Solid-state fermentation with *Eurotium cristatum* HC-18 to improve antioxidant activity of kudzu (*Pueraria lobata*) root

BO ZHANG – ZHOU LU – XIAO XU – MINGSHENG DONG

Summary

The aim of the present work was to investigate, for the first time, the effect of solid-state fermentation with *Eurotium cristatum* HC-18 on antioxidant components and antioxidant activities protection of kudzu (*Pueraria lobata*) root. The effects of fermentation on kudzu root were investigated in terms of total flavonoid and phenolics contents, antioxidant activities, through various solvent (80% methanol, 80% ethanol, water) extracts. Various solvents with different polarities were used to extract antioxidant components and reversed phase high-performance liquid chromatography (HPLC) analysis was conducted to determine the major individual flavonoid and phenolic compounds of the kudzu root samples. The results showed that, compared to the unfermented samples, fermented kudzu root extracts had higher total flavonoid and phenolics contents, as well as greater antioxidant activities. Among various extracts examined, the 80% methanol extracts of fermented kudzu root showed the highest 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) radical-scavenging activities, 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activities, Fe^{2+} chelating ability, reducing power and activities determined by the silver nanoparticle spectrophotometric method. The performed reversed phase HPLC analysis revealed contents of daidzein, genistin and genistein to significantly increase during fermentation. Both correlation analysis and principal component analysis indicated that contents of total phenolics and flavonoids were closely related with antioxidant activities.

Keywords

non-fermented kudzu root; *Eurotium cristatum* fermented kudzu root; total flavonoid contents; total phenolics contents; correlation analysis

Root of kudzu (*Pueraria lobata*), which is a member of the pea family, is one of the earliest mentioned and most essential crude herbs in Chinese medicine. Kudzu root contains high amounts of flavonoid compounds [1, 2], which exhibit antioxidant, antibacterial, anti-diabetic, anti-cancer, and anti-hypertensive activities [3–5]. However, most flavonoid compounds in kudzu roots exist as glycosides or other conjugated forms, which reduce their biological activity [6]. Therefore, bioconversion by the activity of microorganisms must be carried out to release the bioactive flavonoid phenols from kudzu roots [7].

Solid-state fermentation (SSF) using food-safety-grade fungus is a unique strategy for improving the nutritional and health properties, as well as to produce functional ingredients for the human body. During SSF, microbial hydrolytic enzymes, such as cellulases, xylanase, pectinases, β-xylosidase, β-galactosidase, α-amylase or esterase, are produced on the low free water substrate. They transform the bound phytochemicals to free phytochemicals, thereby enhancing their bioactivities [8–10]. SSF has recently gained considerable attention as a way to produce and extract antioxidant phenolics from plant materials, mainly from pulses and cereals [11]. Flavonoids are enriched through SSF, as reported in black beans [12], fermented soybeans [13], fermented cranberry pomace [14], fermented fava beans [15] and fermented mung beans [11].

Eurotium cristatum HC-18, commonly known as "Jinhua Jun" in Chinese, is a widely recognized as safe filamentous fungus traditionally used in preparing Fu brick dark tea [16]. This fungus is characterized by its xerophilic growth on substrate at extremely low free water content, which greatly reduces the risk of microbial contamina-

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tion. The xerophilic characteristics of this fungus make it suitable for application to SSF. *E. cristatum* HC-18, which strongly affects tea quality, offers many functional benefits for human health [17, 18]. However, no study has reported on how the flavonoid contents or antioxidant capacities of kudzu roots can be omproved by SSF using *E. cristatum* HC-18.

MATERIALS AND METHODS

Microorganisms and spore inoculum preparation

E. cristatum HC-18 was identified via morphological and molecular methods. The fungus was isolated from Fu brick tea (Hunan Yiyang Tea factory, Changsha, China) after fermentation. The strain was cultured and maintained on potato-dextrose agar (PDA) culture medium (Nanjing Sode Biotechnology, Nanjing, China) for 7 days. The spores of *E. cristatum* HC-18 were collected by washing the agar surface with 8.5 $g \cdot l^{-1}$ of sodium chloride solution. Then, the spore suspension was created by using sodium chloride solution to achieve a concentration of approximately 1×10^8 spores per millilitre. Afterward, the spore suspension was stored as inoculum for subsequent use.

Materials

Kudzu root (*Pueraria, lobata*) was purchased from Nanjing Tongren Tang Hongze Chinese Medicine Science and Technology (Nanjing, China) and authenticated by reliable traditional Chinese herb experts (Nanjing Tongren Tang Hongze Chinese Medicine Science and Technology). The commercial kudzu root was stored at room temperature and used throughout the experiments.

Ascorbic acid, Folin-Ciocalteu reagent and gallic acid were procured from Merck (Darmstadt, Germany). 2,2'-Azino-*bis*(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) diammonium salt and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were acquired from Sigma-Aldrich (St. Louis, Missouri, USA). All the other chemicals and reagents were of analytical grade and were purchased from Sinopharm (Shanghai, China).

Solid-state fermentation

SSF experiments were performed in 500 ml Erlenmeyer flasks containing 100 g of air-dried kudzu root cubes. The flasks were sterilized by autoclaving for 15 min at 121 °C, and 3 ml of the spore suspension (1×10^8 spores per milllilitres) in 8.5 $g \cdot l^{-1}$ NaCl was injected on the surface. Then, the kudzu root and the *E. cristatum* HC-18 spores were mixed and incubated at 28 °C for 20 days. During the fermentation, samples were collected every 2 days and stored at –20 °C for use and analysis.

Extraction of non-fermented and fermented kudzu root

The fermented kudzu roots were lyophilized and ground in an electric grinder (SF 180; Zhouxiang Phamaceutical Machinery, Shanghai, China). The powders were extracted with 80% methanol, 80% ethanol and water with ultrasound treatment (300 W, 40 °C, 30 min) using a UP-250 HE Ultrasound processor (Leijunda Electricity Machinery, Jiangsu, China). Each 10 ml extract of the fermented kudzu root was centrifuged at 9600 $\times g$ for 10 min. Afterwards, the supernatant was filtered through a Whatman No. 1 filter paper (Whatman, Maidstone, United Kingdom) and a membrane filter (0.22 mm pore size) to determine the total flavonoid content (*TFC*), total phenolics content (*TPC*) and the antioxidant activities.

Enzyme extraction and enzyme activity analysis

The enzymes of the fermented kudzu root were extracted every 2 days by using deionized water with a solid-to-liquid ratio of 1:10 (1 milligram per 10 millilitres). Then, the extracts were centrifuged at 15000 $\times g$ (CT15RT Versatile Refrigerated Centrifuge, Techcomp, Shanghai, China) at 4 °C for 10 min. The supernatants were collected and the residues were re-extracted twice under the same conditions. Afterward, the supernatant was assayed for cellulase and β-glucosidase activities.

The cellulase (EC 3.2.1.4) activity was determined by the method described by Zhao et al. [19] with minor modifications. Briefly, 0.5 ml of the warm (40 °C) sample was mixed with 1.5 ml of 0.2% warm (40 °C) carboxymethyl sodium carbonate. Then, the mixture was treated at 40 °C for 10 min. After the reaction, 3 ml of 3,5-dinitrosalicylic acid reagent was added, and the mixture was boiled for 5 min. The absorbance was measured at 550 nm by using a 722S type visible spectrophotometer (Shanghai Precision Scientific Instruments, Shanghai, China). Approximately 1 μmol of reducing sugar (glucose) was liberated under the assay conditions. The enzyme unit (U) of the cellulase activity was defined as the amount of enzyme that catalysed the formation of 1 $mmol \cdot l^{-1}$ of reducing sugar (glucose) per millilitre and per minute under the given test conditions. The results were expressed as enzyme units per gram of the sample dry weight (DW).

β-Glucosidase (EC 3.2.1.21) activity was deter-

mined by the method proposed by HANDA et al. [20] with slight modifications. The sample (0.1 ml) was mixed with 0.9 ml of 0.2 mol·l^{-1} sodium phosphate buffer (pH 5.0). The mixture was treated in a water bath at 50 °C for 10 min, mixed with 1 ml of warm (50 °C) 5 mmol·l^{-1} *p*–nitrophenol-β-galactopyranoside and treated for another 10 min. The reaction was terminated by adding 1 ml of cold (4 °C) 1 mol·l^{-1} sodium carbonate. The absorbance was read at 420 nm by using a Shimadzu UV-160A UV spectrophotometer (Shimadzu, Kyoto, Japan). One unit of β-glucosidase activity was the amount of enzyme that catalysed the formation of 1 mmol·l^{-1} of *o*-nitrophenol per millilitre and per minute under the given test conditions. The results were expressed as enzyme units per gram of the sample DW.

Determination of contents of total phenolics and total flavonoids

TFC was determined by aluminium chloride colorimetric assay as described by JUAN et al. [21], absorbance being measured at 510 nm by using a 722S type visible spectrophotometer. The results were expressed as grams of rutin equivalents (RE) per kilogram of the extracted sample.

TPC was analysed using the Folin-Ciocalteu method described by XIAO et al. [11]. The results were expressed in grams of gallic acid equivalents (GAE) per kilogram of the extracted sample.

Determination of the contents of specific isoflavonoids

The samples were filtered through a membrane (pore size 0.2 μm, Whatman). The samples were analysed by reversed phase high-performance liquid chromatography (HPLC) using a Waters 2695 system (Agilent Technologies, Santa Clara, California, USA) with an A-AORBAX SB-C18 reverse-phase column (Eclipse Plus; 200 mm × 4.6 mm, particle size 5 μm; Agilent Technologies). Empower software (Agilent Technologies) was used to control the instruments for data acquisition and processing. The mobile phase consisted of deionized water as solvent A (containing 0.1% trifluoroacetic acid) and acetonitrile as solvent B. The solvent flow rate was 0.7 ml·min^{-1}. The elution system was as follows: 0 min (95% B), 15 min (85% B), 32 min (75% B) and 45 min (50% B). An automatic injector was used to inject 10 μl of the test solution into the HPLC system. The wavelength used for monitoring was 254 nm. The results were calculated by using different standard curves and expressed as milligrams per kilogram of the extracted sample.

Measurement of antioxidant activities

ABTS radical cation-scavenging assay

The scavenging activity of the test samples for ABTS radical cations ($ABTS^{\bullet+}$) was analysed using the method described by XIAO et al. [9]. $ABTS^{\bullet+}$ was generated by reacting 7 mmol·l^{-1} of aqueous solution of $ABTS^{\bullet+}$ with a 2.45 mmol·l^{-1} of aqueous solution of $K_2S_2O_8$ in the dark at room temperature for 16 h prior to use. The $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm by using a Shimadzu UV-160A UV spectrophotometer and equilibrated at 30 °C. Approximately 1 ml of various concentrations of extracts was mixed with 4 ml of ethanolic solution of $ABTS^{\bullet+}$, and the absorbance was read at 734 nm by using a spectrophotometer after 6 min. Ascorbic acid was used as the positive control. The ability to scavenge $ABTS^{\bullet+}$ was calculated using the following equation:

$$SA = \frac{(A_0 - A_s)}{A_0} \times 100 \quad (1)$$

where SA is the radical-scavenging activity (in percent), A_0 is the absorbance in the absence of the sample, and A_s is the absorbance in the presence of the sample.

DPPH radical-scavenging assay

The antioxidant capacity of kudzu root during *E. cristatum* HC-18 SSF was analysed based on the hydrogen-donating or radical-scavenging abilities by using stable DPPH free radicals according to the method proposed by LEE et al. [22]. The sample solution (2 ml) was mixed with 2 ml of 0.2 mmol·l^{-1} DPPH. Absorbance of the sample solution (A_s) was read at 517 nm by using a Shimadzu UV-160A UV spectrophotometer against a blank after 30 min of incubation. The absorbance of the DPPH solution was also read at 517 nm. Ascorbic acid was used as the positive control. The capability to scavenge DPPH radicals was calculated using Eq. 1.

Ferrous ion-chelating assay

The ferrous ion-chelating ability (CHA) of kudzu root during fermentation by *E. cristatum* HC-18 SSF was assessed by adopting the method described by XIAO et al. [9] with slight modifications. The sample (0.5 ml) was mixed with 1.75 ml of deionized water. Then, the sample was mixed with 0.05 ml of 2 mmol·l^{-1} ferrous chloride and 0.1 ml of 5 mmol·l^{-1} ferrozine. Then, it was allowed to react for 20 min at room temperature. Absorbance at 562 nm was measured using a Shimadzu UV-160A UV spectrophotometer. A lower absorbance indicated a stronger ferrous ion-chelating

ability. The ferrous ion-chelating activity was calculated as follows:

$$SA = 1 - \frac{A_s}{A_0} \times 100 \quad (2)$$

Reducing power assay

The reducing power (RP) of kudzu roots during fermentation by *E. cristatum* HC-18 SSF was determined by the method proposed by YANG et al. [23]. Briefly, 5.5 ml of the reaction mixture containing 0.5 ml of the sample solution in 2.5 ml of phosphate buffer solution (0.2 mol·l^{-1}, pH 6.6) was added to 2.5 ml of 10 g·l^{-1} potassium ferricyanide. The resulting mixture was incubated at 50 °C for 20 min. Then, 2.5 ml of 100 g·l^{-1} trichloroacetic acid was added and the mixture was centrifuged at 420 ×*g* for 10 min. Afterwards, 2.5 ml of the supernatant was mixed with 0.5 ml of 1 g·l^{-1} ferric chloride and incubated for 10 min at room temperature. The absorbance was read at 700 nm against a blank by using a spectrophotometer. A higher absorbance of the reaction mixture indicated a higher reducing power.

Silver nanoparticle method

ÖZYÜREK et al. [24] used silver nanoparticle (AgNP) spectrophotometric method, which is a novel antioxidant assay, to determine the sample antioxidant capacity. The working solution of AgNP, i.e. 1.0 mmol·l^{-1} of 50 ml $AgNO_3$, was boiled for 10 min and dropwise mixed with 5 ml of 10 g·l^{-1} trisodium citrate. In this process, the solution was rapidly and vigorously mixed and heated until the colour of the mixture changed to canary yellow. When the mixture was cooled down to room temperature, 0.2 ml of the sample solution was mixed with 2 ml of the fresh solution of AgNP and 0.6 ml of deionized water, and then incubated in the dark at 25 °C for 30 min. The canary yellow colour of the reaction solutions was determined at 423 nm by using a Shimadzu UV-160A UV spectrophotometer.

Statistical analysis

The experiments were performed in triplicates and average of the triplicates along with the standard deviation was taken as final result. The results were analysed by Origin 8.0 (Origin Lab, Hampton, Massachusetts, USA). The significance was defined at probabilities of 0.05. Correlations among variables were examined using two-tailed Pearson's correlation analysis. Principal component analysis (PCA) was performed by SIMCA-P (version 11.5 Demo; Umetrics, Umeĺ, Sweden).

The half-maximal effective concentration (EC_{50}) values were obtained by interpolation or extrapolation from the linear regression analysis of the data obtained with a dose-response effect.

RESULTS AND DISCUSSION

Enzyme activities during fermentation process

The cellulase and β-glucosidase activities were determined during SSF of kudzu roots. Thus, the enzyme activity could be reflected by the growth of the microorganism on the substrate. In this study, two enzymes (cellulase and β-glucosidase) were selected to be followed, based on the composition of the substrate. Then, *E. cristatum* HC-18 grown on the substrate of the kudzu root during SSF was analysed. The results are presented in Fig. 1. The highest cellulase (703.94 ± 37.32 U·g^{-1} DW) and β-glucosidase (146.76 ± 9.87 U·g^{-1} DW) activities of kudzu root were observed after 10 and 16 days of SSF. Beyond this time, the activities of these two enzymes decreased. The SSF process was prolonged possibly because of the hydrolysis by other secreted enzymes [25]. In the SSF process, the microorganism could use the components of the substrate for its growth and the products resulting from microbial metabolism as well as nutrient depletion inhibited fungal growth and enzyme formation [26], particularly at the later stages of the SSF process [27].

Determination of the contents of total phenolics and flavonoids

TPC and *TFC* of the non-fermented kudzu root and *E. cristatum*-fermented kudzu root ex-

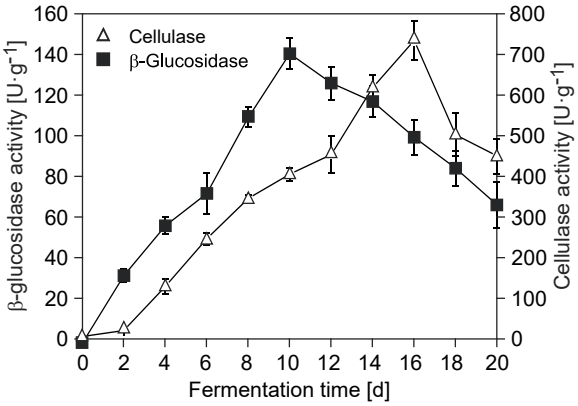

Fig. 1. Dynamics of cellulase and β-glucosidase activities during the solid-state fermentation process of kudzu root with *E. cristatum* HC-18.

Each value represents mean ± standard deviation (n = 3). Enzyme activity is expressed per gram of dry weight.

Tab. 1. Total phenolics and flavonoids contents in extracts from non-fermented and *E. cristatum*-fermented kudzu root.

Solvent	Total phenolics content [g·kg^{-1}]		Total flavonoids content [g·kg^{-1}]	
	Non-fermented kudzu root	Fermented kudzu root	Non-fermented kudzu root	Fermented kudzu root
80% Methanol	1.43 ± 0.47^{Aa}	6.36 ± 0.55^{Ab}	4.86 ± 0.35^{Aa}	13.33 ± 0.82^{Ab}
80% Ethanol	1.37 ± 0.56^{Aa}	6.15 ± 0.62^{Ab}	4.63 ± 0.52^{Aa}	12.95 ± 0.75^{Ab}
Water	1.93 ± 0.89^{Ba}	5.88 ± 0.93^{Bb}	0.79 ± 0.29^{Ba}	6.62 ± 0.43^{Bb}

Each value is expressed as mean ± standard deviation (n = 3). Values with different uppercase letters in superscript within a column demonstrate significant differences ($p < 0.05$). Values with different lowercase letters in superscript within a row indicate significant differences ($p < 0.05$).
Total phenolics content is expressed as gallic acid equivalents, total flavonoids content is expressed as rutin equivalents.

Tab. 2. Isoflavonoid compounds in extracts from non-fermented and *E. cristatum*-fermented kudzu root.

	Content [mg·kg^{-1}]		Fold of increase or decrease
	Non-fermented kudzu root	Fermented kudzu root	
Puerarin	$1\,822.73 \pm 175.39^{a}$	$2\,905.58 \pm 289.43^{b}$	+1.59
Daidzin	226.91 ± 22.58^{a}	15.38 ± 2.35^{b}	–14.75
Glycitin	168.48 ± 19.63^{a}	137.18 ± 20.69^{b}	–1.23
Genistin	126.55 ± 14.14^{a}	70.90 ± 10.89^{b}	–1.78
Daidzein	128.65 ± 12.11^{a}	193.43 ± 25.42^{b}	+1.50
Glycitein	1.98 ± 0.06^{a}	118.35 ± 10.10^{b}	+59.77
Genistein	7.65 ± 0.78^{a}	139.28 ± 13.21^{b}	+18.21

Each value is expressed as mean ± standard deviation (n = 3). Values with different letters in superscript within a row are significantly different ($p < 0.05$).
(+) – indicates increase, (–) – indicates decrease.

tracted with 80% methanol, 80% ethanol and water, respectively, were deterimed. As shown in Tab. 1, depending on the solvent used for extraction, *TPC* (expressed as GAE) ranged from (1.37 ± 0.56) g·kg^{-1} to (6.36 ± 0.55) g·kg^{-1} of the extract, whereas *TFC* (expressed as RE) ranged from (0.79 ± 0.29) g·kg^{-1} to (13.33 ± 0.82) g·kg^{-1} of the extract. Fermented samples presented significantly higher *TPC* and *TFC* values than non-fermented samples regardless of the solvents used for extraction. The 80% methanol extracts displayed the highest *TPC* and *TFC* values, whereas the water extracts had the lowest values. Microbial fermentation significantly increased *TPC* and *TFC*. A similar phenomenon was observed previously for soybeans fermented with fungi [12, 28]. SSF with filamentous fungi was found to be an alternative technique to improve the bioactive phenol content and antioxidant potential in fermented foods [12]. Lin et al. [13] reported that, compared with non-fermented soybeans, soybeans fermented with two different fungi exhibited significantly increased *TPC* and *TFC* values as well as increased antioxidant activities. Our results are consistent with those obtained by Bajalan et al. [17], who reported that *TPC* of an extract by 54% ethanol from wheat was markedly increased by fermentation with *Aspergillus oryzae* and *A. awamori*. Both species are widely recognized as safe filamentous fungi. Yoon et al. [29] reported that SSF can be used to enhance the release of phenols and flavonoids that are related to enzyme activity. Xu et al. [10] demonstrated that the enzyme activity was mainly responsible for the accumulation and simultaneous extrusion as well as hydrolysis of *TFC* and *TPC* during bioconversion.

Contents of specific isoflavonoids

Seven compounds, namely, puerarin, daidzin, glycitin, genistin, daidzein, glycitein and genistein were identified by HPLC in the kudzu root extracts before and after fermentation, data being shown in Tab. 2. During the fermentation, the levels of phenolic and isoflavone compounds were different when 80% methanol, 80% ethanol and water were used for extraction. As shown in Tab. 2, the aglycone contents including daidzin, glycitin and genistin were significantly reduced in fermented samples. For example, contents of daidzin and glycitin were significantly decreased to

(15.38 ± 2.35) mg·kg^{-1} and (137.18 ± 20.69) mg·kg^{-1} of the extract, respectively ($p < 0.05$).

By contrast, higher levels of aglycone isoflavones were determined after fermentation. For example, the daidzein and glycitein contents were (193.43 ± 25.42) mg·kg^{-1} and (118.35 ± 10.10) mg·kg^{-1} of the extract, respectively. Therefore, the highest contents of puerarin in the fermented kudzu root was (2905.58 ± 289.43) mg·kg^{-1} of the extract. Data (Tab. 2) indicated that the isoflavonoid contents determined by HPLC did not match the results obtained by assays of *TPC* and *TFC*. The probable reason for this discrepancy could be that a number of the minor phenolic and isoflavonoid compounds could not be identified by HPLC or could not be determined by the chemical method. WANG et al. [1] described the flavonoid composition of *Pueraria mirifica* and *P. lobata*. Puerarin, daidzin, genistin, daidzein and genistein were found to be the five main flavonoids identified in all of the tested samples, and their contents varied according to locations and cultivars. The accumulation of isoflavones, specifically puerarin, daidzein, glycitein and genistein, during SSF (Tab. 2) was due to microbial activities. The accumulation of phenolic acid and isoflavonoids indicated the enhanced antioxidant status of the kudzu root. PANDEY et al. [30] reported that phenolic acid and isoflavonoids might be major contributors to antioxidant activity.

Antioxidant activities

In view of the differences in the theoretical basis of different antioxidant measurements, a single antioxidant property model can hardly reflect the antioxidant activities of samples [9, 24]. In the present study, five complementary methods with different approaches and mechanisms were used, namely, ABTS radical-scavenging activity, DPPH radical-scavenging activity, ferrous ion CHA, RP and AgNP spectrophotometric methods, to analyse the antioxidant activities of kudzu root before and after SSF with *E. cristatum* HC-18 (Fig. 2–6).

As shown in Fig. 2, both extracts from fermented and non-fermented samples exhibited strong ABTS$^{\bullet+}$ scavenging activity regardless of the extraction solvent. For example, at 1.2 mg·ml^{-1}, the ABTS$^{\bullet+}$ scavenging effect of kudzu root extracts was above 60% regardless of the extraction solvent. Moreover, the extracts from fermented samples exhibited a significantly higher ($p < 0.05$) ABTS radical-scavenging activity than the corresponding extracts from non-fermented samples. For example, the scavenging abilities for ABTS radical of the 80% methanol extracts of non-fermented samples and fermented samples at 0.8 mg·ml^{-1} were 72.3% and 99.8%, respectively. Furthermore, as shown in Tab. 3, both extracts from non-fermented samples and fermented samples with different solvents exhibited low EC_{50} values that were less than 4 mg·ml^{-1}, further verifying their strong ABTS$^{\bullet+}$ scavenging activities.

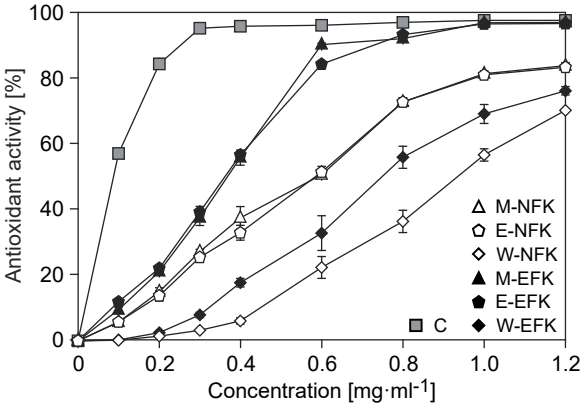

Fig. 2. ABTS radical-scavenging activities of extracts from non-fermented and *E. cristatum*-fermented kudzu root.

Each value represents mean ± standard deviation ($n = 3$). C – ascorbic acid; M-NFK, E-NFK, W-NFK – non-fermented kudzu root extracted with 80% methanol, 80% ethanol and water, respectively; M-EFK, E-EFK, W-EFK – *E. cristatum*-fermented kudzu root extracted with 80% methanol, 80% ethanol and water, respectively.

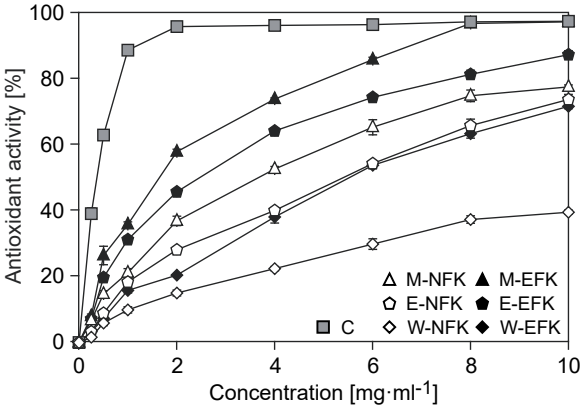

Fig. 3. DPPH radical-scavenging activities of extracts from non-fermented and *E. cristatum*-fermented kudzu root.

Each value represents mean ± standard deviation ($n = 3$). C – ascorbic acid; M-NFK, E-NFK, W-NFK – non-fermented kudzu root extracted with 80% methanol, 80% ethanol and water, respectively; M-EFK, E-EFK, W-EFK – *E. cristatum*-fermented kudzu root extracted with 80% methanol, 80% ethanol and water, respectively.

Tab. 3. Half-maximal effective concentration values of antioxidant activities determined by different methods in extracts from non-fermented and *E. cristatum*-fermented kudzu root.

Method	EC_{50} [mg·ml^{-1}]					
	80% Methanol		80% Ethanol		Water	
	Non-fermented kudzu root	Fermented kudzu root	Non-fermented kudzu root	Fermented kudzu root	Non-fermented kudzu root	Fermented kudzu root
ABTS	1.78 ± 0.33^{Aa}	0.86 ± 0.07^{Ab}	1.73 ± 0.52^{Aa}	0.82 ± 0.31^{Ab}	3.56 ± 0.29^{Ba}	2.43 ± 0.68^{Bb}
DPPH	2.21 ± 0.28^{Aa}	1.56 ± 0.13^{Ab}	2.87 ± 0.45^{Ba}	1.79 ± 0.27^{Bb}	4.58 ± 0.34^{Ca}	2.59 ± 0.19^{Cb}
CHA	8.57 ± 0.69^{Aa}	5.43 ± 0.71^{Ab}	7.69 ± 0.66^{Ba}	5.72 ± 0.35^{Ab}	12.56 ± 0.87^{Ca}	9.33 ± 0.45^{Bb}
RP	6.54 ± 0.38^{Aa}	3.32 ± 0.41^{Ab}	6.28 ± 0.39^{Aa}	3.06 ± 0.71^{Ab}	9.36 ± 0.62^{Ba}	5.42 ± 0.59^{Bb}
AgNP	9.89 ± 0.78^{Aa}	5.88 ± 0.85^{Ab}	8.83 ± 0.57^{Ba}	6.04 ± 0.42^{Bb}	13.54 ± 0.69^{Ca}	9.88 ± 0.81^{Cb}

Values are presented as mean ± standard deviation ($n = 3$). Values in the same column with different lowercase letters in superscript at the same solvent extraction are significantly different ($p < 0.05$) between non-fermented and fermented samples. Values in the same column with different uppercase letters in superscript are significantly different ($p < 0.05$).
ABTS – ABTS radical-scavenging activity assay, DPPH – DPPH radical-scavenging activity assay, CHA – ferrous ion-chelating ability assay, RP – reducing power assay, AgNP – silver nanoparticle spectrophotometric assay.

The antioxidant activity of natural antioxidants is involved in terminating free radical reactions [12]. The extracts from non-fermented samples and fermented samples might react with free radicals, particularly peroxyl radicals, which are the main propagators of the oxidation chain of fat, thereby terminating the chain reaction [31].

Similarly, the DPPH radical-scavenging activity of extracts of both non-fermented and fermented samples increased with increasing dosage (Fig. 3). Extracts from fermented samples exhibited considerably higher scavenging abilities for DPPH radicals than those from non-fermented samples, regardless of the extraction solvent used. The DPPH radical-scavenging abilities of the extracts from non-fermented samples by 80% methanol, 80% ethanol and water at 6 mg·ml^{-1} were 68.5%, 56.9% and 26.2%, respectively, whereas their corresponding values for the same solvents from fermented samples at 6 mg·ml^{-1} were 89.4%, 74.4% and 53.5%, respectively. As shown in Tab. 3, EC_{50} values of the extracts from fermented samples were significantly lower than those from non-fermented samples, regardless of the extraction solvent. For instance, the 80% methanol extract of non-fermented samples had an EC_{50} of 2.21 mg·ml^{-1}, which was greater than that of the 80% methanol textract of fermented samples (1.56 mg·ml^{-1}). Therefore, the improvement of the DPPH radical-scavenging activity was mainly attributed to the fermentation process and to the use of different extraction solvent.

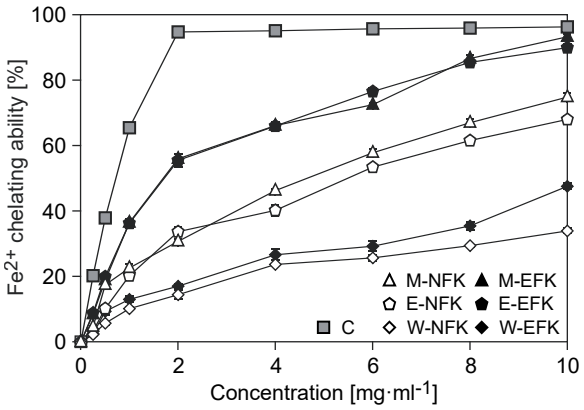

Fig. 4. Ferrous ion-chelating ability of extracts from non-fermented and *E. cristatum*-fermented kudzu root.

Each value represents mean ± standard deviation ($n = 3$).
C – ethylenediaminetetraacetic acid; M-NFK, E-NFK, W-NFK – non-fermented kudzu root extracted with 80% methanol, 80% ethanol and water, respectively; M-EFK, E-EFK, W-EFK – *E. cristatum*-fermented kudzu root extracted with 80% methanol, 80% ethanol and water, respectively.

Bajalan et al. [17] reported that phenolic acids were highly correlated with the DPPH radical-scavenging activities. Thus, the highest levels of flavonoid and phenolic compounds contributed to the highest antioxidant activity. Xiao et al. [11] reported that traditional Chinese fermented mung bean products displayed a stronger ABTS$^{\bullet+}$ scavenging activity than unfermented mung beans. Xiao et al. [9] demonstrated that the enhanced DPPH radical-scavenging activity was due to an increase in *TFC* and aglycone isoflavones. Juan et al. [21] and Kuppusamy et al. [32] showed that extracts from black soybeans fermented with *Bacillus subtilis* exhibited higher DPPH radical-scavenging activity than unfermented samples because of higher *TFC* of the fermented black

soybeans. SINGH et al. [28] reported that phenolic and flavonoid compounds were involved in terminating free-radical reactions and promoting the scavenging effects for free radicals. Therefore, the enrichment of *TFC* and *TPC* during SSF of kudzu root with *E. cristatum* HC-18 probably contributed to the improvement of the DPPH free radical-scavenging abilities.

As shown in Fig. 4, CHA of the extracts, regardless of fermentation, increased with increasing dosage. Chelating agents may inhibit the formation of radicals by stabilizing the transition metals, thereby reducing free radical damage. In addition, chelating agents are effective secondary antioxidants because they can reduce the redox potential to stabilize the oxidized form of the metal ions [4]. Although SSF positively affects CHA of samples, the degree of influence depends on the microorganism species and on the extraction solvents [21]. As shown in Fig. 4, extracts from fermented samples exhibited a significantly higher ($p < 0.05$) CHA than extracts from non-fermented samples. In addition, variations in CHA with EC_{50} values of 7.69–12.56 mg·ml^{-1} and 5.43–9.33 mg·ml^{-1}, respectively, were observed in extracts from non-fermented and fermented samples. Transition metal ions, such as iron and copper, are important catalysts for generating free radicals that initiate the radical chain reaction or the radical-mediated lipid peroxidation [28, 31]. Chelating agents may inhibit the formation of radicals by stabilizing the transition metals, thereby reducing free radical damage. In addition, chelating agents are effective secondary antioxidants as they reduce the redox potential, thereby stabilizing the oxidized form of the metal ions [33, 34].

RP and AgNP of the extracts, regardless of fermentation, increased with increasing dosage. As shown in Fig. 5 and Fig. 6, extracts from fermented samples exhibited a significantly higher ($p < 0.05$) RP than extracts from non-fermented samples at concentrations ranging from 4 mg·ml^{-1} to 10 mg·ml^{-1}. For example, RP of the extract from non-fermented samples at the concentration of 10 mg·ml^{-1} was 1.66, whereas that from fermented samples was 2.01. As shown in Fig. 6, antioxidant activity determined by AgNP spectrophotometric method, regardless of the extraction solvent used or fermentation, increased with increasing dosage, and the highest value was detected for the 80% ethanol extracts from non-fermented samples. YANG et al. [23], XIAO et al. [11] and LEE et al. [35] reported that RP and AgNP, which are closely related to the hydrogen-donating ability of the contained reductones, might increase after fermentation with microorganisms. DEY et al. [36]

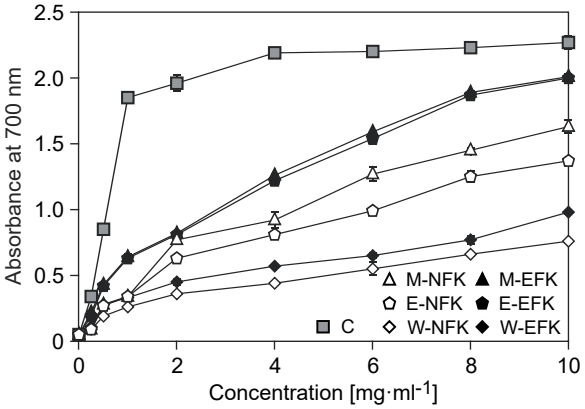

Fig. 5. Reducing power of extracts from non-fermented and *E. cristatum*-fermented kudzu root.

Each value represents mean ± standard deviation ($n = 3$). C – ascorbic acid; M-NFK, E-NFK, W-NFK – non-fermented kudzu root extracted with 80% methanol, 80% ethanol and water, respectively; M-EFK, E-EFK, W-EFK – *E. cristatum*-fermented kudzu root extracted with 80% methanol, 80% ethanol and water, respectively.

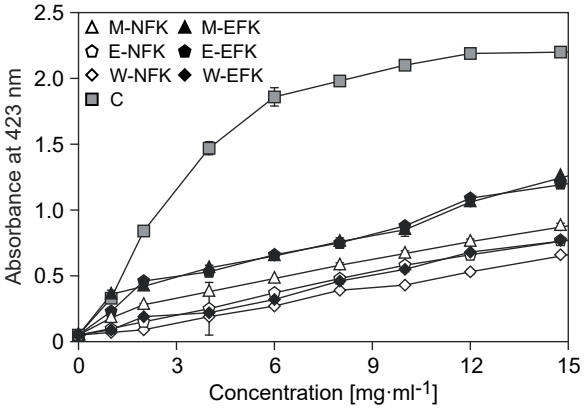

Fig. 6. Antioxidant activity of extracts from non-fermented and *E. cristatum*-fermented kudzu root determined by silver nanoparticle spectrophotometric assay.

Each value represents mean ± standard deviation (n = 3). C – ascorbic acid; M-NFK, E-NFK, W-NFK – non-fermented kudzu root extracted with 80% methanol, 80% ethanol and water, respectively; M-EFK, E-EFK, W-EFK – *E. cristatum*-fermented kudzu root extracted with 80% methanol, 80% ethanol and water, respectively.

demonstrated that SSF can enhance the flavonoid compounds of kudzu root. The compounds reacted with free or active radicals to produce inactive and stable products, thereby contributing to the increase in RP and AgNP or terminating the radical chain reaction. However, the antioxidant capacity of kudzu root decreased with prolonged fermentation time. These results were undesirable from a nutrition perspective. The anti-

oxidative bioactivity of by-products, such as kudzu root, could be enhanced via SSF with *E. cristatum* HC-18. However, the duration of fermentation must be limited, because excessive fermentation may reduce the bioactivity. The changes in the antioxidative capacity of kudzu root exhibited a regular tendency: an initial increase, followed by slow growth until stability. Several researchers indicated that SSF can effectively increase the anti-oxidative activity. Other researchers investigated the changes of antioxidative activity due to bioactivity compounds via SSF to clarify the effect of fermentation time [36].

The antioxidant properties of all assayed samples are provided in Tab. 3. The results were normalized and expressed as EC_{50} values for comparison. The effectiveness of the antioxidant properties was inversely related to their EC_{50} values. For RP, the 80% ethanol extracts from fermented samples had the lowest EC_{50} value of 3.06 mg·ml^{-1}, followed by 3.32 mg·ml^{-1} and 5.42 mg·ml^{-1} for the 80% methanol and water extracts from fermented samples. EC_{50} of AgNP was estimated to be 5.88 mg·ml^{-1}, 6.04 mg·ml^{-1} and 9.88 mg·ml^{-1} for the 80% methanol, 80% ethanol and water extracts from fermented samples, respectively, which were much lower than those of the corresponding extracts from non-fermented samples. These data demonstrate that SSF with *E. cristatum* HC-18 can enhance the antioxidative activities of kudzu root.

Correlation analysis

Tab. 4 presents the results of Pearson's correlation analysis, which was performed to explain the relationship among *TFC*, *TPC*, contents of isoflavonoids and antioxidant capacity.

TFC, *TPC* and antioxidant capacity were significantly related ($p < 0.01$). For instance, Pearson's correlation coefficients from 0.928 to 0.995, gradually approaching 1, were applied to clarify the significant correlation coefficients among *TFC*, *TPC* and antioxidant activities. The strong correlationship could be closely related to the contents of isoflavonoids and phenolic compounds in the extracts.

Moreover, Tab. 4 shows the significant positive correlation among the results of ABTS, DPPH, CHA, RP and AgNP assays, particularly with contents of isoflavonoids. Highly positive correlations between contents of aglycone isoflavonoids, and the antioxidant activities determined by various assays, *TFC* and *TPC* ($p < 0.01$) were expressed.

Principal component analysis

PCA was applied to investigate the reason for the componential change tendency during SSF. As shown in Fig. 7, the score plots of PCA expressed the otherness of the kudzu root samples during SSF, and the different points expressed *TFC*, *TPC* and antioxidant activity. PC1 and PC2 plots revealed the correlation among *TFC*, *TPC* and antioxidant capacities (ABTS, DPPH, CHA, RP, and AgNP assays), which could explain their relationship. Chan et al. [34] reported that flavonoids, primarily isoflavones, contribute to the antioxidant capacity. The less effective component occupied a small proportion of the total variability and was the remaining principal component. The same PC loading plots expressed the positive correlation between PCA and the original variables, whereas the different PC loading plots explained the negative correlation between PCA and the original

Tab. 4. Pearson's correlation coefficients.

	Antioxidant capacity				
	ABTS	DPPH	CHA	RP	AgNP
Total phenolics contents	0.967**	0.989**	0.936**	0.976**	0.928**
Total flavonoids contents	0.968**	0.995**	0.964**	0.981**	0.957**
Puerarin	–0.385	–0.572**	–0.228	–0.563**	–0.329
Daidzin	–0.549*	–0.778**	–0.894**	–0.432	–0.677**
Glycitin	–0.901**	–0.396	–0.632**	–0.565*	–0.772**
Genistin	–0.973**	–0.938**	–0.984**	–0.678**	–0.875**
Daidzein	0.948**	0.867**	0.839**	0.893**	0.799**
Glycitein	0.638	0.923**	0.469	0.384	0.369
Genistein	0.967**	0.771**	0.743**	0.893**	0.766**

** – correlation was significant at $p = 0.01$ level, * – correlation was significant at $p = 0.05$ level.
ABTS – ABTS radical-scavenging activity, DPPH – DPPH radical-scavenging activity, CHA – ferrous ion-chelating ability, RP – reducing power, AgNP – antioxidant activity determined by silver nanoparticle spectrophotometric assay.

variables. In addition, 67.3 % of the PC1 loads demonstrated the good correlation among ABTS, DPPH, CHA, RP and AgNP assays. By contrast, the lower percentage of the second principal components (PC2) expressed the antioxidant capacity evaluation indices, including the similarity of *TFC*, *TPC* and antioxidant capacity to the results of the correlation analysis. The flavonoid compounds were the main radical scavengers and reducing agents. Thus, the relationships between the contents of flavonoid and phenolic compounds, and antioxidant capacity of kudzu root could be well explained by PCA.

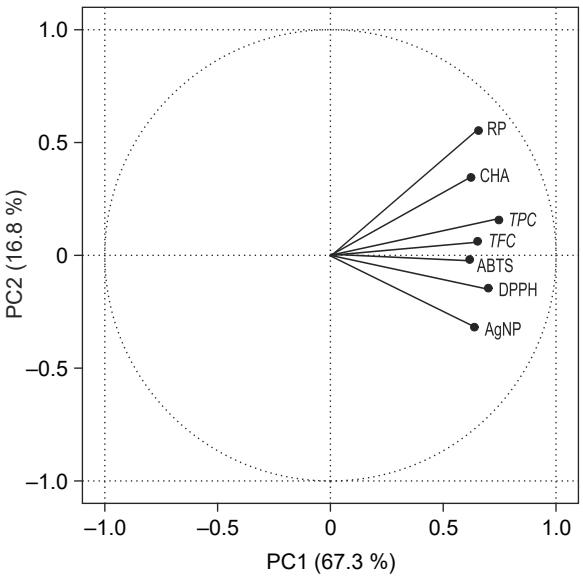

Fig. 7. Principal component analysis loading plot of total phenolics content, total flavonoids content, and antioxidant activities of extracts from non-fermented and *E. cristatum*-fermented kudzu root.

Each value represents mean ± standard deviation (n = 3). *TPC* – total phenolics contents, *TFC* – total flavonoids contents, ABTS – ABTS radical-scavenging activity, DPPH – DPPH radical-scavenging activity, CHA – ferrous ion-chelating ability, RP – reducing power, AgNP – antioxidant activity determined by silver nanoparticle spectrophotometric assay..

CONCLUSIONS

TFC, *TPC* and antioxidant activity of kudzu root can be enhanced via SSF with *E. cristatum* HC-18, and data on enzyme activities showed that the fungus could well use the kudzu root to grow. The Pearson's correlation coefficients and PCA of *TFC*, *TPC* and antioxidant activities during the SSF process suggested that 14 days of fermentation may be optimal.

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