ISOLATION OF ENDOPHYTIC FUNGI FROM *THERMOPSIS LANCEOLATA* AND THEIR ANTIOXIDANT ACTIVITY

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ABSTRACT

Introduction*: Plant endophytic fungi resources is nearly a decade of research hot spots, endogenous fungal resources development and utilization will be fruitful.*

Aim*: Aim of this study is isolation of endophytic fungi strains from healthy tissues of Thermopsis lanceolata and evaluation of antioxidant activity of fermented extracts of these isolated endophytic fungi strains.*

Materials and methods*: Root, stem and leaf of Thermopsis lanceolata were used to isolate endophytic fungi strains. Reducing power and DPPH free radical scavenging assays were used to evaluate the antioxidant activity of fermented extracts from isolated endophytic fungi strains from Thermopsis lanceolata. Meanwhile, Total phenolic content (TPC) of fungal extracts was tested by Folin-Ciocalteau reagent based assay.*

Results*: 16 endophytic fungi strains were isolated by tissue culture from healthy root, stem and leaf of Thermopsis lanceolata; Results of antioxidant activity evaluate indicates that there are 4 samples showed a good reduction force, especially ethyl acetate extract of Tlc-R-7 has the highest reducing power, it's absorbance value reached 0.528 (ascorbic acid, 0.588). Moreover, ethyl acetate extracts had generally higher antioxidant activity than extracts of n-butanol and methanol; Result of DPPH free radical scavenging activity showed there are 3 extracts samples showed inhibition rate of more than 90%. 2 extracts showed inhibition rate of more than 85%, among them, Tlc-R-7 ethyl acetate extract has the highest inhibition rate of 93%; The results indicate that the higher the phenol content in the sample, the stronger the antioxidant capacity of the sample; Most of the higher activity extracts of the fungal was concentrated in the roots.*

Conclusion*: There are abundant endophytic fungi resources in Thermopsis lanceolata, and the endophytic fermentation product has a good prospect of antioxidant drug development.*

Keywords*: Endophytic fungi, Thermopsis lanceolata, Antioxidant activity.*

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Introduction

Thermopsis lanceolata is mainly grown in the northwest of China. It is a kind of plant which is widely distributed in the desert region and grassland[(1)]. This plant is often used for eliminating phlegm and relieving cough.

Endophytic fungi are a kind of microorganism. It exists in the living tissues of various plants, but does not cause any symptoms of the disease[(2)]. Studies have shown that some endophytic fungi can produce special secondary metabolites to protect the host against pathogenic bacteria, insects and herbivores damage[(3)]. At the same time it can produce some bioactive compounds which have the research value[(2,4-5)].

Free radicals are reactive oxygen and nitrogen through a variety of physiological processes in the human body. It is not controlled by the production. The result is that the free radicals attack lipid mem-

branes, proteins, enzymes and DNA leading to oxidative stress and cell death[6]. Reactive oxygen species (ROS) play an important role in many human diseases such as diabetes, cancer, neurodegenerative diseases, Alzheimer's disease, Parkinson's disease, atherosclerosis, aging and inflammation[7]. Antioxidants are a stable molecule that can provide an electron to an active free radical, and terminate the chain reaction before the important molecules are damaged. Elimination of free radicals can delay or inhibit cellular damage[8].

At present, there are many studies focus on the antibacterial, anticancer, insecticidal and antidiabetic activity of endophytes, but there are few studies on the antioxidant activity.

Materials and methods

Isolation of endophytic fungi

Healthy plant materials were collected from the Gannan Tibetan Autonomous Prefecture of China in 2014. Plant tissues (root, stem and leaf) were rinsed completely in running tap water, followed by immersion in 0.1% mercuric chloride for 30s, in 75% ethanol for 3min and rinsed three times in sterilized distilled water. And then the samples were cut into 0.5cm (roots), 0.5cm (stems) and1cm2 (leaves) pieces and placed on Petri dishes containing potato dextrose agar supplemented with 100U/mL streptomycin to suppress bacterial growth. Plates were incubated at 28°C until the mycelium appeared. The growing tips of mycelia were transferred to new PDA plates for pure culture. All fungi were identified by morphology.

Preparation of extracts

The endophytes were transferred to potato dextrose broth media for 15d. All cultures were incubated at 28±1°C on a rotary shaker at 160 rpm. The fermentation broth of each endophyte was filtered by Whatman No1 filter paper. The endophytes mycelium were filtered and dried. The fermentation broth was extracted by equal volume of ethyl acetate and n-butanol 3 times in proper order. The dried mycelium powders were extracted with methanol (1:10, M/V) for 3 times by using cold percolation, each time 1 h. The organic solvent was evaporated and obtained the crude extract.

Antioxidant assays

In this study, Reducing power and DPPH free radical scavenging assays were used to determine the antioxidant potential of endophytic fungal extracts. The ascorbic acid was used as the positive control. All experiments were done in triplicate.

Reducing power assay

The extracts were dissolved in distilled water. The sample and standard (0.1mg/mL, 2.5mL) were mixed with phosphate buffer (2.5mL, 0.2mol/L, pH6.6) and potassium ferricyanide (2.5mL, 1% w/v). Mixture was placed in water bath at 50°C for 20min and in cold water for quick chilling. Then the solution was centrifuged for 10min at 4000r/min. The upper layer of solution (2.5mL) was mixed with trichloroacetic acid (2.5mL, 10%w/v) and ferric chloride (1mL, 0.1%w/v). The value measured at 700nm using spectrophotometer after 10 minutes. The higher the absorbance value, the better the reducing power.

DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging assay

DPPH is a stable free radical which appears purple color in ethanol solution. When the reducing agent was present, the DPPH can be reduced to a yellow substance, diphenyl picryl hydrazine. The extracts were dissolved in anhydrous ethanol. The sample and standard (0.1mg/mL, 2mL) were added to react with DPPH reagent (0.05mg/mL, 2mL) for 30min at room temperature in the dark condition. The value measured at 517nm using spectrophotometer. The percentage of the DPPH free radical scavenging was calculated as follows:

$$\text{Inhibition}(\%)=(A_i-A_t)/A_i\times 100\%$$

A_i is the absorbance of the DPPH free radical, A_t is the absorbance of DPPH free radical+ sample/standard.

Total phenolic content assay

The total phenolic content of the extract was determined according to the method of Halliwel[9]. The extracts were dissolved in methanol (1mg/mL) and Folin-Ciocalteau reagent (500μL, 50%). Then the mixture was added with Na2CO3 (1.5mL, 20%). After 3min the final volume was reached to 5mL by adding distilled water. The reaction solution was incubated at 160rpm on the rotary shaker at room temperature for 30min. Measure the value at 765nm using UV-vis spectrophotometer. Total phenolic content value was expressed as gallic acid equivalent (mg/g) using the regression equation:

y=6.74x+0.0322, R2=0.9971. From 20μg/mL to100μg/mL gallic acid solutions used as standard for calibration curve.

Results

Isolation and identification

16 strains of endophytic fungi were isolated from the root[9], stem[5] and leaf[2] of *Thermopsis lanceolata*. All fungi were identified by colony, morphology and spore characteristics. The results showed that 16 fungi strains belonged to Genus Alternaria, Aspergillus, Cladospora and Fusarium. The experimental data is shown in table 1.

Name of genus	Fungi number		
	Root	Stem	Leaf
Alternaria	5	3	2
Aspergillus	2	2	0
Chaetomium	1	0	0
Fusarium	1	0	0
Total(percentage)	9(56.3%)	5(31.2%)	2(12.5%)

Table 1: endophytic fungi strains isolated from different organ of *Thermopsis lanceolata.*

Antioxidant activity

Reducing power assay showed there are 4 ethyl acetate extracts shows greater reducing power of absorbance value more than 0.1. In particular Tlc-R-7, showed the highest absorbance value of 0.528 which close to ascorbic acid (0.588). The absorption values of Tlc-R-6, Tlc-R-2 and Tlc-R-4 were 0.486, 0.366 and 0.149, respectively. Extracts of n-butanol and methanol has 2 and 1samples respectively which absorbance value more than 0.1. Ethyl acetate extracts had generally higher reducing power than extracts of n-butanol and methanol. On the other hand, extracts which had the better potential were more concentrated in the roots. The experimental data are shown in table 2.

DPPH free radical scavenging experiments showed that 3 ethyl acetate extracts had a clearance rate of more than 90%, 2 extracts had a clearance rate of more than 85%. Tlc-R-7 ethyl acetate extract has the highest clearance rate of 93%. The clearance rate of Tlc-R-6 and Tlc-R-2 were 92% and 91%. Ethyl acetate extracts of Tlc-R-3 and Tlc-R-4 had a clearance rate of more than 85%, other 2 extracts has remarkable clearance rate are Tlc-R-4 methanol(> 80%) and Tlc-R-2 n-butanol(> 75%). The experimental data is shown in Figure 1.

Source organ	Strain number	Absorbance value(0.1mg/mL)		
		Ethyl acetate	n-butanol	methanol
Root	Tlc-R-1	0.033±0.005	0.012±0.004	0.003±0.002
	Tlc-R-2	0.366±0.007	0.131±0.003	0.168±0.006
	Tlc-R-3	0.078±0.007	0.033±0.005	0.015±0.003
	Tlc-R-4	0.149±0.007	0.017±0.007	0.010±0.002
	Tlc-R-5	0.040±0.008	0.016±0.006	0.008±0.001
	Tlc-R-6	0.486±0.005	0.121±0.009	0.067±0.004
	Tlc-R-7	0.528±0.008	0.070±0.013	0.022±0.011
	Tlc-R-8	0.006±0.002	0.035±0.006	0.018±0.003
Stem	Tlc-S-1	0.037±0.005	0.043±0.005	0.014±0.002
	Tlc-S-2	0.030±0.004	0.024±0.005	0.016±0.006
	Tlc-S-3	0.054±0.007	0.036±0.005	0.028±0.006
	Tlc-S-4	0.048±0.005	0.027±0.006	0.017±0.003
	Tlc-S-5	0.038±0.004	0.025±0.002	0.012±0.004
Leaf	Tlc-L-1	0.079±0.004	0.040±0.007	0.038±0.006
	Tlc-L-2	0.024±0.003	0.036±0.004	0.007±0.003
Ascorbic acid		0.588±0.012		

Table 2: The absorbance value of samples and Ascorbic acid in reducing power assay.

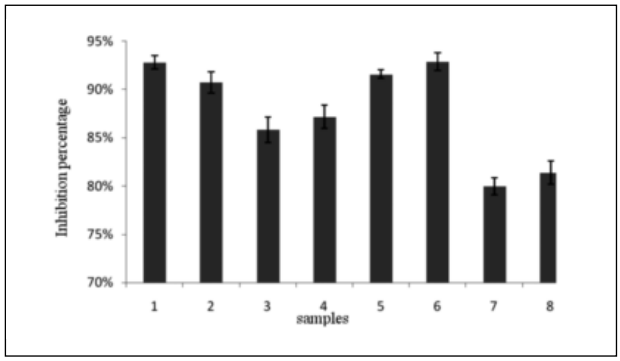

Fig. 1: DPPH free radical scavenging activity of samples and ascorbic acid.

Samples: 1: ascorbic acid, 2: Tlc-R-2 ethyl acetate, 3: Tlc-R-3 ethyl acetate, 4: Tlc-R-4 ethyl acetate,
5: Tlc-R-6 ethyl acetate, 6:Tlc-R-7 ethyl acetate, 7: Tlc-R-2 n-butanol, 8: Tlc-R-4 methanol

The screening result of DPPH free radical scavenging activity showed that a total of 7 extract clearance rates of more than 75%, of which 3 more than 90%, 2 more than 85%, 1 more than 80%, 1 more than 75%. In this study, we further determined the EC50 value of the 3 fungal which clearance rates more than 90%. The results show that EC50 values of Tlc-R-7 ethyl acetate extract DPPH radical scavenging assay is 3.27mg/mL, most closing to that of the ascorbic acid (2.76mg/mL). The EC50 values of the ethyl acetate extracts of Tlc-R-2 and Tlc-R-6 were 5.97 and 6.42 mg/mL, respectively, which were weaker than those of Tlc-R-7 by contrast. In summary, the values indicated that ethyl acetate extracts of three endophytic fungi had greater potential in DPPH free radical scavenging activity. The experimental data is shown in Table 3.

	DPPH free radical scavenging activity
	EC50 value(mg extract/mL)
Tlc-R-2 ethyl acetate	5.97±0.013
Tlc-R-6 ethyl acetate	6.42±0.011
Tlc-R-7 ethyl acetate	3.27±0.023
Ascorbic acid	2.76±0.016

Table 3: EC50 value of DPPH free radical scavenging assay of 3 extracts which have the best activity.

Total phenolic content

The total phenolic content of extracts from 4 fungi strains which showed higher effectiveness in two antioxidant assays (Table 4) shows that ethyl acetate extracts were higher than that of n-butanol. The highest content of total phenolic was found in ethyl acetate extract from Tlc-R-7 (70.23 mg/g), followed by Tlc-R-6 (62.16 mg/g), Tlc-R-2(58.22 mg/g) and Tlc-R-4 (35.87 mg/g).

Strains	Total phenolic content (mg/g)	
	ethyl acetate	n-butanol
Tlc-R-2	58.22±0.15	23.68±0.32
Tlc-R-4	35.87±0.11	11.34±0.25
Tlc-R-6	62.16±0.18	25.75±0.20
Tlc-R-7	70.23±0.25	4.34±0.20

Table 4: Total phenolic content of extracts from 4 fungi strains isolated from *T. lanceolata.*

Discussion

The endophytic fungi widely distributed in higher plants[10-12]. They often produce secondary metabolites similar to their host plants, some of which can produce novel active compounds[13-14]. As a result, some of the metabolites of endophytes showed antioxidant, antibacterial, antitumor and antiinflammatory activity[15]. The study on endophytes had received more and more attention in the last decades. *Thermopsis lanceolata*, a plant that grows in the northwest of China. So far, the research focused on the separation of chemical components, the promotion of plant growth and the pharmacological activity of chemical constituents[16-17]. However, endophytic fungi and their antioxidant activity were still not involved.

Antioxidants in addition to inhibit protein, DNA and cell membrane damage, also were reported to have antiinflammatory, antiatherosclerosis, antitumor, antimutation, antibacterial and antiviral activity to a more or less extent[18-20]. In this study, we found that the greater antioxidant potential was concentrated in the roots of *Thermopsis lanceolata*.

Furthermore, ethyl acetate extracts activities were better than that of n-butanol and methanol. The experimental results of antioxidant activity of current research shows, Tlc-R-7 had showed the highest activity (0.528, 93%), followed by Tlc-R-6 (0.486, 92%) and Tlc-R-2 (0.366, 91%) in sequence. Thus it was consistent with previous reports, that Chaetomium sp. isolated from *Eugenia jambolana* Lam. showed 80% antioxidant capacity[6] and antioxidant activity of Alternaria sp. isolated from *Tabebuia argentea* was 90%[21].

Meanwhile the scavenging activity of Aspergillus sp. isolated from *Rhodiola crenulata* reached a high phenolic content (24.75±0.002) mg/GAE[22]. In the determination of total phenolic content, four fungal with good antioxidant activity also showed high concentrations of phenolic content. Tlc-R-7 (Chaetomium sp.) ethyl acetate sample reached 70.23mg/g. Tlc-R-6 (Alternaria sp.), Tlc-R-2 (Aspergillus sp.) and Tlc-R-4 (Alternaria sp.) ethyl acetate sample reached 62.16 mg/g, 58.22 mg/g and 35.87 mg/g, respectively[23-25].

The results showed that the antioxidant activity was positively correlated with total phenolic content. So, phenolic compounds play an important role in the antioxidant activity of extracts which fermented from endophytic fungi of *Thermopsis lanceolata*.

Conclusion

The results of this study show that *Thermopsis lanceolata* is rich in endophytic fungi. Fungi fermentation products have good antioxidant activity, and their antioxidant activity is positively correlated with the content of phenolic compounds. It suggesting that its endophytic fungi resources and phenolic compounds therein have a good research value.

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