Evaluation of the Antimicrobial Potential and Berberine Production from Endophytic Populations of Medicinal Plant *Berberis aristata*

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Abstract

Endophytes isolated from medicinal plants are a valuable source of antimicrobial agents, owing to their capacity to harbor a wide array of bioactive compounds. Indian berberry, also known as *Berberis aristata*, has long been used in traditional medicine. The plant was selected for the isolation of fungal and bacterial endophytes. A total of 174 endophytes were isolated, of which 112 were fungal and 62 were bacterial. 10 fungal isolates and 6 bacterial isolates were selected for antimicrobial potential. Fungal endophyte FBL-5 and bacterial endophyte BBL-13 were found to have the greatest antimicrobial activity. The growth and synthesis of an antibacterial compound by these endophytes using a batch culture method were optimized. FBL-5 was identified as *Talaromyces verruculosus*, and BBL-13 was identified as *Stenotrophomonas maltophilia*. An HPLC study showed that FBL-5 and BBL-13 also produce Berberine, which is used commercially in several formulations in the health sector. For the first time, a study examined the capacity of the plant's bacterial endophytes to produce berberine and their antibacterial activity. These results suggest that endophytes from *B. aristata* could be a promising reservoir of biologically active secondary metabolites with potential applications in the pharmaceutical sector and as a means to conserve medicinal plants that are frequently exploited for drugs and are endangered.

Background

Endophytes are organisms that inhabit the tissues of their host plants and engage in symbiotic relationships [1]. Generally, endophytes include bacteria, fungi, archaea, and protists, with fungal endophytes the best studied, followed by bacterial endophytes. A distinctive feature of these endophytes is their ability to synthesize numerous novel bioactive compounds of plant origin with applications in pharmacy, agriculture, and other areas. Today, endophytes are being explored for the production of secondary metabolites with potential as anticancer, antioxidant, antimicrobial, insecticidal, immunosuppressive, and antidiabetic agents, which are quite similar to those produced by their host plants [2–4]. Endophytic fungi and bacteria inhabit novel biotopes [5] and are prime candidates for researchers seeking to discover novel secondary metabolites. Their potential to produce novel bioactive compounds offers several advantages, including faster, simpler production of valuable compounds that can be scaled up to industrial levels [6]. In turn, it has been demonstrated that these metabolites exert a variety of biological effects, bringing us a step closer to providing the scientific basis for the use of herbs and medicinal plants in traditional medicine systems [7].

According to the WHO, herbal medicines are used by 4 billion people worldwide as an alternative medicine because of their minimal side effects compared to chemical drugs. Common names for *Berberis aristata* include Chitra, Daruharidra, Daru Haldi, Indian barberry, and Tree Turmeric. It is a spiny, hard, yellowish herb in the family Berberidaceae. The plant can be found throughout the Himalayan region, including Bhutan, Nepal's hilly regions, and Sri Lanka. It is also found especially in the Chamba region of Himachal Pradesh at an altitude of 2000-3000m [8]. In traditional medicine, the plant is used to treat skin diseases, menorrhagia, diarrhea, eye problems, inflammation, and wound healing. Rasaunth is a well-known formulation of this plant that acts as a tonic, blood purifier, and treatment for ulcers and ophthalmic diseases [8]. Many of its pharmacological actions, including antimicrobial, antipyretic, anti-inflammatory, hepatoprotective, antioxidant, anticancer, and immunomodulatory activities, have been demonstrated by extensive research on this medicinal herb. According to phytochemical investigations, the plant *B. aristata* primarily includes alkaloids such as Berberine, oxyberberberine, berbamine, aromoline, karachine, a protoberberine alkaloid, palmatine, oxycanthine, taxilamine, tannins, sugar, and starch. Berberine, mostly found in the plant's roots, is its principal active ingredient [9–11]. Because of the tremendous medicinal value of *Berberis aristata*, we chose this plant for our study. There are very few reports on endophytes isolated and evaluated for bioactive compounds from this plant [12]. In this study, we isolated endophytic microbes from the roots and leaves of *B. aristata.* Bacterial and fungal endophytes are morphologically characterized and identified by DNA sequencing. The antimicrobial properties of isolated endophytes were evaluated using the agar diffusion method. Various parameters, such as pH, temperature, and medium, were optimized to produce bioactive compounds from potential bacterial and fungal isolates.

Furthermore, these bioactive-producing endophytes are tested for Berberine production using HPLC. This is the first study of bacterial endophytes from *B.aristata* and their antimicrobial activity against human pathogens. Also, we are the first to report the production of Berberine by fungal and bacterial endophytes of *B. aristata*. Additionally, the current investigation highlights the potential of endophytes as a substitute source for producing Berberine and other antimicrobial substances, rather than relying on medicinal plants that are frequently overused and at risk of extinction. Consequently, by conducting such research, we can identify novel antimicrobial agents from endophytes while also preserving our biodiversity.

Materials and Methods

2.1 Collection of plant material

For the study, random leaf and root samples from mature, disease-free shrubs growing in their native habitat in the Chamba district of Himachal Pradesh were collected. In sterile bags, the plant material was sent to the lab and stored there at 4°C. The plant material was collected from the cultivated herbal garden of the Research Institute of Indian System of Medicine (RISM), Jogindernagar, Himachal Pradesh, India, with the necessary permission from the Director of the Institute.

2.2 Surface sterilization of the plant material

The plant material was collected and surface-sterilized using the technique described by Regina et al. [13], with slight modifications. The plant's leaves and roots were cut into small segments and washed for 15 minutes under running water. The plant material was then dried between the layers of sterile filter paper. After being thoroughly cleaned, the plant material was rinsed for 15 minutes in a conical flask with water and sterile glass beads. The plant material was then washed three times with sterile water (each wash lasting five minutes), followed by washings with 70% alcohol (lasting one to two minutes), sterile water (lasting two to three minutes), and 0.5% NaOcl solution (lasting 30 seconds to one minute). The plant material was then rinsed with sterile water.

2.3. Herbarium deposition

In the current study, healthy Berberis aristata plant material was obtained from the herbal garden of the Research Institute of Indian System of Medicine (RISM), Jogindernagar, Himachal Pradesh, India, where the species is maintained under cultivation. The plant material was taxonomically identified, Research Institute of Indian System of Medicine (RISM), Jogindernagar, Himachal Pradesh, India. A voucher specimen was deposited in the Herbarium of Arni University under voucher specimen number AU 00983–2020/08.

2.3 Isolation and morphological characterization of endophytes

Arnold et al. [14] modified approach was used to isolate fungal endophytes. On Potato dextrose agar (PDA) plates supplemented with 0.5 g/ml chloramphenicol to prevent bacterial growth, the surface-sterilized segments were inoculated. The plates were then covered with Parafilm and incubated for 7–15 days at 28 ± 2°C. The surface-sterilized segments were placed on Nutrient agar (NA) plates supplemented with 25 g/ml amphotericin B to prevent fungal growth, and the sealed plates were incubated at 35 ± 2°C for 5–10 days to isolate bacterial endophytes [15]. Until a single isolate was obtained, developing bacterial and fungal colonies were subcultured repeatedly on NA and PDA, respectively. Pure isolates were stored in 10% glycerol at -80°C for preservation.

2.4 Fermentation and Extraction of bacterial and fungal extract

The endophytes thus obtained were grown in potato dextrose broth (PDB) and nutrient broth (NB) for 20 days, respectively. Following the incubation period, ethyl acetate (EtOAc) was used three times to remove the growth media and bacterial and fungal biomass. The dried extracts were placed in glass vials and stored at -20°C until the solvent was removed under reduced pressure.

2.5 Antimicrobial assay

During the primary screening, endophytic isolates were screened against eight MTCC bacterial strains, namely *Bacillus cereus* (MTCC 430), Bacillus subtilis (MTCC 441), *Escherichia coli* (MTCC 40), *Enterococcus faecalis* (MTCC 439), *Klebsiella pneumonia* (MTCC 109), *Pseudomonas aeruginosa* (MTCC 1934), *Pseudomonas alcaligenes* (MTCC 493), and *Staphylococcus aureus* (MTCC 3160), and three fungal strains, viz. *Aspergillus niger* (MTCC 281), *Candida albicans* (MTCC 183), and *Penicillium chrysogenum* (MTCC 6795) by using the perpendicular method [16]. The Microbial Type Culture Collection (MTCC) was procured from Chandigarh, India. For secondary screening, 10 fungal and 6 bacterial isolates that showed good inhibitory activity in primary screening were selected and tested using the agar well diffusion method. Dimethyl sulphoxide (DMSO 100% v/v) was employed as the positive control, and each extract was evaluated at a concentration of 1 mg/mL [17]. 50µl of the bacterial, fungal, and control extracts were added to the wells. Then these plates were incubated at room temperature for 60 minutes to allow the extracts to diffuse into the agar medium. The positive controls for bacteria and fungi were gentamycin (10 g/mL) and ketoconazole (50 g/mL), respectively. The size of the well (6 mm) was subtracted from the data obtained after the measurement of the Zone of Inhibition (ZOI) to obtain the real value. The mean ZOI was calculated and reported after this was done in triplicate.

2.6 Molecular identification of endophytes

The molecular identification of endophytes was performed using DNA sequencing of the 16S rRNA gene and the internal transcribed spacer region, and the Basic Local Alignment Search Tool (BLAST). DNA was isolated using the method described by Saghai and

Maroof (1984) [18]. The fungal and bacterial biomass growing in the PDB and NB, respectively, were ground in liquid nitrogen, transferred to a centrifuge cup containing pre-heated CTAB (10 ml), and incubated in a water bath maintained at 60℃ for 45 minutes, with gentle mixing every 5 minutes. After 45 minutes, a mixture of 24:1 chloroform and isoamyl alcohol was added, twice as much CTAB was added, and the mixture was gently mixed. The suspension was then centrifuged for 7 minutes at 7000 rpm. After collecting the upper aqueous phase into a separate centrifuge cup, cold DNA-grade ethanol was added. The resulting spool was then recovered and cleaned with 70% ethanol. DNA was purified using 1 µl RNase treatment. DNA quantification was performed by comparing the band intensities of isolated DNA samples from endophytic isolates with those of the Lambda uncut marker (300 ng/µl) on a 0.7% agarose gel.

Using eukaryote-based ITS-specific primers, ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5'-TCCTCCGCTTA TTGATATGC-3'), isolated fungal DNA samples were amplified using PCR. To amplify the bacterial DNA, 27F (5' AGAGTTTGATCMTGGCTCAG3') and 1492R (5'TACGGYTACCTTGTTACGACTT3') primers were used. The following thermal cycling conditions were used for the PCR reaction: primer annealing at 50°C for 60 s, primer extension at 72°C for 60 s, and final extension at 72°C for 5 min. The DNA template was first denatured at 94°C for 30s. The Montage PCR Clean-up kit (Millipore) was employed to purify the PCR products. The purified products were then sequenced on an ABI 3730xl sequencer (Applied Biosystems).

The ITS sequence of FBL-5 and the 16S rDNA sequence of BBL-13 were used as queries in NCBI BLAST. Following multiple sequence alignments using CLUSTAL X [19], the phylogenetic analysis was performed using the Molecular Evolutionary Genetics Analysis (MEGA) software package version 11 [20]. A phylogenetic tree was then constructed using the neighbor-joining method of Saitou and Nei [21].

2.8 Standardization of Fermentation Conditions for Maximal Production of Secondary Metabolites

The selected endophytic isolates were fermented and optimized to maximize their production of bioactive metabolites by modifying the growth medium, incubation temperature, and culture medium pH.

The broths were tested for their ability to support antimicrobial metabolite production through the use of eight different broth media: Czapek Dox (CD), Sabouraud Dextrose (SD), Potato Dextrose (PD), Malt Extract (ME), Starch Casein (SC), Glycerol Yeast Extract (GYE), Nutrient Broth (NB), and Tryptone Soy Broth (TSB). Each medium's antimicrobial activity was determined by dispensing 100ml of broth into 250ml Erlenmeyer flasks and inoculating each flask with 1ml of actively growing fungal or bacterial endophyte cultures, incubating them for 20 days at 30°C, and shaking at 160rpm. After incubation, the antimicrobial activity from each flask's culture filtrate was measured against selected bacterial and fungal pathogens (adsorbed on agar) using an agar well diffusion assay as described by Pundir et al. [22]. The medium demonstrating the greatest antimicrobial activity will be chosen for subsequent optimization studies.

To optimize the temperature of the fungal isolate used in this project, the isolate was grown in Potato Dextrose Broth (PDB) & the bacterial isolate was grown in Nutrient Broth (NB), with both media adjusted to pH 7. Fungal and bacterial cultures were incubated at varying temperatures (22 to 37°C) for 20 days, shaking at 160 rpm using an orbital shaker. Metabolite production was evaluated after incubation using the agar well diffusion assay; therefore, the temperature yielding the highest antimicrobial activity was designated as the optimal temperature for metabolite production.

To optimize the pH of the fungal cultures used for this study, the pH of the PDB and NB was adjusted to 3–10 using either 1 M HCl or 1 M NaOH before sterilization. The fungal and bacterial isolates were then grown separately in their respective optimally pH-corrected culture media and incubated for 20 days at 30°C at 160 rpm. After incubation, antimicrobial activity was assessed using the agar well diffusion assay; therefore, the pH that yielded the highest antimicrobial metabolite production was identified as optimal for further studies.**2.9 Thin-layer chromatography (TLC) detection**

Using silica gel Thin-layer chromatography (TLC) plates, chemical analysis of bacterial and fungal extracts for the presence of berberine was carried out (silica gel 60 F254, Merck). Ten microliters of the dried extract were put onto the TLC plate and developed in system A: Ethyl acetate: acetone: formic acid: water (8:2:2:1) and system B: Ethyl acetate: methanol: formic acid: water (8:2:2:1) after the dried extract was made in 10 mg/ml (10:1). Following the visualization of separated chemical components under UV light of 254 and 366 nm, a staining reagent was sprayed on them.

2.10 HPLC analysis of potent endophytes

2.10.1 Sample preparation

The sample's ethyl acetate extract was run through the Whatman No. 1 filter paper. The filtrate so collected was evaporated until it was completely dry. After vortexing, the dried material was resuspended in 1 mL of HPLC-grade methanol. For HPLC analysis, the samples were stored at 4°C and then filtered through a 0.45-mm Millipore membrane. Using HPLC-graded methanol, berberine (Sigma) standards were created and used for analysis.

2.10.2 Chromatographic conditions

An Agilent Series 1100 HPLC system (Agilent, Waldbronn, Germany) is used as the detector for the HPLC process, with a wavelength set to 254 nm. The autosampler injected 20 µl of the prepared sample solution. Methanol was used as the mobile phase, combined with HPLC-grade distilled water and 1% acetic acid (80:20:1), and delivered at a flow rate of 0.3 ml/min under low pressure. Based on a comparison between the retention time (Rt) of the sample and the Rt of the standard under equivalent chromatographic conditions, the presence of berberine in the endophytic extracts was established.

2.11 Statistical analysis

All experiments were performed in triplicate, and results are expressed as mean ± standard deviation (SD). Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test to compare differences among groups. A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant. Statistical analysis was performed using SPSS version 16.0. The assumptions of normality and homogeneity of variance were assessed before analysis to ensure the validity of statistical comparisons.

Result

3.1 Isolation and Morphological Characterization of Endophytes

In the present study, a total of 174 endophytes were isolated from *B. aristata*, including 62 bacterial and 112 fungal isolates obtained from healthy plant tissues. The observed differences in antimicrobial activity among isolates were statistically significant ($p < 0.05$), indicating variability in bioactive potential among the endophytes.

Among fungal endophytes, 78 were isolated from leaves and 34 from roots. Among the bacterial endophytes, 38 are from leaves, and 24 are from roots.

3.2 Antimicrobial assay

During the primary screening of endophytes for antimicrobial properties, out of 112 fungal endophyte isolates, 10 fungal endophytes were designated as FBL-1 to FBL-6 (from leaves) and FBR-1 to FBR-4 (from roots), which were found to be active against selected pathogens. Similarly, from 62 bacterial isolates, 6 bacterial endophytes designated as BBL-11 to BBL-13 (from leaves) and BBR-11 to BBR-13 (from roots) were found to have antimicrobial potential against selected Gram-positive and Gram-negative pathogens. The strongest antibacterial activity was observed among three fungal endophytes: FBL-5, FBL-6, and FBR-1. Among bacterial endophytes, BBL-13 recorded excellent antifungal activity against *C. albicans* and *A. niger*. During the secondary screening, all fungal extracts were evaluated for antimicrobial activity using an agar diffusion assay, and FBL-6 and FBR-1 showed broad-spectrum activity against Gram-positive and Gram-negative pathogens. Moreover, FBL-5 exhibited a potent zone of inhibition against S. aureus, C. albicans, and A. Niger, as shown in Tables 1 and 2. Endophytic fungal isolate FBL-5 236 recorded the highest antibacterial activity against *S. aureus*, as the diameter of the zone of inhibition was 25 mm, while fungal endophyte FBR-1 showed activity against *B. cereus*. In the antifungal potential of fungal endophytes, FBR-3 showed maximum activity against *C. albicans* and FBL-5 against *Penicillium chrysogenum.* Bacterial endophyte BBL-13 exhibited the highest antifungal action against *C. albicans.* The recorded zone of inhibition 240 was 22 mm (Table 3). Bacterial endophytes also exhibited antibacterial activity against S. aureus and K. pneumoniae, as shown in Table 4.

Table 1: Antibacterial activity of selected fungal extracts during secondary screening

Test Organisms	Fungal Extracts									
	FBL – 1	**FBL – 2**	**FBL – 3**	**FBL – 4**	**FBL – 5**	**FBL – 6**	**FBR – 1**	**FBR – 2**	**FBR – 3**	**FBR – 4**
Zone of inhibition in mm										
B. subtilis	20±0.40	16±0.65	14±0.60	19±0.12	13±0.81	14±0.60	18±1.43	16±0.34	9±0.5	18±0.21
B. cereus	19±0.30	10±0.21	16±0.13	20±0.23	13±0.11	20±0.56	21±0.32	16±0.11	12±1.25	11±0.34
E. coli	3±0.42	2±0.56	1±0.90	3±1.05	1±1.10	2±1.05	1±0.56	3±1.00	2±1.10	3±0.12
P. alcaligenes	15±0.11	14±1.05	16±1.24	12±0.90	15±0.42	20±0.30	21±0.85	14±0.21	13±1.12	18±0.35
P. aeruginosa	14±0.12	19±0.11	14±0.43	18±1.00	13±0.01	11±0.10	16±0.58	13±0.13	14±0.12	15±1.12
K. pneumoniae	10±0.23	19±0.12	15±0.52	7±0.80	8±0.03	12±0.10	13±0.01	12±0.21	7±1.10	12±1.23
S. aureus	20±0.11	16±0.12	24±0.49	24±1.43	25±0.45	24±0.20	25±0.12	13±0.12	15±1.21	25±0.64
S. epidermidis	23±0.56	17±1.10	20±0.12	22±1.27	9±0.54	24±0.43	24±0.03	9±0.32	9±0.21	20±0.34
LSD p<0.05	0.32	0.22	0.23	0.36	0.31	0.23	0.26	0.25	0.34	0.17
F-ratio	75.61	501.5	351.26	120.32	151.25	351.26	452.25	789.54	184.12	506.23

Mean value ± Standard deviation of triplicates; positive control: Standard chloramphenicol 10 units per disc.

Table 2
Antifungal activity of the selected fungal extracts during secondary screening

Test Organism	Fungal Extracts									
	FBL – 1	**FBL – 2**	**FBL – 3**	**FBL – 4**	**FBL – 5**	**FBL – 6**	**FBR – 1**	**FBR – 2**	**FBR – 3**	**FBR – 4**
Zone of inhibition in mm										
C. albicans	6 ± 0.64	4 ± 0.67	7 ± 0.12	3 ± 0.23	8 ± 1.23	5 ± 0.11	6 ± 1.02	8 ± 1.06	9 ± 1.45	6 ± 0.04
A. niger	5 ± 0.58	4 ± 0.23	1 ± 0.23	3 ± 0.21	7 ± 0.45	5 ± 0.32	5 ± 0.32	1 ± 0.34	4 ± 0.02	5 ± 0.02
P. chrysogenum	7 ± 1.01	5 ± 0.02	8 ± 0.34	9 ± 0.11	9 ± 0.76	6 ± 0.34	7 ± 0.04	3 ± 0.01	3 ± 0.04	9 ± 1.10
LSD p < 0.05	0.21	0.19	0.23	0.35	0.25	0.36	0.27	0.42	0.26	0.35
F- ratio	120.23	73.02	135.23	342.45	356.25	258.32	435.32	354.12	452.75	130.24
Mean value ± Standard deviation of triplicates; positive control: Amphotericin B 10 units per disc.										

Table 3
Antifungal activity of the positive bacterial extracts during secondary screening

Test organisms	Bacterial Extracts					
	BBL-11	**BBL-12**	**BBL-13**	**BBR-11**	**BBR-12**	**BBR-13**
C. albicans	9 ± 0.25	11 ± 1.05	22 ± 1.13	13 ± 1.12	17 ± 1.25	18 ± 0.67
A. niger	15 ± 1.99	10 ± 0.68	18 ± 1.65	11 ± 0.24	13 ± 0.82	15 ± 0.88
P. chrysogenum	11 ± 0.95	12 ± 1.55	7 ± 0.20	15 ± 1.12	12 ± 0.20	11 ± 0.34
LSD p < 0.05	0.38	0.28	0.27	0.45	0.36	0.17
F- ratio	338.32	765.21	120.34	455.54	195.23	495.32
Mean value ± Standard deviation of triplicates; positive control: Amphotericin B 10 units per disc.						

Table 4
Antibacterial activity of the positive bacterial extracts during secondary screening

Test organisms	Bacterial Extracts					
	BBL-11	**BBL-12**	**BBL-13**	**BBR-11**	**BBR-12**	**BBR-13**
P. alcaligenes	4 ± 0.05	6 ± 0.01	9 ± 0.54	9 ± 0.12	7 ± 0.03	5 ± 0.02
K. pneumoniae	5 ± 0.02	4 ± 0.76	7 ± 0.32	8 ± 0.65	5 ± 0.05	6 ± 0.03
S. aureus	4 ± 0.01	7 ± 0.54	3 ± 0.56	4 ± 0.36	6 ± 0.02	6 ± 0.02
LSD $p < 0.05$	0.20	0.35	0.20	0.24	0.18	0.17
F- ratio	506.25	87.12	606.45	354.21	484.65	432.54
Mean value ± Standard deviation of triplicates; positive control: Standard chloramphenicol 10 units per disc.						

3.3 Molecular identification of endophytes

Based on the results of primary and secondary screening, fungal isolate FBL-5 and bacterial isolate BBL-13 were selected for phylogenetic identification by DNA sequencing and for the standardization of the production of the bioactive components from these two endophytes. Using PCR-based DNA markers, FBL-5 and BBL-13 were molecularly characterized. Using Blastn, the partial ITS sequence of FBL-5 and the partial 16S sequence of BBL-13 were matched to the known databases. BBL-13 exhibited 99% homology to *Stenotrophomonas* maltophilia, and FBL-5 displayed 99% homology to *Talaromyces verruculosus*. The ITS sequence of fungal isolate FBL-5 (Talaromyces verruculosus) and the 16S rRNA gene sequence of bacterial isolate BBL-13 (Stenotrophomonas maltophilia) have been deposited in the NCBI GenBank database under accession numbers PV089831 and PV088449, respectively.. The phylogenetic tree of BBL-13 was created using 16S rRNA sequences from closely related species in the NCBI DNA database (Fig. 1A), and the Phylogenetic tree of FBL-5 was created using ITS sequences from closely related species in the NCBI DNA database (Fig. 1B).

3.4 Standardization of Fermentation Conditions for Maximal Production of Secondary Metabolites

Several media, including Czapek's Dox broth, Sabouraud dextrose broth, Potato dextrose broth, and malt extract broth, were examined to study the formation of the desired metabolite from the FBL-5 isolate to optimize the medium. Our bacterial endophyte BBL-13 showed maximum antimicrobial metabolite production in NB media as compared to Tryptone soya broth, Starch casein broth, and Glycerol yeast extract (Fig. 2a). Using PDB, FBL-5 demonstrated maximal metabolite synthesis. When PDB was utilized for metabolite formation, the ZOI was noticeably higher (Fig. 2b).

Temperature-dependent modulation of metabolite synthesis in the FBL-5 and BBL-13 strains was investigated. PDB medium with a chosen FBL-5 isolate and NB medium with a chosen BBL-13 isolate were inoculated on a rotary shaker at different temperatures (22, 25, 27, 30,3,3, 34, and 37°, C). Significant variation in metabolite production was observed under different culture conditions ($p < 0.05$), indicating that environmental factors strongly influence secondary metabolite synthesis. The best temperature for metabolite formation from FBL-5 was 30°C (Fig. 3a), whereas for isolate BBL-13 it was 28°C (Fig. 3b).

One of the key determinants of metabolism and, consequently, the formation of secondary metabolites is the pH of the culture media. For both isolates, the maximum metabolite concentration was produced at pH 7. The effect of pH on antimicrobial metabolite production for fungal and bacterial endophytes is shown in Fig. 4b respectively.

3.5 Thin-layer chromatography (TLC) detection

The plant extract and bacterial isolates were subjected to thin-layer chromatography to detect berberine, compared with the standard berberine (Sigma). The standard berberine showed a relative mobility factor (Rf) of 0.61 when developed on system A used for fungal isolates. Table 5 depicts the Rf value of the plant extract, which was 0.60, and only two fungal isolates were found to show Rf values almost similar to that of the standard, i.e., 0.59 and 0.58, respectively, with system A. In system B, plant extract and just one bacterial isolate, BBL-13, had identical values to the conventional berberine, 0.42 and 0.41, respectively.

Table 5
Rf values of the plant extract, fungal and bacterial endophytes

Sample/Isolate	Rf values with developing system A
Berberine	0.61
Plant extract	0.60
FBL-1	0.84
FBL-2	0.81
FBL-3	0.81
FBL-4	0.68
FBL-5	0.59
FBL-6	0.58
FBR-1	0.72
FBR-2	0.81
FBR-3	0.68
FBR-4	0.58
Sample/Isolate	**Rf values with developing system B**
Berberine	0.43
Plant extract	0.42
BBL-11	0.57
BBL-12	0.63
BBL-13	0.41
BBR-11	0.63
BBR-12	0.59
BBR-13	0.64

3.6 HPLC analysis of potent endophytes

Our studies revealed that the plant extract and standard berberine exhibited Rf values almost identical to those of FBL-5 and BBL-13. The HPLC analysis of the standard berberine and the potent bacterial and fungal isolates confirmed the production of berberine. The potent fungal (FBL-5) and bacterial (BBL-13) isolates showing activity were subjected to reverse-phase C analysis. The standard berberine's HPLC chromatogram revealed a distinctive peak with a retention time of 280 and a duration of 1.998 minutes. (Fig. 5a). The HPLC chromatogram of FBL-5 showed 4–5 peaks, but the characteristic peak was obtained at a time of 1.999 mins (Fig. 5b), marking the presence of berberine in it. During HPLC analysis of BBL-282 13, the chromatogram showed a single, distinct peak with a retention time of 1.997 minutes, indicating the synthesis of berberine. (Fig. 5c).

Discussion

The present study demonstrates that endophytic microbial populations associated with B. aristata exhibit significant antimicrobial potential and the ability to produce pharmaceutically relevant secondary metabolites, such as berberine. The antimicrobial activity observed in both bacterial and fungal endophytes highlights their potential as an alternative source of bioactive compounds, reducing reliance on plant-derived extraction. Notably, this study not only evaluates the antimicrobial potential of these endophytes but also, for the first time, reports that bacterial endophytes from B. aristata can produce berberine. This finding is particularly important as it opens new avenues for sustainable and scalable production of this clinically valuable alkaloid. Furthermore, the higher colonization rate of fungal endophytes in leaves than in roots observed in our study aligns with previous reports [25], suggesting that aerial tissues

may provide a more favorable microenvironment for fungal colonization. However, it is important to note that endophytic colonization is influenced by multiple factors, including host genotype, tissue type, environmental conditions, and seasonal variations [26], which may collectively shape the diversity and metabolic potential of these microbial communities.

Bacterial endophytes have been reported from all parts of plants [27], and in the present study, their colonization was also higher in leaf tissues. However, some studies have reported a greater abundance of bacterial endophytes in root tissues than in aerial parts [28], suggesting that colonization patterns may vary with plant species and environmental conditions. During the assessment of antimicrobial potential, fungal endophytes exhibited stronger activity against bacterial pathogens than against fungal pathogens, particularly against S. aureus, a clinically significant pathogen associated with drug resistance and nosocomial infections. Similar broad-spectrum antimicrobial activity of fungal endophytes from B. aristata has been reported previously [12]. However, activity profiles differed from those reported in earlier studies, in which fungal endophytes were reported to be more active against A. fumigatus; in our study, maximum antifungal activity was observed against *C. albicans* and *P. chrysogenum*. These differences may be attributed to variations in geographical location, host plant physiology, and environmental conditions, all of which influence endophytic diversity and metabolite production. The observed antimicrobial activity further supports the potential of fungal endophytes as a rich source of bioactive compounds, consistent with previous reports on their metabolite-producing capabilities [29].

Furthermore, the bacterial endophytes were evaluated for antimicrobial activity. They were found to be active against C. albicans and A. niger among fungal pathogens, and against S. *aureus and K. pneumoniae* among bacterial pathogens. There are several studies on the antimicrobial potential of bacterial endophytes from medicinal plants [30–32]. The antimicrobial activity observed in this study may be attributed to the production of secondary metabolites that interfere with microbial cell wall integrity, enzyme activity, or nucleic acid synthesis. However, further studies are required to confirm the exact mechanisms. This study represents the first report on bacterial endophytes of *B. aristata*, demonstrating their antimicrobial potential and metabolite production. Various parameters, such as media, temperature, and pH, were optimized to produce bioactive metabolites from endophytes. NB is recorded as the best medium for maximum antimicrobial metabolite production by bacterial endophyte BBL-13. Chavda et al.[33] also reported nutrient broth with plant extract as a basal medium for maximum antimicrobial production by bacterial endophytes. In the present study, we used only NB as the basal medium, as our aim is also to conserve this endangered B. aristata, and at the same time maximizing metabolite production without using the plant. In the case of temperature optimization, our findings fully align with the research by Myo et al. [34], which reported an optimal temperature range of 30–35°C for bacterial endophytes to produce bioactive metabolites. FBL-5 and BBL-13 exhibited maximum production of metabolite at pH 7. The same pH was reported by Hateet et al. [35] for the synthesis of an antibacterial compound by fungi isolated from eucalyptus leaves. There are many reports of berberine production by fungal endophytes from various medicinal plants. Berberine was synthesized by the endophytic fungi HL-Y-3 and was identified as *Alternaria* sp. from the healthy leaves of *Coptis chinensis*. The results of the chromatographic and spectroscopic studies supported the existence of berberine [36]. The endophytic fungus *Fusarium solani* from *Coscinium fenestratum* was also found to produce berberine [37]. Our study also reported berberine production by fungal endophytes, but this is the first-ever study on berberine production by bacterial endophytes found in medicinal plants, as far as we know. There are no reports of the production of berberine from either fungal or bacterial endophytes isolated from *B. aristata*. Berberine production from bacterial and fungal endophytes also provided new insights into the industrial production of this industrially important compound. Since natural antimicrobials from endophytes offer many therapeutic benefits and are relatively safe, they have attracted considerable interest from researchers worldwide. Further research confirms the effectiveness of these endophytes; they could become an important alternative for treating MRSA and other drug-resistant pathogens. In addition, the potential of these endophytes to have other beneficial effects could lead to the development of new formulations and combinations of herbal and synthetic drugs. However, the present study is limited to in vitro antimicrobial evaluation, and further in vivo studies, along with compound purification and characterization, are required to validate the therapeutic potential of these metabolites. Future studies focusing on large-scale fermentation, metabolic profiling, and genetic characterization of these endophytes may provide deeper insights into their industrial and pharmaceutical applications.

Conclusion

Natural antioxidants have many benefits and are very safe to use. Moreover, researchers worldwide are interested in endophytic populations. Research studies highlight significant potential for future therapeutic and industrial applications due to their remarkable pharmacological properties. Moreover, their increasing demand in the agricultural sector is also inevitable. The purpose of our study is to utilize a new technology and discover novel, effective bioactive compounds for human use. So we obtained two novel endophytic

strains from this plant, which have great potential to synthesize antibiotics that can be produced at an industrial scale to address surging drug resistance.

Declarations

Author contributions

Sheetal Sharma: Conceptualization, methodology, investigation, data curation, formal analysis, and writing—original draft preparation. Sarika Sharma: Conceptualization, supervision, project administration, methodology, validation, and writing—review and editing. Andrea Mastinu: Formal analysis, validation, interpretation of data, and writing—review and editing. Jayanthi Barasarathi: Investigation, validation, interpretation of data, and writing—review and editing. Asiya Nazir: Supervision, formal analysis, validation, manuscript coordination, and writing—review and editing. All authors reviewed, revised, and approved the final manuscript.

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Conflict of interest/Competing interest

No, there is no conflict of interest

Ethics approval

The present study did not involve human participants or animals; therefore, formal ethical approval was not required. **The research was conducted in accordance with all applicable institutional and national guidelines governing the use of cultivated medicinal plant materials.**

Consent to publish

All authors are aware of this submission and have consented to the publication of this study.

Consent to participate

All authors have given their consent for participation in this submission and possible publication of this study.

Availability of data and material

The datasets generated and/or analysed during the current study are available in the NCBI GenBank repository. The ITS sequence of Talaromyces verruculosus (FBL-5) has been deposited under accession number PV089831, and the 16S rRNA gene sequence of Stenotrophomonas maltophilia (BBL-13) has been deposited under accession number PV088449. All sequence data are publicly available through the NCBI GenBank database.

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Figures

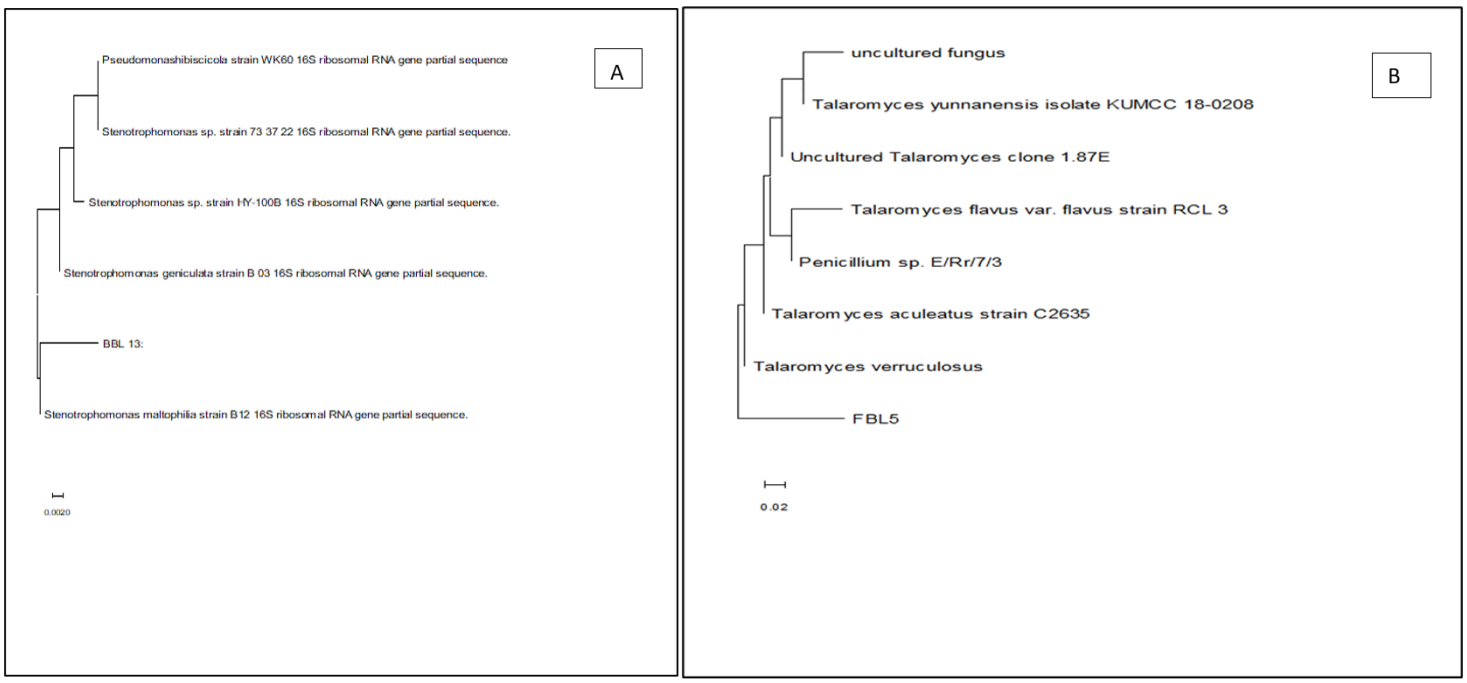

Figure 1

Phylogenetic tree using MEGA 11 and Clustal W 1.86 was used for sequence alignment. A. 16S rRNA gene sequences of BBL-13 and other bacteria from the NCBI DNA database. B. ITS rRNA gene sequences of FBL-5 and other fungi from the NCBI DNA database.

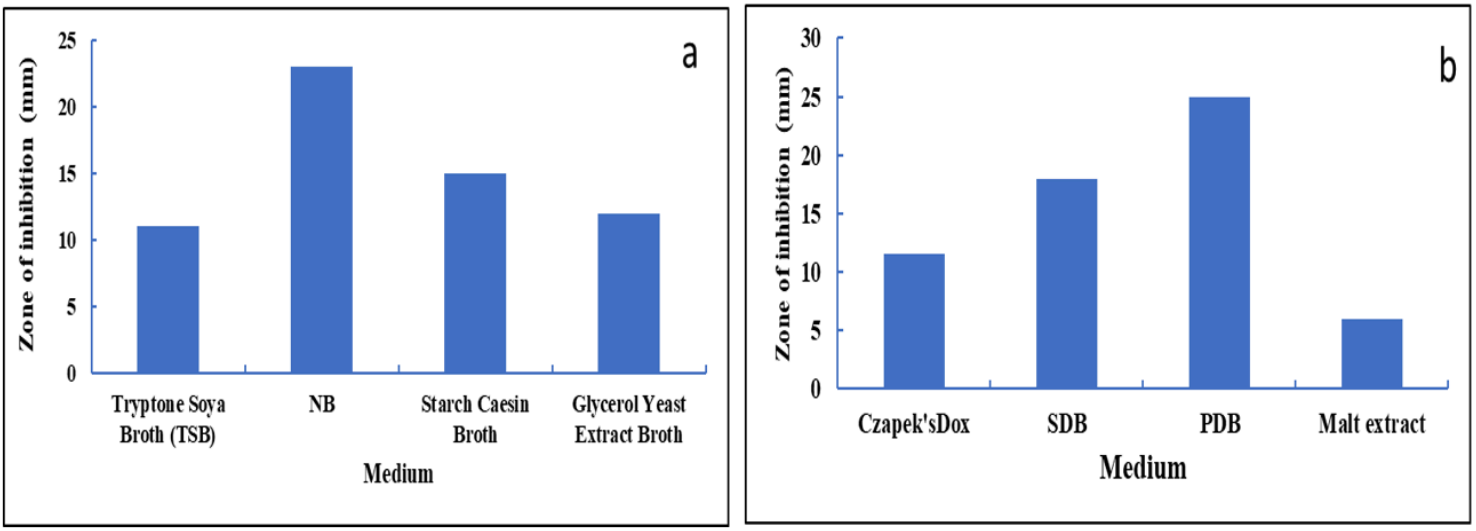

Figure 2

Outcome of various production mediums over metabolite production (a) BBL-13 (b) FBL-5

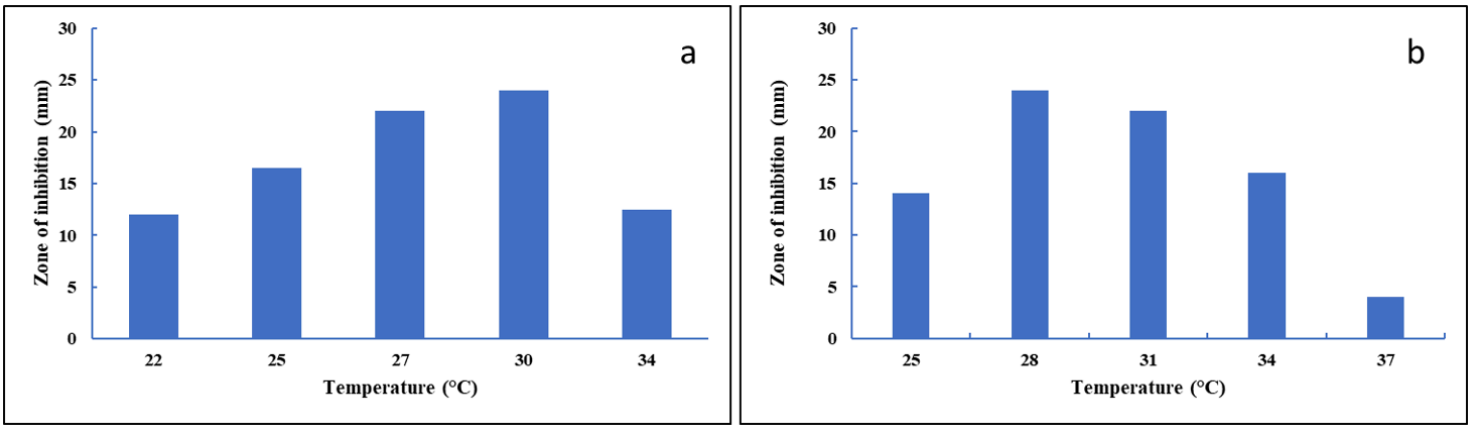

Figure 3

Outcome of various Temperature over metabolite production (a) FBL-5 (b) BBL-13

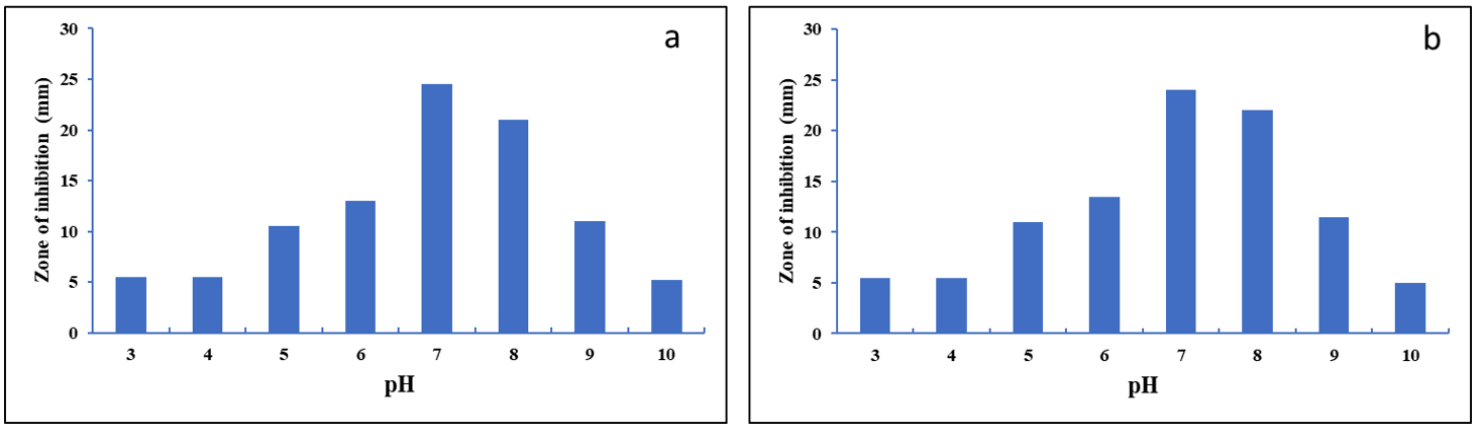

Figure 4

Outcome of various pH levels over metabolite production (a) FBL-5 (b) BBL-1

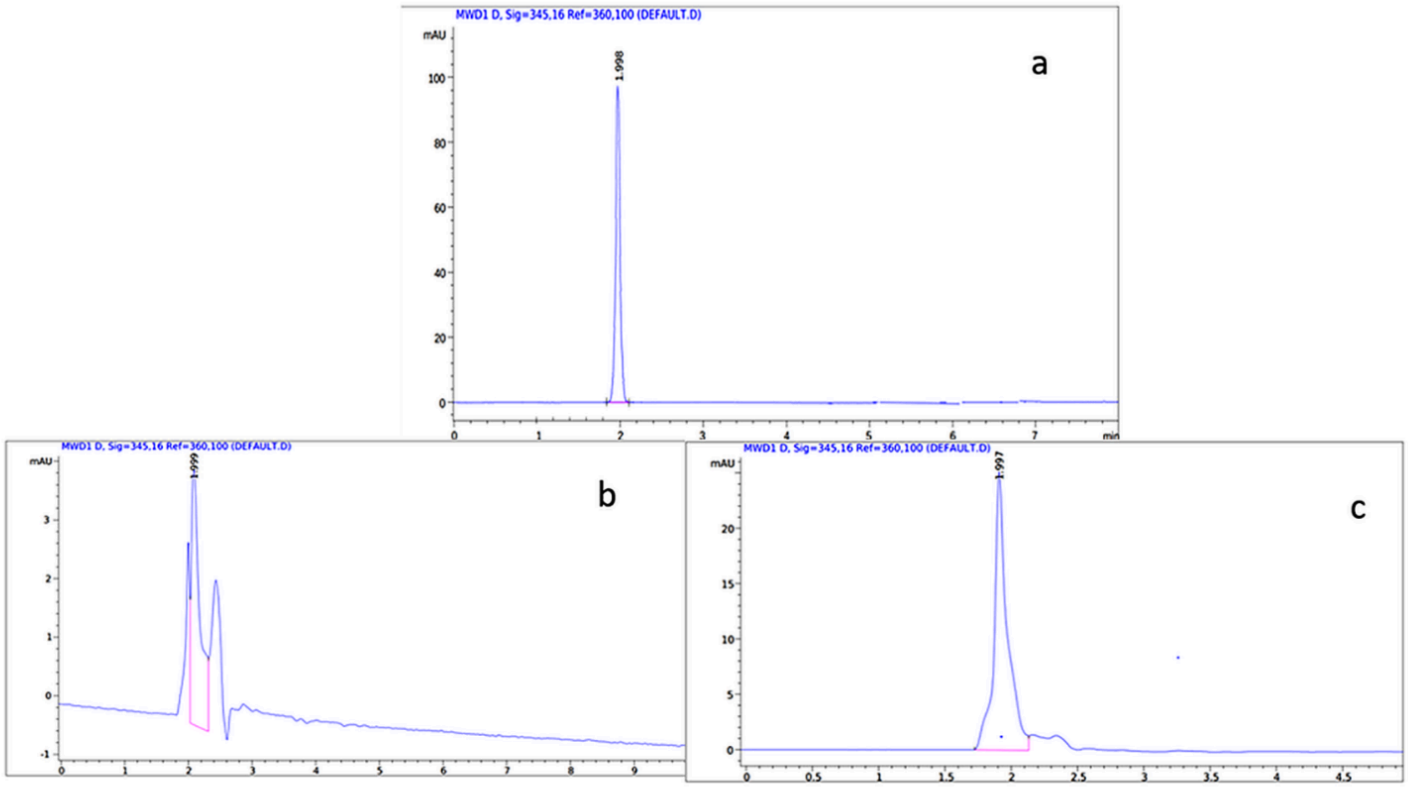

Figure 5

HPLC chromatogram of (a) Standard berberine (b)FBL-5 (c)BBL-13