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Full Length Research Paper

Antibacterial and antioxidant activities of the endophytic fungi from medicinal herb *Trillium tschonoskii*

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Fourteen distinct endophytic fungal isolates were first separated from the healthy underground parts of *Trillium tschonoskii*, which is a traditional medicinal herb mainly distributed in China. They were taxonomically identified by morphological traits and internal transcribed spacer (ITS) rRNA gene sequence analysis. Seven genera, namely, *Acremonium*, *Colletotrichum*, *Fusarium*, *Leptodontidium*, *Paraconiothyrium*, *Plectosphaerella*, and *Volutella*, were identified on the basis of their morphological characterizations. Their ITS-rDNA sequences were compared with those available in the GenBank database to obtain the closest related species by BLAST analysis. A modified thin layer chromatography-bioautography assay was used to detect the antibacterial activity of the *n*-butanol extracts of the mycelia and culture filtrates of the isolates. Most of the fungal isolates were screened to have antibacterial activity, and antibacterial components were examined to mainly exist in the filtrate except isolates Trtsf09 and Trtsf14. Five isolates, that is, Trtsf05, Trtsf06, Trtsf11, Trts12 and Trts13, were also screened to have antioxidant activity. The endophytic fungi isolated from *T. tschonoskii* could be an alternative source to produce antibacterial and antioxidant agents.

Key words: *Trillium tschonoskii*, endophytic fungi, *n*-butanol extract, antibacterial activity, antioxidant activity.

INTRODUCTION

Plant endophytic fungi are micro-organisms that reside in internal tissues of living plants without causing any immediate overt negative effects or external symptoms, but may turn pathogenic during host senescence (Tan and Zou, 2001; Rodriguez and Redman, 2008; Aly et al., 2011). They have been regarded as an important and novel resource of natural bioactive compounds with great potential applications in agriculture, medicine and food industry (Verma et al., 2009; Kharwar et al., 2011; Zhao et al., 2011). In the past two decades, many valuable bioactive compounds with antimicrobial, insecticidal, cytotoxic and anticancer activities have been successfully obtained from the endophytic fungi. These bioactive compounds could be mainly classified as alkaloids, terpenoids, steroids, quinones, isocoumarins, lignans, phenylpropanoids, phenols and lactones (Zhang et al., 2006; Aly et al., 2010; Yu et al., 2010; Zhou et al., 2010).

Trillium tschonoskii Maxim (Trilliaceae), a perennial herb mainly distributed in the Provinces of Tibet, Yunnan, Sichuan and Hubei of China, has been used as an important and traditional Chinese medicine (TCM) for activating blood, hemostasis, antihypertensive, analgesia, detumescence, rheumatism (Yu and Zou, 2008). Many metabolites such as steroid saponins, flavonoids, and sesquiterpenes have been found in this plant so far (Nakano et al., 1983; Ono et al., 1986; Yoshitama et al., 1992; Yu and Zou, 2008). To the best of our knowledge, there is no reported study on the endophytic fungi associated with *T. tschonoskii*. The objective of this study was to isolate and identify the endophytic fungi from the

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underground parts of *T. tschonoskii* as well as, to examine their antibacterial and antioxidant activities.

MATERIALS AND METHODS

Plant materials

The healthy underground parts (including rhizomes and roots) of three-year-old *Trillium tschonoskii* Maxim. (Trilliaceae) were collected from the Shennongjia forest district in Hubei Province of China in June 2009, and were authenticated by Prof. Jiaru Li of Wuhan University, where the voucher specimen of this plant was deposited. The plant samples were stored in the sealed plastic bags at 4°C until required.

Isolation and culture of the endophytic fungi

The underground parts were washed in running water first, then surface sterilized by soaking in 70% ethanol for 30 s, followed by immersing in 0.2% mercuric chloride for 20 min, and finally rinsed in sterile distilled water for three times (that is, 5 min for each time). After surface sterilization, the rhizomes or roots were eliminated epidermis and cut approximately into 5 × 5 × 5 mm cubes, which were placed in a 90 mm diameter Petri-dish containing potato dextrose agar (PDA) supplemented with streptomycin sulfate (500 mg/L) to suppress bacteria growth. After incubation in the dark at 25°C for about 1 month, the cubes were examined per iodically. When fungal colonies developed, they were transferred to new Petri-dishes with PDA; the fungi were isolated and subcultured to get pure cultures at last.

Morphological characterization

The morphological characters of the fungal isolates were observed and described according to the method of Photita et al. (2005). Morphological identification according to the standard taxonomic key included colony diameter, texture, color, the dimensions and morphology of hyphae and conidia (Ainsworth et al., 1973).

DNA extraction, ITS-rDNA amplification and sequence analysis

Total genomic DNA of the fungal isolates was prepared according to a modification of the rapid preparation of DNA from filamentous fungi (Raeder and Broda, 1985). Primers ITS1 (5'-TCCGTAGGTGAACCTGCGG -3') and ITS4 (5'-TCCTCCGCTTATTGATATGC -3'), as well as, ITS-rDNA amplification were referenced by our previous reports (Xu et al., 2008; Li et al., 2008). For identification, the PCR products were purified using the QIA quick Gel Extraction Kits (Qiagen, Hilden, Germany) and sequenced using the primer pair ITS1 and ITS4 on the ABI PRISM 3730 sequencer. Then the sequences were run by BLASTN program against the database (National Center for Biotechnology Information website: http://www.ncbi.nlm.nih.gov), and they were submitted to GenBank where the accession numbers were obtained.

Mycelia suspension culture and *n*-butanol extract preparation

A 1000 ml Erlenmeyer flask containing 300 ml of potato dextrose broth (PDB) was inoculated with 2 to 3 agar plugs containing mycelia taken from the cultures of 14 endophytic fungal isolates purified on PDA. All flasks were incubated on a rotary shaker at 150 rpm and 25°C for 15 days. After suspension culture, the culture broth was filtrated in vacuum to obtain the filtrate and mycelia. Each filtrate was extracted with an equal volume of *n*-butanol for three times to afford filtrate extract. The mycelia were lyophilized and powdered, then extracted with ultrasound in methanol for three times. The concentrated methanol extract was dissolved in water, and then extracted with an equal volume of *n*-butanol for three times to afford mycelia extract.

Detection of antibacterial activity of the extracts

Thin layer chromatography (TLC)-bioautography assay of the samples was carried out according to the method of Zhao et al. (2008). Four bacterial strains *Bacillus subtilis* ATCC 11562, *Escherichia coli* ATCC 25922, *Pseudomonas lachrymans* ATCC 11921, and *Xanthomonas vesicatoria* ATCC 11633 were selected for antibacterial assay. After the TLC plate covered with the test bacterium was incubated at 28°C for 12 h, the color re agent was sprayed with 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, purchased from Amresco, USA), and incubated for another 2 h. The presence of biological activity was determined by the formation of well defined inhibition zones made visible by spraying with MTT that was converted to a formazan dye by the living microorganism (Bernas and Dobrucki, 2000). Antibacterial activity was detected as white inhibition zones against a purple background, and the Rf value of each antibacterial area was determined (Xu et al., 2010). Rf = D_1/D_2, where D_1 is the distance between the antibacterial area and initial sample point, and D_2 is the distance between the developing solvent front and initial sample point on a TLC plate. All tests were performed in triplicate.

Detection of antioxidant activity of the extracts

Both radical scavenging and metal chelating assays were employed to examine antioxidant activity of the extracts. Radical scavenging assay was determined by a micro plate spectrophotometric method based on the reduction of a methanol solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) using the methods of Ono et al. (2008) and Wang et al. (2011). Tests were carried out in triplicate. Butylated hydroxytoluene (BHT) was used as the positive control. The median inhibitory concentration (IC_{50}) value was calculated using linear relation between the extract concentration and probability of the percentage of DPPH inhibition.

Metal chelating activity was determined according to our previous report (Wang et al., 2010). Ethylene diamine tetraacetic acid (EDTA) was used for the positive control. The IC_{50} value calculation for ferrozine-Fe^{2+} complex formation was the same as that for DPPH inhibition assay.

RESULTS AND DISCUSSION

Identification of the endophytic fungi

A total of 56 endophytic fungal isolates were separated from the rhizomes and roots of *Trillium tschonoskii*. According to their morphological features, 14 distinct fungal isolates were selected for further taxonomical identification. They were identical to the members of different genera, that is, *Acremonium*, *Colletotrichum*, *Fusarium*, *Leptodontidium*, *Paraconiothyrium*,

Table 1. Endophytic fungi and their closest relatives based on the data from BLAST analysis and morphological identification.

Fungal isolate	GenBank accession number	Closest related species	Similarity (%)	Macro- and microscopic identification
Trtsf01	GU479898	*Acremonium cellulolyticus*	98	*Acremonium* sp.
Trtsf02	GU479899	*Colletotrichum gloeosporioides*	100	*Colletotrichum* sp.
Trtsf03	GU479900	*Glionectria tenuis*	89	Unidentified
Trtsf04	GU479901	*Volutella ciliata*	99	*Volutella* sp.
Trtsf05	GU479902	Unidentified endopytic fungus 3211	100	Unidentified
Trtsf06	GU479903	*Colletotrichum truncatum*	99	*Colletotrichum* sp.
Trtsf07	GU479904	*Neonectria radicicola*	99	Unidentified
Trtsf08	GU479905	*Fusidium griseum*	90	Unidentified
Trtsf09	GU479906	Unidentified root associated fungus	99	Unidentified
Trtsf10	GU479907	*Paraconiothyrium sporulosum*	99	*Paraconiothyrium* sp.
Trtsf11	GU479908	*Plectosphaerella cucumerina*	99	*Plectosphaerella* sp.
Trtsf12	GU479909	*Fusarium oxysporum*	98	*Fusarium* sp.
Trtsf13	GU479910	*Leptodontidium orchidicola*	99	*Leptodontidium* sp.
Trtsf14	GU479911	*Hymenoscyphus* sp.	95	Unidentified

Plectosphaerella, and *Volutella* according to the results of the macro- and microscopic identification (Table 1), indicating the diversity of the fungi associated with *T. tschonoskii*.

The ITS1-5.8S-ITS4 partial sequences of 14 representative isolates were submitted to the GenBank to obtain their accession numbers, and the closest related species were got by BLAST analysis. The results showed that all the sequences had more than 89% similarity with the species in GenBank. The molecular characters of the endophytic fungi were basically coincident with their morphology (Table 1). Currently, some isolates (that is, Trtsf03, Trtsf05, Trtsf08, Trtsf09 and Trtsf14) remain unidentified because of their nonsporulating or low DNA similarity. It is possible that they are new fungal species which should be further verified. As the present molecular database is not large enough to contain all fungal rDNA sequence information, many fungi are difficult to identify especially the non-sporulating ones.

Detection of antibacterial activity

Antibacterial activities of the mycelia and filtrate extracts were shown in Table 2. Rf values of the antibacterial areas indicated the relative polarity of the active components in the samples, and the diameters can indicate the relatively antibacterial activity of the components. The extracts displayed their antibacterial activity except the filtrate extract of Trtsf14, the mycelia extracts of Trtsf02, Trtsf03, Trtsf04, Trtsf07, Trtsf08 and Trtsf11, and both filtrate and mycelia extracts of Trtsf09. The extracts of Trtsf05, Trtsf06, and Trtsf13 were found to exhibit stronger antibacterial activity than other extracts. Generally, the antibacterial activity of each filtrate extract was stronger than that of its mycelia extract except Trtsf14.

Antioxidant activity

The extracts of the fungal isolates were subjected to a screening for the antioxidant activity by two complementary tests, namely, DPPH free radical scavenging and ferrozine-Fe^{2+} complex formation systems. The extracts of five endophytic fungal isolates (that is, Trtsf05, Trtsf06, Trtsf11, Trtsf12 and Trtsf13) were selected for further examining their IC_{50} values on the basis of primary screening on all extracts for the antioxidant activity. The antioxidant activity results of the extracts were shown in Table 3. All the selected extracts showed stronger ferrozine-Fe^{2+} complex formation inhibitory activity by comparing with the results from the DPPH inhibition assay.

In summary, we first reported the isolated fungi from the medicinal plant *T. tschonoskii*, and the detection of the antibacterial and antioxidant activities of their extracts, some of which were found to have strong antibacterial and antioxidant activities. The endophytic fungi isolated from *T. tschonoskii* could be an alternative source to pro-duce antibacterial and antioxidant agents. Further studies to isolate the antibacterial and antioxidant compounds from these fungi as well as to clarify the physiological and ecological roles of these fungi in their host *T. tschonoskii* are now in progress.

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Table 2. Antibacterial activity of the crude *n*-butanol extracts from the endophytic fungi against different bacteria by TLC-bioautography-MTT test.

Fungal isolate	M/F	Rf value of the antibacterial area (Diameter of the antibacterial area)			
		Bacillus subtilis	***Escherichia coli***	***Pseudomonas lachrymans***	***Xanthomonas vesicatoria***
Trtsf01	M	0.15-0.40(++)	0.33-0.43(++)	0.25-0.60(++)	0.27-0.58(++)
	F	0-0.12(++)	0-0.08(++)	0-0.2(++)	0-0.2(++)
Trtsf02	M	-	-	-	-
	F	0.33-0.41(++)	0.33-0.41(++)	0.33-0.41(++)	0(+),0.30-0.40(++)
Trtsf03	M	-	-	-	-
	F	0-0.22(++), 0.42-0.82(++)	0-0.2(+++), 0.55-0.80(++)	0-0.2(+++), 0.55-0.80(++)	0-0.2(+++), 0.55-0.80(++)
Trtsf04	M	-	-	-	-
	F	0-0.1(+)	0-0.17(++)	0-0.1(+)	0-0.1(+)
Trtsf05	M	0.33-0.60(+)	0.30-0.55(++)	0.33-0.65(++)	0.33-0.67(++)
	F	0-0.65(++)	0-0.65(+++)	0-0.70(+++)	0-0.90(+++)
Trtsf06	M	0-0.67(+++)	0-0.67(+++)	0-0.75(+++)	0-0.75(+++)
	F	0.40-0.65(++)	0.33-0.65(++)	0-0.80(++)	0-0.80(++)
Trtsf07	M	-	-	-	-
	F	0-0.60(++)	0-0.60(++)	0-0.60(++)	0-0.60(++)
Trtsf08	M	-	-	-	-
	F	0.45(+)	0.50-0.60(++)	0.50-0.67(++)	0.50-0.63(++)
Trtsf09	M	-	-	-	-
	F	-	-	-	-
Trtsf10	M	0.17-0.45(++)	-	0.30-0.40(+)	0.33-0.40(++)
	F	0.30-0.50(++)	0.30-0.50(++)	0.30-0.50(++)	0.30-0.50(++)
Trtsf11	M	-	-	-	-
	F	0.50-0.60(+)	0.50-0.60(+)	0.50-0.60(+)	0.50-0.60(+)
Trtsf12	M	0.50-0.60(+)	-	0.50-0.60(+)	-
	F	0.10-0.75(++)	0.10-0.75(++)	0.10-0.75(++)	0.10-0.75(++)
Trtsf13	M	0-0.60(+++)	0-0.60(+++)	0-0.90(+++)	0-0.90(+++)
	F	0-0.60(+++)	0-0.80(+++)	0-0.95(+++)	0-0.60(+++)
Trtsf14	M	0.20-0.37(+)	0.20-0.37(+)	0.20-0.37(+)	0.20-0.37(+)
	F	-	-	-	-

M, mycelia *n*-butanol extract; F, filtrate *n*-butanol extract; Developing solvent system in TLC was chloroform-methanol (10:1, v/v); -, no antibacterial activity was observed; +, the diameter of the antibacterial activity area was 0-5 mm; ++, the diameter of the antibacterial activity area was 5-10 mm; +++, the diameter of the antibacterial activity area was more than 10 mm; The positive control was streptomycin sulfate which was only sampled on the TLC plate and showed antibacterial activity.

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Table 3. Antioxidant activity of the crude *n*-butanol extracts from the selected endophytic fungal isolates.

Sample	DPPH inhibition IC_{50} (µg/ml)	Ferrozine-Fe^{2+} complex formation IC_{50} (µg/ml)
Trtsf05 mycelia *n*-butanol extract	4.52 ± 0.06	0.64 ± 0.05
Trtsf05 filtrate *n*-butanol extract	2.14 ± 0.02	0.34 ± 0.06
Trtsf06 mycelia *n*-butanol extract	2.25 ± 0.05	0.56 ± 0.01
Trtsf06 filtrate *n*-butanol extract	2.86 ± 0.07	0.77 ± 0.02
Trtsf11 mycelia *n*-butanol extract	4.48 ± 0.01	0.72 ± 0.02
Trtsf11 filtrate *n*-butanol extract	2.37 ± 0.10	0.36 ± 0.01
Trtsf12 mycelia *n*-butanol extract	2.58 ± 0.04	0.79 ± 0.03
Trtsf12 filtrate *n*-butanol extract	2.13 ± 0.02	0.50 ± 0.05
Trtsf13 mycelia *n*-butanol extract	2.02 ± 0.04	0.31 ± 0.01
Trtsf13 filtrate *n*-butanol extract	3.34 ± 0.01	0.22 ± 0.01
Positive control	0.026 ± 0.00	0.018 ± 0.00

The positive controls for DPPH inhibition and ferrous ions assays were BHT and EDTA, respectively. The data were expressed as mean ± standard deviation for triplicate experiments. -, no antioxidant activity was observed.

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