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Production of Powerful Antioxidant Supplements *via* Solid-State Fermentation of Wheat (*Triticum aestivum* Linn.) by *Cordyceps militaris*

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Summary

The present study has been conducted to evaluate the antioxidant properties of fermented wheat (*Triticum aestivum* Linn.) extracted using acidified water, 70 % acetone and 70 % ethanol as compared to uninoculated control. Antioxidant activity, measured by the scavenging ability against 1,1-diphenyl-2-picrylhydrazyl radicals and hydroxyl radicals, reducing power and ferrous ion chelating ability was more effective in fermented than unfermented wheat. Furthermore, the extraction yield, total phenolic, total flavonoid and free phenolic acid content were significantly enhanced in fermented wheat. Among the various extracts examined, the acetone extract of the fermented wheat had the highest content of antioxidant compounds (66.37 mg/g as gallic acid equivalent per mass of the extract for polyphenols and 32.27 mg/g as rutin equivalent per mass of flavonoid extract) and antioxidant activity with the lowest EC_{50} values. Thus, fermentation with *Cordyceps militaris* can be used as a tool to develop wheat as a health food or ingredient with multi-functional properties which can be used in the food industry as a natural antioxidant.

Key words: solid-state fermentation, *Cordyceps militaris*, antioxidant supplements, free phenolic acids

Introduction

Solid-state fermentation (SSF) is utilized to produce the desired product in a controlled way. Until now, enzymes such as phytase, amylase, inulinase, cellulase, protease, lipase, tannase, laccase, chitinase or lipase, and metabolites such as lactic acid, citric acid, polyunsaturated acids, iturin A, pigments, hexyl laureate or palatinose, as well as antibiotics such as cephamycin C have been successfully produced employing SSF (*1–3*). Besides the above usage, SSF has also been used to improve product properties. Previous studies have shown that microorganisms start to modify plant constituents during fermentation (*4–6*). Many biochemical changes occur during fermentation, leading to altered ratio of nutritive and antinutritive components of plants, which affects product properties such as bioactivity and digestibility (*7–12*).

It has been reported that oxygen free radicals and other reactive oxygen species can cause oxidative injury to living organisms and thus play an important role in many lifestyle-related diseases such as arthritis, atherosclerosis, emphysema and cancer (*13–15*). Therefore, the search for new products with antioxidative properties is

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very active domain of research. In recent years, several undesirable disorders have developed due to the side--effects of the use of synthetic antioxidants commonly applied in the food and flavouring industries (*16*). This situation has forced scientists to search for new natural antioxidants from spices and herbs.

Wheat (*Triticum aestivum* Linn.) is a major crop and an important component of the human diet. Research has shown that it helps lower the incidence of chronic diseases such as diabetes, cancer and cardiovascular diseases (*17,18*). *Cordyceps militaris*, an entomopathogenic fungus belonging to the class of *Ascomycetes*, which is widely used as a popular tonic and traditional Chinese medicinal mushroom, has been reported to have beneficial biological activities such as hypoglycemic, antitumour, hypolipidemic, anti-inflammatory, antimetastatic, immunomodulatory as well as antioxidant effect (*19–23*). In this regard, we cultivated *C. militaris* on wheat, which might provide plentiful novel nutraceutical compounds derived from the biologically effective components of the two materials.

To our knowledge, there have been no reports on the comparison of the content of active compounds and antioxidant activity between the unfermented wheat and the wheat fermented with *C. militaris*. Therefore, the objective of this work is to assay the influence of *C. militaris* fermentation on the antioxidative activity, total polyphenolic, flavonoid and free phenolic acid content of wheat. Furthermore, the efficiency of the extraction using various solvent systems, including acidified water, 70 % ethanol and 70 % acetone, as well as the subsequent effect on the antioxidant activity was compared for both fermented and unfermented wheat.

Materials and Methods

Materials and chemicals

Fungal strain of *C. militaris* obtained from China General Microbiological Culture Collection Center, Beijing, PR China, was used in the present study. It was cultivated and maintained in potato dextrose agar (Qingdao Haibo Biological Reagent Co. Ltd., Qingdao, PR China). One batch of commercial wheat grain was stored at room temperature and used throughout the experiments. Folin--Ciocalteu reagent, EDTA, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferrozine, ferulic, *p*-coumaric, vanillic, syringic and caffeic acids were purchased from Sigma-Aldrich (St. Louis, MO, USA), acetonitrile and glacial acetic acid were of HPLC grade, and water was purified with Milli--Q water purifier system from Millipore (Bedford, MA, USA). All other chemicals and solvents were of the highest commercial grade and obtained from Huadong Chemical Reagent Co. Ltd. (Hangzhou, PR China).

Fermentation of wheat

Prior to wheat inoculation, the test organism was incubated in a liquid medium containing (in g/L): sucrose 20, KH_2PO_4 3, $MgSO_4$ 1.5, and Bacto™ Casitone 10. Incubation was performed in a 250-mL Erlenmeyer flask, 100 mL of liquid medium were added, the flask was autoclaved at 120 °C for approx. 20 min and then cooled to room temperature. The suspended liquor containing homogenous mycelium was produced after one-week incubation at (25±2) °C. This liquor was used as the seeding culture for wheat fermentation.

Fermentation was conducted in 500-mL Erlenmeyer flask. A mass of 50 g of wheat grain taken as the substrate for each SSF was washed and then soaked in 50 mL of water at room temperature overnight. After decanting the excess medium, the wheat was autoclaved (121 °C, 20 min) in a conical flask and subsequently cooled. The moisture content and pH of the substrate were 45 % and 6.0, respectively. A volume of 10 mL of seeding culture was crushed and added into the flask in the state of asepsis. Wheat was then incubated at (25±2) °C for approx. three weeks, and when the mycelium appeared, its authenticity was confirmed by the method of intersimple sequence repeat (ISSR).

Preparation of solvent extracts

To prepare the solvent extracts of fermented wheat and the unfermented steamed wheat, samples were freeze dried (Freeze Dry System/FreeZone 4.5, Labconco, Kansas City, MO, USA) and then homogenized. The ground powder of the samples was then extracted twice by shaking at 25 °C for 24 h with solvents (1:10, by mass per volume) of various polarities including water (acidified with 1 % HCl, pH=3), 70 % ethanol and 70 % acetone. The solvent extracts obtained with the same solvent were then combined. After filtering through Whatman no. 1 filter paper, the extract was vacuum concentrated and freeze dried.

Total polyphenol and flavonoid assay

The content of total polyphenols in all samples was determined with Folin-Ciocalteu reagent according to the method of Singleton *et al*. (*24*) using gallic acid as a standard. Briefly, an aliquot of 0.1 mL of the extract was mixed with 1.0 mL of Folin-Ciocalteu phenol regent and allowed to react for 3 min. Then, 0.3 mL of 1 M Na_2CO_3 were added and allowed to react for 90 min at room temperature. The absorbance was measured at 760 nm and the total polyphenolic content was expressed as gallic acid equivalent per mass of extract in mg/g. The calibration curve of gallic acid showed good linearity over the range of 0.025–0.400 mg/mL, and the regression equation was as follows:

$$Y=8.1706 \cdot X \quad (R^2=0.9582,\ N=6) \qquad /1/$$

where Y is the absorbance at 760 nm and X is the concentration of gallic acid (in mg/mL).

The content of total flavonoids in the samples was determined using a modified colourimetric method described previously by Jia *et al*. (*25*) and rutin was used as a standard. Extracts or standard solutions (250 μL) were mixed with distilled water (1.25 mL) and 0.5 % $NaNO_2$ solution (75 μL). After resting for 6 min, the mixtures were combined with $AlCl_3$ solution (150 μL). After 5 min, 1 M NaOH (0.5 mL) and distilled water (275 μL) were added to the mixture. The absorbance of the solutions was then measured at 510 nm. The results were expressed as mass of rutin equivalent per mass of extract in mg/g. The calibration curve of rutin showed good

linearity over the range of 0.0375–0.5000 mg/mL, the regression equation was as follows:

$$Y=3.3025\cdot X \quad (R^2=0.9975,\ N=6) \qquad /2/$$

where Y is the absorbance at 510 nm and X is the concentration of rutin (in mg/mL).

Hydroxyl radical scavenging assay

The deoxyribose assay was used to determine the hydroxyl radical scavenging activity in an aqueous medium (*26*). The reaction mixture containing $FeCl_3$ 100 μM, EDTA 104 μM, H_2O_2 1 mM and 2-deoxy-D-ribose 2.8 mM was mixed with or without various concentrations of different extracts to 1 mL of final reaction volume, made with 20 mM potassium phosphate buffer (pH=7.4) and incubated for 1 h at 37 °C. The mixture was heated to 95 °C in a water bath for 15 min, followed by the addition of 1 mL of each TCA (2.8 %) and TBA (0.5 % TBA in 0.025 M NaOH containing 0.02 % BHA). Finally, the reaction mixture was cooled on ice and centrifuged at 6000×*g* for 15 min. The absorbance of the supernatant was measured at 532 nm. All readings were corrected for any interference from the brown colour of the fractions or antioxidant by including appropriate controls. The negative control was without any antioxidant or fractions. The percentage of hydroxyl radical scavenging activity of the extracts was compared to the negative control. The hydroxyl radical scavenging activity of ascorbic acid was also assayed for comparison.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

To evaluate the free radical scavenging activity, all samples were allowed to react with a stable free radical, $DPPH^{\cdot}$ (*27*). Briefly, 0.5 mL of test sample at different concentrations (0.05–0.8 mg/mL) was added to 3 mL of DPPH (5 mM) methanol solution. The reaction mixture was incubated at 28 °C. The scavenging activity against DPPH radical was determined by measuring the absorbance at 515 nm every 10 min until the reaction reached the steady state. The antioxidant activity was expressed as a percentage of scavenging activity against DPPH radical:

$$\text{Scavenging activity}=(1-A_1/A_0)\times 100 \qquad /3/$$

where A_0 is the absorbance of the control, and A_1 is the absorbance of the samples. The control contains all reagents except the sample. The DPPH radical scavenging activity of ascorbic acid was also assayed for comparison. All tests were performed in triplicate.

Ferrous ion chelating activity

Iron chelating ability of all samples was investigated in the present study. The chelating of ferrous ions by the extracts and standards was estimated by the method of Dinis *et al.* (*28*). Test samples (0.2 mL) at different concentrations (0.05–0.80 mg/mL) were added to a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL), then the mixture was shaken vigorously and left to rest at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was measured at 562 nm. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was determined using the following formula:

$$\text{Chelating activity}=(1-A_1/A_0)\times 100 \qquad /4/$$

where A_0 is the absorbance of the control, and A_1 is the absorbance of the samples. The control contained $FeCl_2$ and ferrozine, with complex formation molecules. EDTA was used as positive control.

Reducing power

The reduction potential of all samples was determined by the method of Oyaizu (*29*). Different concentrations of test samples (0.05–0.80 mg/mL) were mixed with phosphate buffer (2.5 mL, 0.2 M, pH=6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1 %). The mixture was incubated at 50 °C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10 %) was added to the mixture, which was then subjected to centrifugation (10 min, 1000×*g*). The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1 %), and the absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicated greater reduction potential.

Determination of free phenolic acid composition

Wheat samples were analyzed for their soluble free phenolic acid composition. Acetone/methanol/water (7:7:6, by volume) was used to extract the free phenolic acids, and each extract was redissolved in acetonitrile. Phenolic acid composition in the methanol solution was analyzed by HPLC using a Phenomenex C_{18} column (250×4.6 mm; Torrance, CA, USA) according to an established protocol (*30*). Phenolic acids were separated using a linear gradient elution program with a mobile phase containing solvent A (acetic acid/H_2O, 2:98, by volume) and solvent B (acetic acid/acetonitrile/H_2O, 2:30:68, by volume). The solvent gradient was programmed from 10 to 100 % B in 42 min with a flow rate of 1.0 mL/min. Identification of phenolic acids was accomplished by comparing the retention time of peaks and fragmentation parameters of the mass spectroscopy of the standard compounds. The quantifications of phenolic compounds were performed by the internal standard method.

Statistical analysis

All tests were conducted in triplicate. Data were reported as mean value±standard deviation. Analysis of variance and significant differences among the mean values were tested by one-way ANOVA using SPSS software (v. 13.0 for Windows, SPSS Inc., Chicago, IL, USA). The experimental data were subjected to an analysis of variance for a completely random design to determine the least significant difference among the mean values at the level of 0.05.

Results and Discussion

Solvent extraction yield

It has been suggested that no single solvent can extract all the antioxidants from food because of their vari-

ation in solubility and polarity (*31*). In the present study, solvents with different polarities including acidified water, ethanol (70 %) and acetone (70 %) were used as solvents to extract antioxidants from wheat before and after the fermentation with *C. militaris*.

Table 1 shows the extraction yields of various solvents. In accordance with the observation of Sun and Ho (*31*), the extraction yield was found to be dependent on the solvent. The extraction yields of unfermented wheat ranged from 5.47–34.32 %, while the fermented wheat had an extraction yield ranging from 6.59 to 43.52 %. Among the tested solvents, acidified water showed the highest extract yield of 34.32 and 43.52 % respectively, with unfermented and *C. militaris*-fermented wheat. The yields of water extracts from the two products might be due to the fact that they contained more water-soluble substances. Fermentation-induced structural breakdown of wheat cell walls may occur, leading to the enhancement of the extraction yield.

Contents of total polyphenols and total flavonoids

Table 1 also shows the total polyphenolic and flavonoid content in the unfermented and fermented wheat extracts expressed as mass of gallic acid equivalent per mass of extract (in mg/g) and mass of rutin equivalent per mass of extract (in mg/g), respectively. A total polyphenolic content ranging from 29.37 to 54.16 and from 32.79 to 66.37 mg/g, respectively, was noted in the extracts of unfermented and fermented wheat. The acetone extract showed the highest total polyphenolic content, which was similar to the results of Juan and Chou (*32*). On the other hand, the total polyphenolic content observed in the water extract was the lowest.

Similar to phenols, the content of total flavonoids was also found to vary with the solvent used for the extraction, with ethanol extract containing the highest flavonoid content among the various extractions of the unfermented and fermented wheat. Furthermore, it was also found that the total polyphenolic and flavonoid contents of the fermented wheat extract are significantly higher than the respective extracts of the unfermented wheat.

C. militaris can catalyze the release of total polyphenols and flavonoids from the wheat substrate during fermentation or produce these antioxidants by itself, which can thus lead to an increase in the content of these compounds. Similar results were also found in black soybeans fermented with fungi (*32*).

DPPH radical scavenging effect

DPPH$^{\cdot}$ is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The decrease in the absorbance of DPPH radical is caused by antioxidants, which react with the radical. The model of scavenging the stable DPPH radical is a widely used method to evaluate antioxidant activities in a relatively short time compared to other methods. The addition of the extracts to the DPPH solution caused a rapid decrease in the absorbance at 517 nm. The degrees of discoloration indicate the scavenging capacity of the extract. Table 2 shows the dose-response effect of the various solvent extracts of unfermented wheat and *C. militaris*-fermented wheat on the DPPH radical scavenging activity. It was found that the DPPH$^{\cdot}$ scavenging effect of wheat extracts, regardless of the extraction solvent and fermentation, increased with the increase of their dosage. As shown in Table 2, ethanol and acetone extracts of wheat at a concentration of 0.80 mg/mL exhibited more than 98 % scavenging activity of DPPH radical. In comparison, water extracts of unfermented and fermented wheat at this concentration showed only 67.18 and 80.60 % scavenging activity of DPPH radical. Furthermore, it was also found that the DPPH$^{\cdot}$ scavenging activity of water extracts of *C. militaris*-fermented wheat is significantly higher than of the respective extracts of the unfermented wheat. The effect of antioxidants on the DPPH radical scavenging was thought to result from their hydrogen donating ability (*33*). These results revealed that different extracts from unfermented and *C. militaris*-fermented wheat can react with highly active free radicals.

Fe^{2+}-chelating ability

It has been well recognized that transition metal ions such as those of iron and copper are important catalysts for the generation of the free radicals which initiate the radical chain reaction or the radical-mediated lipid peroxidation (*34*).

Chelating agents may inhibit the formation of radicals by stabilizing the transition metals, consequently reducing free radical damage. As shown in Table 2, the Fe^{2+}-chelating ability of both unfermented and *C. militaris*-fermented wheat extracts, regardless of the kind of solvent used to prepare the extract, increased as the dosage of extract increased. It was also found that fermented wheat extract exhibited a significantly higher Fe^{2+}-che-

Table 1. Various solvent extraction yields, total polyphenolic and total flavonoid content of unfermented and *Cordyceps militaris*-fermented wheat (*Triticum aestivum* Linn.)

Solvent	Extraction yield/%, by mass		Total polyphenolic content as gallic acid equivalent per mass of extract/(mg/g)		Total flavonoid content as rutin equivalent per mass of extract/(mg/g)	
	unfermented wheat	fermented wheat	unfermented wheat	fermented wheat	unfermented wheat	fermented wheat
70 % ethanol	$(8.63\pm0.28)^{bB}$	$(10.21\pm0.59)^{aB}$	$(36.94\pm2.49)^{bB}$	$(41.63\pm5.66)^{aB}$	$(65.23\pm6.82)^{aC}$	$(72.65\pm5.47)^{aC}$
70 % acetone	$(5.47\pm0.11)^{bA}$	$(6.59\pm0.35)^{aA}$	$(54.16\pm3.84)^{bC}$	$(66.37\pm4.21)^{aC}$	$(29.56\pm3.21)^{aB}$	$(32.27\pm2.36)^{aB}$
water	$(34.32\pm2.36)^{bC}$	$(43.52\pm1.16)^{aC}$	$(29.37\pm2.36)^{bA}$	$(32.79\pm2.11)^{aA}$	$(5.82\pm0.98)^{bA}$	$(13.29\pm1.89)^{aA}$

Values are presented as means±standard deviation (N=3). Means with different lower case letters in the same row are significantly different for the same extract ($p<0.05$) and means with different capital letters within a column are significantly different ($p<0.05$)

Table 2. Antioxidant activity of different extracts of unfermented and *Cordyceps militaris*-fermented wheat (*Triticum aestivum* Linn.)

Antioxidant activity	γ(sample)	Unfermented wheat			Fermented wheat		
	mg/L	70 % ethanol	70 % acetone	water	70 % ethanol	70 % acetone	water
DPPH· scavenging activity/%	0.05	(38.91±1.36)^a	(65.33±3.51)^b	(28.65±1.85)^b	(40.66±2.86)^a	(74.33±4.92)^a	(36.70±2.35)^a
	0.10	(64.52±2.98)^b	(89.23±6.37)^a	(38.27±2.64)^a	(71.21±6.35)^a	(90.20±14.36)^a	(38.72±1.69)^a
	0.30	(78.11±2.41)^a	(93.51±2.96)^a	(44.91±3.86)^b	(79.25±4.85)^a	(94.60±9.81)^a	(51.35±3.57)^a
	0.60	(85.36±5.99)^a	(96.47±9.61)^a	(63.20±3.68)^b	(85.86±9.80)^a	(96.22±5.47)^a	(76.47±6.34)^a
	0.80	(96.18±3.74)^a	(98.29±3.89)^a	(67.18±4.44)^b	(98.24±11.23)^a	(99.11±4.39)^a	(80.60±2.14)^a
hydroxyl radical scavenging activity/%	0.05	(40.10±2.86)^b	(31.26±1.34)^b	(23.53±2.85)^b	(45.25±1.86)^a	(38.66±2.44)^a	(19.93±0.98)^a
	0.10	(43.55±2.65)^b	(48.90±2.89)^b	(32.60±2.68)^a	(49.65±2.59)^a	(59.15±3.15)^a	(38.23±2.54)^a
	0.30	(56.86±3.64)^a	(61.24±0.94)^a	(35.24±1.86)^b	(58.23±6.41)^a	(66.64±6.31)^a	(44.27±3.97)^a
	0.60	(73.48±2.94)^a	(69.24±3.89)^b	(48.16±3.41)^a	(71.85±5.81)^a	(78.29±2.89)^a	(49.52±6.31)^a
	0.80	(75.69±3.57)^b	(74.66±6.65)^a	(50.53±4.86)^b	(87.90±2.89)^a	(81.51±8.31)^a	(58.60±2.89)^a
ferrous ion chelating activity/%	0.05	(9.38±1.06)^b	(24.35±4.31)^b	(14.47±1.35)^a	(14.44±0.65)^a	(33.26±2.13)^a	(15.82±5.47)^a
	0.10	(16.34±2.31)^a	(31.39±3.96)^b	(22.22±2.55)^b	(21.76±5.31)^a	(38.60±3.21)^a	(31.29±3.85)^a
	0.30	(40.35±2.08)^b	(50.21±8.47)^a	(36.80±3.64)^b	(58.66±4.40)^a	(59.25±5.41)^a	(45.94±6.67)^a
	0.60	(49.55±1.57)^b	(67.40±6.54)^a	(41.73±2.89)^b	(68.15±2.86)^a	(66.80±2.98)^a	(52.47±5.96)^a
	0.80	(58.36±2.99)^b	(79.16±3.81)^a	(50.62±6.51)^b	(68.92±3.54)^a	(84.13±6.87)^a	(70.85±2.89)^a
reducing power	0.05	(0.136±0.011)^a	(0.206±0.008)^b	(0.080±0.006)^b	(0.213±0.023)^a	(0.338±0.041)^a	(0.121±0.011)^a
	0.10	(0.216±0.013)^b	(0.322±0.015)^b	(0.101±0.012)^a	(0.297±0.018)^a	(0.456±0.012)^a	(0.120±0.016)^a
	0.30	(0.324±0.020)^a	(0.586±0.021)^b	(0.148±0.018)^b	(0.361±0.017)^a	(0.743±0.020)^a	(0.196±0.009)^a
	0.60	(0.538±0.024)^a	(0.774±0.047)^b	(0.206±0.014)^a	(0.524±0.023)^a	(0.825±0.023)^a	(0.233±0.023)^a
	0.80	(0.613±0.016)^b	(0.851±0.057)^a	(0.251±0.020)^a	(0.699±0.036)^a	(0.861±0.031)^a	(0.269±0.028)^a

Values are presented as means±standard deviation (N=3) and means with different lower case letters within a row are significantly different ($p<0.05$)

lating ability than did the respective extract of unfermented wheat. At the same dosage level, the acetone extract of wheat, regardless of fermentation, exhibited the highest Fe^2-chelating ability followed by ethanol and acidified water extracts. Ferrous ions can stimulate lipid peroxidation by Fenton reaction, and also accelerate peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals, which can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation (*14*). Chelating agents may serve as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ions (*35*). Accordingly, it is suggested that the ferrous ion chelating effects of these extracts can be somewhat beneficial in protection against oxidative damage.

Reducing power

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (*36*). Table 2 shows the dose response of the various solvent extracts of unfermented and *C. militaris*-fermented wheat for reducing power. It was found that *C. militaris*-fermented wheat extract exhibited significantly higher reducing power ability than did the respective extract of unfermented wheat. At the same dosage level, the acetone extract of wheat, regardless of fermentation, exhibited the highest reducing power followed by ethanol and water extracts in descending order.

Different studies have indicated that the reducing power of bioactive compounds is associated with antioxidant activity (*37*). Therefore, the antioxidant activity of the tested samples might partially be a result of their reducing power. Okuda *et al*. (*38*) mentioned that the reducing capacity of tannins prevented liver injury by inhibiting the formation of lipid peroxides. Furthermore, the reducing compounds can react directly with peroxides and also with certain precursors and, thereby prevent peroxide formation (*39*). The reducing capacity of various examined extracts might be due to their hydrogen-donating ability. Therefore, the examined samples might contain reducing compounds, which can react with free radicals to stabilize and terminate radical chain reactions.

Hydroxyl radical scavenging

The hydroxyl radicals, generated by the Fenton reaction in the system, were scavenged by different solvent extracts of unfermented and fermented wheat. The scavenging effects of all samples are shown in Table 2. Among the six samples, 70 % acetone extract of fermented wheat exhibited the strongest scavenging activity against hydroxyl radical. Regarding unfermented wheat, at the concentration of 0.05–0.80 mg/mL, the scavenging effect was 23.53–50.53 % of water extract, 31.26–74.66 % of acetone extract, and 40.10–75.69 % of ethanol extract. All extracts showed dose-dependent scavenging activity. At the same dosage level, the hydroxyl radical scavenging activity of the *C. militaris*-fermented wheat extract was higher than the respective extract of the unfermented wheat.

Hydroxyl radicals are the most reactive and predominant radicals generated endogenously during aerobic metabolism among the free radicals, which can be formed from superoxide anion and hydrogen peroxide, in the presence of metal ions, such as copper or iron, and cause the ageing of human body and some diseases (*37*), interact with the purine and pyrimidine bases of DNA as well as abstract hydrogen atoms from biological molecules, leading to the formation of sulphur radicals able to combine with oxygen to generate oxysulphur radicals, a number of which damage biological molecules (*14*).

For hydroxyl radicals, there are two types of antioxidative mechanisms: one suppresses the generation of hydroxyl radicals, and the other scavenges the generated hydroxyl radicals. In the former, the antioxidant activity may ligate to the metal ions, which react with H_2O_2 to give the metal complexes.

EC_{50} values of wheat extracts

As shown in Table 3, the EC_{50} values of the tested samples were calculated for comparison. With regard to the scavenging ability against DPPH radicals, acetone extracts were more effective than ethanol and acidified water extracts, as evidenced by lower EC_{50} values (<0.05 mg/mL). In addition, the fermented wheat was more effective than the unfermented wheat. With regard to hydroxyl radical scavenging activity, the effectiveness of the three extracts was in a descending order: acetone extract>ethanol extract>acidified water for both the unfermented and fermented wheat. For the acetone extract, the EC_{50} values were 0.12 mg/mL of unfermented and 0.07 mg/mL of fermented wheat. It is obvious that the hydroxyl radical scavenging activity of fermented products was greatly enhanced as mycelia grew. With regard to chelating ability of ferrous ions, the acetone extract showed the highest activity with the lowest EC_{50} values, and the fermented products showed higher activity than the unfermented wheat. Regarding the effectiveness of the reducing power of the tested samples (Table 3), the acidified water extract of the unfermented and fermented wheat showed the lowest activity (EC_{50} values>0.80 mg/mL) and there was no significant difference between the ethanol extracts of unfermented and fermented wheat.

Free phenolic acid composition

Ferulic, *p*-coumaric, vanillic, syringic and caffeic acids were detected in unfermented and *C. militaris*-fermented wheat; ferulic acid showed the highest content, followed by *p*-coumaric, vanillic, syringic and caffeic acid (Fig. 1). It was interesting to find that the content of the above five phenolic acids in *C. militaris*-fermented wheat was significantly higher than in the unfermented wheat, except for caffeic and vanillic acids. Phenolic acids and flavonoids represent the most common forms of phenolic compounds found in whole grains, and they are among the major and most complex groups of phytochemicals in the cereal grain, with a number of types that exist as soluble free compounds, soluble conjugates that are esterified to sugars and other low molecular mass components, and insoluble bound forms. The latter are the major form in wheat and are involved in the crosslinking of polymers, particularly arabinoxylans in the grain cell walls. There are maybe two reasons for the above results, one is that the enzyme of *C. militaris* catalyzes the release of phenolic acid from the insoluble bound forms, and the other is that the phenolic acid may be the secondary metabolite of *C. militaris*.

The above results can be explained by the fact that levels of bioactive compounds can be modified during

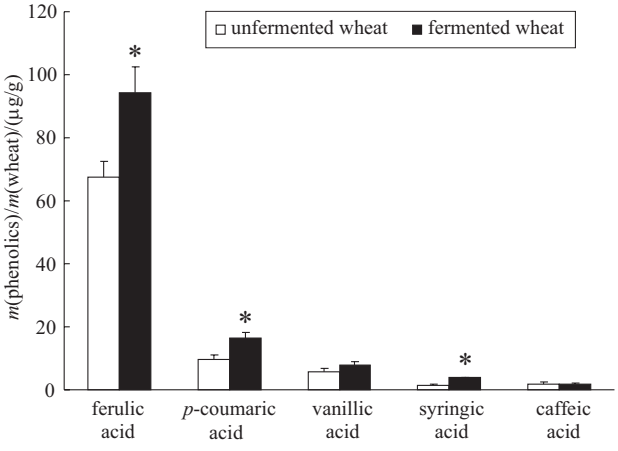

Fig. 1. Free phenolic acid content in unfermented and *Cordyceps militaris*-fermented wheat (*Triticum aestivum* Linn.). Values are presented as means±standard deviation (*N*=3). *p<0.05 compared with the unfermented wheat

Table 3. EC_{50} values of different extracts from unfermented and *Cordyceps militaris*-fermented wheat (*Triticum aestivum* Linn.)

	EC_{50} value/(mg/mL)					
	Unfermented wheat			Fermented wheat		
	70 % ethanol	70 % acetone	water	70 % ethanol	70 % acetone	water
DPPH$^{\cdot}$ scavenging activity	$(0.08\pm0.01)^{aA}$	$<0.05^{B}$	$(0.38\pm0.06)^{aC}$	$(0.06\pm0.02)^{aA}$	$<0.05^{B}$	$(0.32\pm0.03)^{aC}$
hydroxyl radical scavenging activity	$(0.21\pm0.03)^{aA}$	$(0.12\pm0.02)^{aB}$	$(0.76\pm0.06)^{aC}$	$(0.11\pm0.01)^{bB}$	$(0.07\pm0.01)^{bD}$	$(0.61\pm0.05)^{bE}$
ferrous ion chelating activity	$(0.61\pm0.06)^{aA}$	$(0.29\pm0.04)^{aB}$	$(0.79\pm0.09)^{aC}$	$(0.26\pm0.02)^{bB}$	$(0.21\pm0.05)^{aB}$	$(0.44\pm0.04)^{bD}$
reducing power	$(0.52\pm0.04)^{aA}$	$(0.23\pm0.02)^{aB}$	$>0.80^{C}$	$(0.58\pm0.06)^{aA}$	$(0.13\pm0.03)^{bD}$	$>0.80^{C}$

EC_{50}: the effective concentration at which 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals were scavenged by 50 %, hydroxyl radicals were scavenged by 50 %, ferrous ions were chelated by 50 %, and the absorbance was 0.5 for reducing power. EC_{50} value was obtained by interpolation from linear regression analysis.

Each value is expressed as mean±standard deviation (*N*=3). Mean values with different capital letters within a row are significantly different (p<0.05). Mean values with different lower case letters within a column are significantly different (p<0.05)

fermentation by *C. militaris*. Also, fermentation-induced structural breakdown of wheat cell wall may occur, leading to the liberation and/or synthesis of various bioactive compounds. During fermentation, bound phenols can be released by enzymatic treatment of samples prior to extraction (*40*). Other authors have also demonstrated that fermentation has a positive influence on the total phenolic content and antioxidative activity of cereals, but the degree of influence depends on the microorganism species (*41*). The change of the content of free phenolic acid in the unfermented and *C. militaris*-fermented wheat supports the above results.

Furthermore, the difference in the antioxidant activity of the investigated solvent extracts was consistent with the results of Bonoli *et al*. (*42*). The solvent mixtures employed in the present study possessed different polarity. It is reasonable to expect that these solvents will selectively extract antioxidant compounds such as phenols depending on their chemical structures, polarities and solubilities (*43*). Such interactions may thus have led to the variation in antioxidant effects observed among the various solvent extracts used.

Conclusions

Results obtained in the present study demonstrate that fermentation with *C. militaris* can enhance the contents of total polyphenols and flavonoids as well as the antioxidant activity of wheat. Furthermore, this study also revealed that the antioxidant activity of the extracts varied depending on the extraction solvent. The acetone extract exhibited the highest antioxidant activity followed by ethanol and water extract. Therefore, fermentation with *C. militaris* can be applied as a tool to develop wheat as a health food or health food ingredient possessing multifunctional properties including antioxidant activity, which might be used as an alternative supplement to synthetic antioxidants since it is environmentally friendly and safe for consumption.

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References

1. K.C. Sekhar Rao, N.G. Karanth, A.P. Sattur, Production of nigerloxin, an enzyme inhibitor and a free radical scavenger, by *Aspergillus niger* using solid state fermentation, *Process Biochem. 40* (2005) 2517–2522.
2. C. Botella, A. Diaz, I. de Ory, C. Webb, A. Blandino, Xylanase and pectinase production by *Aspergillus awamori* on grape pomace in solid state fermentation, *Process Biochem. 42* (2007) 98–101.
3. C.H. Lin, Y.T. Wei, C.C. Chou, Enhanced antioxidative activity of soybean koji prepared with various filamentous fungi, *Food Microbiol. 23* (2006) 628–633.
4. P. McCue, K. Shetty, Role of carbohydrate-cleaving enzymes in phenolic antioxidant mobilization from whole soybean fermented with *Rhizopus oligosporus*, *Food Biotechnol. 17* (2003) 27–37.
5. D.A. Vattem, K. Shetty, Ellagic acid production and phenolic antioxidant activity in cranberry pomace (*Vaccinium macrocarpon*) mediated by *Lentinus edodes* using a solid-state system, *Process Biochem. 39* (2003) 367–379.
6. G.C. Yen, Y.C. Chang, S.W. Su, Antioxidant activity and active compounds of rice koji fermented with *Aspergillus candidus*, *Food Chem. 83* (2003) 49–54.
7. K. Katina, A. Laitila, R. Juvonen, K.H. Liukkonen, S. Kariluoto V. Piironen *et al.*, Bran fermentation as a means to enhance technological properties and bioactivity of rye, *Food Microbiol. 24* (2007) 175–186.
8. C.H. Liang, J.L. Syu, Y.L. Lee, J.L. Mau, Nonvolatile taste components of solid-state fermented adlay and rice by *Phellinus linteus*, *LWT-Food Sci. Technol. 42* (2009) 1738–1743.
9. C.H. Liang, J.L. Syu, J.L. Mau, Antioxidant properties of solid-state fermented adlay and rice by *Phellinus linteus*, *Food Chem. 116* (2009) 841–845.
10. S.J. Huang, S.Y. Tsai, S.Y. Lin, C.H. Liang, P.Y. Lian, J.L. Mau, Preparation of culinary-medicinal Maitake mushroom, *Grifola frondosa* (Dicks.:Fr.) S.F. Gray (Aphyllophoromycetideae)-fermented wheat and its antioxidant properties, *Int. J. Med. Mushr. 13* (2011) 61–71.
11. C.H. Liang, S.Y. Tsai, S.J. Huang, Z.C. Liang, J.L. Mau, Taste quality and antioxidant properties of medicinal mushrooms *Phellinus linteus* and *Sparassis crispa* mycelia, *Int. J. Med. Mushr. 12* (2010) 141–150.
12. C.H. Liang, Y.L. Lee, H.C. Kuo, T.P. Wu, S.Y. Jian, W.L. Huang, Preparation of novel culinary-medicinal mushroom products using solid-state fermentation and their taste quality, *Int. J. Med. Mushr. 11* (2009) 141–156.
13. D.R. Jacobs Jr., K.A. Meyer, L.H. Kushi, A.R. Folsom, Is whole grain intake associated with reduced total and cause-specific death rates in older women? The Iowa women's heath study, *Am. J. Public Health, 89* (1999) 322–329.
14. B. Halliwell: The Use of Antioxidants in Foods. In: *Free Radicals and Food Additives*, O.I. Aruoma, B. Halliwell (Eds.), Taylor & Francis Ltd, London, UK (1991) pp. 121–150.
15. J.P. Kehrer, Free radicals as mediators of tissue injury and disease, *Crit. Rev. Toxicol. 23* (1993) 21–48.
16. G.M. Williams, M.J. Iatropoulos, J. Whysner, Safety assessment to butylated hydroxyanisole and butylated hydroxytoluene as antioxidant food additives, *Food Chem. Toxicol. 37* (1999) 1027–1038.
17. K.A. Meyer, L.H. Kushi, D.R. Jacobs, J.E. Slavin, T.A. Sellers, A.R. Folsom, Carbohydrates, dietary fiber, and incident type 2 diabetes in older women, *Am. J. Clin. Nutr. 71* (2000) 921–930.
18. S. Liu, M.J. Stampfer, F.B. Hu, E. Giovannucci, E. Rimm, J.E. Manson *et al.*, Whole-grain consumption and risk of coronary heart disease: Results from the Nurses' Health study, *Am. J. Clin. Nutr. 70* (1999) 412–419.
19. B.K. Yang, J.Y. Ha, S.C. Jeong, S. Das, J.W. Yun, Y.S. Lee *et al.*, Production of exo-polymers by submerged mycelial culture of *Cordyceps militaris* and its hypolipidemic effect, *J. Microbiol. Biotechnol. 10* (2000) 784–788.
20. S.Y. Won, E.H. Park, Anti-inflammatory and related pharmacological activities of cultured mycelia and fruiting bodies of *Cordyceps militaris*, *J. Ethnopharmacol. 96* (2005) 555–561.
21. K. Nakamura, Y. Yamaguchi, S. Kagota, Y.M. Kwon, K. Shinozuka, M. Kunitomo, Inhibitory effects of *Cordyceps sinensis* on spontaneous liver metastasis of Lewis lung carcinoma and B16 melanoma cells in syngeneic mice, *Jpn. J. Pharmacol. 79* (1999) 335–341.

22. J.K.H. Cheung, J. Li, A.W.H. Cheung, Y. Zhu, K.Y.Z. Zheng, C.W.C. Bi *et al.*, Cordysinocan, a polysaccharide isolated from cultrued *Cordyceps*, activates immune responses in cultured T-lymphocytes and macrophages: Signaling cascade and induction of cytokines, *J. Ethnopharmacol. 124* (2009) 61–68.
23. R. Yu, Y. Yin, W. Yang, W. Ma, L. Yang, X. Chen *et al.*, Structural elucidation and biological activity of a novel polysaccharide by alkaline extraction from cultured *Cordyceps militaris*, *Carbohydr. Polym. 75* (2009) 166–171.
24. V.L. Singleton, R. Orthofer, R.M. Lamuela-Raventós, Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent, *Methods Enzymol. 299* (1999) 152–178.
25. Z. Jia, M. Tang, J. Wu, The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals, *Food Chem. 64* (1999) 555–559.
26. B. Halliwell, J.M.C. Gutteridge, O.I. Aruoma, The deoxyribose method: A simple 'test-tube' assay for determination of rate constants for reactions of hydroxyl radicals, *Anal. Biochem. 165* (1987) 215–219.
27. C. Sánchez-Moreno, J.A. Larrauri, F. Saura-Calixto, A procedure to measure the antiradical efficiency of polyphenols, *J. Food Sci. Agric. 76* (1998) 270–276.
28. T.C.P. Dinis, V.M.C. Madeira, L.M. Almeida, Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers, *Arch. Biochem. Biophys. 315* (1994) 161–169.
29. M. Oyaizu, Studies on product of browning reaction prepared from glucose amine, *Jpn. J. Nutr. 44* (1986) 307–315.
30. L. Yu, S. Haley, J. Perret, M. Harris, J. Wilson, M. Qian, Free radical scavenging properties of wheat extracts, *J. Agric. Food Chem. 50* (2002) 1619–1624.
31. T. Sun, C.T. Ho, Antioxidant activities of buckwheat extracts, *Food Chem. 90* (2005) 743–749.
32. M.Y. Juan, C.C. Chou, Enhancement of antioxidant activity, total phenolic and flavonoid content of black soybeans by solid state fermentation with *Bacillus subtilis* BCRC 14715, *Food Microbiol. 27* (2010) 586–591.
33. J. Baumann, G. Wurn, V. Bruchlausen, Prostaglandin synthetase inhibiting O_2^- radical scavenging properties of some flavonoids and related phenolic compounds, *N-S. Arch. Pharmacol. 308* (2002) R27.
34. W.W. Nawar: Lipids. In: *Food Chemistry,* O.R. Fennema (Ed.), Marcel Dekker, New York, NY, USA (1996) pp. 255–278.
35. M.H. Gordon: The Mechanism of Antioxidant Action *in vitro*. In: *Food Antioxidants*, B.J.F. Hudson (Ed.), Elsevier, London, UK (1990) pp. 1–18.
36. S. Meir, J. Kanner, B. Akiri, S. Philosoph-Hadas, Determination and involvement of aqueous reducing compounds in oxidative defense systems of various senescing leaves, *J. Agric. Food Chem. 43* (1995) 1813–1819.
37. P. Siddhuraju, K. Becker, The antioxidant and free radical scavenging activities of processed cowpea (*Vigna unguiculata* (L.) Walp.) seed extracts, *Food Chem. 101* (2007) 10–19.
38. T. Okuda, Y. Kimura, T. Yoshida, T. Hatano, H. Okuda, S. Arichi, Studies on the activities of tannins and related compounds from medicinal plants and drugs. I. Inhibitory effects on lipid peroxidation on mitochondria and microsomes of liver, *Chem. Pharm. Bull. 31* (1983) 1625–1631.
39. T. Shimada, K. Fujikawa, K. Yahara, T. Nakamura, Antioxidant properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion, *J. Agric. Food Chem. 40* (1992) 945–948.
40. B. Bartolomé, C. Gómez-Cordovés, Barley spent grain: release of hydroxycinnamic acids (ferulic and *p*-coumaric acids) by commercial enzyme preparations, *J. Sci. Food Agric. 79* (1999) 435–439.
41. S. Kariluoto, M. Aittamaa, M. Korhola, H. Salovaara, L. Vahteristo, V. Piironen, Effects of yeasts and bacteria on the levels of folates in rye sourdoughs, *Int. J. Food Microbiol. 106* (2006) 137–143.
42. M. Bonoli, E. Marconi, M.F. Caboni, Free and bound phenolic compounds in barley (*Hordeum vulgare* L.) flours – Evaluation of the extraction capability of different solvent mixtures and pressurized liquid methods by micellar electrokinetic chromatography and spectrophotometry, *J. Chromatogr. A, 1057* (2004) 1–12.
43. H. Zhao, J. Dong, J. Lu, J. Chen, Y. Li, L. Shan *et al.*, Effects of extraction solvent mixtures on antioxidant activity evaluation and their extraction capacity and selectivity for free phenolic compounds in barley (*Hordeum vulgare* L.), *J. Agric. Food Chem. 54* (2006) 7277–7286.