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ORIGINAL PAPER

Studies on a chlorogenic acid-producing endophytic fungi isolated from *Eucommia ulmoides* Oliver

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Abstract *Eucommia ulmoides* Oliver is a traditional medicinal plant of China, and it is one of the main sources of chlorogenic acid. Chlorogenic acid is an ester of caffeic acid, quinic acid, and a phenolic compound that has antibacterial, antifungal, antioxidant, and antitumor activities. The purpose of this study was to determine whether endophytic fungi isolated from *Eucommia ulmoides* Oliver had the same ability to produce chlorogenic acid. Primary screening was done by antibacterial and antifungal reactions, and the strain reselection was done with high-performance liquid chromatography (HPLC) to identify the fermentation products of the selected strains. Extracts of the leaf and cortex of *Eucommia ulmoides* Oliver were also deteted by HPLC, then positive results of HPLC were analyzed by GC-MS and LC-MS. In this study, 29 strains were isolated from *Eucommia ulmoides* Oliver. Most of them had antibacterial activity, and a few of them had antifungal activity. One ingredient of the B5 extract had a retention time identical to that of authentic chlorogenic acid. With GC-MS, other ingredients, isocoumarin and p-chlorocinnamide, were found. With LC-MS, chlorogenic acid and geniposide related to *Eucommia ulmoides* Oliver were found. The strain B5 was identified as *Sordariomycete* sp. Thus, endophytic fungi may produce the bioactive compound chlorogenic acid, as their host plant does, and could be used for the production of chlorogenic acid by fermentation in the future.

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Keywords Medicinal plant · *Eucommia ulmoides* Oliver · Endophytic fungi · Chlorogenic acid

Introduction

Eucommia ulmoides Oliver, also called Du-Zhong, is a living-fossil plant, and the only species both in its genus and in its family [8]. Its cortex has been used as one of the oldest tonic herbs in traditional Chinese medicine for thousands of years. Modern scientific research has shown that the leaves also have some pharmacological effects similar to the cortex [5, 6, 19]. Previous investigations of the roasted leaves identified 22 constituents. The principal components are geniposidic acid and chlorogenic acid [9, 10, 14, 18]. Nowadays, chlorogenic acid is widely used for its antimicrobial, anti-inflammatory, antioxidant, anticancer, and anti-hepatitis B virus activities [3, 13, 20, 23, 24]. Chlorogenic acid has now been officially recorded in the National Pharmacopoeia of China [2].

This natural medicinal resource is now in short supply because of the over-collection of the wild plant, which is now protected in China. This plant can be cultivated in China, but because it needs 20 years to mature before being used for medicinal applications, and the time period for obtaining high yields of chlorogenic acid from the cortex is only in July and November, the conflict between supply and demand is a major problem [17]. Therefore, it is important to find an alternative way to produce its active constituents to satisfy the pharmaceutical demand.

In 1993, a striking finding raised hope concerning this medicinal plant, which is extinct in the wild: an endophytic fungi isolated from *Taxomyces andreanae* produced the bioactive compounds, taxol and taxane, just like the host plant [15]. Since then, many reports have come

out about endophytic fungi and other microorganisms that can produce active compounds like their host plant that are potentially useful for modern medicine, agriculture, and industry [4, 16].

It is well known that a microbial source of a valuable product is usually easier and more economical to produce. Therefore, if by fermentation an endophytic fungi isolated from *Eucommia ulmoides* Oliver can produce the same bioactive compounds, such as chlorogenic acid, just like its host plant, this will protect the plant from extinction. Based on this theory, this study was carried out to isolate endophytic fungi from *Eucommia ulmoides* Oliver and to find out whether any of these fungi can produce chlorogenic acid. In this way, the isolated fungi with positive results can be processed by fermentation to produce the bioactive compounds, and the natural resource *Eucommia ulmoides* Oliver can be protected.

Materials and methods

Plant material

Fresh stems of *Eucommia ulmoides* Oliver were collected at Sichuan University, Chengdu, Sichuan Province, China.

Authentic drug

Authentic chlorogenic acid was bought from National Institute for the Control of Pharmaceutical and Biological Products, China. Pure cholorogenic acid is a white crystal and can be easily dissolved in water, methanol, ethanol, and other polar solvents; the molecular formula is: $C_{16}H_{18}O_9$; molecular weight: 354.1029 (Fig. 1i).

Medium

PDA medium (200 g potato, 20 g D-glucose, 10 g agar, 1,000 ml deionized water) was used for culturing endophytic fungi. Czapek's medium (10 g peptone, 30 g sucrose, 1 g K_2HPO_4, 0.5 g KCl, 0.5 g $MgSO_4$, 0.01 g $FeSO_4$, 1,000 ml deionized water) was used for the fermentation of endophytic fungi. All media were sterilized by autoclaving [21].

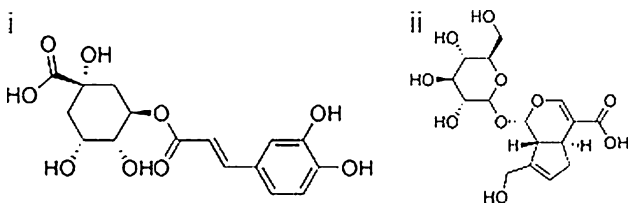

Fig. 1 The structures of chlorogenic acid (i) and geniposide (ii)

Isolation of endophytic fungi

The general procedures adopted for isolation of the microorganisms followed the methodology described by Weber et al. [22]. After collection, the plant material was washed with water and surface sterilized by immersion in 70% aqueous ethanol (3 min), followed by 5% aqueous sodium hypochlorite (60 s, 90, s and 5 min), and finally with 70% aqueous ethanol (1 min). After these procedures, the leaves and roots were rinsed with sterilized water. This water was incubated in Petri dishes to ensure the elimination of all epiphytic microorganisms. Small pieces of the leaves and roots were excised and placed on agar in Petri dishes containing potato dextrose agar (PDA) medium at 30°C. Individual hyphal tips of the emerging fungi were removed and placed on PDA slants. The endophytic fungal strains were isolated by routine microbiological methods, then numbered.

Fermentation of the isolated endophytic fungi

Endophytes obtained were inoculated into 250-ml Erlenmeyer flasks, each containing 100 ml Czapek's medium, and autoclaved at 121°C for 20 min. Biomass was removed by filtration after incubation for 5 days at 28°C on rotary shakers at 200 rpm, and fermentation broth was extracted by ethanol (pH 3) on the evaporator concentrate. The extracts were dried at 50°C.

Antibacterial and antifungal activity of the metabolites

The above extracts were dissolved in sterilized water (concentration 1 g/ml), and the test bacteria were *Escherichia coli*, *Staphyloccocus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Salmonella Lignieres*. Test fungi were *Fusarium gramminearum, Fusarium Maize*, *Magnaporthe grisea, Sclerotinia sclerotiorum, Mucor racemosus, Rhizoctonia solani*, and *Bipolaris maydis*. The method used was agar diffusion tests [12]. Test bacteria were coated on the surface of the LB medium plate, and the test fungi were mixed with PDA in each plate. Three apertures were done: one for water and the other two for extract solution (100 µl). Czapek's medium extract without fungi was used as negative control, and chlorogenic acid (50 µg/µl) was used as positive control. Then, all plates were put in an incubator at 37°C overnight. The next day, we measured the diameter of the inhibition zone.

Extraction of the cortex and leaf of *Eucommia ulmoides* Oliver

The method of extraction was approved by the Editorial Board of the Pharmacopoeia of the People's Republic of

China (2005). For the leaf, 10 g of leaf powder was placed into the extraction apparatus, heated, and recirculated with 200 ml methanol for 30 min. The obtained liquid was centrifuged at 12,000 rpm for 10 min. The supernatant was prepared for HPLC. For the cortex, 30 g of cortex powder was placed into the extraction apparatus, heated, and recirculated with chloroform for 6 h. Then the powder was dried at room temperature. After that, the pretreated cortex powder was heated and recirculated with 200 ml methanol for 6 h, then centrifuged at 12,000 rpm for 10 min. The supernatant was prepared for HPLC analysis.

High-performance liquid chromatography (HPLC) analysis

The extracts of the strains that had obvious antibacterial and antifungal activity were dissolved in water (20 mg/ml) and centrifuged at 12,000 rpm for 10 min. The supernatant was concentrated for HPLC analysis.

HPLC was done with a LC-6AD system and a Kromasil C18 chromatographic column (200 mm*4.6 mm, 5 μm) at 30°C with mobile phase methanol/phosphate (20:80), a sample injection volume of 20 μl, flow rate: 1.0 ml/min, and detection wavelength 327 nm [2].

GC-MS analysis of the extract of the selected strain

In order to find out whether other ingredients existed with similar bioactivity to chlorogenic acid, the extract sample of the strain selected by HPLC was analyzed by GC-MS. An HP 6890 GC system, coupled with an HP MD5973 quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA), was used to analyze the compounds. The extracted compounds were separated on an HP-5MS capillary column (30 m length, 0.25 mm ID, 0.25 μm film thickness). Splitless injection was employed. The column oven temperature was programmed to rise from an initial temperature of 60°C (3 min) to 180°C at 8°C min^{-1}, then to 250°C at 15°C min^{-1}, and then to 280°C at 10°C min^{-1}, staying at 280°C for 2 min. The injection temperature and ion source temperature were 280 and 200°C, respectively. Helium was used as the carrier gas with a flow rate of 1 ml min^{-1}. The ionizing energy was 0.7 kV. All data were obtained by collecting the full-scan mass spectra within the scan range 30–450 amu. Compounds were identified using the Wiley 6.0 (Wiley, New York, NY) Mass Spectral Library [1].

LC-MS analysis of the extract of selected strain

UPLC was performed using a Waters ACQUITY UPLC system (Waters Corp., Milford, MA) equipped with a binary solvent delivery system, autosampler, and a PDA detector. Chromatography was performed on a Waters ACQUITY BEH C_{18} column (100 × 2.1 mm, 1.7 μm). The mobile phase consisted of (A) 0.1% formic acid in water and (B) methanol. The UPLC eluting conditions were optimized as follows: 90% A and 10% B (0–10 min), linear gradient from 90% to 30% A, and 10% to 70% B (10–20 min). The flow rate was 0.15 ml/min. The column and autosampler were room temperature.

The Q-TOF/MS analysis was performed on an electrospray ionization source. Mass calibration and resolution adjustments were performed on Q-TOF by an infusion of Naformate before MS experiments. In the analysis, a Lock-MS mode was applied for the experiment to calibrate the molecular weight real time using reserpine (611.2814). The electrospray ionization source was operated in positive ion mode with a spray voltage of 2.8 kV. Source temperature and desolvation temperature were set at 90°C and 200°C, respectively. Desolvation gas flow was set at 400 l/h. Mass accuracy was set less than 5 ppm for all the ions detected in the MS scan.

Identification of the selected strain

The selected strain was identified by microscopic morphologic characteristics and the analysis of the 18S rDNA sequence. The fungus strain was grown on the surface of PDA medium at 30°C for 5 days for identification based on the morphology of the fungal culture. The characteristics of the spores were scanned by light microscope. The colony of the selected strain was grown in 100-ml Erlenmeyer flasks containing 50 ml potato dextrose liquid medium at 28°C with 200 rpm; after 3 days, its genomic DNA was extracted using the SDS/CTAB method to amplify the 18S rDNA sequence. A PCR reaction mixture was made containing 30 to 100 ng genomic DNA of the strain, 100 μM each 18S-F1 (5′-GGAAGGGRTGT ATTTATTAG-3′) and 18S-R1 (5′-TCCTCTAAATGACCA AGTTTG-3′), 150 μM dNTPs in l*PCR reaction buffer, and 2.5 U *Taq* DNA polymerase, and the total volume was brought to 50 μM with deionized water. The thermal cycling condition was hot start for 5 min at 95°C followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, 1.5 min at 72°C, and 10 min at 72°C. The amplified DNA fragments were cloned into the sequencing vector pMD-19 and sequenced by Invitrogen Biotechnology Technology and Service Co., Ltd.

Results

Isolation and antibacterial activity

Twenty-nine endophytic fungal strains were isolated on the basis of morphology, and they were divided into six groups (N, B, S, C, A, E). The metabolites of most of them had

antibacterial activity, and a few of them had antifungal activity. Tables 1 and 2 show the results.

In the two tables, only a few strains had both antibacterial and antifungal activity. We chose N1, N4, B5, A10, #2, S8, and C5 for HPLC analysis.

High-performance liquid chromatography (HPLC) analysis

The HPLC chromatograms showed that only the sample B5 and authentic chlorogenic acid had the same retention time of about 24.906 min, and the chlorogenic acid of leaf and cortex also had the same retention time as sample B5 and authentic chlorogenic acid. By comparison of B5's *t*R and UV/PAD spectra with chlorogenic acid standard analyzed under identical chromatographic conditions, we could say that the extract of B5 may have contained chlorogenic acid. To make sure that B5 contained chlorogrnic acid, authentic chlorogenic acid was diluted with sample B5 40 times; at the retention time, a heightened peak could be found (Fig. 3). So, on the basis of the above results, it is highly likely that the strain B5 produces chlorogenic acid like its host does by comparing extracts of B5 with the extracts of leaf and cortex of *Eucommia ulmoides* Oliver Fig. 2.

GC-MS analysis of the extract

In GC-MS analysis of the extract of B5, isocoumarin, another substance with antibacterial activity, could be found. p-Chlorocinnamide, the intermediate of chlorogenic acid, was also found in the GC-MS analysis (Fig. 4).

Table 1 Antibacterial activity (mm)

Fungal isolate[a]	*Escherichia coli*[b]	*Staphyloccocus aureus*[b]	*Bacillus subtilis*[b]	*Pseudomonas aeruginosa*[b]	*Salmonella Lignieres*[b]
N1	12[c]	16.5	15	13	13
N2	9	13.5	17	13	11
N3	12.5	17.5	16	17	13
N4	12	18	18	19	14
N5	12	20	12	15.5	10
N6	12	10.5	10.5	16	11
B1	13.5	12	15.5	16	11.5
B3	11	15	12	13	13.5
B5	14.5	23.5	22	21	16
B6	11.5	17	15	16	10
B7	14	17.5	18.5	15.5	13
B8	10	15	11.5	12	–[d]
C2	14.5	15	19.5	15.5	14
C3	9	10	–	–	11.5
C5	11.5	14.5	18	18	11
C6	10	9	16.5	–	–
A2	12	16.5	16	15.5	11.5
A4	12.5	–	13	9.5	–
A6	–	–	–	–	–
A7	10.5	15.5	9.5	12	8.5
A10	12	18.5	18	17	10.5
S2	10.5	16.5	15	15	10.5
S3	11.5	16	15	16	10
S5	13.5	17.5	15	14.5	11.5
S6	14	15.5	14	14.5	12.5
S7	12	17.5	15.5	16	9
S8	11.5	17	13	14.5	10.5
E1	11.5	16	12	15	10.5
#2	13.5	8	17	9.5	15
Negative control	–	–	–	–	–
Positive control	10	13	12.5	8.5	10.5

Table 2 Antifungal activity (mm)

Fungal isolate[a]	*Fusarium gramminearum*[e]	*Fusarium Maize*[e]	*Magnaporthe grisea*[e]	*Sclerotinia sclerotiorum*[e]	*Mucor racemosus*[e]	*Rhizoctonia solani*[e]	*Bipolaris maydis*[e]
N1	–	20	15	22	16	21	27
N2	–	–	–	15	–	–	–
N3	–	–	–	–	–	–	–
N4	–	–	–	26	–	17	19
N5	–	–	–	22	–	20	20
N6	–	15	–	16	–	21	23
B1	–	–	–	11	–	–	–
B3	–	15	–	–	–	–	15
B5	20	21	19	23	15	18	20
B6	14	17	–	19.5	–	–	–
B7	–	19	20	7	–	–	22
B8	–	–	–	18	–	11	–
C2	–	–	10	–	–	–	–
C3	12	16	–	–	–	–	17
C5	18	20	–	15	–	–	23
C6	–	–	–	–	–	–	13
A2	–	–	–	7.5	–	–	–
A4	–	–	–	19.5	–	–	15
A6	–	–	–	–	–	–	–
A7	15	18	–	21.5	–	12	15
A10	–	18	16	–	14.5	13	21
S2	–	–	–	14.5	–	–	–
S3	–	–	–	21	–	–	22
S5	–	–	–	–	–	–	–
S6	–	–	–	10	–	13	18
S7	–	–	–	–	–	–	–
S8	–	–	17	28	16	–	–
E1	–	–	–	–	–	–	–
#2	16	–	–	29	14	19	27
Negative control	–	–	–	–	–	–	–
Positive control	10	12	11.5	12	10.5	12.5	13

[a] Isolates were cultivated from *Eucommia ulmoides* Oliver

[b] Test bacteria

[c] Number is the diameter of the inhibition zone; the bigger, the better bioactivity

[d] "–" means no activity

[e] Test fungi

LC-MS analysis of the extract

UPLC-MS analysis of the extract of B5 was carried out. Abundant positive $[M-H]^+$ ions (data not shown) were produced, and the molecular mass of chlorogenic acid could be easily determined based on the detection of product ion of m/z 355.1040 (theoretical 355.1029). And in LC-MS analysis, another ingredient called geniposide was found; it is also the secondary metabolite of *Eucommia ulmoides* Oliver. The detection of production ion is m/z 389.1466 (Figs. 1ii, 5).

Identification of strain B5

According to the morphology of the culture and light microscope scanning, B5 could be identified as *Sordariomycete sp*, which was identified by Prof. Shaorong Ge (College of Life Science, Sichuan University, China), and

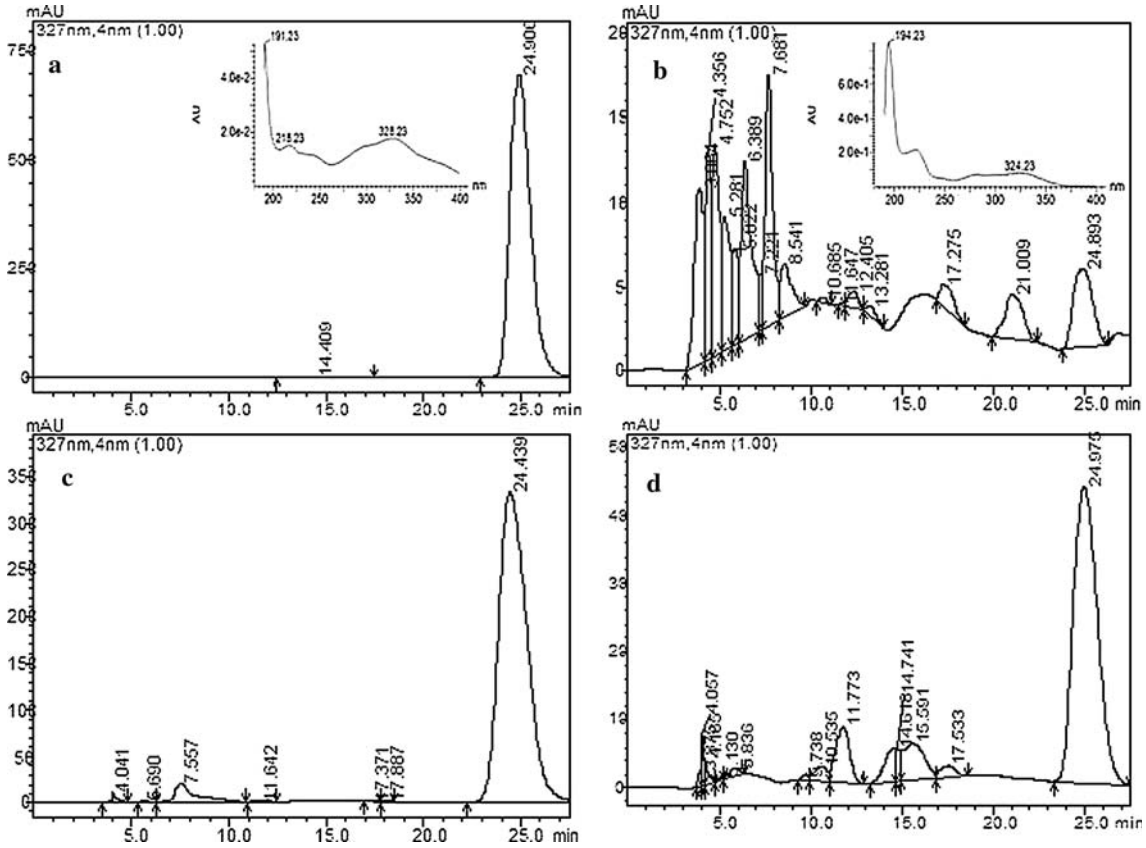

Fig. 2 **a** HPLC chromatogram of authentic chlorogenic acid and its representative UV/PAD spectra; **b** HPLC chromatogram of B5 extract and its representative UV/PAD spectra; **c** HPLC chromatogram of the leaf of *Eucommia ulmoides* Oliver; **d** HPLC chromatogram of the cortex of *Eucommia ulmoides* Oliver

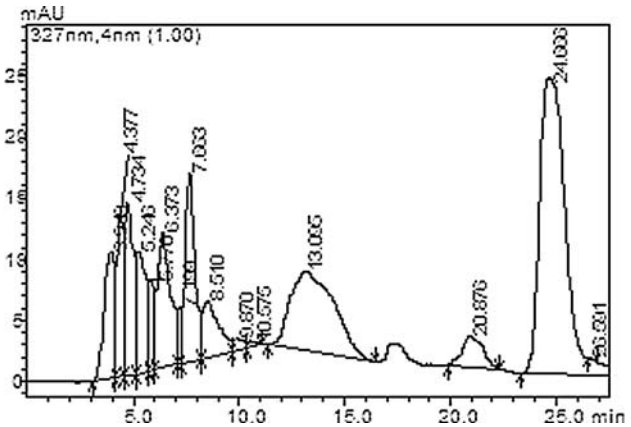

Fig. 3 The chromatograms of co-injection of authentic chlorogenic acid with B5 extract

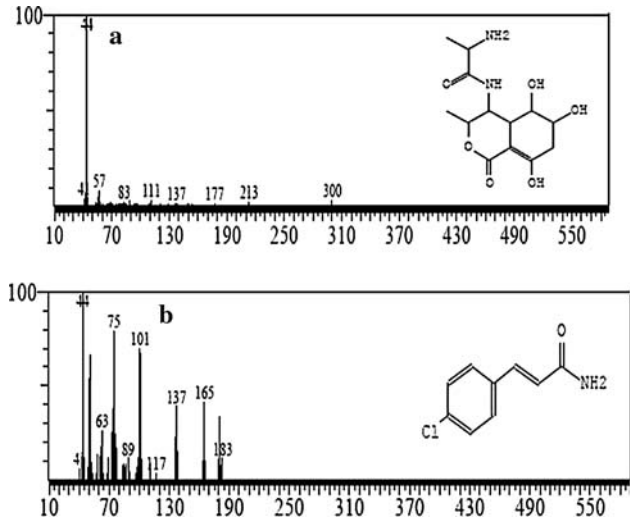

Fig. 4 **a** The analysis of isocoumarin; **b** the analysis of p-chlorocinnamide

the partial 18S rDNA sequence from strain B5 (ggaaggggt gtatttattagattaaaaaccaatgcccttcggggctcactggtgattcataataacttc tcgaatcgcatggccttgtgccggcgatggttcattcaaatttctgccctatcaactt tcgacggctgggtcttggccagccgtggttacaacgggtaacggagggttagggc ttgaccccggagaaggagcctgagaaacggctactacatccaaggaaggcagca ggcgcgcaaattacccaatcccgactcggggaggtagtgacaataaatactgatac agggctcttttgggtcttgtaattggaatgagtacaatttaaatcccttaacgaggaac aattggagggcaagtctggtgccagcagccgcggtaattccagctccaa

tagcgtatattaaagttgttgcagttaaaaagctcgtagttgaaccttgggcctggct ggccggtctgcctcaccgcatgcactggtccggccgggcctttccctctgggga gccgcatgcccttcactgggtgtgtcggggaaccaggacttttactgtgaaaaaat tagagtgttcaaagcaggcatatgctcgaatacattagcatggaataatagaatag gacgtcgcggttctattttgttggtttctaggaccgccgtaatgattaatagggacag

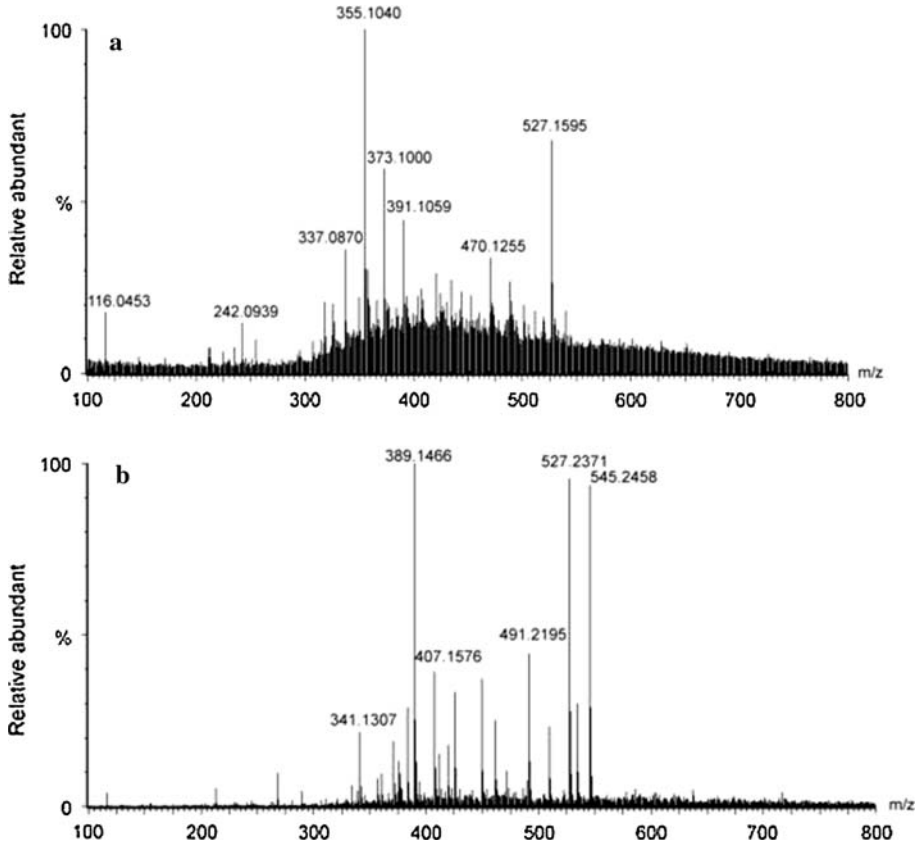

Fig. 5 **a** LC-MS mass spectra of chlorogenic acid from the B5 extract; **b** LC-MS mass spectra of geniposide from the B5 extract

Fig. 6 Picture of the culture and spore of strain B5

tcgggggcatcagtattcaatcgtcagaggtgaaattcttggatcgattgaagacta actactgcgaaagcatttgccaaggatgttctcattaatcaggaacgaaagttagg ggatcgaaaacgatcagataccgttgtagtcttaaccataaactatgccgactagg gatcgggcggtgttatttcttgacccgctcggcaccttacacgaaagtaaagttttt gggttctgggggagtatggtcgcaaggctgaaacttaaagaaattgacggaag ggcaccacaaggggtggagcctgcggcttaatttgactcaacacggggaaactc accaggtccagacacaactaggattgacagattgagagctctttcttgattttgtgg gtggtggtgcatggccgttctcagttggtggagtgatttgtct) showed that it shares 99% identity with *Sordariomycete sp*. Fig. 6.

Discussions

The isolation of bioactive components from endophytic fungi has only been considered since the end of the last century. It has been demonstrated that these endophytes produce many valuable substances and have significant influence because of the production of relative components and the protection of medicinal plants. The prospect of using endophytes as new sources of natural products for medicine, agriculture, and industry is exciting [4, 16].

In our study, endophytic fungi were isolated and selected based on the basic function of chlorogenic acid, antimicrobal activity. Then the samples with positive results were analyzed by HPLC, GC-MS, and LC-MS. In HPLC, sample B5 and authentic chlorogenic acid had the same retention time of about 24.906 min, as did the cortex and leaf of *Eucommia ulmoides* Oliver. The result of LC-MS confirmed that the extract of B5 might contain chlorogenic acid, too. With GC-MS analysis, the discovery of isocoumarin and p-chlorocinnamide could indicate that the pathway of chlorogenic acid synthesis exists in this strain, because coumarin and p-chlorocinnamide are intermediates in chlorogenic acid biosynthesis, while isocoumarin is the isomer of coumarin [11]. In previous research, isocoumarin had similar bioactivity to chlorogenic acid, such as antimicrobial and antitumor activity [7]; thus, it can be exploited as another new biological resource. Although we found geniposide in the extract of B5, it could not be proven that geniposide indeed existed in the extract just based on molecular mass. Other methods must be used to obtain

further confirmation, for geniposide has anti-inflammatory activity, and it was found to be the main hypotensive compound in *Eucommia ulmoides* Oliver [24].

Therefore, it is highly likely that the strain B5 could produce the bioactive component, chlorogenic acid, just like its host, *Eucommia ulmoides* Oliver, does. Thus, the strain B5 could be used as a source of chlorogenic acid by fermentation, and this antitumor medicine could be obtained easily without over-collecting plant resources. In HPLC analysis, the yield of chlorogenic acid of strain B5 was relatively low and was not quite suitable for its production on an industrial scale.

Therefore, the next goal is to confirm whether geniposide exists in the extract of B5 in order to optimize the fermentation condition of strain B5 and regulate the key enzyme of chlorogenic acid biosynthesis to gain large quantities of chlorogenic acid or other similar ingredients, such as isocoumarin, for medicinal use. Before such a bioactive substance can be used in applications, its security must be checked. In this way, we hope to lay a sound foundation for a new method for producing these important bioactive compounds.

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