

## Antioxidant activity of secondary metabolites and mycelium extracts of endophytic fungi isolated from *Astragalus monadelphus*

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13 strains of endophytic fungi were isolated from *Astragalus monadelphus*. The radical scavenging activity assay of secondary metabolites and mycelium extracts from these strains were investigated. Results showed that secondary metabolites and mycelium extracts from these strains had significant antioxidant activities. Reducing ability of H401 strain mycelium extracts were stronger than the ethyl acetate extract parts, and its EC<sub>50</sub> value was 0.0034 mg/mL. Ethyl acetate parts of H802 strain exhibited strong ·OH scavenging effect, the IC<sub>50</sub> value was 0.0384 mg/mL. the DPPH radical scavenging activity test indicated that endophytic fungi can be a good source of radical scavenger. Besides, the compounds extracted from H401 secondary metabolites belong to β-sitosterol based on the results of NMR analysis and literature data.

**Key words:** Endophytic fungi, Secondary metabolites, Antioxidant activity

### INTRODUCTION

*Astragalus monadelphus* belongs to phylum of Angiospermae, class of Magnoliopsida, order of Fabales, family of Leguminosae sp., genus of *Astragalus*. It grows at a high altitude of 3000-4000 meters in central and southwest of Gansu Province, southeast of Qinghai Province, northwest of Sichuan Province.

Most of researches on the genus *Astragalus* previously focused on the identification of its main chemical compositions. With the development of biology and molecular biological techniques, more and more compounds have been separated. Later, secondary metabolites of genus *Astragalus* callus were studied by some scholars. However, the genus *Astragalus* endophytic fungi was few reported, and the relative researches mainly focused on determination of antimicrobial activities of endophytic fungi. Zhou et al. [1] isolated *Aspergillus fumigatus* from the root of *Astragalus membranaceus*<sup>1</sup>, and the ethyl acetate extract of the culture exhibited significant antimicrobial activity. Ma et al. isolated four strains of endophytic fungi (strains 16, 17, 23 and 75) from *A. Mongholicus* [2], but the fourth isolated endophytic fungi did not produce astragalosides I-IV, flavonoids or polysaccharides. Due to rich contents of active constituents such as glucuronic acid, β-sitosterol, astragalosides, isoflavone and asparagines, *Astragalus membranaceus* is recognized one of the

most important herbs [3].

Endophytic fungi have been known to be a rich repository of medicinally important compounds since the discovery of penicillin. Some endophytic fungi could produce bioactive compounds the same as their hosts that exemplified by taxol [4], subglutinol A and B [5], and peptide leucinostatin A [6]. By now, few study reported that endophytic fungi isolated from *Astragalus monadelphus* and their secondary metabolites. In this work, isolation, identification and determination of the activities of the endophytic fungi from *Astragalus monadelphus* were investigated.

### EXPERIMENTAL

#### Materials

Plants sample were collected in September 2012 from Longxi County, Gansu Province, China, identified by Yanlin Professor, Lanzhou University of technology, and belonged to *Astragalus Monadelphus*.

#### Methods

**Isolation of endophytic fungi:** The endophytic fungi were isolated as described by Fernandes et al.<sup>7</sup>. The growing mycelia were purified on PDA plates and then maintained at 4 °C on PDA slopes for further study.

**Fermentation** For coarse screening, the fermentation of endophytic fungi was conducted at 28 °C for two weeks. Then, the culture broth was centrifuged at 12000 rpm for 10 min. Mycelia were broken down by ultrasound, dried at 50°C, powdered. And triple volumes of anhydrous ethanol

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was added in, then the system was heated reflux extraction for twice, each time 2 h. The fermentation was successively extracted with the same volume of ethyl acetate and n-butanol for three times, respectively. The above ethyl acetate, n-butanol and ethanol solutions were concentrated in rotary evaporation at 40°C, 65°C, and 55°C to obtain fermentation and mycelia extracts, respectively.

#### Antioxidant Activity

**The total reducing ability assay** Total reducing ability was determined according to Scherer's method [8]. 2.5 mL 0.2 mol/L (pH 6.6) phosphate buffer and 2.5 mL 1% potassium ferricyanide solution were in turn added into 2.5 mL different concentrations of samples. The mixed solution was kept at 50 °C for 20 min, and then cooled rapidly. Then 2.5 mL the supernatant, 2.5 mL distilled water and 0.5 mL 0.1% ferric chloride solution were respectively added into 2.5 mL 10% trichloroacetic acid solution, shook well and stewed for 10 min. The absorbance at 700 nm was measured, with ascorbic acid being the positive control.

**Hydroxyl radical scavenging activity assay** Hydroxyl radical scavenging activity was measured by the Scherer's method [9]. The reaction mixture containing 1 mL FeSO<sub>4</sub> (6 mM), 1 mL salicylic acid-ethanol (6 mM), 1 mL H<sub>2</sub>O<sub>2</sub> (0.1%), were mixed with 1 mL various concentrations samples into 10 mL final reaction volume and incubated for 1 h at 37 °C. The absorbance of the mixture was measured at 510 nm. Ascorbic acid was taken as the positive control. Scavenging rate (SR) was calculated as the formula (1):

$$SR(\%) = \left\{ 1 - \frac{A_{\text{sample}} - A_{\text{control}}}{A_{\text{blank}}} \right\} \times 100\% \quad (1)$$

A<sub>sample</sub> - the absorbance of adding ferrous sulphate, acid - ethanol absorbance, hydrogen peroxide, and the same volume of the sample

A<sub>control</sub> - the absorbance of adding ferrous sulphate, acid - ethanol absorbance, and the same volume of the sample, without hydrogen peroxide

A<sub>blank</sub> - blank solution absorbance values, i.e., the absorbance of only adding to join ferrous sulfate, salicylic acid - ethanol, hydrogen peroxide, without the sample

**1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assay** DPPH radical scavenging activity was assessed according to Ashley et al. [12], and Kimura et al. [13]. And the tested samples of n-butanol extracts from fermentation were added into 2 mL samples with various concentrations, and were mixed in 2 mL freshly prepared 0.025 mg/mL

DPPH ethanolic solution. The mixture were kept at room temperature for 30 min and measured calorimetrically at 517 nm. The control was established by ethanol instead of samples. The DPPH radical scavenging activity of samples was calculated based on the following equation (2):

$$DPPH \text{ scavenging effect } (\%) = \left\{ 1 - \frac{A_{\text{sample}} - A_{\text{control}}}{A_{\text{blank}}} \right\} \times 100\% \quad (2)$$

Where A<sub>control</sub>, A<sub>sample</sub> and A<sub>blank</sub> denoted the absorbance of the control, the sample and DPPH without samples, respectively

**Fermentation, Isolation and structure determination** Secondary metabolites of fungus had a good antioxidant activity. Thin-layer chromatography analysis showed that ethyl acetate parts of H401 contained rich chemical compositions. The fungus was cultured in a 25-liter fermentation system, and selected for further study. Purification was carried out by column chromatography. The structures of the compounds were elucidated primarily by NMR analysis.

## RESULTS AND DISCUSSION

**Isolation of endophytic fungi** 13 strains of endophytic fungi were isolated from *Astragalus monadelphus* in this study. Colony morphologies of some typical strains were shown in Figure 1. And they were all stored in 20% glycerol at -80 °C.

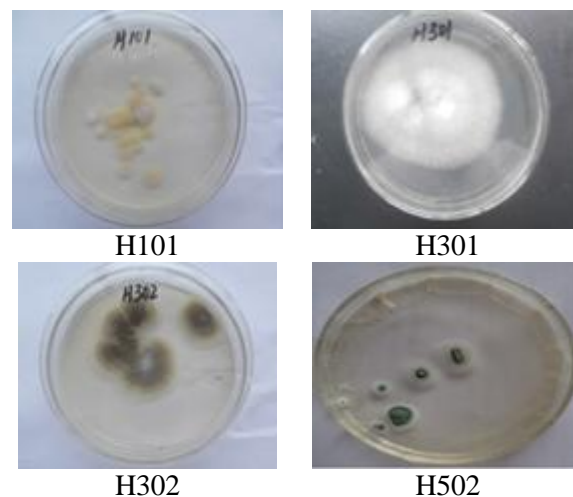


Fig. 1. Colony morphology of some strains.

#### Antioxidant Activity

**The total reducing ability assay** The reducing abilities of the samples were determined according to potassium ferricyanide reduction method. The results (Figure 2a) showed that 39 samples (0.05 mg/mL) have different reducing ability. Two parts of extracts of the sample H401 have strong reducing ability with the absorbance values of

2.555 and 2.565, respectively, which were slightly lower than that of the positive control (Vc, 2.636). On the other hand, it could be seen from Figure 2b that the reducing ability enhanced with the increasing of the sample's concentration. According to half-maximum effective concentration ( $EC_{50}$ ), reducing ability of H401 mycelium were stronger than the ethyl acetate extract parts, and its  $EC_{50}$  value was 0.0034 mg/mL ( $y=134.73x+0.0361$ ,  $R^2=0.9943$ ), but the value was still somewhat weak when compared with the positive control Vc ( $EC_{50}$  value is 0.0019 mg/mL,  $y=233.27x+0.0665$ ,  $R^2=0.9975$ ).

**Hydroxyl radical scavenging activity assay** In this study, spectrophotometric assay was introduced to estimate on  $\cdot OH$  scavenging activities of the samples. The results (Figure 3a) illustrated that the scavenging effects on  $\cdot OH$  of 39 samples (0.25 mg/mL) varied, i.e., the scavenging ratios of 3 samples were between 70% and 80%, as to the ethyl acetate extract parts of H401 and H802, and mycelium part of H301, the scavenging ratios of 4 samples were noticed more than 80%, in addition, the scavenging ratios of the mycelium parts of H302, H601, H802 and H803 were 89.93% which is higher than the positive control Vc (89.75%).

The results (Figures 3b, 3c) implied that within the range of 0.02~0.1 mg/mL, the scavenging abilities of  $\cdot OH$  of the tested materials all enhanced with the increasing concentration of the samples.

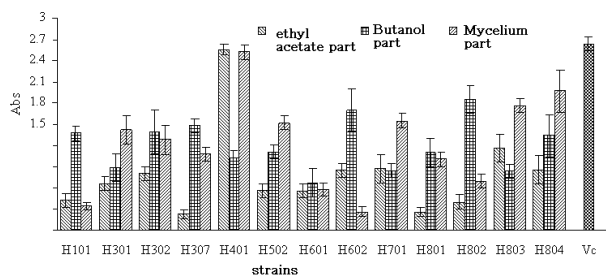


Fig. 2a. The reduction abilities of the samples

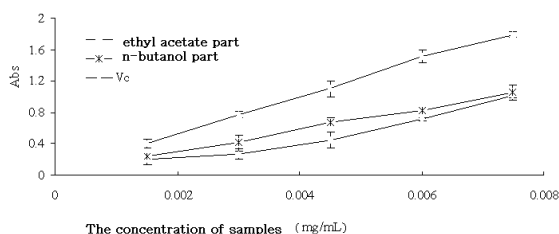


Fig. 2b. The results of concentration gradient experiment.

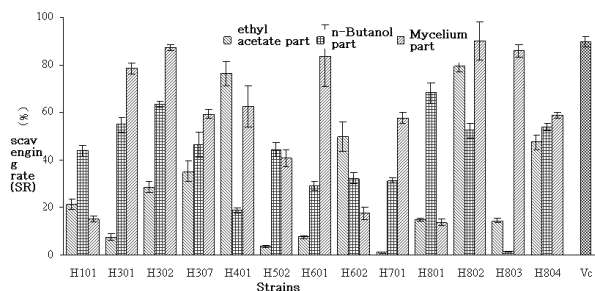


Fig. 3a. The  $\cdot OH$  scavenging ratios of the samples

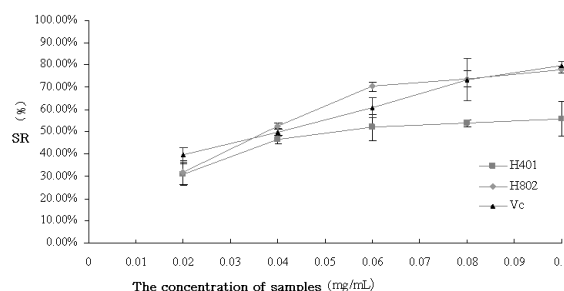


Fig. 3b. The  $\cdot OH$  scavenging ratios of H401 and H802 (Ethyl acetate extract parts)

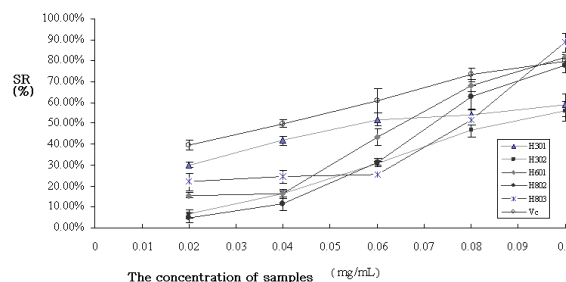


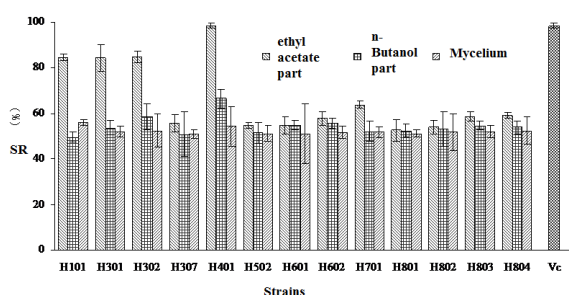
Fig. 3c. The  $\cdot OH$  scavenging ratios of H301, H302, H601, H802 and H803 (mycelium parts)

The results shown in Table 1 showed that ethyl acetate extract parts of H802 has relative strong  $\cdot OH$  scavenging effect ( $IC_{50}$  value was 0.0384 mg/mL), which was slightly stronger than the positive control Vc ( $IC_{50}$  value was 0.0395 mg/mL). While the  $\cdot OH$  scavenging abilities of other samples were not obvious.

**1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assay** The results displayed in Figure 4a that DPPH $\cdot$  scavenging abilities of 39 samples (0.025 mg/mL) were all over 50%, in detail, DPPH $\cdot$  scavenging ratio of ethyl acetate extract of 13 samples were higher than the others, especially, DPPH $\cdot$  scavenging ratio of ethyl acetate parts of H101, H301, H302 and H401 were more than 80%. Among these four samples, the most predominant one was H401 for its scavenging ratio reached 98.52%, which was a little higher than the positive control Vc (98.4%).

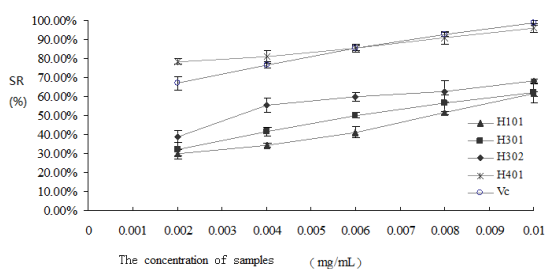
**Table 1.** IC<sub>50</sub> values and linear relationship of the samples

Samples		Concentration range (mg/mL)	linear relationship	IC <sub>50</sub> (mg/mL)
ethyl acetate extract	H401	0.02~0.06	$y=5.325x+0.2183(R^2=0.9224)$	0.0529
	H802	0.02~0.06	$y=9.675x+0.1283(R^2=0.9973)$	0.0384
	H301	0.02~0.06	$y=5.45x+0.1947(R^2=0.9982)$	0.0560
	H302	0.02~0.1	$y=6.74x+0.0365(R^2=0.9982)$	0.0688
mycelium	H601	0.04~0.1	$y=11.02x-0.2469(R^2=0.9831)$	0.0678
	H802	0.04~0.1	$y=11.48x-0.3447(R^2=0.9841)$	0.0738
	H803	0.06~0.1	$y=15.73x-0.7045(R^2=0.9891)$	0.0766
control	Vc	0.02~0.1	$y=5.21x+0.2944(R^2=0.9925)$	0.0395



**Fig. 4a.** The DPPH· scavenging rate of samples.

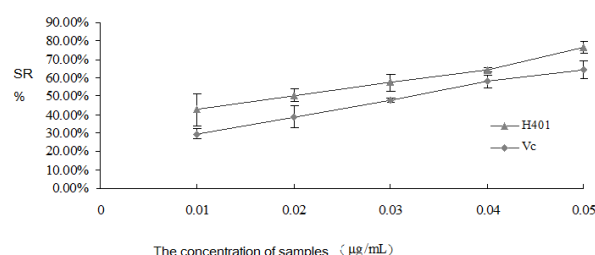
In general, the abovementioned four samples exhibited significant antioxidant activities in the concentration gradient of 0.002, 0.004, 0.006, 0.008, 0.01 mg / mL. The results (Figure 4b) showed that DPPH· scavenging ratio raised with the increasing concentration of the samples. Within a certain range of the concentrations, most of the samples (except H302) represented an obvious linear relationship with the scavenging ratio.



**Fig. 4b.** The DPPH· scavenging ratios of H101, H301, H302 and H401 (ethyl acetate extract parts).

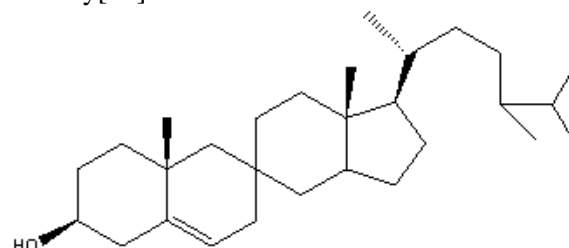
Moreover, within the range of 0.002~0.01 mg/mL, both of the DPPH· scavenging ratios of H401 and Vc were over 50%. The result of further study focused on H401 (Figure 4c) illustrated that within 0.01~0.05 μg/mL, with the increasing of the concentration of the sample, not only the DPPH· scavenging ratio enhanced, but also a linear relationship ( $y=8.15x+0.3381$   $R^2=0.9844$ ) appeared. And the IC<sub>50</sub> value was 0.0198 μg/mL, which was much lower than Vc of 0.0325 μg/mL

( $y=8.86x+0.2112$   $R^2=0.9958$ ).



**Fig. 4c.** The DPPH· scavenging ratios of H401 and Vc.

*Fermentation, isolation and structure forecast*  
In order to obtain and identify the major components, the crude ethyl acetate extract of H401 was screened and purified by column chromatography and TLC. However, only one kind of compound was isolated. The structure was elucidated primarily based on HRMS and NMR analyses and confirmed by comparison with published data. And the biological activities of Compound 1 (Figure 5) exhibited significant bioactivities, including antioxidant and anti-atherogenic effect [15], anti-inflammatory and antipyretic activity [16], anthelmintic and antimutagenic activity [17], immunoregulatory activity [18].



**Fig. 5.** The structure of Compound 1.

**Compound 1** was obtained as white needle-like crystals. Its molecular formula was established as C<sub>29</sub>H<sub>50</sub>O, by ESI-MS (m/z 414 [M<sup>+</sup>]). The <sup>1</sup>H-NMR (600 MHz, CDCl<sub>3</sub>) data showed δ : 3.52 (1H, m, H-3), 5.35 (1H, m, H-6), 0.68 (3H, s, H-18), 1.03 (3H, s, H-19), 0.93 (3H, d, H-21). While the <sup>13</sup>C-

NMR (600 MHz, CDCl<sub>3</sub>) data showed  $\delta$  (ppm) : 33.2 (C-1), 33.1 (C-2), 71.8 (C-3), 42.2 (C-4), 140.7 (C-5), 121.7 (C-6), 31.9 (C-7), 31.7 (C-8), 50.1 (C-9), 36.5 (C-10), 21.2 (C-11), 39.7 (C-12), 42.3 (C-13), 56.0 (C-14), 24.3 (C-15), 28.2 (C-16), 55.9 (C-17), 19.4 (C-18), 11.9 (C-19), 36.1 (C-20), 18.8 (C-21), 39.9 (C-22), 26.1 (C-23), 45.8 (C-24), 29.1 (C-25), 19.8 (C-26), 19.0 (C-27), 23.1 (C-28), 11.2 (C-29). Besides, combining with the previous published data<sup>19</sup>, Compound 1 maybe belongs to  $\beta$ -sitosterol family.

### CONCLUSIONS

Endophytic fungi were studied from *Astragalus monadelphus*, and 13 strains were isolated and identified.

The anti-oxidization activities of secondary metabolites and mycelium extracts were investigated. Results showed that secondary metabolites and mycelium extracts from these strains had some promising antioxidant activity. Reducing ability of H401 mycelium extracts were stronger than the ethyl acetate extract parts, and its EC<sub>50</sub> value is 0.0034 mg/mL, but compared to the positive control Vc (EC<sub>50</sub> value is 0.0019 mg/mL) is weak. Ethyl acetate parts of H802 has strong  $\cdot$ OH scavenging effect, the IC<sub>50</sub> value is 0.0384 mg/mL, and less than the positive control Vc (IC<sub>50</sub> value is 0.0395mg/mL). Especially, in the DPPH radical scavenging activity assay, the IC<sub>50</sub> value (0.0198 $\mu$ g/mL) of strain H401 (ethyl acetate part) is much lower than Vc (0.0325 $\mu$ g/mL). the DPPH radical scavenging activity test indicated that endophytic fungi can be a good source of radical scavenger. Moreover, based on the NMR date and comparison to literature data, we known that the compound belong to  $\beta$ -sitosterol. These may offer a basis for further development and utilization of endophytic fungi from *Astragalus monadelphus*.

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## АНТИОКСИДАНТНА АКТИВНОСТ НА ВТОРИЧНИ МЕТАБОЛИТИ И МИЦЕЛАРНИ ЕКСТРАКТИ ОТ ЕНДОФИТОВИ ГЪБИЧКИ, ИЗОЛИРАНИ ОТ *Astragalus monadelphus*

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(Резюме)

Тринадесет щама от ендофитови фунги са изолирани от *Astragalus monadelphus*. Изследвана е активността на техните вторични метаболити и мицеларни екстракти за отстраняване на свободни радикали. Резултатите показват, че те притежават значителна антиоксидантна активност. Редукционната способност на мицеларния екстракт от Н401 е по-силна, отколкото екстракта с етилацетат, като неговата ЕС<sub>50</sub> стойност е 0.0034 mg/mL. Екстрактът с етилацетат на щама Н802 проявява силна способност да отстранява ·ОН – радикали с IC<sub>50</sub> стойност от 0.0384 mg/mL. DPPH-тестът за антирадикалова активност показва, че ендофитовите фунги могат да бъдат добър източник за анти-радикалови средства. Вторичните метаболити, екстрахирани от щама Н401 принадлежат към групата на β-ситостерола, което е установено с ЯМР-анализ и от литературни данни.