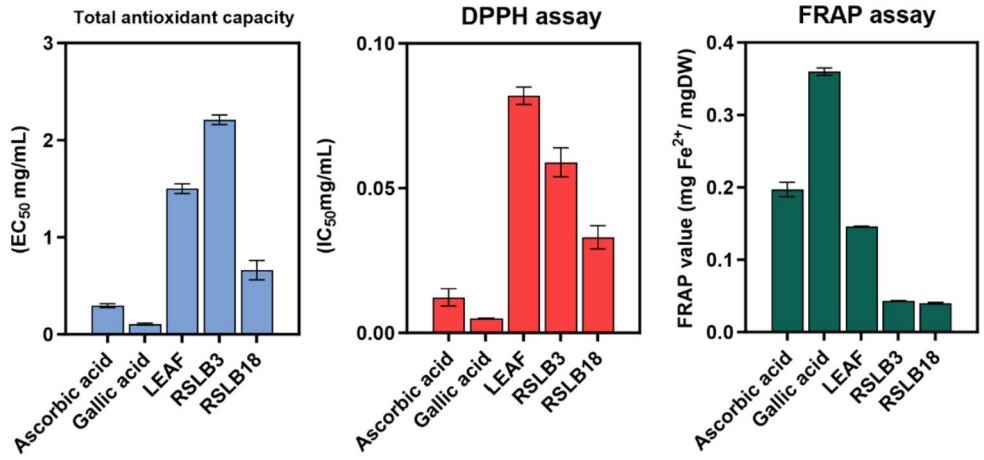

Fig. 4. TAC, DPPH and FRAP assay of *R. serpentina* leaf extract and secondary metabolites of leaf endophytic bacteria RSLB3 AND RSLB18.

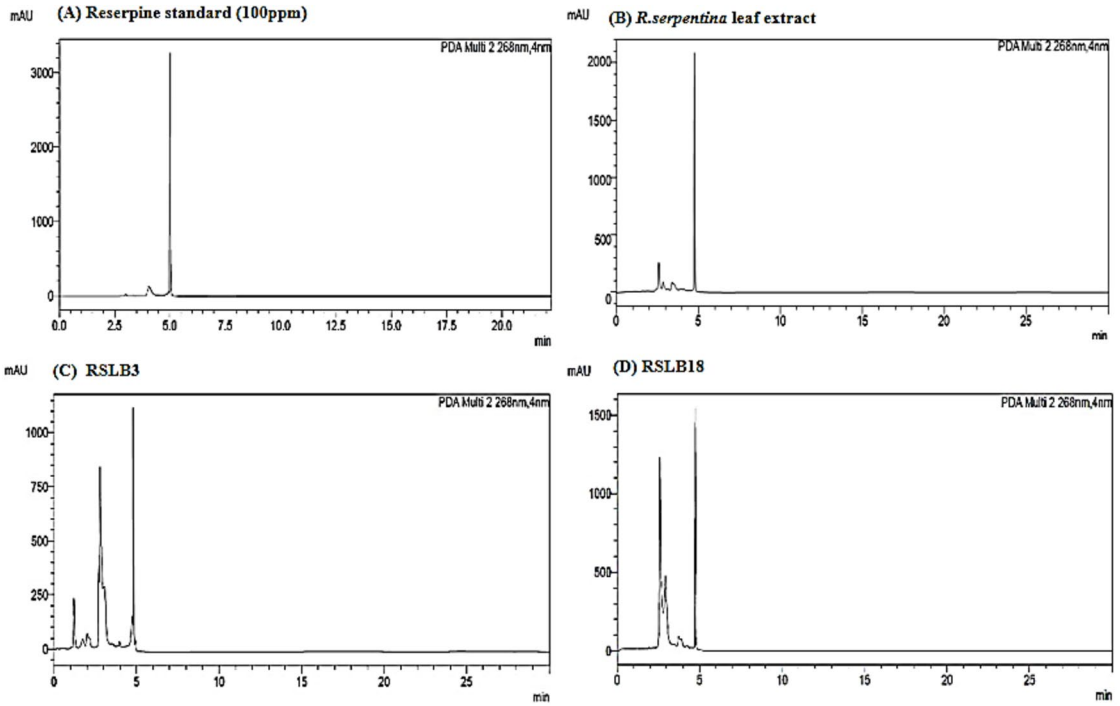

Fig. 5. Chromatogram showing: **(A)** Reserpine standard (100ppm) **(B)** *R.serpentina* Leaf extract (61.91pmm) **(C)** RSLB3 (53.51ppm) and **(D)** RSLB18 (63.02ppm).

to the leaf extract. The data presented in Table 6 collectively suggest that the secondary metabolites produced by the endophytic bacterial isolates RSLB3 and RSLB18 exhibit significant antioxidant and free radical scavenging abilities compared to the *R. serpentina* leaf extract. These findings highlight the potential of these endophytic bacteria as a valuable source of natural antioxidants and bioactive compounds with potential applications in the development of antimicrobial and therapeutic agents[113].

The detection of the host-derived compound reserpine in the secondary metabolites of the endophytic bacteria, as confirmed by HPLC analysis, is a significant finding. The result suggests that the endophytic bacterial isolates acquired or evolved the necessary metabolic pathways to synthesize or accumulate this secondary metabolite. The ability of the endophytic bacteria to produce this bioactive compound, which is structurally similar to their host plant, highlights their potential as a renewable source of valuable natural products. The untargeted metabolomic profiling of the *R. serpentina* leaf extract and the secondary metabolites of the endophytic bacteria using UHPLC-HRMS/MS revealed a diverse range of compounds with known as well as unknown bioactive properties. These include alkaloids, terpenoids, flavonoids, quinones, and other secondary metabolites with

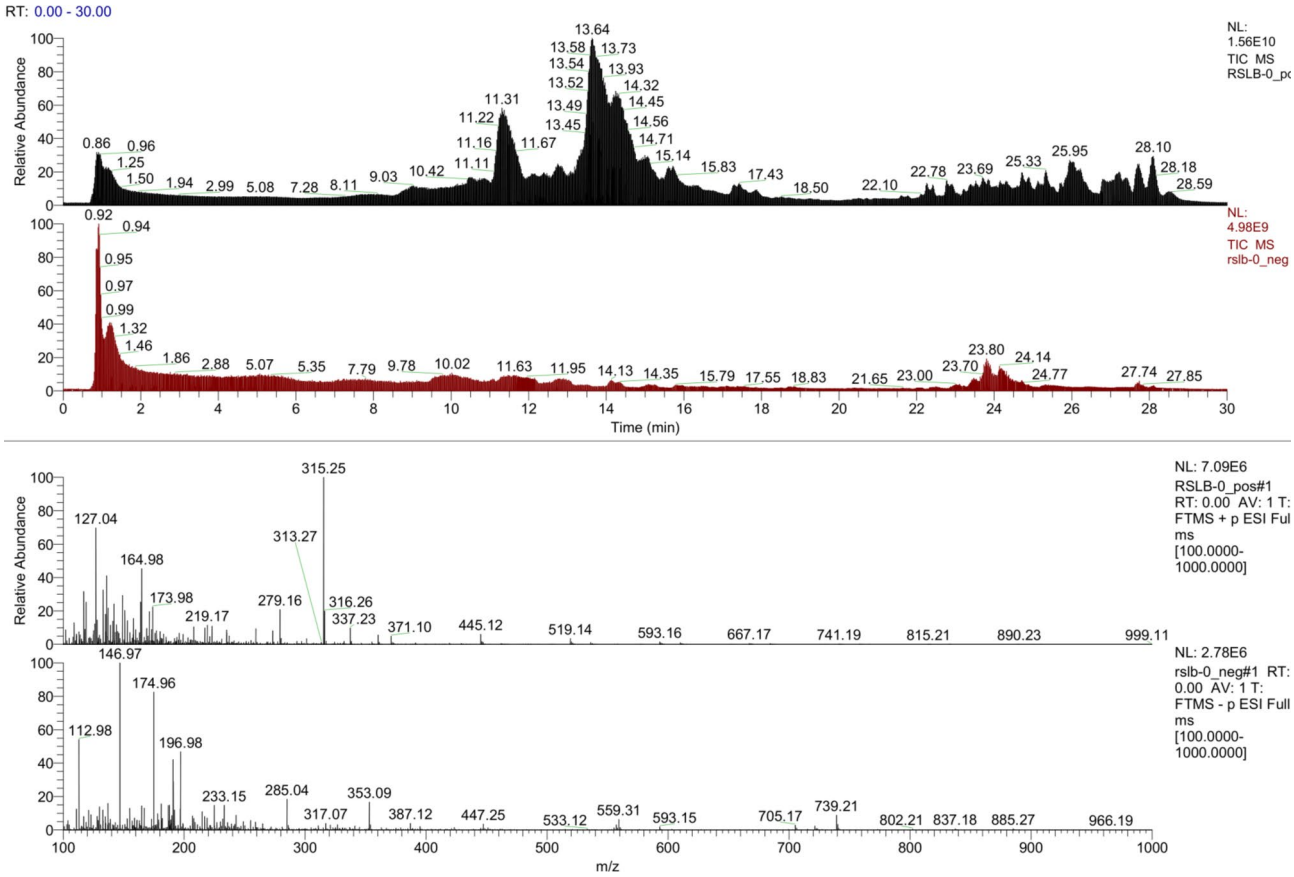

Fig. 6. A base peak total ion chromatogram of leaf extract of *R. serpentina*.

antimicrobial, antioxidant, anticancer, and anti-inflammatory activities. It also allowed identification of various host origin as well as microbial origin secondary metabolites present in bacterial crude metabolite extracts. Some of the major compounds identified in *R. serpentina* leaf and their reported biological activities are noteworthy. Chlorogenic acid, a phenolic acid, exhibits potent antioxidant, anti-inflammatory, neuroprotective, and antidiabetic properties. The flavonoid glycoside kaempferol has been investigated for its antioxidant, anti-inflammatory, and anticancer activities. Fraxetin, a coumarin derivative, has demonstrated antioxidant, anti-inflammatory, and anticancer effects in preclinical studies[114]. Kojic acid, a pyrone compound, is known for its antioxidant, tyrosinase inhibition, and antimicrobial capabilities[115]. The iridoid glycoside loganin has been reported to exhibit neuroprotective, antidepressant, and anticonvulsant effects. Salicin, a phenolic glycoside found in willow bark, has analgesic, anti-inflammatory, and antioxidant applications.

Harmine and the harmala alkaloids detected in the RSLB3 secondary metabolite, have been studied for their antidepressant, antitumor, and antimalarial effects[116]. Phenolic compounds like guaiacol, vanillin, and eugenol have shown antimicrobial and antioxidant activities, while the phenylpropanoid cinnamaldehyde possesses antimicrobial and anti-inflammatory activities. Other notable plant-derived compounds include the analgesic and anti-inflammatory alkaloid capsaicin[47], the stimulant and neuroprotective caffeine, and cardioprotective stilbenoid resveratrol. The extract also contained compounds of microbial origin, including nucleoside antibiotics like sangivamycin produced by various microorganisms. Other microbially-derived compounds include the quaternary ammonium osmolyte betaine and the β-lactam antibacterial clavulanic acid[117]. RSLB18 produced nigakinone, an alkaloid with antimicrobial properties, and cyclo (dehydrophenylalanyl-L-leucyl), a cyclic dipeptide with antimicrobial activity. Tetrahydropapaveroline, an isoquinoline alkaloid, exhibits both antimicrobial and neuroactive effects[118], while phoslactomycin B, a macrolide antibiotic, displays antitumor and antimicrobial activities[119]. This extensive repertoire of secondary metabolites highlights the metabolic versatility of RSLB18 and its potential applications in pharmaceuticals, agriculture, and other biotechnological fields. The analysis of plant and endophyte metabolomes provide a direct insight into the contribution of microbiome toward host plant phenotype, as well as the impact of environment on plant-microbe interactions, thereby serving as sensitive and accurate markers. The application of microbial metabolomics enables the complete analysis of crude extracts as well as the identification of marker metabolites (associated with specific bioactivities), before undergoing tedious and laborious downstream processes[120].

Research on endophytic fungi has had a longer historical context, with many known bioactive compounds derived from fungal sources, such as antibiotics and anticancer agents. For instance, the fungal endophyte *Taxomyces andreanae* was discovered to synthesize paclitaxel (Taxol), the first anticancer bioactive metabolite

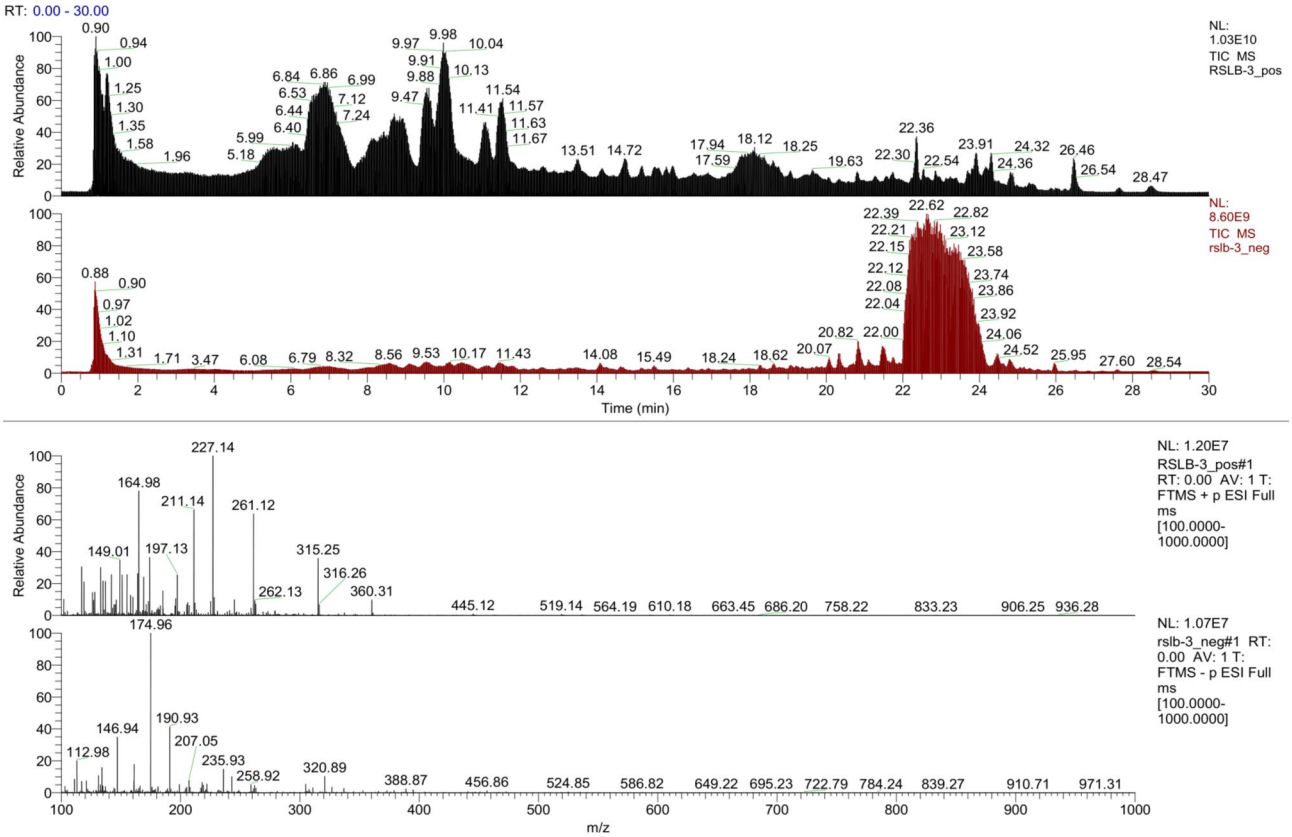

Fig. 7. A base peak total ion chromatogram of crude secondary metabolite of RSLB3 (*B. mojavensis*).

previously found only in Taxus plants. Many genera of endophytic fungi, including *Alternaria*, *Aspergillus*, *Botryodiplodia*, *Botrytis*, *Cladosporium*, *Fusarium*, etc. have been screened for their ability to produce paclitaxel and its analogues[121]. Bacterial endophytes, particularly those within the Bacillus genus, are known for their ability to produce a wide range of secondary metabolites that exhibit various biological activities, including antimicrobial, antioxidant, and anti-inflammatory properties. This metabolic diversity is crucial for bioprospecting novel compounds that may not be found in fungal endophytes[122]. While fungal endophytes are recognized for their complex metabolic capabilities and historical significance in bioactive compound research[123], bacterial endophytes present unique advantages; they are generally easier to culture than fungal endophytes, which often require more complex growth conditions. Bacteria can be rapidly grown in liquid media, allowing for quick scaling and experimentation. Their rapid growth rates and well-established protocols for genetic manipulation facilitate efficient experimentation and analysis, enabling targeted production of desired metabolites[124].

Conclusion

While the roots of *R. serpentina* are well-studied and known to contain over 70 distinct alkaloids with significant biological and therapeutic potential, the leaves may represent an underexplored source of bioactive compounds. Harvesting leaves is generally more sustainable and less destructive to the plant compared to harvesting roots. By focusing on the leaves and related bacterial endophytes, our research promotes conservation and sustainable use of *R. serpentina*, which is crucial for maintaining biodiversity and ecological balance. Investigating the leaves can provide complementary insights into the overall metabolite profile of *R. serpentina*, offering a more comprehensive view of the plant's medicinal potential. Given that the roots are already well-studied, concentrating on the leaves increases the likelihood of discovering new compounds and bioactivities, further enriching our understanding of this valuable plant.

In the symbiotic relationship between endophytic microbes and their host, the host provides nutrients and a suitable habitat for the endophytes, while the endophytes produce bioactive compounds that help the host resist biotic and abiotic stresses and promote its growth. Endophytes influence the chemical makeup of their host plants and promote the production of host-specific bioactive compounds, with genomic pathways involved in this process often distributed among all plant partners. Leveraging the biosynthetic capabilities of these endophytic bacteria could offer advantages in terms of scalability, sustainability, and potentially lower production costs compared to traditional plant-based extraction methods. Our future study could apply advanced fermentation techniques for improving the yield and purity of desired compounds.

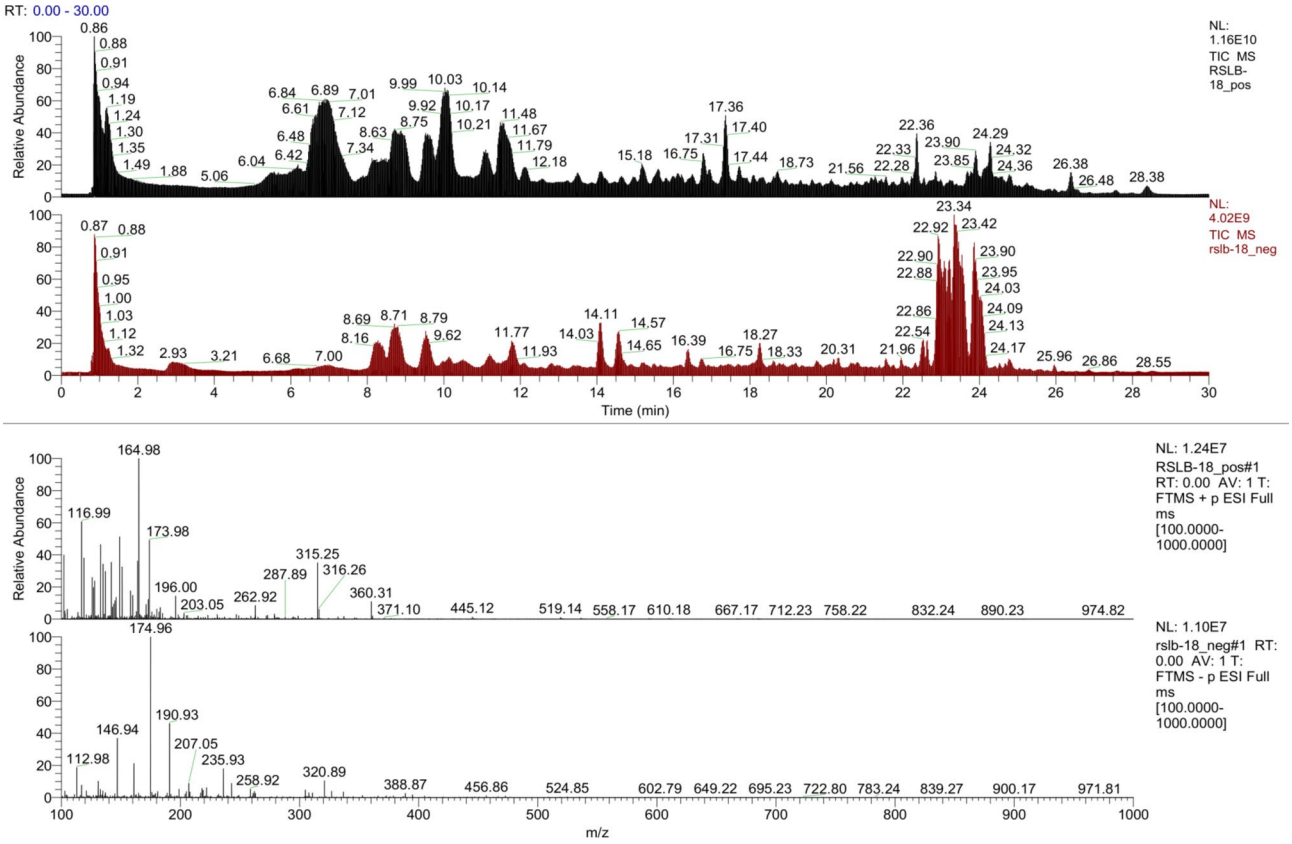

Fig. 8. A base peak total ion chromatogram of crude secondary metabolite of RSLB3 (*B. mojavensis*).

S.no	RT [min]	[a]Ionization mode	m/z	Molecular formula	Compound	Class	Reported activity	References
1.	1.17	+	328.13	C15 H21 N O7	Sesbanimide A	Alkaloids	Anticancer and anti-inflammatory	25
2.	8.395	+	337.19	C21 H24 N2 O2	Catharanthine	Terpene indole alkaloid	Acetylcholinesterase inhibition	26
3.	8.411	+	323.17	C20 H22 N2 O2	Gelsemine	Indole alkaloid	Antinociception, anxiety neurosis	27
4.	9.122	+	531.23	C27 H34 N2 O9	3-alpha(S)-Strictosidine	Indole alkaloid	Intermediate for monoterpene indole alkaloids	28
5.	9.529	+	325.19	C20 H24 N2 O2	Quinine	Cinchona alkaloid	Analgesic, anti-malarial	29
6.	9.854	+	327.20	C20 H26 N2 O2	Ajmaline	Indole alkaloid	Antihypertensive and antiarrhythmic	30
7.	17.767	+	635.29	C35 H42 N2 O9	Rescinnamine	Monoterpenoid indole alkaloid	Antihypertensive and tranquilizer agent, Anticholinergic	
8.	9.896	+	311.17	C19 H22 N2 O2	Sarpagine	Monoterpenoid indole alkaloid	Anticancer, antibacterial, antiarrhythmic, anti-inflammatory, antimalarial	31
9.	10.165	+	355.20	C21 H26 N2 O3	Yohimbine	Indole alkaloid	Anti-inflammatory, Anticancer effect, Antihypotensive, Myocardial protection	32
10.	10.681		391.15	C17 H26 O10	Loganin	Iridoid glycosides	Neuroprotective, Anti-Inflammatory,	33
11.	11.108	+	353.18	C21 H24 N2 O3	1_2-dihydrovomilenine	Indole alkaloid	Precursor for biosynthesis of ajmaline	1
12.	11.882	+	287.05	C15 H10 O6	Kaempferol	Flavonoid	Anticancer, hepatoprotective, neuroprotective, and cardioprotective	34
13.	13.195	+	399.22	C23 H30 N2 O4	Mitragynine	Indole alkaloid	Antinociceptive, antidopaminergic, antimicrobial, anti-inflammatory	35
14.	13.997	+	355.20	C21 H26 N2 O3	Rauwolscine	Rauwolscine	A2-adrenergic antagonist	36
15.	14.685	-	577.15	C27 H30 O14	Kaempferitrin	Flavonoid	Antimicrobial, antiparasitic, hypoglycemic agent, anti-inflammatory and anticancer	37
16.	16.439	+	389.19	C22 H28 O6	Quassin	Quassinoid	Antiulcerogenic and antiplasmodial activity	38
17.	16.64	+	609.28	C33 H40 N2 O9	Reserpine	Monoterpene Indole Alkaloid	Antihypertensive, and an antipsychotic	39
18.	16.996	+	825.40	C46 H56 N4 O10	Vincristine	Tryptophan alkaloids	Hodgkin's lymphoma treatment, an antineoplastic agent	40
19.	18.536	-	361.18	C17 H30 O8	Makomotine C	Sterol	Anti-osteoporotic	41
20.	20.718	+	149.09	C10 H12 O	Cuminaldehyde	Benzaldehyde	Anti-inflammatory, Neuroprotective, Anticancer, Antidiabetic	42
21.	20.906	+	213.12	C14 H12	Stilbene	Diarylethene	estrogenic, bacteriostatic and fungistatic, Anticancer	43
22.	22.543	+	329.21	C21 H28 O3	Pyrethrin I	Irregular monoterpenoids	Potent biopesticide	44
23.	23.214	+	625.35	C33 H52 O11	Brainesteroside A	Phytoecdysteroid glucoside	Anabolic, adaptogenic, antidiabetic, hypolipidemic, and hepatoprotective	45

Table 7. UHPLC-HRMS data of compounds detected in *R. serpentina* leaf extract. [a] Ionization mode: − negative and + positive; Compounds reported with increasing order of RT (retention time).

S.no	RT [min]	[a]Ionization mode	m/z	Formula	Compound	Class	Reported activity	References
1.	0.947	-	187.04	C11 H8 O3	Plumbagin	Naphthalenes and derivatives;	Antioxidant, antiinflammatory, anticancer, antibacterial, and antifungal activities	46
2.	1.125	-	198.07	C9 H13 N O4	Anticapsin	Carboxylic acids and derivatives	Antimicrobial	47
3.	1.61	-	259.10	C14 H16 N2 O3	Maculosin	Dipeptides	Anticancer/antioxidant	48
4.	4.519	+	310.11	C12 H15 N5 O5	Sangivamycin	nucleoside	Antibiotic, antiviral and anti-cancer	49
5.	4.598	+	231.13	C8 H15 N O4	(+) -Castanospermine	Indolizidine alkaloids;	Antiviral agent, antitumor	50
6.	6.928	+	135.08	C9 H10 O	Cinnamyl alcohol	Cinnamic acids and derivatives;	Anti-inflammatory and antimicrobial activities;	51
7.	9.417	-	205.04	C11 H10 O4	Scoparone	Coumarins and derivatives;	Anti-inflammatory, anti-allergic, and anti-tumor activities.	52
8.	10.003	-	211.08	C13 H12 N2 O	Harmine	Carboline alkaloids	Antimicrobial, antifungal, antitumor, cytotoxic, antiplasmodial, antioxidaant, antimutagenic, antigenotoxic and hallucinogenic	53
9.	10.315	-	241.08	C8 H10 N2 O3	Azatyrosine	Alkaloids	Antibiotic; inhibits the tumor growth by deactivating c-Raf-1 kinase and ras signalling pathway	54
10.	11.379	-	315.12	C18 H20 O5	Combretastatin A-4	Monomeric stilbenes	Antimitotic antitumor agents	55
11.	12.192	-	265.06	C15 H10 N2 O3	Nigakinone	Carboline alkaloids	Anti-inflammatory and anti-cancer properties; bile acid (BA) metabolism disorder	56
12.	12.308	-	214.05	C12 H9 N O3	Robustine	Quinoline alkaloids	Cytotoxic activity against MCF-7 cells	57
13.	13.117	+	181.13	C8 H13 N O	(+/-)-Tropinone	Tropane alkaloid	Central intermediate for tropane alkaloid biosynthesis; anticancer drugs	58
14.	13.653	-	257.08	C15 H14 O4	Yangonin	Kavalactones and derivatives	Protects against cholestasis and hepatotoxity	59
15.	14.101	-	329.103	C18 H18 O6	Acetylshikonin	Naphthoquinones	Anticancer, anti-inflammatory, antioxidative, antibacterial, antifungal, lipid-regulatory, antidiabetic, neuroprotective, and antiviral properties	60
16.	14.627	-	360.15	C16 H19 N3 O3	(-)-Febrifugine	Quinazoline alkaloids	Antimalarial agent	61
17.	15.892	-	429.15	C21 H22 O6	B,β-dimethylacrylshikonin	Naphthoquinones	Cytotoxic activity for several cancer cell lines	62
18.	16.081	-	349.15	C21 H22 N2 O3	Vomilenine	Ajmaline-sarpagine alkaloids;	Key Intermediate of the Ajmaline Pathway	63
19.	19.22	+	289.10	C16 H16 O5	Shikalkin	naphthoquinone	Antiinflammatory	64
20.	27.107	+	290.17	C17 H23 N O3	Atropine	Tropane alkaloid	Treatment of myopia, amblyopia and organophosphorus poisoning; M-cholinoblockers	65
21.	27.16	+	609.28	C33 H40 N2 O9	Reserpine	Indole alkaloids	Antihypertensive and an antipsychotic	39
22.	27.163	+	579.26	C32 H38 N2 O8	Deserpidine	Yohimbine-like alkaloids	Antipsychotic and antihypertensive	66
23.	27.766	+	460.26	C26 H37 N O6	Militarinone A	pyridone alkaloid	Neuritogenic properties	67
24.	27.767	+	133.06	C9 H8 O	Cinnamaldehyde	Cinnamic acids and derivatives	Antimicrobial ; Antiviral; Antidiabetic; Anticancer; Anti-neurodegenerative	68
25.	28.124	+	419.24	C24 H34 O6	Phyllanthin	Naphthoquinones	Anti-angiogenic; suppress tumour growth	69
26.	28.452	+	272.16	C17 H23 N O3	()-Hyoscyamine	Tropane alkaloids	Anticholinergic	70
27.	28.932	+	305.17	C18 H24 O4	Prosolanapyrone II	Polyketides	Anti-inflammatory	71
28.	29.073	+	329.21	C21 H28 O3	Pyrethrin I	Irregular monoterpenoids	Potent biopesticide	44
29.	29.621	+	457.33	C29 H44 O4	Callystatin A	Open-chain polyketide	Anti-tumor, antibiotic	72

Table 8. UHPLC-HRMS data of compounds detected in crude secondary metabolite of RSLB3 (*B. mojavensis*). [a]Ionization mode: − negative and + positive; Compounds reported witn increasing order of RT (retention time).

S.No.	RT [min]	[a]Ionization mode	m/z	Molecular Formula	Compound	Class	Reported activity	References
1.	1.088	-	188.09	C8 H15 N O4	(+) Castanospermine	Indolizidine alkaloids	Antiviral agent, antitumor	75
2.	7.441	+	115.05	C4 H6 N2 O2	**Muscimol**	Isoxazoles	GABA agonist	
3.	12.308	-	214.05	C12 H9 N O3	Robustine	Quinoline alkaloids	Potency against human phosphodiesterase 5 (hpde5a)	76
4.	13.235	-	227.07	C14 H12 O3	Resveratrol	Monomeric stilbenes	Anti-obesity, cardioprotective neuroprotective, antitumor, antidiabetic, antioxidant	
5.	13.579	+	134.05	C8 H7 N O	2-oxindole	Quinazoline alkaloids; bicyclic monoterpene alkaloid	Anticancer, gastric ulcers, arthritis, and other mild physical inflammation, antimicrobial, antileishmanial	77
6.	13.863	-	373.15	C17 H26 O9	7-deoxyloganin	A,β-unsaturated carboxylic ester	Reaction intermediate to strictosidine, indole alkaloid biosynthesis	78
7.	14.281		284.09	C16 H15 N O4	Arborinine	Acridone alkaloids	Antimicrobial activity, anticancer activity	79
8.	15.066	+	350.14	C20 H19 N3 O3	Anisotine	Quinazoline alkaloids	Inhibit the proteolytic activity of SARS cov-2; antibacterial activity	80
9.	15.159	-	211.05	C12 H8 N2 O2	Questiomycin A	Phenoxazinone	Cytoprotective anticancer activity	81
10.	15.734	-	300.17	C17 H23 N3 O2	(-)-Indolactam v	Indole alkaloid	Pkc activator antitumor- activity	82
11.	15.753	-	465.15	C13 H10 N2 O	Pyocyanin	Phenazine compound	Antioxidant, antimicrobial, cytotoxic, and anticancer activities	83
12.	16.176	-	271.09	C16 H16 O4	Deoxyshikonin	Hydroxy naphthoquinone.	Colorectal cancer by down-regulating the PI3K/Akt/mtor pathway	84
13.	16.727	-	255.13	C12 H20 N2 O4	Pulcherriminic acid	Cyclic dipeptide	Antimicrobial agent, Siderophore	85
14.	16.811	-	322.1	C19 H17 N O4	Rutacridone epoxide	Acridines and an alkaloid antibiotic	Algicidal and antifungal	86
15.	18.158	+	349.09	C15 H16 N4 O4 S	Anguibactin	Siderophore	Catecholate and a hydroxamate iron-chelating agent with antimicrobial activity	87
16.	18.694	-	331.14	C19 H16 N2	Sempervirene	Indole alkaloid	RNA polymerase I transcription independently from p53 in tumor cells	88
17.	18.962	+	221.07	C14 H8 N2 O	Canthin-6-one	Carboline alkaloids	Antifungal, antileishmanial, antitumor, tryponocidal	89
18.	19.37	-	365.11	C20 H16 N2 O5	10-hydroxycamptothecin	Pyrroloquinoline alkaloids	Inhibits pseudorabies virus (prv); anticancer drug	90
19.	19.436	+	187.08	C11 H10 N2 O	Deoxyvasicinone	Quinazoline alkaloids	Antimicrobial, anti-inflammatory, and anti-depressant activities	91
20.	19.54	+	349.11	C20 H16 N2 O4	Camptothecin	Monoterpene indole alkaloid	Anti-tumor activity	92
21.	20.458	+	289.1	C16 H16 O5	Shikalkin	Naphthoquinone	Antiinflammatory	64
22.	21.136	-	255.1	C16 H16 O3	Pterostilbene	Monomeric stilbenes	Anticancer, antineoplastic antiinflammatory, anti-oxidant agent	93
23.	21.802	-	433.22	C24 H34 O7	Ajugarin I	Diterpenes	Insect antifeedent, anti-neuropahtic pain	94
24.	22.171	+	293.16	C19 H20 N2 O	Vellosimine	Sarpagine alkaloid	Reaction intermediate of sarpagine alkaloid pathway	95
25.	22.209	+	351.16	C21 H22 N2 O3	Vomilenine	Ajmaline-sarpagine alkaloids	Key intermediate of ajmaline pathway	63
26.	22.235	+	353.18	C21 H24 N2 O3	Raubasine/ ajmalicine	Monoterpene indole alkaloid	Antihypertensive agent	96
27.	22.255	+	225.06	C13 H8 N2 O2	Tubermycin B	Phenazine	Active antibacterial compound	97
28.	22.435	+	215.117	C13 H14 N2 O	Harmaline	Carboline alkaloids	Vasorelaxant, Antimicrobial, Antileishmanial, Antiplasmodial	98
29.	22.76	+	318.13	C17 H19 N O5	Piperlongumine	Amide alkaloid	Antiplatelet, Neuroprotective, Anti-diabetic, Anti-inflammatory	99
30.	22.768	+	144.08	C10 H12 N2	Tryptamine	Indole alkaloids	Secologanin and strictosidine biosynthesis; beta-carboline biosynthesis	30
31.	22.858	+	609.27	C33 H40 N2 O9	Reserpine	Monoterpene Indole Alkaloid	Antihypertensive and an antipsychotic	39
32.	22.871	+	369.18	C21 H24 N2 O4	Mitraphylline	Oxindole derivative Indolizidines	Arthritis, heart disease, cancer, and other inflammatory diseases	100
33.	23.134	+	257.16	C16 H20 N2 O	Huperzine B	Alkaloid	Acetylcholinesterase inhibitor	101
34.	23.279	-	529.3	C28 H42 N4 O6	Kukoamine A	Spermine alkaloid	Anti-diabetic, antioxidant and cytoprotective	102
35.	23.741	-	219.17	C15 H24 O	(-)-Caryophyllene oxide	Caryophyllane sesquiterpenoids	Analgesic, anti-infammatory, antitumor,	103
36.	26.782	-	324.17	C15 H22 O5	Artemisinin	Sesquiterpenoids	Antimalarial drug;	104
37.	28.334	+	324.12	C19 H17 N O4	Stylopine	Berberine alkaloid	Block vascular endothelial growth factor receptor 2 (VEGFR2)	105

Continued

S.No.	RT [min]	[a]Ionization mode	m/z	Molecular Formula	Compound	Class	Reported activity	References
38.	28.626	+	290.17	C17 H23 N O3	Atropine	Tropane alkaloid	Treatment of myopia, amblyopia and organophosphorus poisoning; M-cholinoblockers	106
39.	29.301	+	163.03	C9 H6 O3	7-hydroxycoumarine	Coumarin	Anti-hyperglycemic, Antinociceptive, antiedematogenic	107
40.	29.406	+	485.28	C29 H40 O6	Stigmatellin Y	Chromone	Biofilm inhibitory activity, antioxidant	108
41.	29.641	+	457.33	C29 H44 O4	Callystatin A	Polyketide	Anti-tumor antibiotic	72
42.	29.751	+	344.25	C22 H33 N O2	Paravallarine	Steroidal alkaloids	Cytotoxic Activity	109

Table 9. UHPLC-HRMS data of compounds detected in crude secondary metabolite of RSLB18 (*B. Wiedmannii*). [a]Ionization mode: - negative and + positive; Compounds reported witn increasing order of RT (retention time).

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information (Supplementary material S1 and S2).

Received: 13 July 2024; Accepted: 27 December 2024
Published online: 15 January 2025

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Acknowledgements

Authors would like to thank Department of Botany, MMV, BHU, for providing the necessary facilities. RL is thankful to the UGC, New Delhi for financial support as JRF and SRF. We are thankful to IoE, BHU for providing financial assistance in form of incentive and equipment grant and Central Discovery Centre- Banaras Hindu University (CDC-BHU) for providing High-Resolution Mass Spectrometry facility. SKG wants to acknowledge DST-SERB (Govt. of India) for financial aid (EEQ/2016/000555 and EEQ/2020/000485). A special thank goes to Mr. Hemant Kumar for final editing of figures.

Author contributions

The authors confirm contribution to the paper as follows: Lata R has conducted all the experiments, data collection, draft manuscript preparation under guidance of Gond SK has analysed and participated in the interpretation of results. All authors reviewed the results and approved the final version of the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-024-84893-0.

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