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Original Article

Isolation and Screening of Endophytes from *Sarcandra glabra* to Increase the Content of Isofraxidin

Yuxin Chen[1], Tianqing Huang[1], Fei Li[1*] and Ehu Liu[1,2*]

[1]*State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing, Jiangsu, China;* [2]*College of Pharmacy, Nanjing University of Chinese Medicine, Nanjing, Jiangsu, China*

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Abstract

Background and objectives: Isofraxidin, an important coumarin compound found in the medicinal plant *Sarcandra glabra*, is reported to have anti-inflammatory activity. However, its natural concentration is insufficient to meet the existing demand for this valuable molecule. Therefore, biotechnological approaches are necessary to enhance the isofraxidin content.

Methods: Endophytes were isolated from the roots, stems, and leaves of *Sarcandra glabra* and fermented with *Sarcandra glabra*, respectively. The target strains capable of increasing isofraxidin content were screened using high-performance liquid chromatography. Their genes were amplified, and the polymerase chain reaction products were sequenced. BLAST analysis was used to compare the sequences with those in GenBank, and a phylogenetic tree was constructed for species identification.

Results: Fifteen endophytic bacteria and six endophytic fungi were isolated from the roots, stems, and leaves of *Sarcandra glabra*. Among them, *Enterobacter*, *Bacillus wiedmannii*, *Trametes versicolor* from the roots, and *Diaporthe celeris* and *Diaporthe hongkongensis* from the leaves increased the isofraxidin content in *Sarcandra glabra*. The isofraxidin content in *Sarcandra glabra* fermented by endophytes *Enterobacter*, *Bacillus wiedmannii*, *Trametes versicolor*, *Diaporthe celeris*, and *Diaporthe hongkongensis* was 1.37, 1.27, 1.11, 1.40, and 1.16 times higher than in the blank samples, respectively.

Conclusions: The fermentation of *Sarcandra glabra* with specific endophytes can increase its isofraxidin content. These findings provide preliminary scientific evidence for the potential of using microorganisms to enhance the quality of traditional Chinese medicine.

Introduction

Sarcandra glabra is a valuable medicinal plant belonging to the family Chloranthaceae, mainly distributed south of the Yangtze River in China. Phytochemical analysis has shown that *Sarcandra glabra* is rich in coumarins, flavonoids, sesquiterpenes, and phenols, which are reported to have diverse biological activities, including hepatoprotective,[1,2] antitumor,[3] antiviral,[4] anti-inflammatory, and immunomodulatory actions.[5,6] Isofraxidin, a major coumarin compound derived from *Sarcandra glabra*, is considered an index component due to its remarkable anti-inflammatory, anticancer, and antioxidative properties, and multi-target effects.[7] The advantages of a definitive clinical effect, good safety profile, and abundant resources have led to the development of numerous Chinese patent medicines containing *Sarcandra glabra* as a primary ingredient in modern times. Of these, 38 have been approved for marketing by the State Food and Drug Administration of China.[1]

Isofraxidin can attenuate a variety of destructive signaling mediators and therapeutic targets in certain diseases. Mechanistically, isofraxidin has been reported to regulate signaling pathways, exerting anti-inflammatory and anti-cancer effects.[8–10] Additionally, isofraxidin can affect the activity of various enzymes involved in the pathogenesis of certain cardiac and neurological diseases.[11–13] Although chemical synthesis of isofraxidin has been applied due to its wide application, the process is limited by the number of synthesis steps and *the* unmanageable nature of the process.[14]

The term "endophyte" refers to "microbes that colonize living, internal tissues of plants" without causing any immediate, overt negative effects.[15] Endophytes can stimulate the growth and pro-

Keywords: *Sarcandra glabra*; Isofraxidin; Endophyte; Biosynthesis; Fermentation; High-performance liquid chromatography.

[*]**Correspondence to:** Fei Li and Ehu Liu, State Key Laboratory of Natural Medicines, China Pharmaceutical University, No. 24 Tongjia Lane, Nanjing, Jiangsu 210009, China. ORCID: https://orcid.org/0000-0001-8721-5183 (FL), https://orcid.org/0000-0002-7281-210X (EL). Tel: +86-25-83271379, Fax: +86-25-83271379, E-mail: lifeicpu@163.com (FL); liuehu2011@163.com (EL).

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duction of secondary metabolites in host plants. Due to the possibility of genetic recombination between the endophyte and the host,[16] rare compounds that can be produced by the host can also be produced by endophytes, thus reducing the need for harvesting slow-growing and rare plants. Previous studies have indicated that the endophytes of *Taxus* spp. (e.g., *Taxomyces andreanae* and *Pestalotiopsis microspore*) are capable of producing the antileukemic and antitumor drug taxol.[17] Currently, the wild resources of *Sarcandra glabra* plants are decreasing, and cultivated ones contain lower concentrations of isofraxidin. The demand for such important chemical constituents far exceeds what is available from existing natural resources. Endophytes, a less-studied class of microorganisms, have great potential for exploitation in the medical, agricultural, and industrial fields.

Fermentation technology is a new method for obtaining the active ingredients of functional health foods. It uses the powerful enzyme systems of microorganisms to achieve directional biotransformation of certain active ingredients in natural food, thereby improving the yield of these active ingredients. In the present study, to produce isofraxidin in a targeted and efficient way, we isolated and identified endophytes from *Sarcandra glabra*. The isolated endophytes were then co-fermented with *Sarcandra glabra* to induce the production of active metabolites.[18]

Materials and methods

Sampling of Sarcandra glabra

The *Sarcandra glabra* samples were collected from Changsha, Hunan Province, and identified by Professor E-Hu Liu (Department of Pharmacognosy at China Pharmaceutical University). The stems, roots, and leaves were separated using a sterile scalpel, placed in sterile plastic bags, and stored at −20°C.

Isolations of endophytes from Sarcandra glabra

For the isolation of endophytes from *Sarcandra glabra*, portions of lesion-free roots, stems, and leaves were washed in tap water.[19] They were surface sterilized by immersion in 75% ethanol for 3 m under sterile conditions. After allowing the alcohol to evaporate, the roots, stems, and leaves were surface sterilized with 0.1% mercury chloride for 2 m. The samples were rinsed with autoclaved water three to five times and dried with sterile paper towels. The roots, stems, and leaves were then cut into small pieces and inoculated onto potato dextrose agar (PDA) plates containing potato (20%), dextrose (2%), and agar (1.5–2%) (Qingdao Hope Bio-Technology, China) using a sterile scalpel. The inoculated plates were incubated at 28 ± 2°C for seven to ten days, during which the emergence of bacteria and fungi from the tissues was observed. The nascent bacteria were transferred to beef extract peptone agar plates containing peptone (1%), beef extract (0.3%), NaCl (0.5%), and agar (1.5–2.5%) (Qingdao Hope Bio-Technology, China) and incubated at 37°C for one to two days. Individual mycelia were inoculated on each PDA plate supplemented with streptomycin at 100 µg/mL and incubated for seven to ten days at 28 ± 2°C under dark conditions. All cultures were periodically transferred to fresh plates until pure endophyte cultures were obtained. The same procedure was used to blot sterilized roots, stems, and leaves on PDA plates, while a plate coated with 200 µL of sterile water from the last rinse served as a negative control.

Fermentation sample preparation

The bacteria isolated in this study were grown in beef extract peptone medium (Qingdao Hope Bio-Technology, China), and the fungi were grown in potato dextrose water (Qingdao Hope Bio-Technology, China) liquid medium supplemented with streptomycin at 100 µg/mL. The endophyte suspensions were prepared by shaking the cultures at 37 °C and 150 rpm for 24 h.

Five grams of *Sarcandra glabra* root, stem, and leaf powder (sieved through a four-mesh sieve) were accurately weighed respectively and placed in a conical flask, each with 30 mL of sealed growth medium. The samples were sterilized at 121°C for 20 m and then used as substrates. Four milliliters of each bacterial suspension was added to the corresponding substrate containing the respective medium (n = 3) (endophytes isolated from different tissues were only added to the corresponding substrates), respectively, and the samples were lyophilized after fermentation to obtain fermentation samples. Another 4 mL of sterile water was added to the substrate, and the same culture method was used to prepare a blank control sample.

Screening of endophytes to increase isofraxidin content

The endophytic isolates obtained were screened for strains capable of increasing the isofraxidin content. Chromatographic separation was performed using a Thermo BDS Hypersil C18 column (4.6 × 250 mm, 5 µm, Thermo Fisher Scientific, Waltham, MA, USA) at a column temperature of 40°C. The mobile phase consisted of ultra-pure water with 0.1% phosphoric acid (eluent A) and acetonitrile (eluent B), using isocratic conditions of 80% A and 20% B. The compounds were monitored at 344 nm, with a sample injection volume of 10 µL. The flow rate was set at 1.0 mL/m.[20]

A standard solution of isofraxidin was prepared by accurately weighing 10 mg of the standard, dissolving it in methanol to achieve a concentration of 100 µg/mL. This standard solution was further diluted with 50% methanol to obtain final concentrations of 5, 10, 20, 40, and 80 µg/mL, respectively.

Fermentation samples weighing 0.5 g were extracted with 10 mL of 70% methanol using ultrasonic extraction for 30 m. The extract was then centrifuged at 4,000 rpm for 10 m, and the supernatant was passed through a 0.22 µm microporous filtration membrane to obtain the working solution for high-performance liquid chromatography (HPLC) analysis. Each extraction was performed in triplicate.

Validation of methodology

Based on the established HPLC conditions, standard curves were prepared using different concentrations of isofraxidin standard solutions. Precision was assessed by analyzing six replicate sample solutions. The repeatability was determined by evaluating six replicates of a single sample. A stability study was conducted by analyzing a sample at different time points over a day (0, 2, 4, 6, 8, 12, and 24 h). Each sample solution was tested three times in parallel.

Extraction of fungal genomic DNA

Total genomic DNA was extracted using the Ezup Column Fungi Genomic DNA Purification Kit (Sangon Biotech, Shanghai, China). The DNA concentration was determined using a Nano-100 micro-spectrophotometer (AllSheng Instruments, Hangzhou, China) according to the manufacturer's instructions for polymerase chain reaction (PCR) amplification.

Amplification of gene

The universal internal transcribed spacer (ITS) region was amplified using the primers ITS1 (5′-TCCGTAGGTGAACCT-

GCGG-3′) (all primers from Sangon Biotech, Shanghai, China) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) for fungal strain identification. The total PCR mixture volume was 50 µL, consisting of 21 µL dd water, 1 µL of each primer, 25 µL Taq PCR Mastermix (TIANGEN Biotech, Beijing, China), and 2 µL of template DNA. The PCR was programmed with an initial denaturation at 95°C for 5 m, followed by 35 cycles of denaturation at 94°C for 1 m, annealing at 55°C for 30 s, and extension at 72°C for 2 m. The final extension was performed at 72°C for 10 m.[21]

Taxonomic identification of the endophytic bacterial strains was carried out using the universal primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′). The total PCR mixture volume was 25 µL, comprising 9.5 µL dd water, 1 µL of each primer, 12.5 µL Premix Taq™ (Takara Bio, Beijing, China), and 1 µL of endophytic bacterial solution. The PCR was programmed with an initial denaturation at 94°C for 5 m, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 59°C for 45 s, and extension at 72°C for 1 m. The final extension was performed at 72°C for 10 m.[22]

Quality and integrity detection for PCR products

To prepare a 1% (w/v) agarose gel, dilute 50 × TAE Buffer (Sangon Biotech, Shanghai, China) to 1 × with purified water and mix it with the appropriate amount of agarose powder (Baygene, Shanghai, China).

Add 5 µL of GelRed (Sangon Biotech, Shanghai, China) to 1 mL of 10 × Loading Buffer (Vazyme, Nanjing, China). Then, add 1 µL of GelRed to 200 µL of DNA Marker (Takara Bio, Beijing, China), mix well, and store at 4°C. In each well, add 10 µL of PCR products and 1 µL of 10 × Loading Buffer, and place 5 µL of DL 3000 Marker in wells on both sides of the gel electrophoresis tank to indicate the molecular weight. Electrophoresis was conducted at 120 mV for 10 m. Successfully amplified PCR products (single, bright bands) were sequenced by Sangon Biotech Ltd Co.

Sequencing and construction of phylogenetic tree

The sequences were submitted to the GenBank database and compared with existing sequences in GenBank using Blast analysis (MEGA 6.0 software). A phylogenetic tree was constructed using MEGA 6.0 software to determine the relationships and conduct phylogenetic analysis.

Statistical analysis

The means and standard deviations were calculated using MS Excel 2010. Statistical analyses were conducted with GraphPad PRISM version 8.0 (GraphPad Software, La Jolla, CA, USA). Data were presented as mean ± SD. The significance of differences among multiple groups was analyzed by one-way analysis of variance with Tukey's multiple comparison test, and $p < 0.05$ indicated a significant difference.

Results

Isolation of the endophytes

The endophytes were placed on a medium for continuous inoculation passaging to obtain pure cultures. A total of 15 strains of endophytic bacteria and 6 strains of endophytic fungi were isolated from the roots, stems, and leaves of *Sarcandra glabra*. Five endophytic bacteria [*Enterobacter* (BR 1), *Leclercia adecarboxylata* (BR 2), *Burkholderia stabilis* (BR 3), *Bacillus mobilis* (BR 4), and *Bacillus wiedmannii* (BR 5)] and two endophytic fungi [*Trametes versicolor* (FR 1) and *Penicillium rubens* (FR 2)] were found in the roots. Five endophytic bacteria [*Enterobacter* (BS 1), *Lelliottia amnigena* (BS 2), *Priestia aryabhattai* (BS 3), *Paenibacillus humicus* (BS 4), and *Lysinibacillus macrolides* (BS 5)] and one endophytic fungus *Aspergillus aflatoxiformans* (FS 1) were isolated from the stems. Five endophytic bacteria [*Enterobacter* (BL 1), *Pantoea rodasii* (BL 2), *Kluyvera intermedia* (BL 3), *Priestia aryabhattai* (BL 4), and *Lysinibacillus pakistanensis* (BL 5)] and three endophytic fungi [*Diaporthe celeris* (FL 1), *Diaporthe hongkongensis* (FL 2), and *Nodulisporium indicum* (FL 3)] were isolated from the leaves.

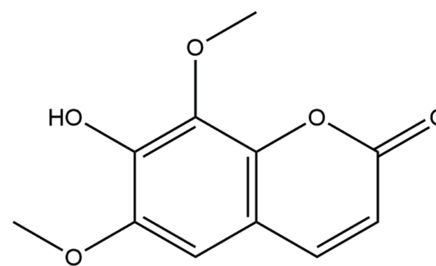

Fig. 1. Structure of isofraxidin.

Estimation of isofraxidin contents in Sarcandra glabra after the fermentation of endophytes

HPLC-based analysis revealed absorption peaks at 344 nm for the samples. The fermented samples displayed the same peak at the same retention time as the raw samples and the isofraxidin standard (Figs. 1 and 2). Compared with the root blank samples, the content of isofraxidin in *Sarcandra glabra* fermented by endophytes BR 1, BR 5, and FR 1 was 1.37, 1.27, and 1.11 times higher than the control, respectively. The content of isofraxidin in *Sarcandra glabra* fermented by endophytes FL 1 and FL 2 was 1.40 and 1.16 times higher than the blank samples, respectively (Tables 1 and 2). These results indicate that two strains of endophytic bacteria (BR 1 and BR 5) and three strains of endophytic fungi (FR 1, FL 1, and FL 2) were able to increase the isofraxidin content in the corresponding fermentation samples, with the endophytic fungus FL 1 having the most significant effect (Fig. 3).

Validation of methodology

The standard curve was plotted with the sample volume (µg) of the standard on the horizontal axis and the peak area on the vertical axis. The regression equation for isofraxidin is $y = 5.9826x + 13.479$, with an r-value of 0.9998. The relative standard deviations for precision, repeatability, and sample stability were 1.82%, 2.34%, and 2.51%, respectively (all less than 3%), indicating that the developed HPLC method is valid and suitable for sample analysis (Table 3).

Identification of the yield-enhancing endophytes

The bacterial fluid of the endophytic bacterium was amplified to obtain a target fragment of approximately 1,500 bp. The total DNA of the endophytic fungi was extracted, and the ITS region was amplified to obtain a target fragment of approximately 550 bp (Fig. 4). The yield-enhancing endophytes (BR 1, BR 5, FR 1, FL 1, and FL 2) were characterized using gene sequencing technology. The nucleotide sequences obtained from the endophytes were submitted to the NCBI database, and their corresponding accession numbers are available in GenBank. Based on the results of

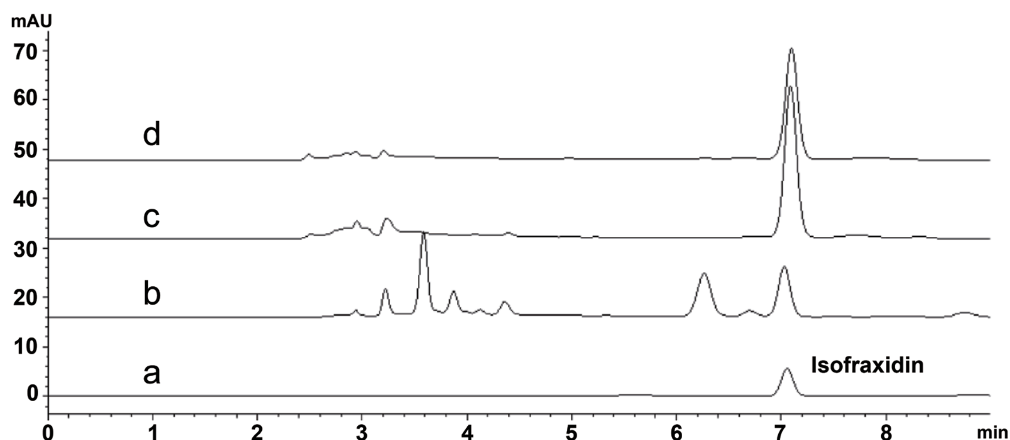

Fig. 2. *High-performance liquid chromatography* chromatogram of endophytic bacterium *Bacillus wiedmannii* (BR 5) before and after fermentation of S. glabra. (a) Isofraxidin standard, (b) *Sarcandra glabra* (raw), (c) Fermented samples of roots with BR 5 and (d) Fermented samples of roots with sterilized water.

sequence alignment conducted on the NCBI BLAST server, two yield-enhancing endophytic bacteria were identified as BR 1 and BR 5). The three yield-enhancing endophytic fungi were identified as FR 1, FL 1, and FL 2) (Figs. 5–7).

Discussion

Sarcandra glabra was chosen as the source plant for isolating endophytes because it has been reported to be the most vital natural source of isofraxidin. The complex pathophysiological mechanisms underlying diseases such as cancer, heart failure, and neurodegenerative disorders have prompted the need for multi-targeted compounds. Given these complexities, isofraxidin might be introduced as an effective multi-target drug. We aimed to isolate endophytes from *Sarcandra glabra* and screen them for their potential to promote isofraxidin production. Isofraxidin, present in herbal sources, has a unique pharmacokinetic profile and low levels of side effects.[23] However, the isolation and purification of isofraxidin are slow and low-yielding, and the chemical synthesis involves multiple steps.[24] To our knowledge, the main pathway

Table 1. Determination of isofraxidin in the fermentation samples of *Sarcandra glabra*

Endophytic bacteria	Content of isofraxidin (mg/g)		
	Fermentation samples of roots	Fermentation samples of stems	Fermentation samples of leaves
BR 1	0.8895 ± 0.0033****		
BR 2	0.6306 ± 0.0158		
BR 3	0.5273 ± 0.0088		
BR 4	0.5704 ± 0.0127		
BR 5	0.8190 ± 0.0045****		
BS 1		0.3746 ± 0.0021	
BS 2		0.3317 ± 0.0026	
BS 3		0.2811 ± 0.0064	
BS 4		0.3764 ± 0.0021	
BS 5		0.2423 ± 0.0050	
BL 1			0.0329 ± 0.0090
BL 2			0.0116 ± 0.0006
BL 3			0.0033 ± 0.0001
BL 4			0.0270 ± 0.0007
BL 5			0.0161 ± 0.0045
Sterilized water	0.6470 ± 0.0036	0.4838 ± 0.0037	0.1128 ± 0.0030
Fermentation solution control		——	

Compared to *Sarcandra glabra* fermented with sterilized water, **** $p < 0.0001$. BR: Bacteria from roots; BS: Bacteria from stems; BL: Bacteria from leaves.

Table 2. Determination of isofraxidin in the fermentation samples of *Sarcandra glabra*

Endophytic fugus	Content of isofraxidin (mg/g)		
	Fermentation samples of roots	Fermentation samples of stems	Fermentation samples of leaves
FR 1	0.7395 ± 0.0003****		
FR 2	0.6578 ± 0.0034		
FS 1		0.5322 ± 0.0096	
FL 1			0.5751 ± 0.0047****
FL 2			0.4754 ± 0.0029****
FL 3			0.3953 ± 0.0021
Sterilized water	0.6639 ± 0.0108	0.5510 ± 0.0010	0.4102 ± 0.0023
Fermentation solution control		——	

Compared to *Sarcandra glabra* fermented with sterilized water, **** $p < 0.0001$. FR: Fungi from roots; FS: Fungi from stems; FL: Fungi from leaves.

of isofraxidin biosynthesis has not been revealed.[25] Thus, there is a need to use biotechnological methods to enhance the yield of isofraxidin. Previous studies have shown that interactions between fungi and plants can enhance secondary metabolites *in vivo* and make plants more resistant to harsh environments.[26] Among the methods to enhance secondary metabolites in plants, microbial fermentation techniques are the most cost-effective. Endophytes have been reported to effectively increase the yield of secondary metabolites.[27]

In this study, five strains of endophytes (BR 1, BR 5, FR 1, FL 1, and FL 2) were isolated, which can increase the content of isofraxidin. The results of the HPLC analysis demonstrated the presence of unidentified compounds in the raw *Sarcandra glabra.* However, these compounds disappeared after fermentation with the production-enhancing endophytes. This suggests that the increased levels of isofraxidin may result from biotransformation by the endophytes. For example, *C. globosum* CGMCC 6882, as reported by Wang, could produce extracellular polysaccharides with excellent inhibitory effects against both *E. coli* and *S. aureus*.[28] Endophytic fungi of *Aspergillus aculeatus*, *Penicillium georgiense*, and *Aspergillus flavus* isolated from *Polygonum cuspidatum* could convert polydatin into resveratrol, thus promoting plant growth and development.[29] Our findings align with these studies, suggesting the application of endophytes to enhance the yield of secondary metabolites. Notably, FL 1 exhibited the greatest capacity to enhance yield. Although we have not confirmed the production of isofraxidin by the endophytes themselves, most studies indicate that endophytic fungi can produce host compounds. For example, a macrolide antibiotic, lasiodiplodin, was isolated from the endophytic fungus Lasiodiplodia pseudotheobromae J-10 associated with the medicinal plant *Sarcandra glabra*.[30] However, the specific mechanisms remain poorly understood.

Future research directions

The limitation of this study is that the optimal fermentation process

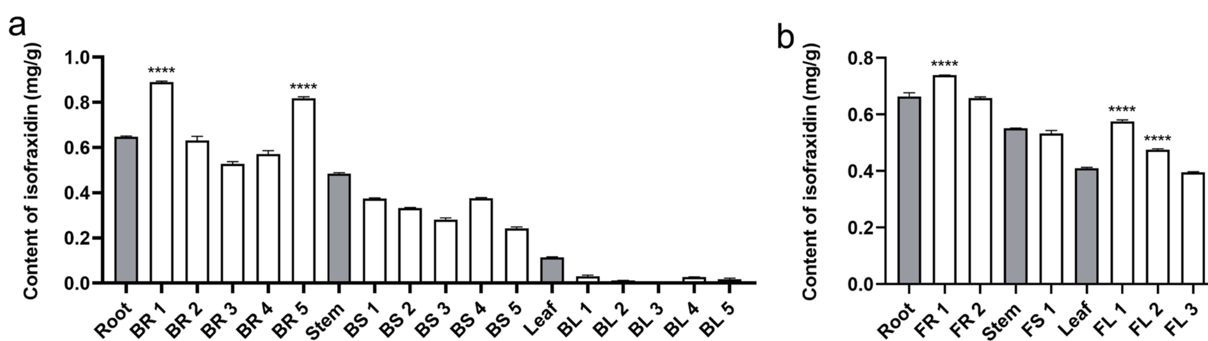

Fig. 3. Determination of isofraxidin in endophytic bacteria (a) and endophytic fungi. (b) fermentation samples. BR: Bacteria from roots; BS: Bacteria from stems; BL: Bacteria from leaves; FR: Fungi from roots; FS: Fungi from stems; FL: Fungi from leaves. For example, BR1, BR2, etc. represent the first, second, etc. bacteria isolated from roots. Compared with *Sarcandra glabra* root and leaf group, respectively, **** $p < 0.0001$.

Table 3. Regression equation, precision, repeatability, and stability of isofraxidin

Sample	Regression equation	R^2	Precision RSD(%)	Repeatability RSD(%)	Stability RSD(%)
			n = 6	n = 6	24 h
Isofraxidin	y = 5.9826x + 13.479	0.9998	1.82	2.34	2.51

RSD: Relative standard deviation.

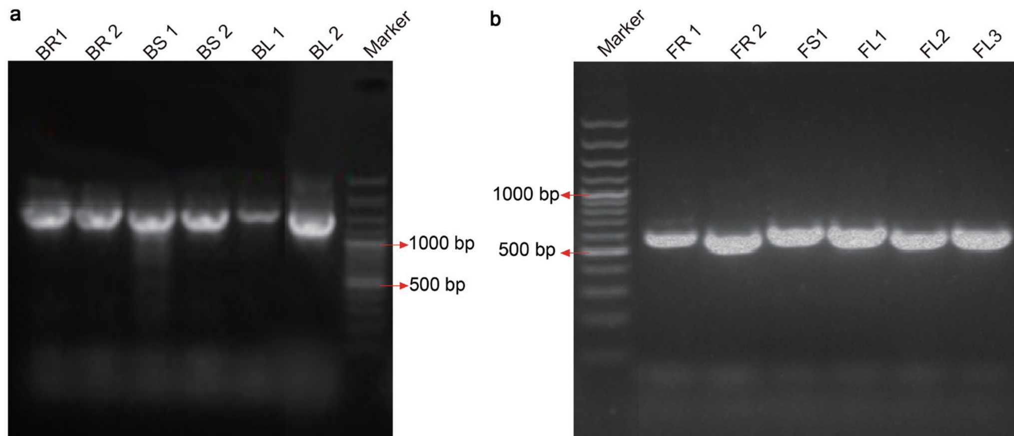

Fig. 4. 16S rDNA amplification products of endophytic bacteria (a) and ITS (Internal Transcribed Spacer) amplification products of endophytic fungi (b). BL: Bacteria from leaves; BR: Bacteria from roots; BS: Bacteria from stems; FL: Fungi from leaves; FR: Fungi from roots; FS: Fungi from stems.

was not identified, despite discovering that five strains increased the content of isofraxidin. The fermentation conditions need to be further optimized to obtain a higher amount of isofraxidin. Meanwhile, the separation of isofraxidin in fermented samples by column chromatography is straightforward and effective, making it suitable for obtaining purified isofraxidin products. Additionally, isolating isofraxidin from fungi and bacteria is a future research goal. Further studies are needed to reveal the mechanisms of interaction among these endophytes and to discover novel active metabolites, which could serve as a foundation for developing new drugs.

Conclusions

The results indicate that five bioactive endophytes (BR 1, BR 5, FR 1, FL 1, and FL 2) were isolated and identified. We found that the effect of the endophytic fungus FL 1 was the most significant, with the isofraxidin content in *Sarcandra glabra* treated with FL 1 increasing by 1.40 times. Therefore, the biotransformation function of endophytes can be utilized to enhance the yield of active ingredients.

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Conflict of interest

The authors declare that they have no conflict of interest that could appear to influence the work reported in this paper.

Author contributions

Conceptualization (YC, FL, EL), methodology (YC, TH, EL), data

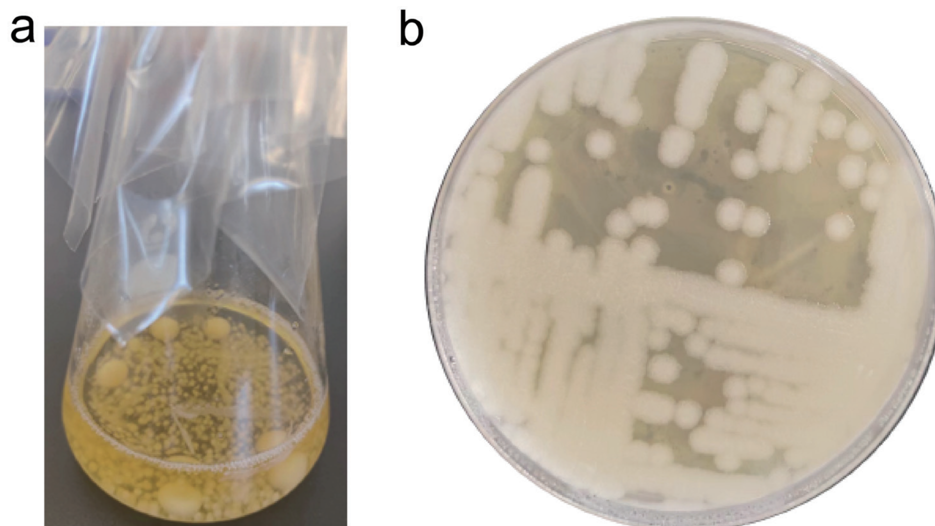

Fig. 5. Endophytic fungi *Diaporthe celeris* (FL 1) grown on Potato Dextrose Water (a) and endophytic bacteria *Bacillus wiedmannii* (BR 5) grown on BPA (Beef Extract Peptone Agar) plate (b).

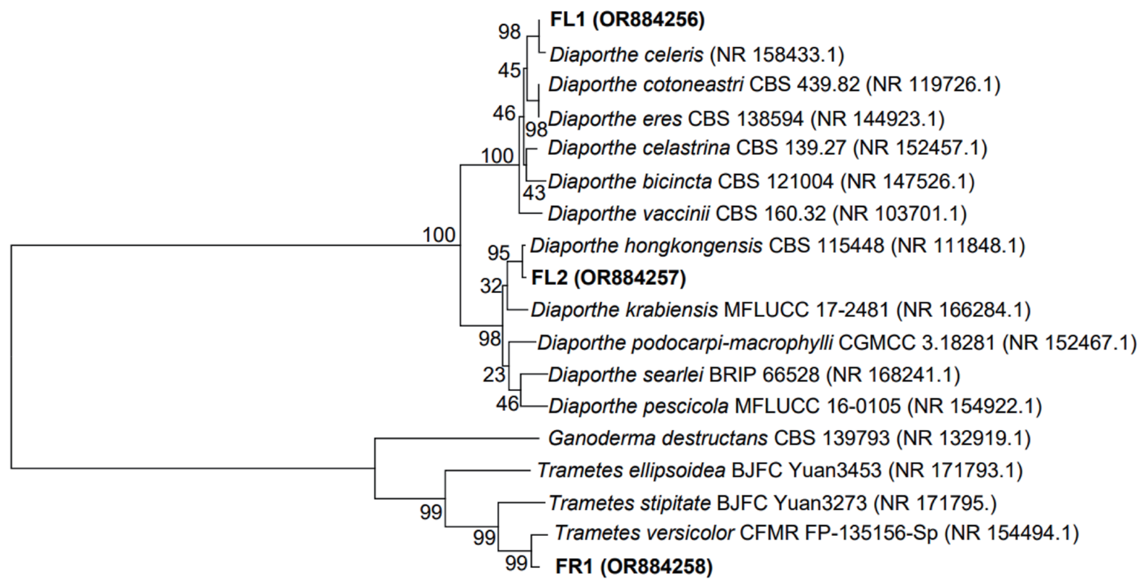

Fig. 6. Neighbour-joining phylogenetic tree based on ITS-rDNA sequences of fungal isolate *Trametes versicolor* (FR 1), *Diaporthe celeris* (FL 1), *Diaporthe hongkongensis* (FL 2) and related sequences from Gen Bank (NCBI).

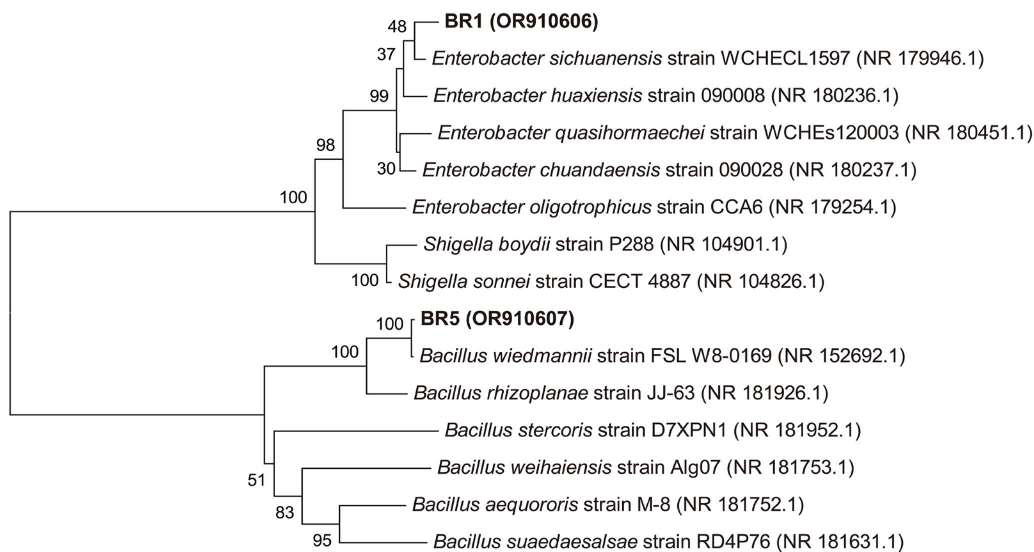

Fig. 7. Neighbour-joining phylogenetic tree based on 16s-rDNA sequences of endophytic bacterial isolate *Enterobacter* (BR 1), *Leclercia adecarboxylata* (BR 2), and related sequences from Gen Bank (NCBI).

curation (YC, TH), writing (YC, EL), reviewing and editing (TH, EL), project administration (FL), and funding acquisition (EL).

Data sharing statement

No additional data are available.

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