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Secondary metabolites in a soybean fermentation broth of *Paecilomyces militaris*

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ABSTRACT

A methanolic extract from a soybean broth fermented by *Paecilomyces militaris* was analysed by HPLC–PDA–HRMS. It mainly contained mannitol, uracil, adenosine, cordycepin, daizein, genistein, and three new isoflavone glycosides. The isoflavonoids were isolated by preparative HPLC and identified with the aid of HPLC–PDA–HRMS, ^{1}H NMR, ^{13}C, H–H COSY, HMBC, and HMQC NMR analysis as: 7,4′-dihydroxyisoflavone-7-O-(4″-O-methyl)-β-D-glucopyranoside, 7,4′-dihydroxy-6-methoxyisoflavone-7-O-(4″-O-methyl)-β-D-glucopyranoside, and 5,7,4′-trihydroxyisoflavone-7-O-(4″-O-methyl)-β-D-glucopyranoside. The three novel compounds are transformation products of soybean isoflavones. The results of metabolites analysis revealed that the fermentation broth reserved the main functional molecules of soybean and *P. militaris* or *Cordyceps militaris*. This suggested that soybean fermented with *P. militaris* may be able to create a combined healthy food. However, isoflavones are also parts of anti-nutrients of soybean products, and cordycepin is a known antibiotic. This means that the fermentation broth may not suit to be used as normal food. At the same time the safety and bioactivity of the novel methylated glycosides need also further approval.

1. Introduction

Soybean fermentation products are traditionally consumed as food in Asia (Liu et al., 2004). Soybean contains various isoflavones. Epidemiologic studies have shown that isoflavone-containing diets such as soybean-based foods may exert protective effects against breast and prostate cancer, cardiovascular disease, osteoporosis, and post-menopausal symptoms (Choi & Rhee, 2006; Chui, 2001; Nieelsen & Williamson, 2007). *Cordyceps sinensis* is a well-known fungal drug used in Traditional Chinese Medicine as a tonic. The pharmacological properties and chemical constituents of *Cordyceps sinensis*, related *Cordyceps* species, and their anamorphs have been extensively studied (Chen, Cao, & Huang, 2005; Schmidt et al., 2003; Zhu, Halpern, & Kenneth, 1998). For that natural *Cordyceps* were becoming less and less recently, they were tried to be replaced with fermentation products of *Cordyceps* related fungi such as *Hirsutella sinensis* and *Paecilomyces militaris*. At present there are more than 40 kinds of products fermented with *Cordyceps* related fungi and used as healthy foods or medicines in Asia (Hu & Li, 2007). Some of the products were fermented by using soybean as culture medium (Ohta et al., 2007; Xiao et al., 2004). Although most of the products have passed toxicity tests before get licence for production, there was no report on secondary metabolites of the soybean fermentation broth yet. Therefore, we embarked on an analysis of the metabolites in a soybean fermentation broth of *P. militaris* with the aim to evaluate nutritional value on the view of small molecules and accumulate chemical knowledge on these kind products.

2. Materials and methods

2.1. Chemicals and equipments

Solvents and chemicals were of analytical or high performance liquid chromatography (HPLC) grade. HPLC–photodiode array detector (PDA)–electrospray ionisation (ESI)–mass spectrometry (MS) analysis was carried out on an Agilent 6210 time-of-flight LC/MS system with a binary high-pressure mixing pump, auto sampler, column oven, PDA, high performance time-of-flight (TOF) MS with an ESI source and an Agilent workstation. Analytical separations were carried out on a Waters ODS2 column (5 μm, 250 × 4.6 mm i.d.), and preparative HPLC separations was performed on a Waters ODS2 column (5 μm, 250 × 19 mm i.d.) with a Younglin preparative HPLC. Liquid samples were concentrated with a Senko R 502 rotary evaporator and dried with a Laboconco freeze dryer. ^{1}H nuclear magnetic resonance (NMR), ^{13}C, H–H COSY, HMBC, and HMQC spectra were recorded with a Bruker Advance 400 spectrometer.

2.2. Fungal strain and preservation

The fungal strain was isolated from a *C. militaris* collected in Anhui Province, China, and identified by two of our colleagues (Huang

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and Li) as *P. militaris*. It has been catalogued as strain RCEF 0927 in the culture collection of the Research Center on Entomogenous Fungi (RCEF), Anhui Agricultural University. The strain is preserved as mycelial pieces on agar slants at 4 °C, in sterile aqua dest. at room temperature, in sterile 10% aqueous glycerol in liquid nitrogen, and as spores in milk sealed in glass tube after freeze drying which are stored at 4 °C.

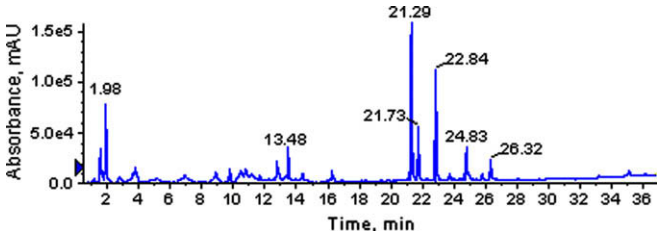


Fig. 1. Chromatogram of the PDA absorbance of the methanol extract.

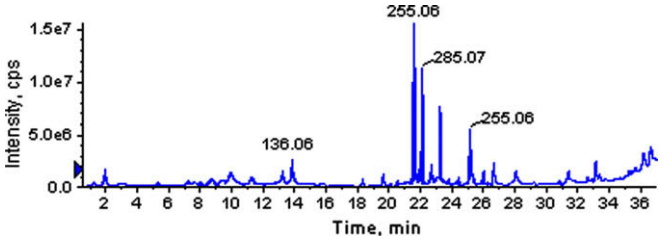


Fig. 2. Total positive ion flow of the methanol extract.

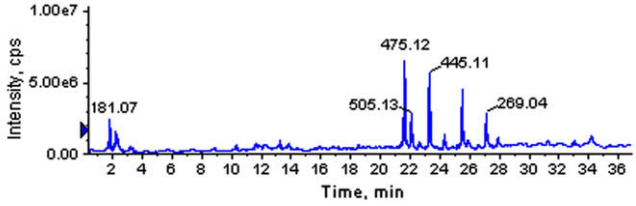


Fig. 3. Total negative ion flow of the methanol extract.

2.3. Culture conditions and work-up of fermentation broth

The mycelium preserved on agar slants with a medium containing 40 g L^{-1} glucose, 10 g L^{-1} peptone, 10 g L^{-1} yeast extract, and 20 g L^{-1} agar was used as starter cultures. The slants were grown in petri dishes on a medium (20 g L^{-1} glucose, 30 g L^{-1} soybean powder, 10 g L^{-1} yeast extract, and 20 g L^{-1} agar) for 15 days at 25 °C. The mycelial pieces were transferred into 30 Erlenmeyer flasks (1000 mL each) with 300 mL of a liquid medium (20 g L^{-1} glucose, 30 g L^{-1} soybean powder, 10 g L^{-1} yeast extract), and cultured for 20 days under shaking at 145 r min^{-1} and 25 °C.

Mycelium and broth were separated by filtration. The broth was concentrated in a rotary evaporator to 1/5 volume and subsequently lyophilised to afford a dried residue (196.3 g). An aliquot 1(50 g) was extracted with 1.5 L methanol, and 6.7 g of crude extract were obtained.

2.4. HPLC–PDA–MS analysis and preparative HPLC

Five microlitre of the methanolic extract (concentration of 1.0 mg mL^{-1}) was injected onto the analytical ODS2 column. Flow rate was 1 mL min^{-1}. The column was eluted with 100% water for the first 3 minutes, followed by a water/methanol gradient from 100:0 to 0:100 over 27 minutes, and 100% methanol for further 15 minutes. The mobile phase contained 2.5 mM ammonium formate to assist ionisation in ESI–MS. Detection with PDA was from 200 to 600 nm.

For preparative HPLC separations, 400 μL of the methanol extract at a concentration of 20.0 mg mL^{-1} were injected. The column was eluted at 18 mL min^{-1} using a MeOH/H_2O step gradient (0%, 30%, 50% and 100% for 15 min each). Fractions were analysed by analytical HPLC. Mixed fractions were rechromatographed to afford compounds 5 (13.4 mg), 6 (7.2 mg), and 7 (8.1 mg). Purity of the compounds was confirmed by HPLC–PDA–MS analysis.

Table 1
HR–ESI–MS data of compounds 1–9 in positive and negative ion mode.

Compound	Rt (min)	Positive ion mode; *m/z* and molecular formula of molecular ions and fragments, and mass accuracy (ppm)	Negative ion mode; *m/z* and molecular formula of molecular ions and fragments, and mass accuracy (ppm)	UV (λmax nm)
1	1.47		181.0716 $C_6H_{13}O_6^{-1}$ $(M-H)^{-1}$ (0.89)	–
2	1.98	113.0344 $C_4H_5N_2O_2^{+}$ $(M+H)^{+}$ (1.36)	111.0199 $C_4H_3N_2O_2^{-1}$ $(M-H)^{-1}$ (0.90)	267
3	12.89	268.1034 $C_{10}H_{14}N_5O_4^{+}$ $(M+H)^{+}$ (2.35) 136.0617 $C_5H_6N_5^{+}$ $(M-C_5H_7O_4)^{+}$ (0.52)	266.0891 $C_{10}H_{12}N_5O_4^{-1}$ $(M-H)^{-1}$ (2.35) 134.0469 $C_5H_4N_5^{-1}$ $(M-C_5H_9O_4)^{-1}$ (0.52)	260
4	13.48	274.0909 $C_{10}H_{13}N_5O_3Na^{+}$ $(M+Na)^{+}$ (0.58) 252.1094 $C_{10}H_{14}N_5O_3^{+}$ $(M+H)^{+}$ (1.12) 136.0614 $C_5H_6N_5^{+}$ $(M-C_5H_7O_3)^{+}$ (2.73)	250.0943 $C_{10}H_{12}N_5O_3^{-1}$ $(M-H)^{-1}$ (0.58) 134.0468 $C_5H_4N_5^{-1}$ $(M-C_5H_9O_3)^{-1}$ (2.73)	260
5	21.29	431.1339 $C_{22}H_{23}O_9^{+}$ $(M+H)^{+}$ (0.55) 883.2419 $C_{44}H_{44}O_{18}Na^{+}$ $(2M+Na)^{+}$ (0.02) 255.0647 $C_{15}H_{11}O_4^{+}$ $(M-C_7H_{11}O_5)^{+}$ (1.90)	429.1193 $C_{22}H_{21}O_9^{-1}$ $(M-H)^{-1}$ (0.45) 859.2447 $C_{44}H_{43}O_{18}^{-1}$ $(2M-H)^{-1}$ (0.92) 475.1246 $C_{23}H_{23}O_{11}^{-1}$ $(M+CHO_2)^{-1}$ (0.03) 253.0503 $C_{15}H_9O_4^{-1}$ $(M-C_7H_{13}O_5)^{-1}$ (1.31)	250
6	21.73	461.1440 $C_{23}H_{25}O_{10}^{+}$ $(M+H)^{+}$ (0.48) 943.2639 $C_{46}H_{48}O_{20}Na^{+}$ $(2M+Na)^{+}$ (0.40) 285.0752 $C_{16}H_{13}O_5^{+}$ $(M-C_7H_{11}O_5)^{+}$ (1.92)	459.1295 $C_{23}H_{23}O_{10}^{-1}$ $(M-H)^{-1}$ (0.37) 919.2653 $C_{46}H_{47}O_{20}^{-1}$ $(2M-H)^{-1}$ (1.43) 505.1347 $C_{24}H_{26}O_{12}^{-1}$ $(M+CHO_2)^{-1}$ (0.89) 283.0610 $C_{16}H_{11}O_5^{-1}$ $(M-C_7H_{13}O_5)^{-1}$ (0.69)	260
7	22.84	447.1283 $C_{22}H_{23}O_{10}^{+}$ $(M+H)^{+}$ (0.61) 915.2315 $C_{44}H_{44}O_{20}Na^{+}$ $(2M+Na)^{+}$ (0.34) 271.0599 $C_{15}H_{11}O_5^{+}$ $(M-C_7H_{11}O_5)^{+}$ (0.73)	445.1143 $C_{22}H_{21}O_{10}^{-1}$ $(M-H)^{-1}$ (0.62) 891.2347 $C_{44}H_{43}O_{20}^{-1}$ $(2M-H)^{-1}$ (0.69) 491.1193 $C_{23}H_{23}O_{12}^{-1}$ $(M+CHO_2)^{-1}$ (0.40) 269.0453 $C_{15}H_9O_5^{-1}$ $(M-C_7H_{13}O_5)^{-1}$ (0.91)	260
8	24.83	255.0648 $C_{15}H_{11}O_4^{+}$ $(M+H)^{+}$ (1.51) 531.1043 $C_{30}H_{20}O_8Na^{+}$ $(2M+Na)^{+}$ (1.39)	253.0504 $C_{15}H_9O_4^{-1}$ $(M-H)^{-1}$ (0.91)	250
9	26.32	271.0603 $C_{15}H_{11}O_5^{+}$ $(M+H)^{+}$ (0.73) 293.0425 $C_{15}H_{10}O_5Na^{+}$ $(M+Na)^{+}$ (1.21)	269.0457 $C_{15}H_9O_5^{-1}$ $(M-H)^{-1}$ (0.56)	260

3. Results and discussion

3.1. HPLC–PDA–MS analysis

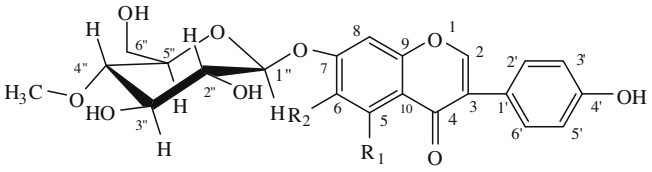
From the PDA and total ion chromatograms (Figs. 1–3), the extract was found to contain nine major compounds. MS and PDA data of these metabolites are listed in Table 1.

From the accurate mass analysis (Table 1), their molecular formula were calculated as $C_6H_{14}O_6$ (compound 1), $C_4H_4N_2O_2$, (compound 2), $C_{10}H_{13}N_5O_4$ (compound 3), $C_{10}H_{13}N_5O_3$ (compound 4), $C_{22}H_{22}O_9$ (compound 5), $C_{23}H_{24}O_{10}$ (compound 6), $C_{22}H_{22}O_{10}$ (compound 7), $C_{15}H_{10}O_4$ (compound 8), and $C_{15}H_{10}O_5$ (compound 9). Compounds 3 and 4 had only one oxygen difference in their molecular formula and both showed a MS fragment of $C_5H_5N_5$ attributable to adenine. We can conclude that the compounds were adenosine and cordycepin, respectively, which are typical metabolites of *Paecilomyces militaris* or *Cordyceps militaris* (Chen, 2001; Chen et al., 2005; Hu & Li, 2007; Zhu et al., 1998). Compounds 8 and 9 had the same molecular formula and UV spectral data as daizein and genistein, respectively, which are well-known isoflavonoids in soybean (Choi & Rhee, 2006; Chui, 2001; Nieelsen & Williamson, 2007; Wang & Murphy, 1994). Database searches with the molecular formula and UV spectral data indicated that compounds 1 and 2 were most likely mannitol, uracil, respectively. The two metabolites are well-known metabolites from entomogenous fungi (Chen et al., 2005; Hu & Li, 2007; Hu, Schmidt, Li, & Hamburger, 2002). HPLC–PDA–MS analysis of authentic samples of mannitol, uracil, adenosine, cordycepin, daizein and genistein showed that they had the same retention times, mass and UV spectral data as compounds 1, 2, 3, 4, 8, and 9 and thus confirmed the identity of these metabolites. Molecular formula and MS fragments indicated that compounds 5, 6, and 7 were glycosides with a similar sugar moiety. Comparable UV spectral data with 8 and 9, and higher polarity suggested that compounds 5 and 7 were glycosides of these two isoflavones (Table 1). UV and MS data of compound 6 suggested a glycoside of glycitein. Although the aglycons of 5, 6, and 7 are known from soybeans, a database search indicated that no isoflavones matched the molecular formulas of the three compounds.

3.2. Isolation and structure elucidation of isoflavonoid glycosides

Glycosides 5, 6, and 7 were isolated by preparative HPLC and analysed by extensive 1D and 2D-NMR analysis, including ^{1}H NMR, ^{13}C, H–H COSY, HMBC, and HMQC spectra.

The data of compounds 5, 6, and 7 (Table 2) were consistent with those of daidzin, genistein, and glycitein, respectively (Chen, 2001; Hirota et al., 2004; Langat, Song, Hu, Simons, & Murphy, 2003), with the exception that there was a methoxyl group (^{1}H δ 3.46–3.50; ^{13}C δ 59.3–59.5) attached to the sugar moiety. HMBC correlations between CH_3O-4″ and H-4″ with C-4″ indicated the position of attachment. The NMR data of the 4″-O-methylglucopyranosyl moiety were in good agreement with those reported for 4-O-methyl-β-glucopyranosides from entomogenous fungi (Hu et al., 2002; Kikuchi, Takahashi, & Oshima, 2004). The structures thus were established as 7,4′-dihydroxyisoflavone-7-O-(4″-O-methyl)-



Compound 5: R_1=H R_2=H

Compound 6: R_1=H R_2=OCH_3

Compound 7: R_1=OH R_2=H

Fig. 4. Structures of compounds 5–7.

Table 2
NMR spectral data of compound 5, 6, and 7 (400 MHz, recorded in *DMSO-d_6*).

Pos.	Compound 5			Compound 6			Compound 7		
	δ_C	δ_H (J_{HH} in Hz)	HMBC ^{1}H to ^{13}C	δ_C	δ_H (J_{HH} in Hz)	HMBC ^{1}H to ^{13}C	δ_C	δ_H (J_{HH} in Hz)	HMBC ^{1}H to ^{13}C
1									
2	153.4	8.37 (s)	3,4,9,1′	153.3	8.19 (s)	3,4,9, 1′	153.9	8.41 (s)	3,4,9,1′
3	123.6			124.1			122.2		
4	174.5			175.3			180.1		
5	126.6	8.04 (d 8.9)	4,6,7,8,9	104.6	7.61(s)	4,6,7,8,9	161.3		
6	115.2	7.14 (dd 8.9, 2.3)	7,8,10	148.1			99.2	6.46 (d 2.0)	5,7,8,10
7	161.6			152.2			162.6		
8	102.3	7.22 (d 2.3)	6,7,9,10	100.7	7.31 (s)	6,7,9,10	94.1	6.70 (d 2.0)	6,7,9,10
9	156.5			152.1			156.8		
10	118.2			118.3			105.7		
1′	122.0			122.8			120.6		
2′/6′	129.8	7.39 (d 8.6)	3,6′/2′,3′/5′,4′	129.9	7.39 (d 8.6)	3,6′/2′,3′/5′,4′	129.8	7.39 (d 8.5)	3,6′/2′,3′/5′,4′
3′/5′	114.6	6.81 (d 8.6)	1′,2′/6′,5′/3′,4′	114.7	6.82 (d 8.6)	1′,2′/6′,5′/3′,4′	114.8	6.82 (d 8.5)	1′,2′/6′,5′/3′,4′
4′	156.9			157.3			157.1		
6-O-Me				55.3	3.84 (s)	6			
1″	99.4	5.12 (d 7.8)	2″,3″,7	99.8	5.11 (d 7.7)	2″,7	99.1	5.08 (d, 7.8)	2″,5″,7
2″	73.3	3.29 (m)	1″,3″	73.3	3.29 (m)	1″, 3″	72.9	3.26 (m)	1″,3″
3″	76.3	3.44 (m)	2″	76.3	3.45 (m)	2″	75.6	3.42 (m)	2″
4″	79.2	3.04 (dd 9.2,7.9)	5″,6″,7″	79.0	3.07 (dd 9.3,8.0)		78.6	3.03 (dd 9.0, 9.0)	5″,6″,4″,7″
5″	75.9	3.51 (m)	1″,3″,4″,6″	76.0	3.50 (m)	1″,3″,4″,6″	75.4	3.49 (m)	1″,3″,4″,6″
6″	59.9	6″a: 3.64 (dd 10.0, 4.5)	6″a: 5″,4″	60.2	6″a: 3.71 (dd 11.0, 4.8)	6″a: 5″,4″	59.9	6″a: 3.63 (dd 11.0, 4.8)	6″a: 5″, 4″
		6″b: 3.51 (m)	6″b: 5″,4″		6″b: 3.92 (dd 10.2, 5.0)	6″b: 5″,4″		6″b: 3.49 (m)	6″b: 5″, 4″
4″-O-Me	59.3	3.46 (s)	4″	59.5	3.50 (s)	4″	59.3	3.45 (s)	4″
4-OH								12.93 (s)	5,6,10
4′-OH		9.55 (s)	3′,4′,5′		9.60 (s)	3′,4′,5′		9.61 (s)	3′,4′,5′
2″-OH		5.50 (d 5.0)	1″,2″,3″		5.49 (d 5.0)	1″		5.47 (d 5.0)	1″
3″-OH		5.30 (d 5.0)	2″,3″,4″		5.31 (d 5.0)	2″,3″,4″		5.29 (d 5.0)	2″,3″,4″
6″-OH		4.73 (dd 5.7, 5.0)	5″,6″		4.74 (dd 6.0, 4.8)	5″,6″		4.72 (dd 6.0, 4.8)	5″,6″

β-D-glucopyranoside (compound 5), 7,4′-dihydroxy-6-methoxyisoflavone-7-O-(4″-O-methyl)-β-*d*-glucopyranoside (compound 6) and 5,7,4′-trihydroxyisoflavone-7-O-(4″-O-methyl)-β-D-glucopyranoside (compound 7), respectively (Fig. 4). A database search confirmed that the compounds are new metabolites, despite their widely occurring aglycons (Chen, 2001; Chui, 2001; Wang & Murphy, 1994). Given that daidzin, genistein, and glycitein occur in the soybean medium, it appears that the 4″-O-methylglucosides are transformation products of fungal fermentation with *Paecilomyces militaris*.

4. Conclusions

Above analysis revealed that soybean fermentation broth of *Paecilomyces militaris* reserved the main secondary metabolites of soybean and *P. militaris* such as daizein, genistein, mannitol, adenosine, and cordycepin, and contained three novel methylglucosides which have the same functional aglycons as daidzin, genistein, or glycitein. For that soybean isoflavones, mannitol, adenosine, and cordycepin are the main functional molecules of soybean and *Paecilomyces militaris* or *C. militaris* (Chen et al., 2005; Choi & Rhee, 2006), it seemed that soybean fermented with *P. militaris* could combine the bioactivities of the two healthy foods. However, the isoflavones are also parts of anti-nutrients of soybean products (Choi & Rhee, 2006; Nieelsen & Williamson, 2007), and cordycepin is a known antibiotic (Chen, 2001; Chen et al., 2005; Hu & Li, 2007). This means that the fermentation broth may not suit to be used as a normal food. Additionally, there is accumulating evidence showed that methylation was widely occurred in fermentation of *Cordyceps* related fungi (Hu et al., 2002; Kikuchi et al., 2004). Considering that fermentation products of *Cordyceps* related fungi are consumed more and more popularly, the methylation properties of the fungi and safety of the novel methylated products are worthy of further studies in the future.

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