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Research Article

Active anti-acetylcholinesterase component of secondary metabolites produced by the endophytic fungi of *Huperzia serrata*

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ABSTRACT

Background: An endophytic fungus lives within a healthy plant during certain stages of, or throughout, its life cycle. Endophytic fungi do not always cause plant disease, and they include fungi that yield different effects, including mutual benefit, and neutral and pathogenic effects. Endophytic fungi promote plant growth, improve the host plant's resistance to biotic and abiotic stresses, and can produce the same or similar biologically active substances as the host. Thus, endophytic fungal products have important implications in drug development.
Result: Among the numerous endophytic fungi, we identified two strains, L10Q37 and LQ2F02, that have anti-acetylcholinesterase activity, but the active compound was not huperzine A. The aim of this study was to investigate the anti-acetylcholinesterase activity of secondary metabolites isolated from the endophytic fungi of *Huperzia serrata*. Microbial cultivation and fermentation were used to obtain secondary metabolites. Active components were then extracted from the secondary metabolites, and their activities were tracked. Two compounds that were isolated from endophytic fungi of *H. serrata* were identified and had acetylcholine inhibitory activities. In conclusion, endophytic fungal strains were found in *H. serrata* that had the same anti-acetylcholinesterase activity.
Conclusion: We isolated 4 compounds from the endophytic fungus L10Q37, among them S1 and S3 are new compounds. 6 compounds were isolated from LQ2F02, all 6 compounds are new compounds. After tested anti acetylcholinesterase activity, S5 has the best activity. Other compounds' anti acetylcholinesterase activity was not better compared with huperzine A.

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1. Introduction

Alzheimer's disease is a neurodegenerative disease of the central nervous system. The first clinical manifestation is recent memory dysfunction, which is followed by persistent intellectual impairment, loss of judgment and reasoning abilities, aphasia, and movement dysfunction [1]. A study found that of the 10–15% of elderly people with different degrees of dementia, approximately 60–70% of the cases are due to Alzheimer's disease [2]. However, the pathogenesis of senile dementia is not clear. Cholinergic nerve injury is the most accepted hypothesis of Alzheimer's disease pathogenesis, and if this is true, acetylcholinesterase inhibitors could be developed to effectively improve Alzheimer's disease treatment.

Huperzine A is used as an effective drug to treat senile dementia [3, 4]. Because chemical synthesis has limitations that are difficult to overcome [5], the main method to obtain huperzine A is to isolate it from *Huperzia serrata* [6]. Many natural compounds from plants and plant endophytic fungi are closely related, including some secondary metabolites of endophytic fungi [7,8]. Natural products with medicinal value obtained from endophytic fungi can overcome both the lack of resources and the long plant regeneration cycle. Industrial fermentation can be used to obtain naturally active compounds on a large scale with low costs and no pollution production. Stierle et al. [7] first isolated paclitaxel from *Taxus brevifolia* in 1993. As a result, research on plant secondary metabolites of endophytic fungi became more feasible. Thus, components that inhibit cholinesterase activity could be extracted from the endophytic fungi of *H. serrata* instead of extracting active components from plants. This allowed us to identify new compounds with anti-acetylcholinesterase activities, which may solve the *H. serrata* resource exhaustion crisis. We studied metabolites of the endophytic fungi L10Q37 and LQ2F02, which were isolated from *H. serrata*, to obtain acetylcholinesterase inhibitors.

2. Materials and methods

Wild *H. serrata* plants were obtained from Tianmu Mountain, Zhejiang Province, China. All of the isolates were grown and purified as single colonies on potato dextrose agar (PDA) solid

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media containing 200 g/L potato, 20 g/L glucose, and 18 g/L agar. Potato dextrose (PD) liquid medium was used to cultivate strains.

2.1. Isolation of endophytic fungi

We used the surface disinfection method that was described by Zeng et al. [9]. Fresh *H. serrata* roots were washed, soaked in 70% ethanol for 4 min, washed five times in sterile water, placed in 1% sodium hypochlorite solution for 5 min, and washed five times with distilled water. Then, the roots were cultivated on PDA medium. After 2 weeks at 28°C, 1% sodium hypochlorite solution was used to sterilize explants without fungal contamination for 15 min, and cuttings were inoculated on PDA media. Hyphae were inoculated on PDA plates at 28°C for purification. The mycelial tips were transferred to PDA plates. This purification method was repeated more than five times. Mycelia were then saved on a test tube slant.

2.2. Screening of anti-acetylcholinesterase activity

The thin-layer chromatography (TLC) bioautographic method is a combination of TLC and biological activity testing with a drug screen. The specific method was described by Marston et al. [10] and Wang et al. [11]. Briefly, acetylcholinesterase (500 U) was dissolved in 75 mL 0.05 M Tris buffer (pH 7.8) with 75 mg bovine serum albumin to protect the protein and enzyme activity. The solution was stored at 4°C. About 2.5 μL of the standard and sample solutions were absorbed on the same thin silica gel plate with chloroform:methanol:acetone:water (4:4:1.5:0.05) as the developing agent. The TLC plate was immersed in a solution of enzyme to allow leaching of plate surface vessels. The plate was then removed and dried. The TLC plate was placed at 37°C in an insulated water bath for 20 min to maintain surface moisture. Alpha naphthalene ethyl acetate (25 mg) was dissolved in 10 mL methanol, and 50 mg Fast Blue B Salt was dissolved in 20 mL distilled water. Alpha naphthalene ethyl ester solution (5 mL) was combined with 20 mL Fast Blue B solution, and surface vessels were soaked in acetylcholinesterase enzyme liquid, alpha naphthalene ethyl ester solution, and Fast Blue B Salt solution. After immersion, the plate was removed and dried. After 1–2 min, the TLC plate developed a purple background and was viewed with a visible light camera system.

Fig. 1. TLC-bioautography result. Lane 1, huperzine A; lane 2, crude extract from LQ2F02; and lane 3, crude extract from L10Q37.

2.3. Active strain and species analysis

Active strains were cultured in PD liquid medium at 28°C and 150 rpm. Active strains and species were analyzed using morphological observations and the internal transcribed spacer (ITS)-rDNA method. The colony morphology was observed by light microscopy, and electron microscopy allowed for detailed observations of spores, spore stalks, spore sizes, lengths, colors, surface conditions, and aggregation. Molecular analyses using 5.8S and 18S rDNA gene sequencing were performed. The fungal 5.8S rDNA was amplified using universal primers ITS1 (5′-AACT CGGCCATTTAGAGGAAGT-3′) and ITS4 (5′-TCCTCCGCTTATTGATAT GC-3′), and 18S rDNA was amplified using universal primers NS1 (5′-GTAGTCATATGCTTGTCTC-3′) and NS8 (5′-TCCGCAGGTTCACC TACGGA-3′) (Synthesized by Shanghai Jierui Bioengineering Co., Ltd., Shanghai, China). PCR amplifications of 5.8S rDNA were performed in an Eppendorf thermal cycler (Corbett Research UK, Ltd., Shanghai, China) using the following conditions: initial denaturation at 94°C for 300 s; 34 cycles of 30 s at 94°C, 30 s at 50°C, and 60 s at 72°C, and a final extension step of 600 s at 72°C. PCR amplifications of 18S rDNA were performed in an Eppendorf thermal cycler as follows: initial denaturation at 94°C for 300 s; 34 cycles of 60 s at 94°C, 45 s at 53°C, and 90 s at 72°C; and a final extension step of 600 s at 72°C. PCR products were analyzed by electrophoresis in 1% agarose gels. DNA sequences obtained from active strains were aligned by CLUSTALW [12] using MEGA 4.0 software. Assembled DNA sequence data were analyzed using the nucleotide basic local alignment search tool (BLASTn) and were compared with the non-redundant nucleotide sequence database at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Multiple alignments were generated with DNA sequences obtained using CLUSTALW. Phylogenetic distance trees were inferred by neighbor-joining analyses using MEGA 4.0. The confidence in topologies was assessed using 1000 bootstrapped replicates.

2.4. Determination of anti-acetylcholinesterase activity

Strains isolated from *H. serrata* were preliminarily screened using a TLC bioautographic method to confirm that the endophytic fungi had anti-acetylcholinesterase activities. Then, active strains were cultured in 100 mL liquid PD medium in an incubator shaker at 28°C and 150 rpm for 7 d. The fermentation products were filtered, and the fermentation broth and mycelium were separated. The fermentation broth was concentrated by vacuum rotary evaporation. The soluble fermentation liquid concentrate was dissolved in 10 mL methanol, and the insoluble impurities were removed using a 2.2 μm filter membrane. The obtained solution was used directly to determine the anti-acetylcholinesterase activity. The mycelium was dried in a 55°C oven, mixed with methanol (1:20 w/v), extracted ultrasonically three times for 30 min each, and then combined with the methanol extract. Vacuum distillation was used to dry the extract, all of the methanol extract was dissolved in 10 mL methanol, and the insoluble impurities were removed by a 2.2 μm filter membrane, leaving the mycelium extract that was used for the anti-acetylcholinesterase activity test.

This activity was measured using the method of Ellman et al. [13]. The sample solution (10 μL) was combined with 120 μL 0.1 M PBS, 50 μL 0.4 U/mL acetylcholinesterase, and 20 μL 7.5 mM color developing agent 5,5′-dithiobis-(2-nitrobenzoic acid) solution and added to 96 well plates. The mixture was incubated at 37°C for

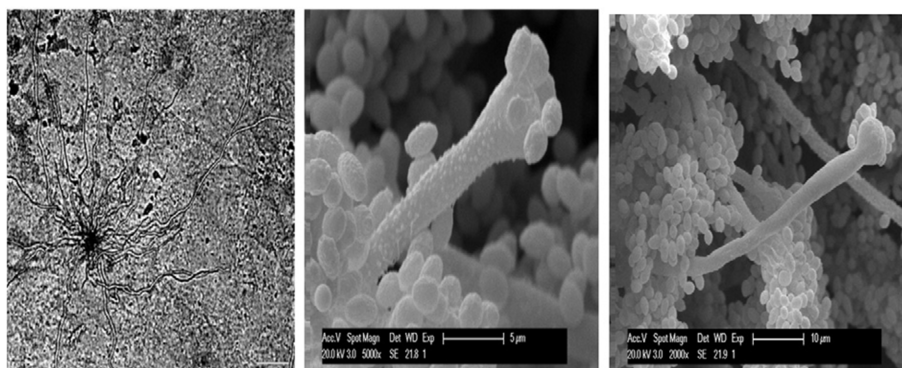

Fig. 2. Strain LQ2F02 mycelial morphology and spore microstructure.

30 min. Next, 50 μL 7.5 mM substrate acetylthiocholine iodide solution was added to start the reaction. The progress of the reaction was monitored using a microplate reader with detection at 412 nm every 30 s. The optical density at 412 nm was read 10 times (300 s). A blank control of 0.1 M phosphate buffer was monitored at the same time. The above experiment was repeated three times. A linear relationship between time and absorption value was determined, and the difference in the slope between the blank and sample indicated the acetylcholinesterase inhibition rate.

2.5. Fungal growth curve and its relationship with activity

Using the mycelial growth rate method, we determined the fungal growth curves. Every 2 d, five bottles of culture fluid were selected from the incubator at random. After filtering using a Buchner funnel, the mycelium was dried in a 60°C oven. After 3 h, the mycelial weight was measured every 20 min. The mycelial dry weight was determined when three consecutive measurements differed by less than 0.01 g. The activity was measured using a modified Elleman spectrophotometry method. We combined the growth and activity data to determine the optimum culture time.

2.6. Sample preparation and determination of the active ingredient's polarities

For fermentation, the seed solution was prepared as follows: a slant-activated strain was aseptically inoculated in PD liquid medium and incubated at 28°C and 150 rpm for 48 h. The fermentation liquid was filtered through four layers of sterile gauze to obtain a homogeneous first-level seed solution. Next, 10% of the solution was inoculated in liquid PD medium and cultured at 28°C and 150 rpm for 48 h to obtain a second-level seed solution. Then, 20 L PD liquid medium was added to the 30 L aerated stirring fermentation tank, sterilized at 121°C for 20 min, and cooled to 28°C. One percent of the second-level seed liquid was fermented. The fermentation parameters were as follows: ventilation, 1:0.2; temperature, 28°C; speed, 150 rpm; and duration, 10 d. The fermentation products were filtered, and the fermentation broth and mycelium were separated. The fermentation solution was concentrated to 1/10th of the original volume. Then, it was extracted three times each in the same volume of petroleum ether, chloroform, ethyl acetate, and n-butanol. The extracts were combined by vacuum drying the petroleum ether, ethyl acetate, chloroform, and n-butanol extracts. The concentration was adjusted to 5 μg/mL, and the anti-acetylcholinesterase activities of the extracts were measured.

2.7. Alkaloids analysis in metabolites

Endophytic fungi living in plants, which interact with plants for a long time, often produce the same or similar chemical compounds as the host [14,15,16]. Alkaloids are the main materials with anti-acetylcholinesterase activities in *H. serrata*. To determine whether the strains with anti-acetylcholinesterase activities contain alkaloids having this activity, fermented liquid samples from the active strains were analyzed using TLC. The TLC expansion agent was chloroform:methanol:acetone:ammonia (4:4:1.5:0.05). After drying, plates were sprayed with Dragendorff's chromogenic reagent. Dull red spots indicated the presence of alkaloids in the fermentation liquid.

2.8. Extraction and isolation of active compounds

Alkaloids were extracted using the acid water method [17,18]. Briefly, 100 mL fermentation broth was extracted with an equal volume of ethyl acetate three times. The extracts were combined, vacuum dried to obtain the ethyl acetate extract, and dissolved in 200 mL 2% hydrochloric acid solution adjusted to pH 2. Petroleum ether (200 mL) was added three times to remove filtrate impurities. Then, the filtrate was combined with a 5% NaOH solution adjusted to pH 10–11 and extracted three times using an equal volume of

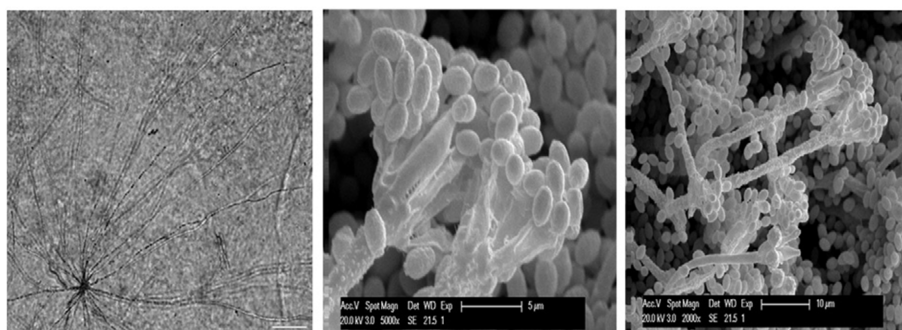

Fig. 3. Strain L10Q37 mycelial morphology and spore microstructure.

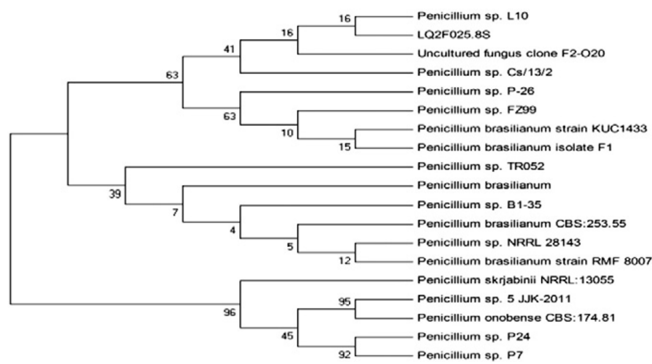

Fig. 4. LQ2F02 5.8S rDNA phylogenetic tree.

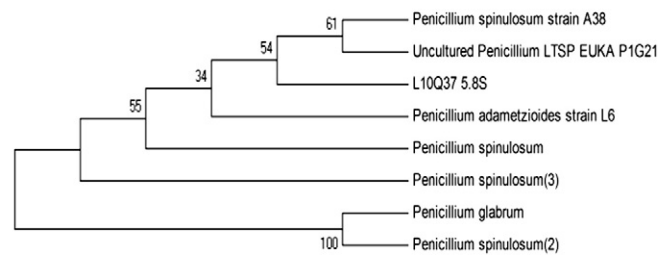

Fig. 6. L10Q37 5.8S rDNA phylogenetic tree.

chloroform. The chloroform extracts were combined and evaporated to obtain alkaloids.

To extract non-alkaloids, endophytic fungi of *H. serrata* were fermented, the fermentation products were filtered, and the fermentation broth and mycelium were separated. The fermentation solution was concentrated to 1/10th the original volume, extracted five times with an equal volume of ethyl acetate, and concentrated. The ethyl acetate and total alkaloids parts were separated using a prepared Shiseido C18 column (MGS5 20 mm × 250 mm) with a mobile phase of acetonitrile and water. The flow rate was 12 mL/min, and 0.3 mL samples were analyzed. The column temperature was 25°C, and the detection wavelengths were 220, 260, 280, and 310 nm.

2.9. Anti-acetylcholinesterase activity and structure identification

Activity was measured using the modified Elleman spectrophoto metric method described above. Structure identification was determined using NMR (Germany Bruker) and LC-MS (Waters Synapt G2 UPLC-Q-TOF). Mass spectrometric conditions were described previously [19] as follows: negative electron ionization mode, spray voltage of 3.8 kV, precipitation temperature of 300°C, ion source temperature of 150°C, ion energy of 0.5 V, cone voltage of 90 V, and scanning range from 100 to 800 m/z. In positive electron ionization mode, spray voltage of 3.8 kV, precipitation temperature of 300°C, ion source temperature of 150°C, ion energy of 0.5 V, cone voltage of 90 V, and scanning range from 100 to 800 m/z.

3. Results

3.1. Endophytic fungal separation and screening results

Because fungi are eukaryotic organisms, widespread heterokaryons, and multi-core phenomena, their genetic traits are not stable. Impure strains were purified using the continuous culture method. In this way we isolated 460 endophytic fungi that were roughly identified as belonging to 166 strains. We then used TLC-biological screening to obtain two strains, L10Q37 and LQ2F02, that are resistant to acetylcholinesterase activity (Fig. 1). After light and scanning electron microscopic observations, morphological descriptions of strains L10Q37 and LQ2F02 were composed [20,21,22]. Strain LQ2F02: first aerial hyphae colorless; gray gradient finally becoming green; colonies close, circular, margin irregular, tan on the back; electron microscopy; hyphae unbranched; conidium terrier coarse, diaphragm, about 1.6–2.8 μm in diameter; top capsule globose; small terrier radiated, produced small ovoid, conidia. Strain L10Q37: first aerial hyphae light; blue gradient; colonies close, circular, margin not neat, amber on the back; hyphae unbranched, conidium terrier coarse; small terrier with several rounds of asymmetry; small terrier radiated; small, oval at the top, conidia. Light microscopy and scanning electron microscopy images are shown in Fig. 2 and Fig. 3.

Using the BLAST algorithm, we identified 99 strains with sequence similarities of 99% or more. Sequences were aligned using Clustal X, which was used to construct a phylogenetic tree. Bootstrap ratings were calculated for each branch using 1000 repeated samplings. If the bootstrap support rate was low, then trees were recalculated. Results are shown in Fig. 4, Fig. 5, Fig. 6, and Fig. 7.

According to the morphological description, we determined that LQ2F02 is an unknown fungus. The ITS 5.8S ribosomal gene sequence indicated that LQ2F02 is phylogenetically related to *Penicillium*, displaying the highest sequence similarity (18%) with *Penicillium* sp. L10. An analysis of the ITS 18S ribosomal gene sequence also showed that LQ2F02 is phylogenetically related to *Penicillium*, displaying the highest sequence similarity (81%) with *Penicillium malachiteum*. Therefore, LQ2F02 is most likely a *Penicillium* sp.

According to the morphological description, we determined that L10Q37 is an unknown fungus. The ITS 5.8S ribosomal gene sequence indicated that L10Q37 is phylogenetically related to *Penicillium*, displaying the highest sequence similarity (54%) with *Penicillium spinulosum* and uncultured *Penicillium* LTSP EUKA P1G21. An analysis of the ITS 18S ribosomal gene sequence also indicated that L10Q37 is phylogenetically related to *Penicillium*, displaying the highest sequence similarity (87%) with *Penicillium* sp. 1 F and *Chromocleista* sp. 12F. Therefore, this strain was most likely a *Penicillium* sp.

3.2. Acetylcholinesterase inhibitor screening

The relationship between growth and acetylcholinesterase inhibition activity is shown in Fig. 8 and Fig. 9. Strains that resist acetylcholinesterase activity are positively correlated with growth. The main active ingredient was investigated in the logarithmic and

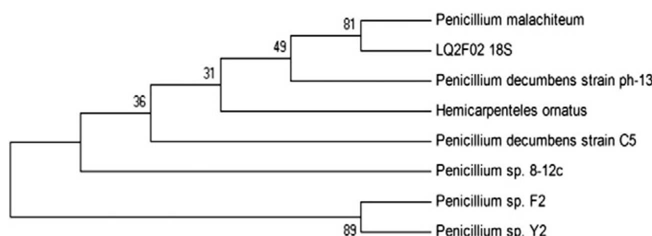

Fig. 5. LQ2F02 18S rDNA phylogenetic tree.

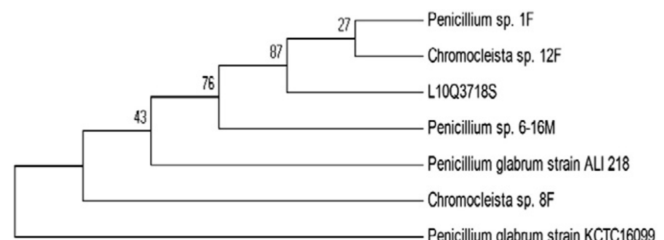

Fig. 7. L10Q37 18S rDNA phylogenetic tree.

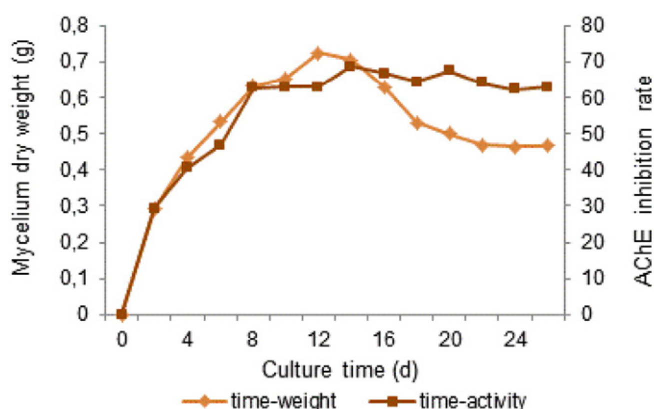

Fig. 8. Relationship between culture time, anti-acetylcholinesterase activity, and cell dry weight of strain LQ2F02.

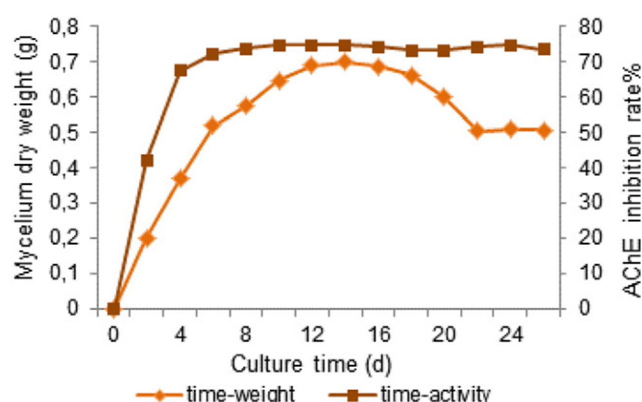

Fig. 9. Relationship between culture time, anti-acetylcholinesterase activity, and cell dry weight of strain L10Q37.

stationary phases. The active ingredient appears to be an exocrine-type product. With an incubation time of 14 d for L10Q37 and 13 d for LQ2F02, the growth of strains and secondary metabolites that resist acetylcholinesterase activity is maximized.

The analysis results of active ingredient polarities are shown in Table 1. The two strains have polarities that resist acetylcholinesterase activity, and under medium polarity, their active ingredients are mainly concentrated in ethyl acetate. The active ingredient is the main component that can resist acetylcholinesterase activity. This component was mainly found in the fermented liquid (Fig. 10).

The results of the total secondary metabolites and total alkaloid composition activity analyses are shown in Fig. 11 and Table 2. The secondary metabolites of both strains produce the same spots as huperzine A, which indicates that the secondary metabolites have alkaloid compositions. Additionally, the acetylcholinesterase activity

Table 1
Acetylcholinesteration rates of fermentation products of different polarity periods.

Polarity section	Inhibition rate (%)
LQ2F02 petroleum ether	35.12 ± 1.72
LQ2F02 chloroform	34.34 ± 2.21
LQ2F02 ethyl acetate	61.77 ± 1.29
LQ2F02 butanol	42.81 ± 1.42
L10Q37 petroleum ether	29.82 ± 1.02
L10Q37 chloroform	32.81 ± 0.71
L10Q37 ethyl acetate	66.66 ± 1.51
L10Q37 butanol	31.38 ± 1.22

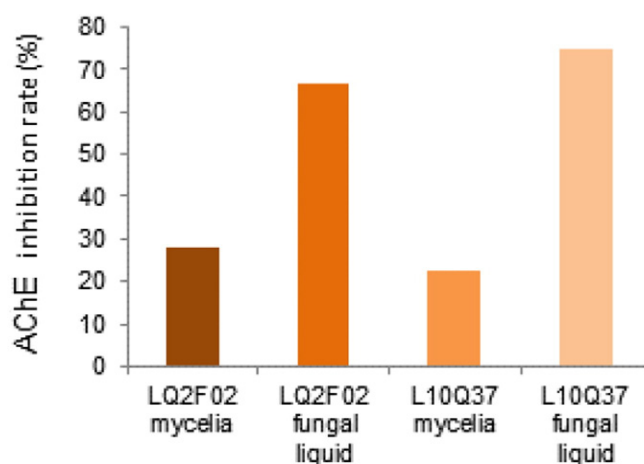

Fig. 10. The acetylcholinesterase inhibitory rate of mycelia and fungal liquid.

test indicated that the main active ingredients were in the alkaloid portions of LQ2F02 and L10Q37.

3.3. Isolation, identification, and activity determination of acetylcholinesterase inhibitors

Using preparative high-performance liquid chromatography (HPLC) separation compounds, we obtained four compounds from L10Q37 metabolites (S1–S4) and six compounds from LQ2F02 metabolites (S5–S10) (Fig. 12):

S1 ESI-MS: 165.0547 [M + Na], calculated value for 142.06. ^{1}H NMR (CD3OD-d6, 600 MHz): 9.56 (1H, s, H-4), 7.40 (1H, d, H-5), 7.05 (1H, d, H-3), 4.63 (3H, d, H-6), 3.77 (1H, d, H-OH), and 3.33 (3H, d, H-7). ^{13}C NMR (CD3OD-d6, 151 MHz): 156.79 (C-1), 130.87 (C-2), 116.12 (C-4), 64.60 (C-5), 51.82 (C-3), 51.21 (C-6), and 39.42 (C-7).
Noesy: 2D NOESY experiments were performed for CD3OD-d6. Inter-molecular cross peaks between H-4 and H-5, H-5 and H-OH, H-5 and H-6, and H-6 and H-7 appear in the contour plots of the 2D spectra, indicating that cross peaks between protons are separated from each other by less than 0.5 nm.
S2 ESI-MS: 137.0595 [M-H], calculated value for 138.07. ^{1}H NMR (CD3OD-d6, 600 MHz): 7.05 (1H,s,H-3), 7.03 (1H, s, H-3′), 6.72 (1H, s, H-2), 6.71 (1H, s, H-2′), 4.87 (1H, s, H-OH), 3.71 (2H, t,

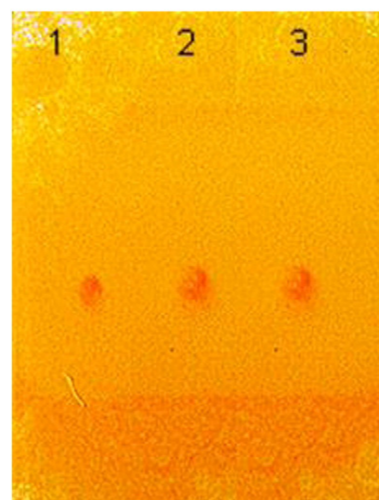

Fig. 11. Alkaloid TLC analysis. Lane 1, huperzine A; lane 2, crude extract from LQ2F02; and lane 3, crude extract from L10Q37.

Table 2
Anti-acetylcholinesterase activity analysis.

Component	Activity (IC$_{50}$)
LQ2F02 fermented liquid total alkaloid components	12.23 ± 1.32 μg/mL
LQ2F02 fermented liquid of non-alkaloid components	51.82 ± 1.81 μg/mL
L10Q37 fermented liquid total alkaloid components	39.47 ± 1.11 μg/mL
L10Q37 fermented liquid of non-alkaloid components	11.03 ± 1.42 μg/mL

H-6), 2.74 (2H, t, H-5), and 1.34 (1H, t, H-OH). ^{13}C NMR (CD3OD-d6, 151 MHz): 179.43 (C-1), 163.22 (C-2), 153.93 (C-2′), 130.87 (C-4), 116.12 (C-3), 110.88 (C-3′), 57.64 (C-6), and 39.42 (C-5).

S3 ESI-MS: 178.0531 [M + H], calculated value for 177.04. ^{1}H NMR (CD3OD-d6, 600 MHz): 7.05 (1H, s, H-3), 7.03 (1H, s, H-3′), 6.72 (1H, s, H-2), 6.71 (1H, s, H-2′), 4.87 (1H, s, H-OH), 3.71 (2H, t, H-6), 2.74 (2H, t, H-5), and 1.34 (1H, t, H-OH). ^{13}C NMR (CD3OD-d6, 151 MHz): 174.56 (C-1), 157.59 (C-2), 131.30 (C-2′), 126.33 (C-4), 116.26 (C-3), 52.36 (C-3′), and 40.89 (C-6).

Noesy: 2D NOESY experiments were performed for CD3OD-d6. Inter-molecular cross peaks between H-1 and H-2, H-1 and H-10, H-2 and H-3, H-2 and H-7, H-3 and H-4, H-4 and H- OH, H-5 and H-9, and H-10 and H-1 appear in the contour plots of the 2D spectra, indicating that cross peaks between protons are separated from one another by less than 0.5 nm.

S4 ESI-MS: 137.0599 [M-H], calculated value for 138.07. ^{1}H NMR (CD3OD-d6, 600 MHz): 6.15 (2H, s, H-2,5), 4.89 (2H, d, H-OH), 2.15 (3H, s, H-8), and 1.99 (3H, t, H-4). ^{13}C NMR (CD3OD-d6, 600 MHz): 157.09 (C-1, 6), 136.83 (C-3), 108.31 (C-2, 5, 7), 21.27 (C-4), and 8.19 (C-8).

S5 ESI-MS: 125.0630 [M + H], calculated value for 124.05. ^{1}H NMR (CD3OD-d6, 600 MHz): 6.86 (1H, s, H-3), 5.99 (1H, d, H-4), 3.84 (3H, s, H-6), and 2.05 (3H, s, H-7). ^{13}C NMR (CD3OD-d6, 151 MHz): 183.50 (C-1), 160.66 (C-5), 145.32 (C-2), 134.54 (C-3), 108.00 (C-4), 56.91 (C-6), and 15.33 (C-7).

S6 ESI-MS: 165.0547 [M + Na], calculated value for 142.06. ^{1}H NMR (CD3OD-d6, 600 MHz): 9.56 (1H, s, H-4), 7.40 (1H, d, H-5), 7.05 (1H, d, H-3), 4.63 (3H, d, H-6), 3.77 (1H, d, H-OH), and 3.33 (3H, d, H-7). ^{13}C NMR (CD3OD-d6, 151 MHz): 156.79 (C-1), 130.87 (C-2), 116.12 (C-4), 64.60 (C-5), 51.82 (C-3), 51.21 (C-6), and 39.42 (C-7).

S7 ESI-MS: 301.1160 [M-H], calculated value for 302.12. ^{1}H NMR (CD3OD-d6, 600 MHz): 6.08 (1H, s, H-15), 4.87 (2H, s, H-OH), 3.88 (3H, s, H-17), 2.13 (3H, sH-8, 10, 13), and 1.97 (1H, d, H-OH). ^{13}C NMR (CD3OD-d6, 151 MHz): 188.72 (C-5), 184.03 (C-1), 160.41 (C-16), 154.82 (C-11), 150.88 (C-14), 144.35 (C-4), 143.33 (C-2), 133.21 (C-7), 116.74 (C-9), 115.10 (C-6), 111.16 (C-12), 108.34 (C-15), 57.00 (C-17), 17.15 (C-8), 13.26 (C-3), 12.23 (C-10), and 9.36 (C-13).

S8 ESI-MS: 165.0513 [M + Na], calculated value for 302.12. ^{1}H NMR (CD3OD-d6, 600 MHz): 6.39 (1H, d, H-5), 4.39 (1H, t, H-4), 3.77 (3H, d, H-7), 2.62 (1H, sext, H-2), 2.24 (1H, multiplet, H-OH), and 1.22 (3H, q, H-3). ^{13}C NMR (CD3OD-d6, 151 MHz): 205.76 (C-1), 158.21 (C-6), 127.62 (C-5), 74.21 (C-4), 57.67 (C-7), 51.09 (C-2), and 13.26 (C-3).

S9 ESI-MS: 353.2625 [2 M + Na], calculated value for 166.07. ^{1}H NMR (CD3OD-d6, 600 MHz): 4.05 (2H, d, H-7), 3.33 (1H, d, H-8), 2.59 (2H, d, H-4), 2.59 (1H, q, H-5), and 1.32 (3H, d, H-3). ^{13}C NMR (CD3OD-d6, 151 MHz): 197.52 (C-1), 187.67 (C-6), 147.75 (C-2), 66.99 (C-8), 51.73 (C-7), 49.43 (C-5), 36.52 (C-3), and 18.06 (C-4).

S10 ESI-MS: 349.1817 [M + H], calculated value for 348.11. ^{1}H NMR (CD3OD-d6, 600 MHz): 5.31 (3H-5, s), 4.87 (1H-2, d), 3.95 (1H-11, s), 3.33 (1H-1-OH, s), 1.917 (1H-2-OH, d), 1.916 (1H-3-OH, s), 1.83 (2H-8, d), 1.46 (1H-10, s), 1.30 (1H-14, s), and 0.79 (3H-7, d). ^{13}C NMR (CD3OD-d6, 151 MHz): 216.61 (C-13), 181.60 (C-4), 173.09 (C-1), 108.11 (C-3), 106.06 (C-11), 89.57 (C-2), 85.51 (C-9), 77.07 (C-12), 60.68 (C-5), 43.47 (C-8), 36.72 (C-6), 24.26 (C-10), 17.18 (C-7), and 15.90 (C-14).

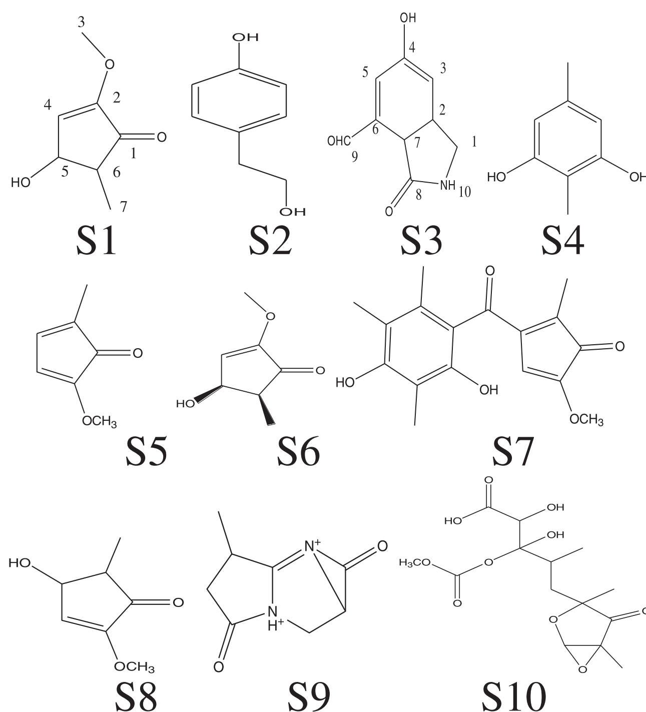

Fig. 12. Structure of all the compounds isolated from LQ2F02 and L10Q37.

The compounds obtained inhibited acetylcholinesterase activity are shown in Table 3.

This experiment isolated four compounds from the endophytic fungus L10Q37. We compared the compounds with those in the SCI-Finder database to confirm that compounds S1 and S3 were two new compounds. All six compounds isolated from LQ2F02 were previously unidentified. For anti-acetylcholinesterase activity, S5 is the best compound; however, there may be other components with anti-acetylcholinesterase activity. There may also be a variety of active ingredient combinations that interact to produce a greater anti-acetylcholinesterase activity.

4. Discussion

The excessive and the unmanaged use of some traditional Chinese medicinal resources, has led to environmental deterioration, with

Table 3
Anti-acetylcholinesterase activity.

Sample name	Activity (IC$_{50}$)
S1	29.35 ± 2.33 μg/mL
S2	16.66 ± 1.04 μg/mL
S3	32.18 ± 1.75 μg/mL
S4	34.42 ± 2.94 μg/mL
S5	5.23 ± 0.28 μg/mL
S6	16.68 ± 1.46 μg/mL
S7	15.14 ± 1.57 μg/mL
S8	16.68 ± 0.85 μg/mL
S9	43.26 ± 2.73 μg/mL
S10	20.71 ± 1.43 μg/mL
Huperzine A	0.213 ± 0.021 μg/mL

large vegetative areas destroyed and a decline in medicinal plant resources. To protect such resources, the ability to obtain effective endophytic fungal components from medicinal plants has become a major research focus. Until now, compounds with anti-acetylcholinesterase activity produced by the endophytic fungi of *H. serrata* were seldom reported. Su et al. [23] isolated 14 endophytic fungi from *H. serrata*, screened 8 strains that produced alkaloids, and identified 1 strain that was screened with the Xylariaceae fungus SY202 and could produce huperzine A under fermentation conditions. We first screened for alkaloids, and then we determined the activity and structures of the compounds. Li et al. [24] isolated two compounds from the *H. serrata* endophytic fungus G324-4. These compounds were identified as a nuclear plexus of penicillin and pencolide. The nuclear complex of penicillin had anti-acetylcholine activity, suggesting that some non-alkaloid compounds are also worth research attention. Ju and Wang [25] isolated two strains that produced huperzine A from *Phlegmariurus cryptomerianus* (Maxim.) Ching. This was the first report on the isolation of an endophytic fungus that could produce huperzine A from *P. cryptomerianus* (Maxim.) Ching. Dong et al. [26] isolated 42 strains of endophytic fungi from 9 species from Long Island. Among them, 24 strains had anti-acetylcholinesterase activity. This indicates that marine endophytic fungi are important resources for acetylcholinesterase inhibitors. Therefore, endophytic fungi are useful resources containing species with anti-acetylcholinesterase activity.

We isolated endophytic fungi from *H. serrata*, cultured them in liquid medium, separated the components with anti-acetylcholinesterase activities from the metabolites, and identified its active components. However, the concentrations of active components in endophytic fungal metabolites were very low, greatly reducing the usefulness of the strains. Future research will optimize fermentation conditions to improve strains growth and optimize metabolic regulation to increase the yields of active secondary metabolites. Only then can endophytic fungi truly become a source of medicinal compounds.

Conflict of interest

None.

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