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Research article

Isolation and identification of endophytic fungi from *Alhagi sparsifolia* Shap. and their antibacterial activity

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ABSTRACT

In order to explore the endophytic resources of *Alhagi sparsifolia* Shap. and identified novel antibacterial substances. Thirty endophytic fungal strains were isolated from the stems and roots of *A. sparsifolia* Shap. Morphological and molecular biology methods were used to identify ten strains of fungi, including four strains of *Aspergillus niger*, three strains of *Alternaria alternata*, two strains of *Aspergillus flavus*, and one strain of *Fusarium incarnatum*. All these strains were isolated from *A. sparsifolia* Shap. for the first time, and of these *Aspergillus* was the dominant genus. Antibacterial activity of the ten strains against *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, and *Pseudomonas aeruginosa* were evaluated using the disc diffusion method. The results demonstrated that the metabolites from all the strains had inhibitory effects on at least one indicator bacterium. Notably, the endophytic fungi AFJ3 and AFG6 demonstrated strong broad-spectrum antibacterial activity, particularly against *E. coli*, with inhibition zones measuring 32.0 ± 0.3 and 31.3 ± 0.3 mm, respectively. The three endophytic fungi (AFG1, AFG2, and AFG3) isolated from the roots demonstrated significant antibacterial activity against *P. aeruginosa* forming an inhibition zone of diameter 31.3 ± 0.1, 25.6 ± 0.2, and 25.6 ± 0.2 mm, respectively. However, the strains of endophytic fungi demonstrated no significant inhibitory effects on *C. albicans*. Ultra-high performance liquid chromatography with quadrupole time-of-flight mass spectrometry/mass spectrometry (UPLC-QTOF-MS/MS) analysis depicted that the ethyl acetate phase of AFJ3 and AFG6 fermentation broth predominantly contained organic acids, phenolic acids, flavonoids, and fatty acids. These secondary metabolites often exhibited good antibacterial activity. This study broadens our understanding of endophytic fungi in *A. sparsifolia* Shap. The antibacterial activity of some strains of endophytic fungi was significant, making it worthy of further research on their active material.

1. Introduction

Alhagi sparsifolia Shap. is a perennial lignified herbaceous plant with strong salinity, drought, and stress resistance. This plant is predominantly distributed throughout North Africa and Eurasian desert areas, such as Russia and Mongolia. In China, these plants are

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common in Xinjiang, Inner Mongolia, Gansu, and Qinghai [1]. *A. sparsifolia* Shap. is commonly used in Uyghur medicine. Various parts of the plant—including leaves, petals, stems, and roots—are utilized to treat ailments such as dysentery, diarrhea, abdominal bloating, and pain [2]. Early references of its leaf secretion thorn sugar, also known as thorn honey, is found in 'Bencao Shiyi' literature. According to the drug standard 'Uygur medicine sub-volume' issued by the Chinese Ministry of Health, thorn sugar is used to remove abnormal biliary fluid and regulate body fluid [3]. Phytochemical and pharmacological studies have demonstrated that *A. sparsifolia* Shap. contains flavonoids, alkaloids, terpenoids, and other chemical components that reveal significant anti-bacterial, anti-tumour, anti-inflammatory, and gastrointestinal functions [4,5].

Throughout history, plants have been used to treat a wide range of ailments. Currently, scientists are increasingly focused on the microbial communities associated with medicinal plants and their role in synthesizing secondary metabolites. These microbiomes hold great promise for producing biologically active compounds. As a result, researchers have begun to investigate the endophytic fungi residing within medicinal plants, opening new avenues in bioprospecting due to their potential as antioxidants and antimicrobial agents. In the realm of plant symbiotic microorganisms, endophytic fungi have garnered significant attention for their crucial role in modern drug development [6]. Endophytic fungi are a group of fungal communities that live in symbiosis with their host plants, colonizing intercellular or intracellular spaces within the plant tissues. They provide benefits to the host while gaining survival advantages in return. Recent studies have revealed the biodiversity of endophytic fungi, their broad ecological distribution, and their complex interactions with host plants and other microbial communities along a symbiotic continuum [7]. The close relationship between endophytes and the host plants enables endophytes to take advantage of the multiple secondary metabolites produced [8]. Due to the large number of endophytic species present, the host plant demonstrates strong resistance to various stress conditions. During the stress response process, active components with diverse skeletons are produced, demonstrating strong ecological advantages. On average, a single plant hosts about 4–5 distinct endophytic fungal species, suggesting that the global count of these fungi could exceed one million, given the approximately 250,000 recognized plant species [9]. Endophytic fungi activate secondary metabolic pathways in host plants and regulate the transformation and synthesis of active substances by secreting fungal elicitors, such as polysaccharides, glycoproteins, and oligosaccharides, during their prolonged symbiotic evolution with the plants [10,11]. Simultaneously, they can produce a variety of structurally diverse and biologically active compounds that assist host plants in resisting environmental stress factors, including pests and drought [12,13]. The ability of endophytic fungi to produce secondary metabolites that closely resemble those of their host plants underscores their promising role in the development of new pharmaceuticals [14–17].

In the 1990s, *Stierle* et al. [18] successfully isolated an endophytic fungus, *Taxomyces andreanae,* capable of producing taxol, from the branches of *Taxus chinensis (Pilger) Rehd* for the first time. This groundbreaking discovery sparked a significant increase in the exploration of endophytic fungi, particularly those associated with medicinal herbs. *Adiyadolgor Turbat* et al. [19] isolated fifteen distinct strains of endophytic fungi from various parts of *Sophora flavescens*, a significant medicinal plant in Mongolia and China, including *Alternaria*, *Didymella*, *Fusarium*, and *Xylogone*. Phosphate-increasing activity and siderophore secretion was demonstrated by five and twelve strains, respectively. However, there are few reports on the anti-bacterial activities of endophytic fungi isolated from *A. sparsifolia* Shap [20]. Infections caused by bacteria are among the most challenging to treat due to their increasing resistance to commonly used antibiotics. Currently, the rise of antibiotic-resistant pathogenic bacteria poses a significant global health risk, leading to severe infections characterized by high rates of morbidity and mortality in medical treatment settings. Worldwide, approximately 1.7 billion cases of diarrhea are reported each year [21], with 9.4 % of these attributed to *E. coli*, which is a common cause of diarrhea [22]. To safeguard public health, researchers and the pharmaceutical industry are increasingly focused on discovering new therapies to combat pathogens [23]. Scientists are now exploring the development and utilization of endophytic fungi found in medicinal plants as a novel approach for discovering new pharmaceuticals [24].

Recent research has demonstrated that metabolites from endophytic fungi exhibit significant biological activity, including antimicrobial effects, cancer prevention, pest repellent properties, and the ability to combat malaria. *Aruna Vigneshwari* et al. [25] have reported that fifty-eight *Juniperus communis* strains demonstrated antibacterial activity against at least one organism in the anti-bacterial test. A total of 6.67 % of the strains demonstrated antimicrobial activity, of which ten strains revealed significant activity against yeast and eleven strains revealed significant activity against fungi. Among these, *Aspergillus* emerged as the most dominant genus, serving as a rich source of biologically active compounds with diverse chemical structures and pharmacological properties [26]. *Aruna Vigneshwari* et al. isolated endophytic fungi from various parts of *Hypericum perforatum* and concluded that the extract of *Aspergillus niger* mycelium exhibited good antibacterial activity [27].

Aspergillus ochraceus is considered to have high antibacterial activity owing to the presence of a large quantity of fatty acids [28]. Endophytic fungi, such as *Alternaria tenuissima* strains ZP28 and ZM148, derived from the twigs of *Loranthus tanakae Franch*. & Sav., have demonstrated potent antibacterial properties [29]. Gannan navel orange endophytic fungi efficiently produced secondary metabolites with significant antimicrobial effects [23]. Here, we systematically isolated and identified endophytic fungi from *A. sparsifolia* Shap. We explored the diversity of these fungi, conducted a preliminary evaluation of the antibacterial activity of metabolites from each strain, and analyzed the fermentation extracts to guide future research and exploitation of these fungal resources.

2. Materials and methods

2.1. Instruments and reagents

Benchtop JJ-CJ2FD (Suzhou Jinjing Purification Equipment Technology Co., Ltd.); High-pressure sterilizer GR60DA (Zhiwei Xiamen Instrument Co., Ltd.); Rotary evaporator R-300EL(Swiss Buqi Co., Ltd); Electron microscope SMZ25 (Shanghai Qianxin Instrument Co., Ltd.); −80 °C Refrigerator BC/BD-233H (Qingdao haier co.,ltd.); PCR C1000 (Jiangsu Vanke Science and Education

Instrument Co., Ltd.); Ultra performance liquid chromatography AB ExionLC Tandem high resolution mass spectrometry AB TripleTOF 6600 plus (AB Sciex, USA).

Rapid Extraction Kit of Fungal Genomic DNA、ITS5 (5′–GGAAGTAAAAGTCGTAACAAGG–3″) and ITS4 (5′–TCCTCCGCTTATTGATATGC–3′) primers, Universal PCR kits were purchased from Shanghai Biological Engineering Co., Ltd.

2.2. Plant material

A. sparsifolia Shap. was collected from Yumin County, Tacheng, Xinjiang in October 2022, it was identified as *A. sparsifolia* Shap. by Professor Yang Xiaorong, College of Biological and Geographical Sciences, Yili Normal University., sealed in a fresh-keeping bag at −80 °C refrigerator.

2.3. The strains tested

Four bacteria, including *Escherichia coli* (CGMCC1.1103), *Staphylococcus aureus* (CGMCC1.8721), *Candida albicans*(ATCC10123) and *Pseudomonas aeruginosa*(CMCC10104)were provided by the Key Laboratory of Microbial Resources Protection and Development and Utilization of Yili Normal University for testing the antibacterial activity.

2.4. Medium

Potato Dextrose Agar (PDA), Potato Dextrose Broth (PDB) and LB medium were purchased from Haibo Biological Co., Ltd. for the isolation and purification of endophytic fungi from *A. sparsifolia* Shap. and for the activation of the test bacteria.

2.5. Isolation and purification of endophytic fungi from A. sparsifolia Shap

Endophytic fungi of *A. sparsifolia* Shap. was isolated and purified by tissue isolation method. The collected samples were rinsed to eliminate surface contaminants. Under sterile conditions, the surface of the samples was disinfected according to the surface disinfection method. The samples were rinsed with sterile water for 3 times on a sterile operation table, then treated with a 0.1 % mercuric chloride solution for 5 min, followed by a rinse with an 8 % sodium hypochlorite solution for 5 min. Afterward, they were washed three more times with sterile water, soaked in 75 % ethanol for 5 min, and finally rinsed again with sterile water three times. Under sterile conditions, the samples were cut into small pieces measuring 0.2 cm × 0.2 cm and inoculated onto PDA medium, respectively. The colonies were cultured at 28 °C until colonies appeared. The hyphae were taken from the edge of the colonies and inoculated on fresh PDA, respectively. The purified strains were obtained by repeated transfer and stored for use. To ensure complete surface disinfection, the final washing water was plated on PDA and cultured at 37 °C for 24 h.

2.6. Morphological and molecular biological identification of endophytic fungi from A. sparsifolia Shap

The activated strains were inoculated in the center of the medium and cultured at 24 °C for 7 days to observe the colony morphology. The taxonomic status of the strains was determined by referring to the 'Fungal Identification Manual 1979' [30] and 'Modern Medical Fungal Identification Manual' [31] and other related fungal taxonomic monographs.

The genomic DNA of the endophytic fungi was extracted using fungal genomic extraction kit. The Internal Transcribed Spacers (ITS) of the rRNA gene were amplified from fungal genomic DNA using the Polymerase Chain Reaction (PCR) with universal primers ITS1 and ITS4. PCR reaction system: 1 × PCR Buffer, 22 mmol/L $MgCl_2$, 200 μL dNTP, 20 pmol ITS4 and ITS5 primers, 0.25 U Taq DNA polymerase, 10 ng DNA template.

PCR Reaction Conditions: The reaction included pre-denaturation at 94 °C for 3 min, followed by denaturation at 94 °C for 30 s, annealing at 51 °C or 56 °C for 1 min, and extension at 72 °C for 1 min. This cycle was repeated for 40 cycles, with a final extension at 72 °C for 10 min. The PCR products were analyzed using 1.0 % agarose gel electrophoresis and subsequently sent to Shenzhen Huada Gene Technology Co., Ltd. for sequencing.

The measured ITS sequence was compared with the BLAST program in the NCBI database, and the obtained sequence was analyzed with the Clustal X 8.1 software for multiple sequence comparison and manual correction. The software MEGA 10.0 was used to construct the phylogenetic tree according to the N–J method.

2.7. Antibacterial activity test of fermentation broth of endophytic fungi from A. sparsifolia Shap

Culture of Test Strains: The four indicator bacteria—*Escherichia coli* (CGMCC1.1103), *Staphylococcus aureus* (CGMCC1.8721), *Candida albicans* (ATCC10123), and *Pseudomonas aeruginosa* (CMCC10104)—were inoculated into 5 mL of LB liquid medium and cultured at 37 °C for 18 h. The bacterial solution was then diluted to 1 × 10^5 CFU/mL with fresh culture solution. and 100 μL bacterial solution was coated on LB medium.

Preparation of Fermentation Broth: The endophytic fungi of *A. sparsifolia* Shap. were inoculated into 100 mL potato liquid medium and cultured at 28 °C with shaking at 180 rpm for 5–7 days. The culture was then centrifuged at 4000 r/min for 15 min to collect the supernatant.The 100 mL fermentation broth was concentrated to 1 mL by a rotary evaporator.

Bacteriostatic Test: The antibacterial activity of the fermentation broth from the endophytic fungi against the four indicator

bacteria was assessed using the disk diffusion method. Specifically, a filter paper disk (6 mm diameter) was placed on a plate inoculated with the indicator bacteria, and 20 μL of fermentation broth was added. Ampicillin sodium (at a concentration of 50 mg/mL) served as a positive control, while PDB medium was used as a negative control and sterile water as a blank control. The plates were incubated at 28 °C for 1–2 days, after which the diameter of the inhibition zones was observed and measured.

2.8. Chromatographic analysis of secondary metabolites of endophytic fungi from A. sparsifolia Shap

The fermentation broth of endophytic fungi AFJ3 and AFG6 with broad-spectrum antimicrobial activity was extracted three times with equal volume of ethyl acetate, the extracts were combined and dried by rotary evaporator, and a small amount of the sample was dissolved in methanol. The samples dissolved in methanol were passed through a 0.22 μm organic microporous membrane, and a small amount of the sample passed through the membrane was taken for backup detection. Chromatographic conditions: chromatographic column: ACQUITY UPLC BEH C18 (100 mm × 2.1 mm, 1.8 μm, Waters); column temperature: 30 °C; injection volume: 10 μL; detection wavelength: 190–600 nm; flow rate: 0.3 mL/min; mobile phase A: 0.1 % formic acid-water, B: acetonitrile; elution gradient: 0–3 min, 98 % A, 3–6 min, 98 % A to 30 % A; 6–12 min, 30 % A to 20 % A; 12–21 min, 20 % A; 22–42 min, 20 % A to 0 % A. Mass spectrometry conditions: ESI as the ion source, using negative ion mode. The molecular weight scanning range of primary mass spectrometry: 100–2000 Da; the secondary mass spectrometry collision energy range: 40 ± 20 eV. The best source parameters: carrier gas pressure 50 psi, ion source temperature 550 °C, atomization gas N_2 and dry gas N_2 pressure 50 psi.

3. Results and analysis

3.1. Morphological identification of endophytic fungi from A. sparsifolia Shap

Thirty distinct strains of endophytic fungi were separated from the stems and roots of *A. sparsifolia* Shap. by a surface disinfection method. Multiple isolation and purification were carried out based on morphological classification, and six strains of endophytic fungi with significant differences in colony morphology and mycelial microscopic characteristics were obtained from roots, numbered AFG1–AFG6. Additionally, four types of endophytic fungi were collected from the stems., numbered AFJ1–AFJ4 (Figs. 1–3 and Table 1).

3.2. Molecular biological identification of endophytic fungi from A. sparsifolia Shap

3.2.1. The PCR amplification and sequencing of the ITS fragment of endophytic fungi in A. sparsifolia Shap

Amplification of DNA bands, approximately 550 bp in size, from the genomic DNA of ten endophytic fungi of *A.sparsifolia* Shap., was performed by PCR using primers ITS1 and ITS4(Fig. 4), Sequence analysis revealed that the ITS sequences for strains AFG1–AFG6 were 566, 564, 565, 560, 571, and 516 bp, respectively. Whereas the ITS sequences of AFJ1–AFJ4 were 542, 542, 567, and 535, respectively, and the accession numbers in the GeneBank database were OR064103-OR064112, respectively.

3.2.2. Similarity analysis of ITS sequence of endophytic fungi from A. sparsifolia Shap

The homology of the ITS sequences of the endophytic fungi was analyzed using the Basic Local Alignment Search Tool (BLAST) It can be seen from Table 2 that the similarity between the ITS sequences of ten strains of endophytic fungi and the related strains in the

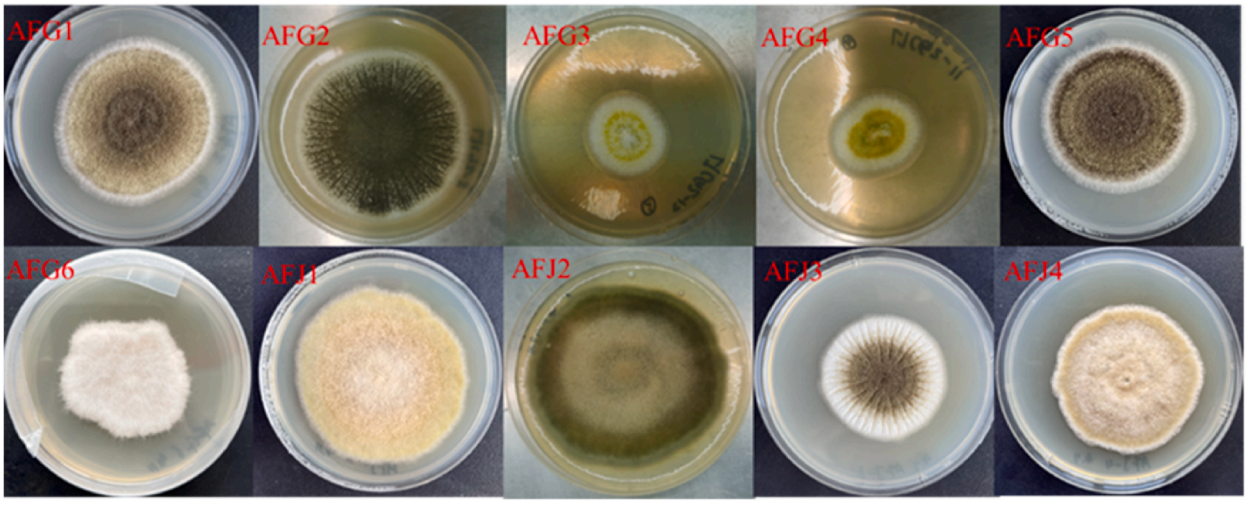

Fig. 1. The colony morphology of endophytic fungi, isolated from both the roots and stems of *A. sparsifolia* Shap., were observed after cultivation on PDA medium for a week at a temperature of 24 °C within an incubator.

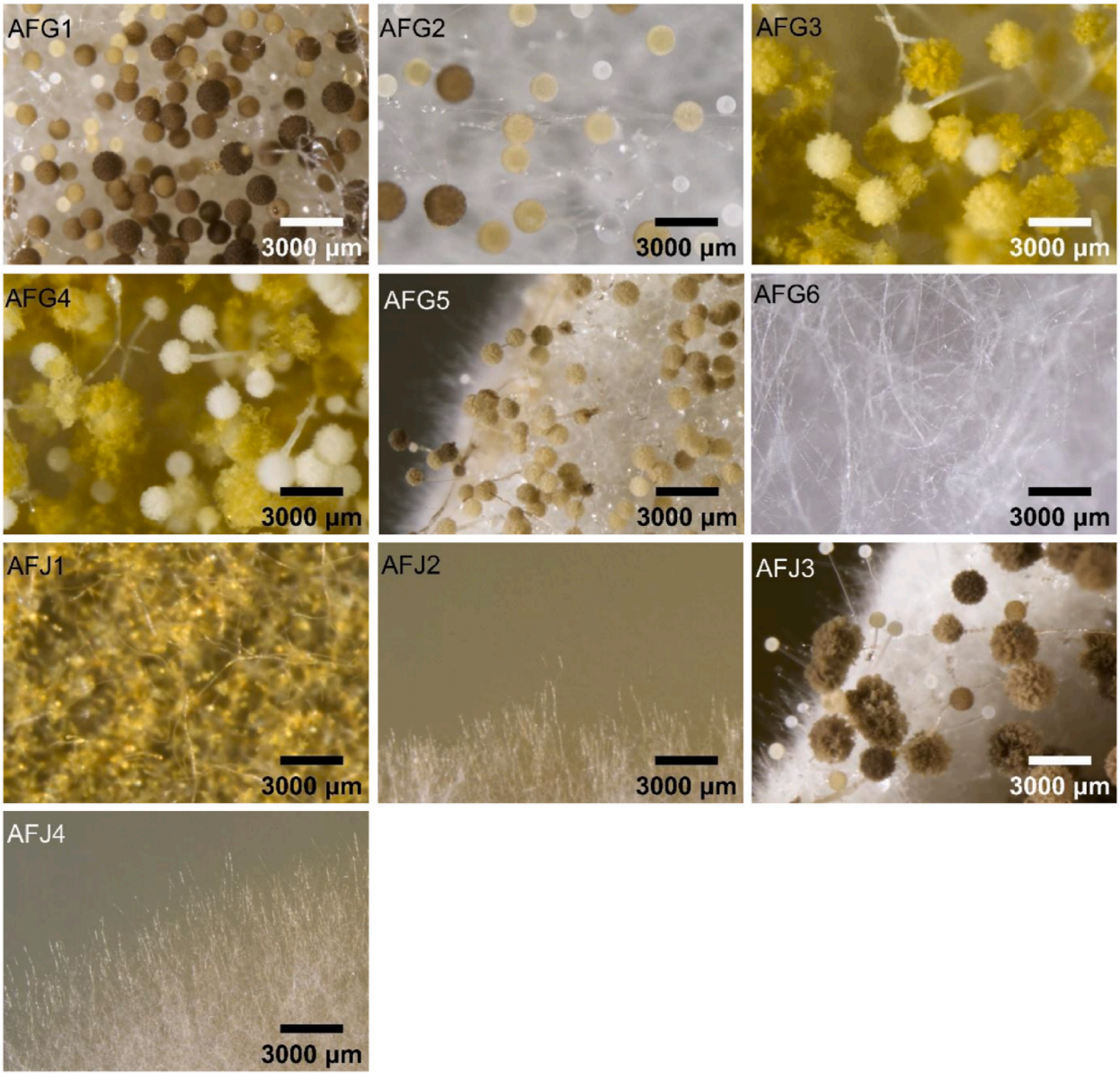

Fig. 2. The microstructure of the colony of endophytic fungi from the roots and stems of *A. sparsifolia* Shap. after being cultured on PDA medium in an incubator at 24 °C for 7 days. (The scale bars of the images are all 3000 μm)

Genbank database ranged from 92 % to 100 %.

3.2.3. Phylogenetic tree analysis of endophytic fungi from A. sparsifolia Shap

As can be seen from Fig. 5, AFG1, AFG2, AFG5, and AFJ3 were clustered within the group *Aspergillus* and were grouped in a branch with *Aspergillus niger* strain AUMC-16067 (OQ930379), which showed 92 % similarity in their ITS sequences. AFG3 and AFG4 were clustered within the group *Aspergillus* and were grouped in a branch with *Aspergillus flavus* isolate smaple-307 (OQ422930), which showed 100 % similarity in their ITS sequences. AFG6 was grouped in the *Fusarium incarnatum* group and separated from *Fusarium incarnatum* isolate Diyala2 (OQ357847) in a clade with 100 % similarity in ITS sequence. AFJ1, AFJ2, and AFJ4 were clustered within the group *Alternaria alternata* and were divided into a branch with *Alternaria alternata* strain NL-333-B (OQ561208), which had 100 % similarity in their ITS sequences. Therefore, the strains were preliminarily identified as AFG1, AFG2, AFG5 and AFJ3, and they were identified as *Aspergillus niger*. Strains AFG3 and AFG4 were identified as *Aspergillus flavus*. Strain AFG6 was identified as *Fusarium incarnatum*. Strains AFJ1, AFJ2 and AFJ4 were identified as *Alternaria alternate*.

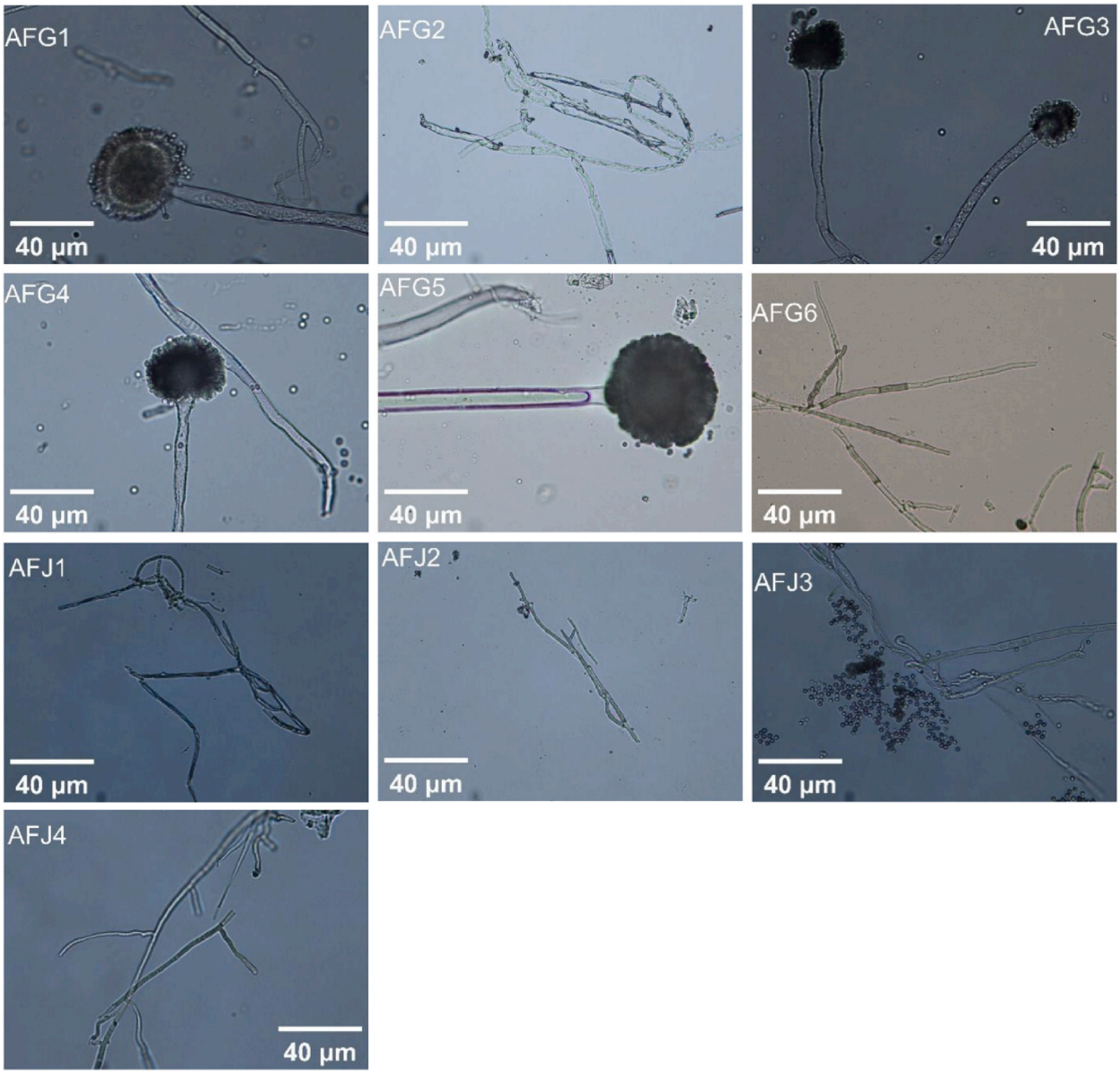

Fig. 3. The morphological features of the ten strains were observed under a Nikon (10 × 40) microscope (Scale bars of the images are all 40 μm).

3.3. Bioactive screening of fermentation broth of endophytic fungi derived from A. sparsifolia Shap

Su Yinquan et al. [32] classified strains based on their inhibition zone diameters: <6.0 mm as having no inhibitory effect, 6–10 mm as light, 10–15 mm as moderate, and ≥15 mm as potent inhibitory activity. The antibacterial activity of ten endophytic fungi strains isolated from A. sparsifolia Shap. against the four indicator bacteria were determined using disc diffusion (K–B method). As depicted in Table 3, eight endophytic fungi demonstrated moderate antibacterial activity against *E. coli*, six against S. aureus and *P. aeruginosa*, and five against *C. albicans*. The positive control demonstrated strong inhibition against *E. coli*(26.8 ± 0.3 mm), *S. aureus*(40.0 ± 0.2 mm) and *C. albicans*(37.1 ± 0.3 mm), but showed no inhibition against *P. aeruginosa*. Negative control and blank control had no antibacterial effect. The two endophytic fungi, AFG6 (diameter of the inhibitory zone was 31.3 ± 0.3 mm) and AFJ3 (diameter of the inhibitory zone was 32.0 ± 0.3 mm) demonstrated significant antibacterial activity against *E. coli,* and were stronger than the positive control (diameter of the inhibitory zone was 26.8 ± 0.3 mm). (Fig. 6). Additionally, three endophytic fungi (AFG1, AFG2, and AFG3) were separated from the root systems of *A. sparsifolia* Shap. exhibited substantial antimicrobial activity against *P. aeruginosa*. However, none of the endophytic fungi displayed significant inhibitory activity against *C. albicans*.

Table 1
Morphology of endophytic fungi from *A. sparsifolia* Shap.

Strain code	Morphology of fungal	Microstructure description	Genus
AFG1, AFG2, AFG5, AFJ3	Colonies are black with dense mycelium. Short downy and sparse mycelium.	A part of the aerial mycelium forms a long and rough conidiophore, and many pedicels are produced on the surface. The pedicels are covered with a string of rough spherical conidia. Conidiophore, apical produce subglobose apical sac, pedicel and conidia synthesize spore head.	*Aspergillus*
AFG3, AFG4	The colony is filamentous, the colony grows faster, the structure is loose, the surface is yellowish green, and the back is colorless or slightly brown. The mycelium has many complex branching hyphae. The surface of the newly grown hyphae is milky white, and the color gradually becomes yellow as it matures.	The fungus has many complex branching hyphae, long and straight. The hyphae have septations, conidiophores, and flask-shaped or nearly spherical apical sacs are produced at the top.	*Aspergillus flavus Link*
AFG6	Colonies white, mycelium dense, short fluffy, strains of aerial mycelium growing well on PDA agar, cottony or spidery, usually white.	Small conidia are borne in aerial mycelium, capitate inserted, or in mucilaginous spore clusters with smooth, tufted surfaces.	*Fusarium*
AFJ1, AFJ2, AFJ4	Colonies flocculent, just out of the mycelium for the wall growth, to the surrounding has a spiny form of diffusion, with the development of maturity, the color gradually become darker.	Under the microscope, the mycelium is colorless and transparent, uniform in thickness and fineness, septate, forked, conidia solitary or some like beads strung together like dendrites, irregular cylindrical to streamlined, mycelium has a long slightly curved, aerial mycelium abundant.	*Alternaria*

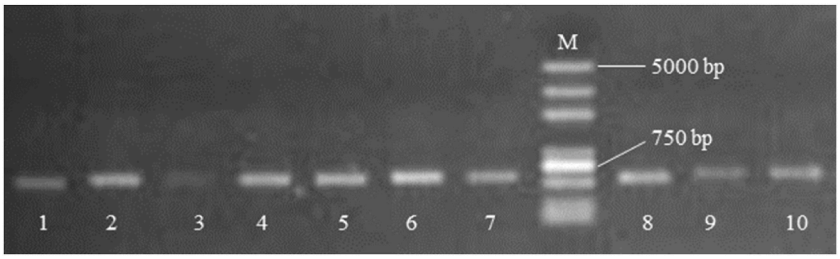

Fig. 4. PCR amplification of ITS fragments of endophytic fungus from *A. sparsifolia* Shap. Note: 1–4 correspond to AFG1–AFG4, 5 correspond to AFG6, 6 correspond to AFG5, 7–10 correspond to AFJ1–AFJ4. M is DL5000 DNA marker.

Table 2
ITS sequence similarity of endophytic fungi from *A. sparsifolia* Shap.

Strains	GenBank accession No.	ITS sequences of reference strains (Accession No.)	Length (bp)	Similarity (%)
AFG1	OR064103	Aspergillus niger strain AUMC-16067 (OQ930379)	566	92 %
AFG2	OR064104	Aspergillus niger strain AUMC-16067 (OQ930379)	564	92 %
AFG3	OR064105	Aspergillus flavus isolate smaple-307 (OQ422930)	565	100 %
AFG4	OR064106	Aspergillus flavus isolate smaple-307 (OQ422930)	560	100 %
AFG5	OR064108	Aspergillus niger strain AUMC-16067 (OQ930379)	571	92 %
AFG6	OR064107	Fusarium incarnatum isolate Diyala2 (OQ357847)	516	100 %
AFJ1	OR064109	Alternaria alternata strain NL-333-B (OQ561208)	542	100 %
AFJ2	OR064110	Alternaria alternata strain NL-333-B (OQ561208)	542	100 %
AFJ3	OR064111	Aspergillus niger strain AUMC-16067 (OQ930379)	567	92 %
AFJ4	OR064112	Alternaria alternata strain NL-333-B (OQ561208)	535	100 %

3.4. Preliminary analysis of secondary metabolites of endophytic fungi isolated from A. sparsifolia Shap

The secondary metabolites of endophytic fungi isolated from *A. sparsifolia* Shap. were analyzed using UPLC-ESI-QTOF-MS/MS, as shown in Fig. 6. The major chemical components of strains AFJ3 and AFG6 were identified using ChemSpide, PubChem, and MassBank. The molecular formulas, relative molecular masses, and mass spectrometry (MS/MS) fragments were utilized to characterize the primary chemical constituents of the endophytic fungi's secondary metabolites. A total of 26 distinct compounds were detected in the strains, with 22 compounds identified in AFJ3 and 15 in AFG6. These compounds included six organic acids, two flavonoids, nine phenolic acids, and nine fatty acids. (Table 4).

Among these, compound 10 ($C_{16}H_{18}O_9$), identified as chlorogenic acid, has its fragmentation pattern shown in Fig. 7. Here, *m/z* 191.0586 [quinic acid-H]$^-$, denotes the loss of 162 Da (caffeoyl) from the parent ion at *m/z* 353.087, *m/z* 135.0447 denotes the loss of one molecule of CO from [quinic acid-H]$^-$, and *m/z* 179.03 indicates that the caffeoyl group is attached to the 3-OH position of quinic acid [33,34]. The molecular ion peak of compound 5 was *m/z* 181.0512, and the characteristic fragment ions *m/z* 163.0399, 135.0447,

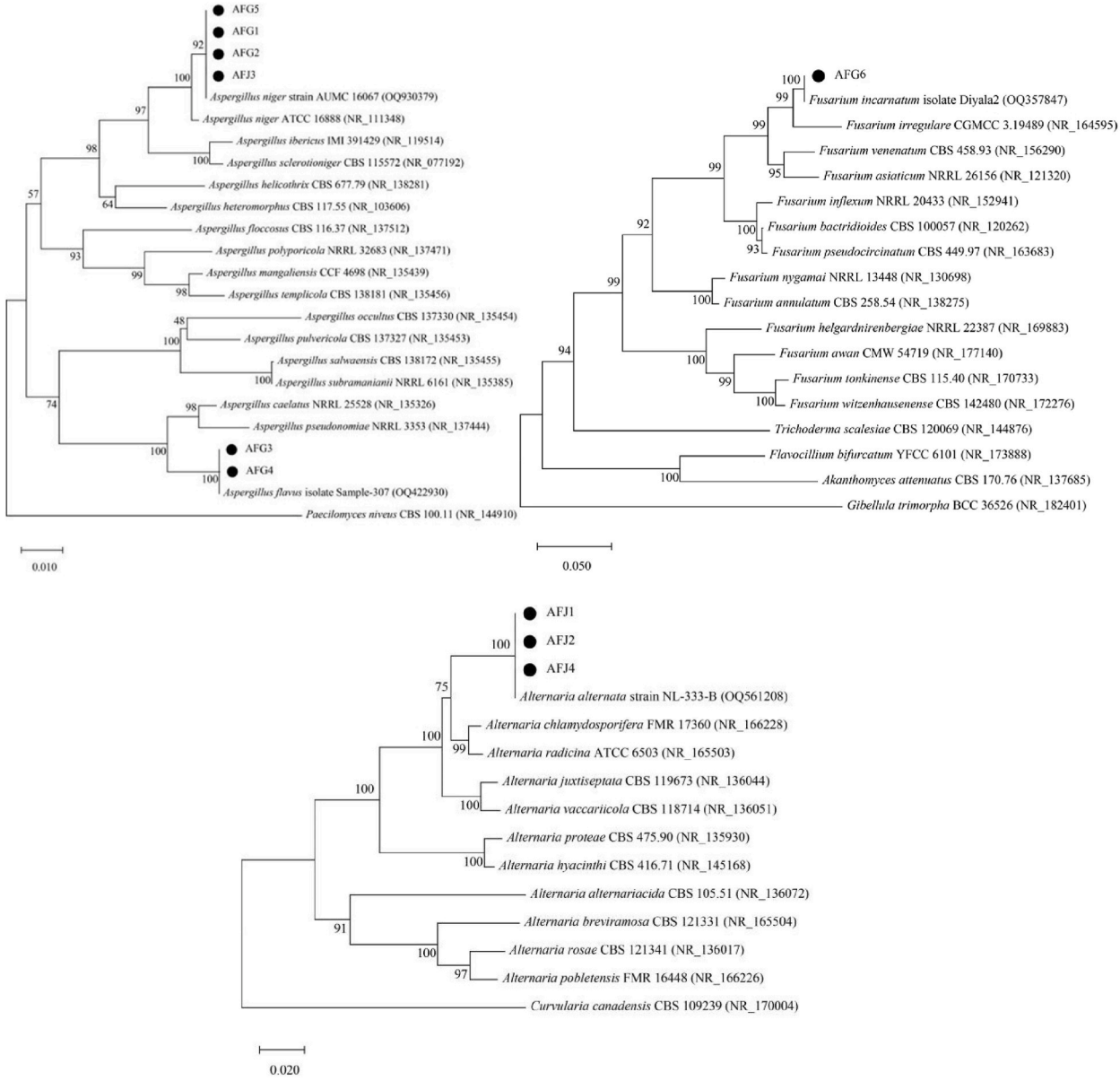

Fig. 5. The phylogenetic tree of ITS sequence of endophytic fungi from *A. sparsifolia* Shap. was constructed using the neighbor-joining approach. (1000 bootstrap replications).

and 119.0514 were denoted as $[M\text{-}H\text{-}H_2O]^-$, $[M\text{-}H\text{-}HCOOH]^-$, and $[M\text{-}H\text{-}H_2O\text{-}CO_2]^-$, respectively, and were identified as dihydrocaffeic acid [35]. The parent ion *m/z* of compound 8 was 301.0345 ($C_{15}H_{10}O_7$) and its MS/MS ions were 178.9965, 151.0045, and 107.0073, respectively. Thus, the compound was identified as quercetin [36,37]. The parent ion of compound 26 was $[M-H]^-$ *m/z* 329.2353. The fragment ions *m/z* 311.2233 and *m/z* 293.2147 indicated that the parent ion was produced by the loss of $1H_2O$ and $2H_2O$, respectively. Additionally, the fragment ion *m/z* 171.1030 resulted from the cleavage of the C9-C10 bond. Based on previous studies [37,38], compound 26 was identified as 9,12,13-trihydroxyoctadecenoic acid (see Fig. 8).

4. Discussion

A. sparsifolia Shap., a perennial herb belonging to the Alhagi genus within the Leguminosae family, is predominantly found in the desert regions of China, including Inner Mongolia, Gansu, Qinghai, and Xinjiang [50]. A variety of pharmacologically active secondary metabolites can be extracted from *A. sparsifolia* Shap., including flavonoids, alkaloids, steroids, pseudalhagin A, phospholipids and polysaccharides [51]. According to previous studies, the plant also contains saponins, essential oils, tanning agents, organic acids, vitamins, sugars, resins and waxes [52], phenolic acid [53], etc. They exhibit diverse biological effects, such as antioxidant,

Table 3
Antibacterial activity of endophytic fungal fermentation broth from the root of *A. sparsifolia* Shap. (diameter of antibacterial zone mm).

Strains	Bacteriostatic zone diameter			
	E. coli	*S. aurus*	P. aeruginosa	*C. albicans*
AFG1	19.7 ± 0.1	11.6 ± 0.1	31.3 ± 0.1	–
AFG2	19.3 ± 0.2	13.0 ± 0.2	25.6 ± 0.2	11.3 ± 0.1
AFG3	12.3 ± 0.2	10.3 ± 0.1	25.6 ± 0.2	13.0 ± 0.1
AFG4	–	–	–	11.0 ± 0.1
AFG5	–	17.6 ± 0.2	–	–
AFG6	31.3 ± 0.3	15.0 ± 0.2	28.6 ± 0.1	12.3 ± 0.1
AFJ1	11.6 ± 0.2	–	–	–
AFJ2	10.6 ± 0.2	–	–	–
AFJ3	32.0 ± 0.3	15.0 ± 0.1	27.6 ± 0.1	11.6 ± 0.1
AFJ4	13.6 ± 0.1	–	10.0 ± 0.2	–
S	26.8 ± 0.3	40.0 ± 0.2	–	37.1 ± 0.3

Note: "–" means no antibacterial activity, inhibition zone of diameter <6.0 mm, between 6 and 10 mm, between 10 and 15 mm, and ≥15 mm as having no inhibitory effect, light, moderate, and potent inhibitory actions, respectively, S denotes ampicillin sodium.

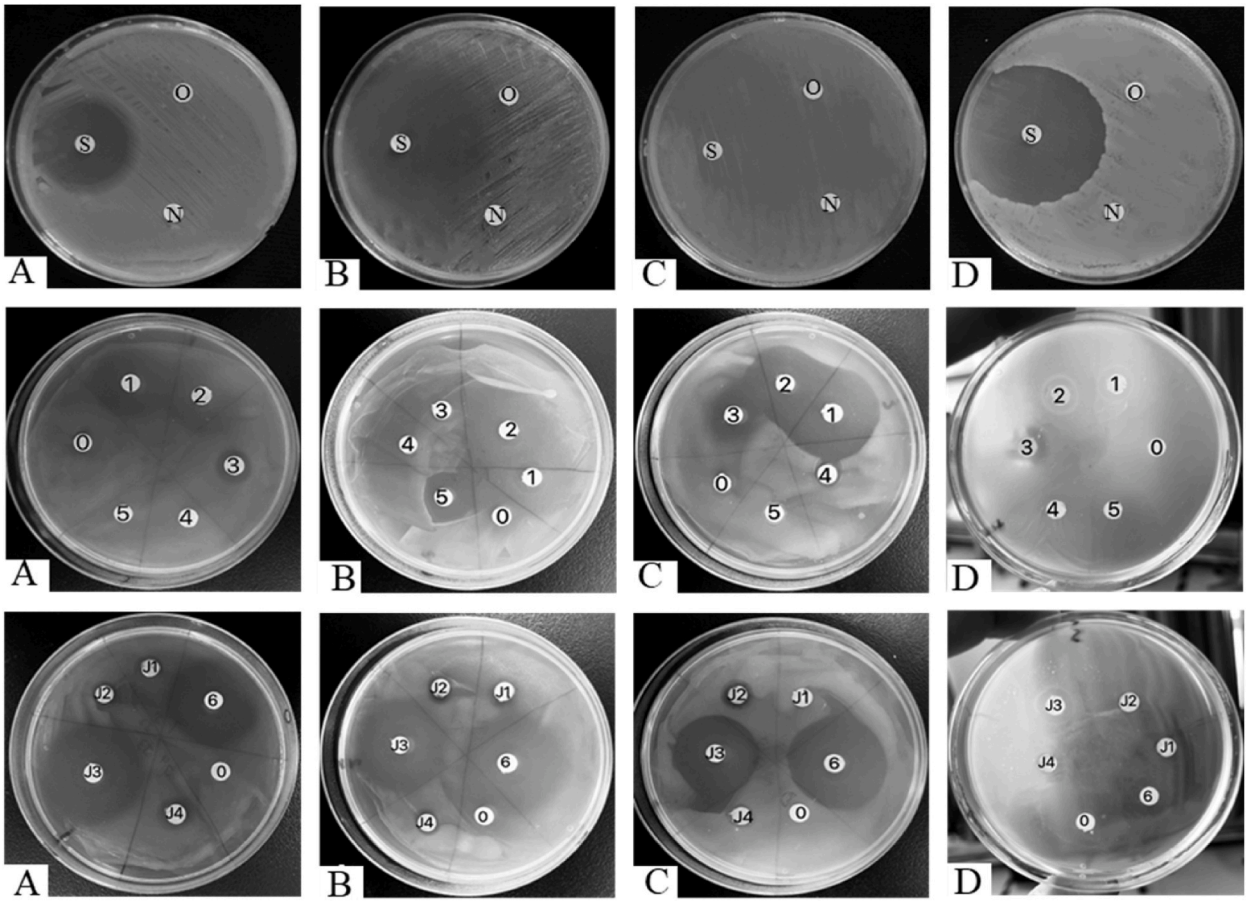

Fig. 6. Antibacterial activity of endophytic fungal fermentation broth from *A. sparsifolia* Shap. Note: A–D means the inhibiting effects on *E. coli*, *S. aurus*, *P. aeruginosa*, *C. albicans* respectively. 0 correspond to ddH_2O, S represents the positive control, and N represents the negative control. 1–6 correspond to AFG1–AFG6, J1–J4 correspond to AFJ1–AFJ4.

cardiovascular, anti-ulcer, hepatoprotective, antispasmodic, anti-diarrheal, anti-inflammatory, antipyretic, antirheumatic, antibacterial, and antifungal activities [2,51]. Additionally, as a typical xerophyte, *A. sparsifolia* Shap. significantly contributes to the stabilization and enhancement of the delicate desert ecosystem. Strong biological activity of *A. sparsifolia* Shap. indicates that its endophytic fungi may also be of interest for future studies.

Recently, the extensive use of synthetic insecticides has increased resistance in pathogenic microorganisms. Endophytic fungi with

Table 4
UPLC-QTOF-MS/MS analysis of chemical constituents in AFJ3 and AFG6 fermentation broths.

NO.	RT/ min	Molecular formula	Found at *m/z*	MS/MS	Proposed compounds	AFJ3	AFG6	Ref.
Organic acids								
1	1.193	$C_6H_8O_7$	191.0197	191.0201, 111.0079, 87.0081	Citric acid	+	+	[39]
2	3.856	$C_4H_6O_5$	133.0141	115.0076, 71.0127	Malic acid	+	+	[40]
3	6.750	$C_8H_8O_3$	151.0402	107.0501, 106.0427	4-p-Hydroxyphenyl acetic acid	+	–	[41]
4	5.880	$C_9H_{17}NO_5$	218.1038	218.1036, 146.0834, 88.0408	Pantothenic acid	+	–	[42]
5	6.295	$C_9H_{10}O_4$	181.0507	181.0512, 163.0399, 135.0447, 119.0514	Dihydrocaffeic acid	+	+	[35]
6	4.387	$C_6H_{10}O_5$	161.0459	161.0463, 101.0250, 71.0494	Dimethyl(R)- (+)-malate	+	–	[43]
Flavonoids								
7	7.063	$C_{15}H_{10}O_6$	285.0601	285.1707, 205.0887, 171.1023	Kaempferol	+	–	[44]
8	7.176	$C_{15}H_{10}O_7$	301.0345	178.9965, 151.0045, 107.0073	Quercetin	–	+	[36, 37]
Phenolic acids								
9	6.58	$C_9H_6O_4$	177.0209	177.0200, 149.0245, 133.0294, 105.0341, 81.0353, 77.0388	6,7-Dihydroxycoumarin	–	+	[37]
10	6.282	$C_{16}H_{18}O_9$	353.0874	353.0875, 191.0561, 179.0347, 135.0447	Chlorogenic acid	+	+	[33, 34]
11	6.698	$C_7H_{12}O_6$	191.0198	191.0207, 111.0073, 87.0071	Quinic acid	+	+	[43]
12	4.815	$C_7H_6O_4$	153.0192	153.0197, 108.0214	Protocatechuic acid	+	–	[45]
13	6.621	$C_{17}H_{20}O_9$	367.1023	367.1021, 193.0507, 173.0458, 161.0242, 135.0447	3-Feruloylquinic acid	+	–	[42]
14	6.539	$C_8H_8O_3$	151.0407	136.0193, 108.0215	Vanillin	–	+	[37]
15	6.621	$C_9H_8O_4$	179.0347	179.0347, 135.0444, 107.0505	Caffeic acid	+	+	[43]
16	5.476	$C_{13}H_{16}O_9$	315.0728	315.0727, 152.0107, 108.0210	Maplexin A	+	–	[46]
17	10.168	$C_{13}H_{12}O_8$	296.2313	296.2310, 128.1136	Coutaric acid	+	–	[47]
Fatty acids								
18	20.888	$C_{18}H_{36}O_2$	283.2644	283.2651	Stearic acid	+	+	[43]
19	17.833	$C_{18}H_{34}O_2$	281.2481	281.2482, 219.0265, 116.9341	Oleic acid	+	+	[43]
20	15.783	$C_{16}H_{32}O_2$	255.2328	255.2334	Palmitic Acid	+	–	[43]
21	13.594	$C_{18}H_{32}O_2$	279.2329	279.9297, 261.2245	Linoleic acid	+	–	[43]
22	12.515	$C_{14}H_{28}O_2$	227.2013	227.2014	Myristic acid	+	+	[43]
23	12.381	$C_{16}H_{32}O_3$	271.2280	271.2296, 253.2224, 225.2234	3-Hydroxyhexadecanoic acid	+	+	[35]
24	12.162	$C_{20}H_{30}O_2$	301.2173	301.0191, 255.2299, 146.0392	(−)-Abietic acid	+	–	[48]
25	11.944	$C_{18}H_{32}O_2$	277.2169	277.2173, 259.2051	α-Linolenic acid	+	+	[49]
26	7.455	$C_{18}H_{34}O_5$	329.2	293.2147, 229.1468, 211.1358, 183.1400, 171.1041, 127.1138, 119.1134	9,12,13-Trihydroxyoctadecenoic acid	–	+	[37]

Note: "+" and "−" indicate the presence or absence of the identified compounds in AFJ3 and AFG6 samples.

unique genetic and metabolic diversity are a new source for the screening of novel antibacterial active substances and have broad prospects for future research [54]. Identifying fungi can be quite challenging due to their vast diversity and the similarities in appearance among different species. Consequently, alongside morphological identification, employing molecular phylogenetic techniques is crucial for accurate fungal classification. DNA barcoding, which utilizes specific DNA sequences for species classification, often relies on the internal transcribed spacer (ITS) region as a widely accepted and reliable molecular marker for identifying a broad array of fungi [55]. Here, ten endophytic fungal strains were isolated and identified using a combination of traditional morphological methods and sequence analysis of the ITS region. Endophytes were successfully isolated from the roots and stems of the plant species *A. sparsifolia* Shap., including four strains of *Aspergillus niger*, three identified as *Alternaria alternata*, two belonging to *Aspergillus flavus*, and one strain of *Fusarium incarnatum*. All the strains were isolated from *A. sparsifolia* Shap. for the first time, which highlights the diversity of fungal species in *A. sparsifolia* Shap.

Among them, *Aspergillus* has become the dominant species of the plant due to its strong environmental adaptability. *A. sparsifolia* Shap. is a typical deep-rooted desert plant, and *Aspergillus* has a high heat resistance, capable of growing under extreme conditions, showing an opportunistic survival strategy. *Beever* and *Laracy* et al. [56] reported that *Aspergillus* has drought resistance. The high antibacterial activity of *Aspergillus* may be related to its production of specific secondary metabolites. These metabolites, such as pyruvate, phytosterols, alkaloids, cyclic peptides, and phenolic acids, can inhibit or kill other microorganisms, thereby providing a competitive advantage within the plant's ecosystem [26]. Around the world, there has been a broad focus on studying the variety and prevalence of endophytic fungi to harness their potential. Numerous studies confirm that these fungi are a rich source of biologically active compounds. They can produce not only analogous or identical active constituents to those of their host plants but also novel active ingredients. A variety of chemical groups have been identified for the classification of metabolites of endophytic fungi, including but not limited to flavonoids, terpenoids, alkaloids, coumarins, steroids, lignans, quinones, and others [57]. For instance, fungi residing within the roots of *Taxus wallichiana* can generate a range of organic acids, including ascorbic, citric, lactic, malic, oxalic, pyruvic, and succinic [58]. Research by Pan et al. [59] reveal that Fritillaria-sourced endophytic fungi create phenolic substances, among them caffeic acid, gallic acid, chlorogenic acid, ferulic acid, and marinic acid. Ladoh Ymeda et al. identified the existence of flavonoids, phenols, and coumarins in the initial extract of *Phragmanthera capitata* [60]. The secondary metabolites isolated from the Jamblang plant branch by Shirly et al. mainly contain fatty acids and phenolic compounds [61]. Endophytic fungi from mangrove

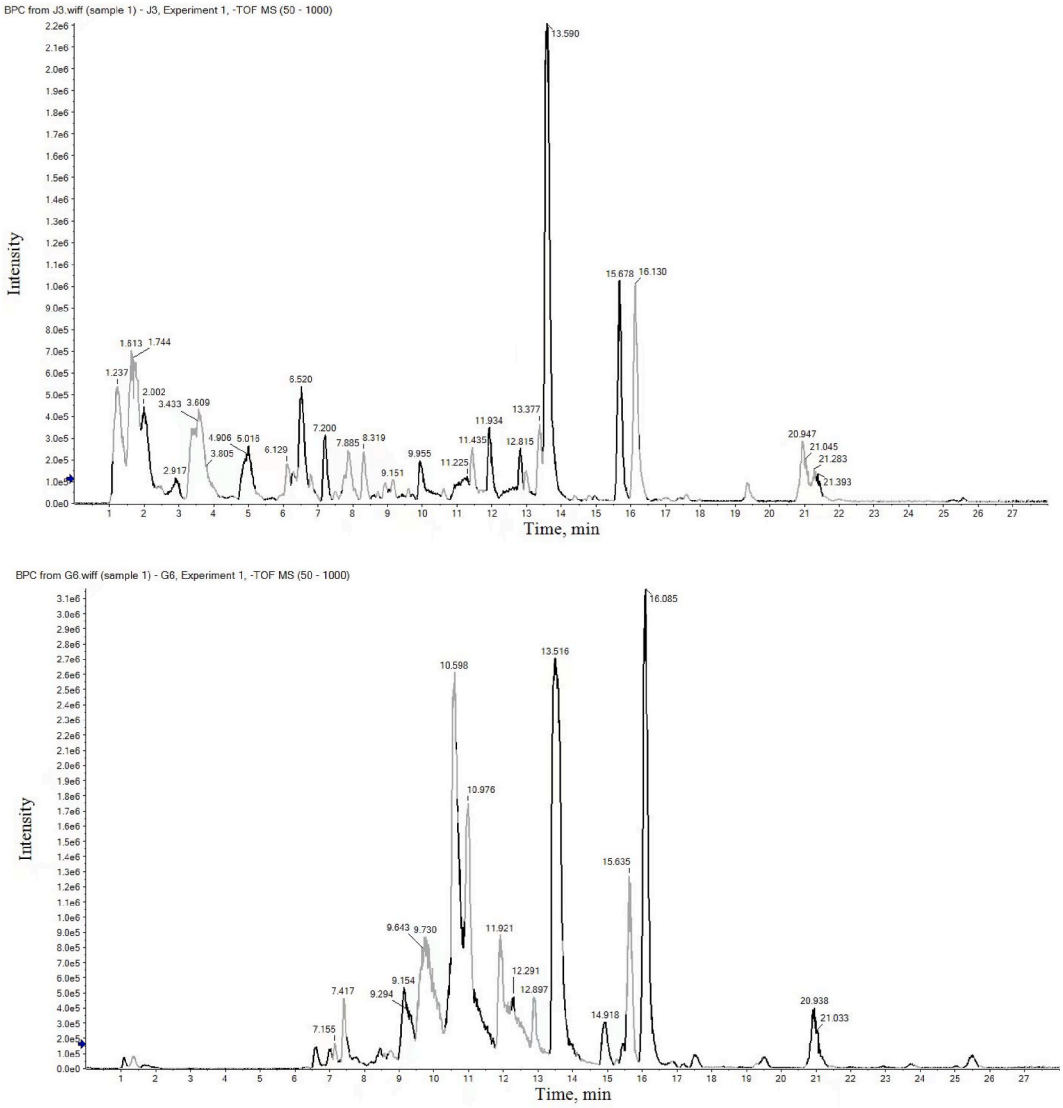

Fig. 7. Base peak chromatograms of AFJ3 and AFG6 ethyl acetate phases in negative ion mode.

sources, owing to their unique genetic backgrounds and metabolic pathways, have produced secondary metabolites with significantly novel structures and notable activities, such as polyketides, alkaloids, and terpenoids [57]. Liquid chromatography-mass spectrometry (LC-MS) is a cutting-edge spectroscopic technique well-suited for the rapid detection of metabolites. It can identify numerous precursor ions in crude extracts, providing significant information about the chemical framework and facilitating the discovery of various compounds [62]. This article reports a rapid method for identifying chemical constituents like organic acids, phenolic acids, flavonoids, and fatty acids in the fermentation broths of endophytic fungi AFJ3 and AFG6 using UPLC-QTOF-MS/MS.

5. Conclusions

In this research, ten distinct strains of endophytic fungi were successfully separated and identified from the roots and stems of *A. sparsifolia* Shap., and their fermentation broth was screened for bacteriostatic activity. Notably, the fermentation broths from the endophytic fungi AFJ3 (from *Aspergillus*) and AFG6 (from *Fusarium*) demonstrated significant antimicrobial effects against all four tested indicator bacteria. The ethyl acetat crude extract of the two fermentative liquids were analyzed in depth for their chemical composition by UPLC-QTOF-MS/MS technique, and 26 different compounds were identified. However, elucidating the material basis of their antimicrobial activities requires further detailed isolation, purification and identification work on a large number of fermentation products under the activity-oriented principle.

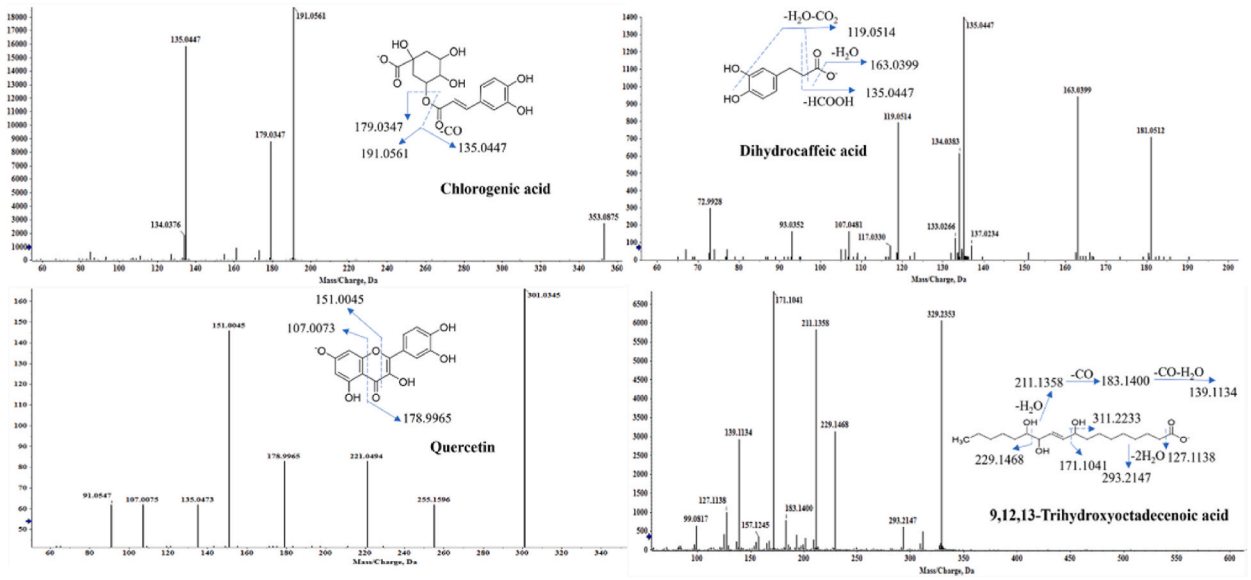

Fig. 8. MS/MS spectra and major fragmentation pathways of chlorogenic acid, dihydrocaffeic acid, quercetin, and 9,12,13-trihydroxyoctadecenoic acid.

Data availability

All data included in the current study are available upon request by contact with the corresponding author.

CRediT authorship contribution statement

Mayila Tuerdibieke: Writing – original draft, Data curation. **Xue Tian:** Writing – original draft, Data curation. **Xuerui An:** Data curation. **Yaping Feng:** Software, Data curation. **Wei Liu:** Writing – review & editing, Project administration, Investigation, Funding acquisition.

Declaration of competing interest

The authors declare that there are no financial or other relationships that might lead to a conflict of interest of the present article.

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