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Improving the antioxidant activity and enriching salvianolic acids by the fermentation of *Salvia miltiorrhizae* with *Geomyces luteus*[*]

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Abstract: The antioxidant activities and total phenolic content of fermented *Salvia miltiorrhiza* with fungus *Geomyces luteus* were investigated. The results revealed that *G. luteus* fermentation could significantly improve the antioxidant activity and total phenolic content of *S. miltiorrhiza*. The main antioxidant constituents were characterized by spectroscopic analysis as salvianolic acids. High-performance liquid chromatography (HPLC) quantification also showed the enhanced content of salvianolic acid B after fermentation. The present study suggests that *G. luteus* fermentations are effective in the *S. miltiorrhiza* salvianolic acids' enrichment process.

Key words: *Salvia miltiorrhiza*, *Geomyces luteus*, Salvianolic acids, Antioxidant, Fermentation
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1 Introduction

Salvia miltiorrhiza Bunge (Labiatae), known as Danshen and first recorded in Shennong Bencaojing (an ancient herbalism in China), has long been used to treat cerebrovascular and cardiovascular diseases. It is listed in the Chinese Pharmacopeia. Modern pharmacological studies revealed that *S. miltiorrhiza* could activate blood circulation to dissipate blood stasis, alleviate pain, and treat inflammatory diseases (Zhou *et al.*, 2005), which may be because of its large number of bioactive ingredients, including diterpene, chinone, and phenolic acids (Li, 1997). Salvianolic acids, the main polyphenolic acids in *S. miltiorrhiza* (Sun *et al.*, 2009), produce a variety of pharmacological activities such as reducing atherosclerosis, inhibiting formation of low-density lipoprotein, and prohibiting the aggregation of platelets, which has long stimulated scientists' strong interest (Yang *et al.*, 2010). Plant phenolics, particularly phenolic acids, are known to be potent antioxidants, and can effectively scavenge free radicals and cut off radical chain reactions (Duan *et al.*, 2006). Previous studies revealed that a certain amount of phenolics possessing significant antioxidant activity exist in *S. miltiorrhiza* (Zhang *et al.*, 2010), and these be vital contributors to the pharmacological activity of *S. miltiorrhiza*.

Traditional Chinese medicine (TCM) has played a significant role in healing for a very long time, but recently scientific evidence regarding the better utilization of the resources of TCM has been needed. Fermented traditional medicine (FTM) has garnered attention for its enhanced biological activity compared to traditional medicine (Wei *et al.*, 2007; Okabe *et al.*, 2011). Several studies using single herbs and fungi support the availability and efficacy of FTM.

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Chen and Chen (2000) had reported that yeast elicitors increased the cryptotanshinone production of the *S. miltiorrhiza* extracts. *Coprinus comatus* fermentation enhanced the anti-inflammatory properties of *Sophora flavescens* (Han *et al*., 2011).

In the present study, *S. miltiorrhiza* was fermented with *Geomyces luteus* and 19 other fungi. The antioxidant activities of fermented *S. miltiorrhiza* were evaluated with 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) assays. Subsequently, the total phenolic contents (TPCs) of fermented *S. miltiorrhiza* were measured. Extensive spectroscopic analysis (ultraviolet (UV), infrared (IR), and nuclear magnetic resonance (NMR)) and the high-performance liquid chromatography (HPLC) method were employed for the characterization and quantitative analysis of the main antioxidant constituents of fermented *S. miltiorrhiza*. This paper explores a new method to enrich the salvianolic acids in *S. miltiorrhiza* by microorganism fermentation.

2 Materials and methods

2.1 Materials, chemicals, and instruments

Trolox, salvianolic acid B, ABTS, and DPPH were obtained from J&K Chemical (Beijing, China). Gallic acid and rutin were purchased from Aladdin-Reagent (Shanghai, China). Deuterated methanol (CD_3OD) and dimethylsulfoxide (DMSO) were obtained from Sigma-Aldrich (Shanghai, China). Deionized water (resistivity ≥18.25 MΩ/cm) was acquired with a Youpu purity water system (Chengdu, China). Acetonitrile and CH_3OH (HPLC grade) were supplied by Fisher Scientific (NJ, USA). All other reagents were of analytical grade.

Roots of *S. miltiorrhiza* were obtained from Kunming City, China in October 2013. The sample was authenticated by Professor Shu-gang LU at the School of Life Science in Yunnan University. A voucher specimen (No. DS01) has been deposited in the Key Laboratory of Medicinal Chemistry for Natural Resources, Yunnan University, China.

2.2 Microorganisms and fermentation

G. luteus and 19 other fungi (*Talaromyces purpurogenus*, *Penicillium vancouverense*, *Alternaria tenuissima*, *Chaetomium globosum*, *Penicillium swiecickii*, *Arthrinium phaeospermum*, *Stagonosporopsis cucurbitacearum*, *Arthrinium arundinis*, *Scytalidium lignicola*, *Fusarium oxysporum*, *Clonostachys rogersoniana*, *Alternaria compacta*, *Cladosporium cladosporioides*, *Arthrinium sacchari*, *Stagonosporopsis chrysanthemi*, *Botrytis cinerea*, *Fusarium avenaceum*, *Helminthosporium maydis*, *Trichophyton verrucosum*) were inoculated in potato dextrose agar slant media in a constant temperature incubator at 28.0 °C for 7 d. Dried and powdered roots of *S. miltiorrhiza* (5.0 g) were added to 150 ml Erlenmeyer flask to form the solid fermentation culture medium. A total of 11 ml distilled water was added to the medicine to achieve a moisture circumstance, and autoclave-sterilized at 120 °C for 30 min. Then the mature-inoculated slants were added and incubated with *S. miltiorrhiza* at 28.0 °C for a month, together with a blank fermentation (without added microbe). Each of the samples had 3 other parallel controls.

The original material and microorganism-fermented *S. miltiorrhiza* were immersed in 50 ml of 80% (v/v) CH_3OH-H_2O for 24 h, and the extracts were filtered after ultrasonic treatment for 30 min. The extraction was repeated 3 times. The extracts of the samples and parallel controls were concentrated under vacuum to dryness using a rotary evaporator at 60 °C (Heidolph, Germany). The non-fermented *S. miltiorrhiza* (original material) and *S. miltiorrhiza* fermented with *G. luteus* were respectively obtained as NFS (2.5015 g) and GFS (0.7030 g).

2.3 DPPH assay

The DPPH assay was performed according to the method described by He *et al.* (2015). Briefly, DPPH· solution (3.9 ml, 0.075 mmol/L) was mixed with 0.1 ml of the sample at gradient concentrations (10, 20, 25, 30, 35, 40, 50, and 60 μg/ml). The mixture was shaken and rested at 25 °C for 30 min. The absorbance was determined at 515 nm. The DPPH· scavenging effect was calculated using the following equation: inhibition=$(1-A_s/A_o)\times100\%$, where A_s is the absorbance of the sample mixture and A_o is the absorbance of the mixture without the sample. The 50% absorbance reduction (IC_{50}) was estimated based on a curve relating concentration to the absorbance of a sample. Rutin was used as a positive reference.

2.4 ABTS assay

The ABTS assay was performed according to the method described by Re *et al.* (1999). Briefly, 7 mmol/L ABTS solution was mixed with 2.5 mmol/L $K_2S_2O_8$ solution and incubated in the dark for 12–16 h to achieve an $ABTS^{+}$· solution. The $ABTS^{+}$· solution was diluted with phosphate buffer saline (PBS; 200 mmol/L, pH=7.4) to acquire an initial absorbance of 0.70±0.02 at 734 nm. The 100-μl sample extract was added to 3.9 ml of $ABTS^{+}$· working solution. After resting at room temperature for 6 min, the absorbance was measured at 734 nm. A standard curve was acquired using Trolox solution at different concentrations in the PBS buffer solution. The $ABTS^{+}$· scavenging effect was evaluated using the following equation: inhibition=$(1-A'_s/A'_o)\times100\%$, where A'_s is the absorbance of the sample solution mixed with $ABTS^{+}$· and A'_o is the absorbance of blank reaction where the sample was replaced by the PBS buffer solution. The antioxidant capacity was obtained by the standard curve and expressed as Trolox equivalent antioxidant capacity (TEAC). Rutin was used as reference standard.

2.5 Total phenolic content

The TPC was measured by the Folin-Ciocalteau method (Gursoy *et al.*, 2009). Folin-Ciocalteau reagent was prepared by the method described by Huang *et al.* (2005). Briefly, Folin-Ciocalteau reagent (4.5 ml) was mixed with a 1.0-ml proper concentration sample. After 5 min, 3.0 ml of 7.5% (0.075 g/ml) Na_2CO_3 was added. After resting at room temperature for 30 min, the absorbance was measured at 765 nm. Gallic acid was used as a standard to prepare for a calibration curve (4, 8, 12, 16, 20, and 24 μg). The TPC was expressed as mg gallic acid equivalents/g dry extract (mg GAE/g Ex).

2.6 Spectroscopic analysis

UV spectra of samples (0.05 mg/ml in 50% (v/v) CH_3OH-H_2O) were acquired with a Shimadzu UV-Vis 2550 spectrometer (Kyoto, Japan) within the wavelength range of 200 to 600 nm. IR spectra of solid dry samples (0.5 mg) were acquired with a Nicolet Avatar 360 FT-IR spectrometer (Madison, USA) with KBr pellets within the range of 400–4000 cm^{-1}. ^{1}H and ^{13}C NMR spectra were collected using a Bruker AM-500 spectrometer (Karlsruhe, Germany) in CD_3OD-D_2O (1:1, v/v).

2.7 HPLC analysis

The quantifications of crude extracts of GFS and NFS were achieved by an HPLC system. NFS (2.00 mg/ml), GFS (1.00 mg/ml), and salvianolic acid B (0.20 mg/ml) were dissolved with 50% CH_3OH-H_2O. Before injection, the samples were passed through a filter (0.45 μm). Mobile phase A was 0.1% aqueous formic acid, and mobile phase B was acetonitrile. The recorded HPLC spectrogram was linearly graduated: 0–10.0 min, 10.0%–20.0% (v/v) solvent B; 10.0–27.0 min, 20.0%–33.0% solvent B; 27.0–30.0 min, 33.0%–70.0% solvent B; 30.0–50.0 min, 70.0%–85.0% solvent B. The column temperature was 20 °C, the flow-rate was 1.0 ml/min, the injection volume was 10 μl, and the UV detection wavelength was 280 nm (Qu *et al.*, 2007).

2.8 Statistical analysis

The results were expressed as the mean±standard deviation (SD) of three replicates for each sample. Statistical analyses were performed with SPSS Version 19.0 (SPSS Inc., Chicago, IL, USA). A significant difference was assessed at a level of $P<0.05$.

3 Results and discussion

3.1 Antioxidant activity

Two different models of *S. miltiorrhiza*, DPPH and ABTS assays, were used to assess the antioxidant activity of extracts of *S. miltiorrhiza* fermented with 20 fungi. As observed in Table 1, the GFS (IC_{50}, (25.0±0.6) μg/ml) exhibited higher DPPH· scavenging capacity than NFS (IC_{50}, (30.9±1.1) μg/ml), but less than the positive control rutin (IC_{50}, (4.32±0.15) μg/ml), which implied that the DPPH· scavenging effect of the *S. miltiorrhiza* was increased after fermentation by *G. luteus*.

Table 1 Total phenolic contents and antioxidant activities

Sample	TPC (mg GAE/g Ex)	IC_{50} of DPPH· (μg/ml)	TEAC of $ABTS^{+}$· (μmol Trolox/g Ex)
NFS	87.9±3.8	30.9±1.1	435.1±15.3
GFS	167.8±4.6	25.0±0.6	1006.6±23.6
Rutin		4.32±0.15	4546.8±79.5

Each value is presented as mean±SD (n=3)

The antioxidant activity of extracts of fermented *S. miltiorrhiza* was also determined as Trolox equivalents using the $ABTS^{+}\cdot$ assay. As shown in Table 1, the TEACs of $ABTS^{+}\cdot$ of NFS, GFS, and rutin were (435.1±15.3), (1006.6±23.6), and (4546.8±79.5) μmol Trolox/g Ex, respectively. Similar to the results of the DPPH· scavenging effect, the higher $ABTS^{+}\cdot$ scavenging activity was obtained after fermentation by *G. luteus*. In addition, the results indicated that the GFS showed an ABTS value more than twice as high as that of NFS. In conclusion, the above methods gave similar results that the fermentation by *G. luteus* could effectively enhance the antioxidant activity of *S. miltiorrhiza*.

3.2 Total phenolic content

Previous studies have revealed that phenolics are the major bioactive components in *S. miltiorrhiza* (Zhao *et al*., 2006). In this research, 20 fermentation extracts of *S. miltiorrhiza* with different fungi were screened for their ability to improve the phenolic content, and the *G. luteus* fermentation extract of *S. miltiorrhiza* showed notable improvement in phenolic content. The results revealed that GFS possessed the highest TPC ((167.8±4.7) mg GAE/g Ex), which was about twice as high as that of NFS ((87.9±3.8) mg GAE/g Ex), indicating that some biotransformation might be taking place to improve hydroxyls in the chemical constituents of *S. miltiorrhiza*.

The improvement of phenolic content in *S. miltiorrhiza* might be responsible for the increase in antioxidant activity. Previous studies have demonstrated the positive correlation between antioxidant constituents and phenolic content in different plant extracts (Socha *et al.*, 2009), which suggested that the increased antioxidant activity might result from the increasing of phenolic content after fermentation.

3.3 Characterization of the antioxidant compounds of *S. miltiorrhiza* fermented with *G. luteus*

The UV-Vis spectrum was used for the characterizations of NFS and GFS (0.05 mg/ml in H_2O). As shown in Fig. 1a, the absorption peak at maximal wavelength (λ_{max}) 285 nm was the signal of $\pi\rightarrow\pi^{*}$ electron transition of phenolic acid (Fang and Tan, 2005; Zhang *et al.*, 2009). The Fig. 1b shows that the absorbance (λ_{max}=285 nm) of GFS was much higher than that of NFS, indicating the higher content of phenolic acid in GFS, which was consistent with the results of the TPC and antioxidant activity.

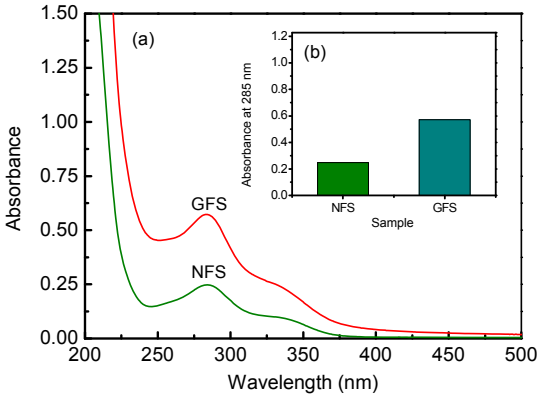

Fig. 1 UV-Vis spectra (a) and the absorbance at 285 nm (b) of NFS and GFS

To clarify more biotransformation details of *S. miltiorrhiza* caused by *G. luteus* fermentation, the IR spectrum was used for the chemical characterization of GFS compared with NFS. The IR spectrum (Fig. 2) revealed that the band at 1042 cm^{-1} was the change angle vibration of O–H and the stretching vibration of C–O–C (Chen *et al.*, 2012). The absorption peak at 1042 cm^{-1} weakened significantly after fermentation, indicating that the hydrolyzation of glucoside and metabolism of saccharides might take place in fermentation processing.

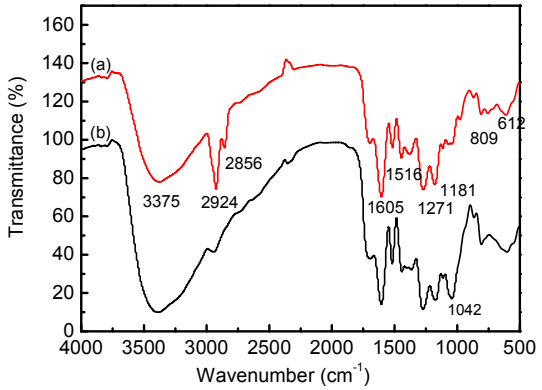

Fig. 2 FT-IR spectra of extracts of GFS (a) and NFS (b)

More exploration was performed to identify and characterize the major components of GFS and NFS by NMR spectroscopy (Pieri *et al.*, 2011). The NMR spectra of GFS indicated the presence of the characteristic signals of salvianolic acids (Figs. 3 and 4). In its ^{1}H NMR spectrum (Fig. 3a), δ_H 6.0 (d, 1H, J=16 Hz) and 7.1 (d, 1H, J=16 Hz) were assigned to the *trans* double bond protons of H-7 and H-8, respectively, in unit A of the salvianolic acids (Fig. 5). Its ^{13}C NMR spectrum (Fig. 4a) showed the signals of C-7 and C-8

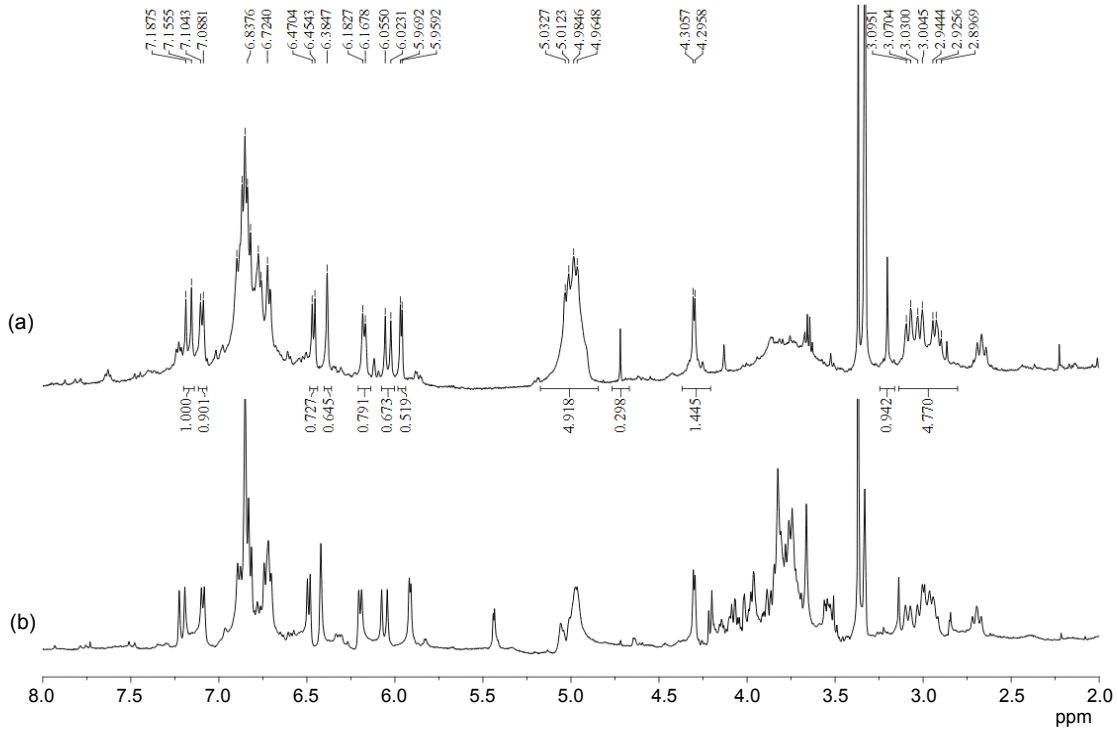

Fig. 3 ^{1}H NMR spectra (400 MHz, CD_3OD-D_2O) of extracts of GFS (a) and NFS (b) at the same concentration of 20 mg/ml (ppm: δ, 10^{-6})

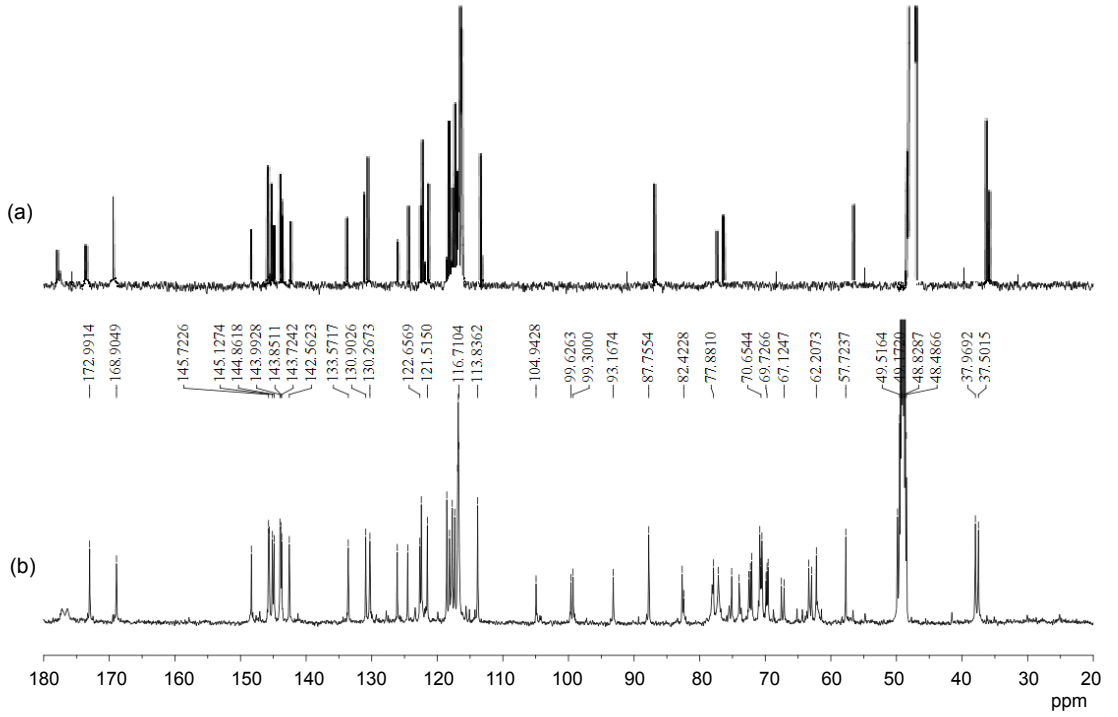

Fig. 4 ^{13}C NMR spectra (500 MHz, CD_3OD-D_2O) of extracts of GFS (a) and NFS (b) at the same concentration of 50 mg/ml (ppm: δ, 10^{-6})

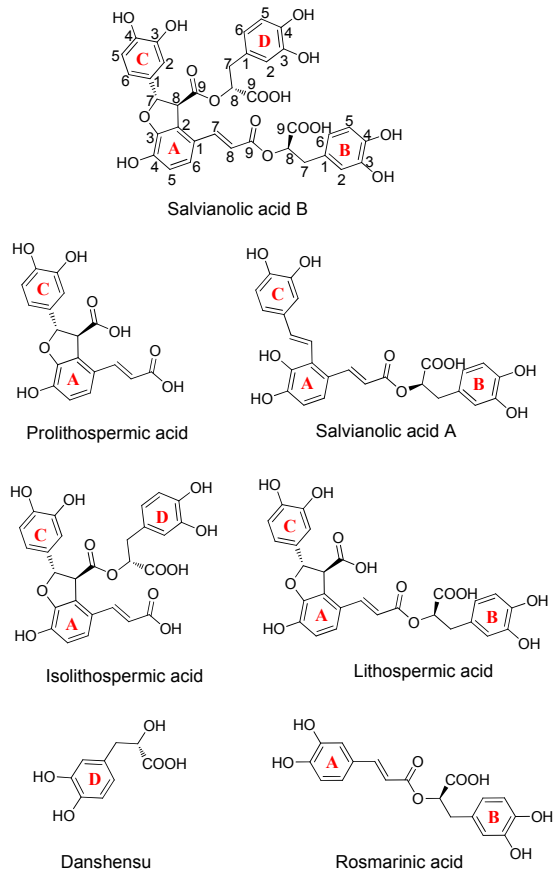

Fig. 5 Structures of salvianolic acids

in unit C at δ_C 87.8 and 57.7, respectively. The signals at δ_C 38.0, 37.5, 77.8, and 77.1 were assigned to C-7 and C-8 in units B and D, respectively. The chemical shifts at δ_C 143.7–148.6 were the typical signals of C-3 and C-4 in units A, B, C, and D, while the signals of δ_C 168.9–176.3 were ascribed to C-9 in units A, B, C, and D.

Therefore it can be concluded that GFS was mainly composed of salvianolic acids, including salvianolic acids A and B, prolithospermic acid, isolithospermic acid, lithospermic acid, danshensu, and so on (Fig. 5). Similarly, the NMR spectra of NFS (Figs. 3b and 4b) showed the presence of the signals of salvianolic acids. However, the NMR spectra of NFS revealed the presence of the obvious saccharide signals at δ_H 3.5–4.2, 5.1 and δ_C 62.2–78.0, 93.2–105.0, which were not observed in the NMR spectra of GFS. These results implied that *G. luteus* fermentation caused the deglycosylation of phenolic glycosides in *S. miltiorrhiza*, which led to the improvement of the TPC and antioxidant activity.

3.4 Quantification of GFS and NFS

The chromatographic profiles of GFS and NFS are shown in Fig. 6. The HPLC quantitative data were acquired from the calibration curve. The content of

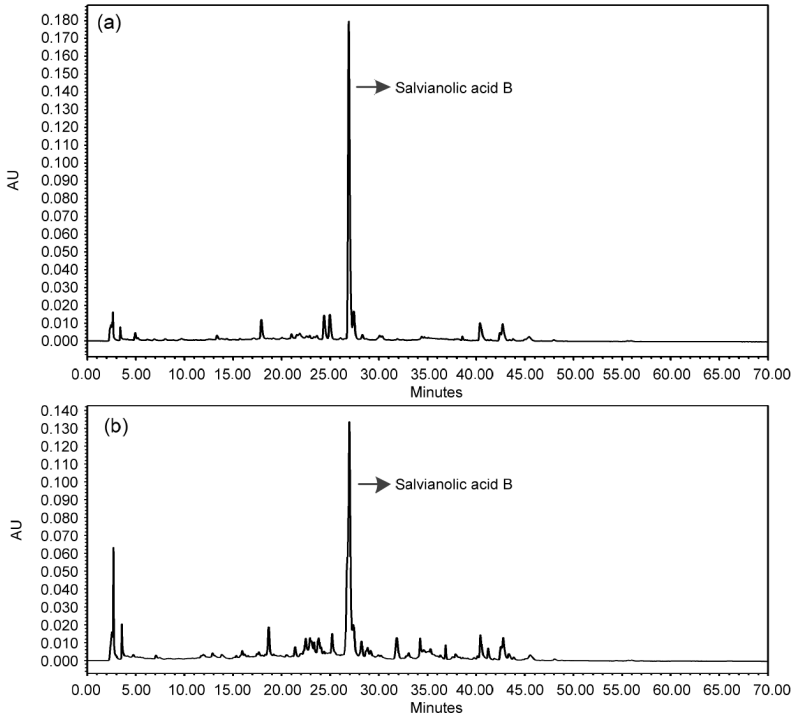

Fig. 6 HPLC chromatograms of NFS (a) and GFS (b) at the concentrations of 2 and 1 mg/ml, respectively

salvianolic acid B in GFS ((95.2±2.5) mg/g Ex) is nearly twice as high as that in NFS ((59.0±1.2) mg/g Ex). The improvement of salvianolic acid B after fermentation may account for the increased TPC of *S. miltiorrhiza*. The antioxidant activity of salvianolic acid B was determined using DPPH assay with rutin as the positive control. Salvianolic acid B exhibited significantly high DPPH· scavenging activity (IC_{50}, 2.71 μmol/L) and was more effective than rutin (IC_{50}, 7.08 μmol/L), which indicated that the enrichment of salvianolic acid B had a significant contribution to the improvement of the antioxidant abilities.

4 Conclusions

G. luteus fermentation could significantly improve the antioxidant activity and TPC of *S. miltiorrhiza*, suggesting that *G. luteus* fermentation was effective in *S. miltiorrhiza* processing. This might be attributed to the deglycosylation of phenolic glycosides in *S. miltiorrhiza*. The main antioxidant constituents were characterized by spectroscopic analysis as salvianolic acids, and the content of salvianolic acid B improved. This study is the first report on the enrichment of salvianolic acids by microbial fermentation.

Compliance with ethics guidelines

Yun XING, Le CAI, Tian-peng YIN, Yang CHEN, Jing YU, Ya-rong WANG, and Zhong-tao DING declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文概要

题　目：*Geomyces luteus* 发酵丹参实现抗氧化活性的增加和丹酚酸的富集

目　的：研究真菌固态发酵对丹参抗氧化活性的影响及探索丹酚酸富集的途径。

创新点：该研究发现真菌 *G. luteus* 固态发酵可增加丹参的抗氧化活性及有效地富集丹酚酸 B，为丹参更有效的开发利用提供了新思路。

方　法：采用 20 种真菌对丹参进行固态发酵，以两种抗氧化模型（二苯代苦味酰基自由基（DPPH•）和 2,2'-联氮基双（3-乙基苯并噻唑啉-6-磺酸）二铵盐自由基（$ABTS^{+}$•））测定发酵物的抗氧化活性；采用 Folin-Ciocaltea 比色法测定发酵物的总酚含量；以多种波谱学方法（紫外光谱（UV）、红外光谱（IR）和核磁共振波谱（NMR））和高效液相色谱法（HPLC）表征发酵前后丹参化学成分的变化。

结　论：抗氧化活性测试和总酚测定结果表明，*G. luteus* 发酵可有效地增加丹参的抗氧化活性和总酚含量。UV、IR 及 NMR 等波谱表征发现，在丹参经 *G. luteus* 发酵后，丹酚酸类化合物的含量提高了；同时 HPLC 测定也发现，丹酚酸 B 含量显著增加。这些结果表明，*G. luteus* 发酵可实现丹酚酸 B 的富集。

关键词：丹参；*Geomyces luteus*；丹酚酸；抗氧化；发酵