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Comparative Study of the Effects of Solid-State Fermentation with Three Filamentous Fungi on the Total Phenolics Content (TPC), Flavonoids, and Antioxidant Activities of Subfractions from Oats (*Avena sativa* L.)

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ABSTRACT: The aim of present work was to investigate the effect of solid-state fermentation with filamentous fungi (*Aspergillus oryzae* var. *effuses*, *Aspergillus oryzae*, and *Aspergillus niger*) on total phenolics content (TPC), flavonoids, and antioxidant activities of four subfractions of oat, namely, *n*-hexane, ethyl acetate (EA), *n*-butanol, and water, and compare them to their corresponding subfractions of unfermented oat. The TPC and total flavonoids increased dramatically, especially in EA subfractions ($p < 0.05$). The levels of antioxidant activity of subfractions were also significantly enhanced ($p < 0.05$). The highest antioxidant activities were also found in the EA subfractions. The polyphenols in EA were analyzed by high-performance liquid chromatography at 280 nm. Most polyphenols were increased remarkably, especially ferulic and caffeic acids. There was a clear correlation between the TPC and antioxidant activity. In conclusion, fungi fermentation is a potential bioprocess for increasing the TPC, flavonoids, and antioxidant activities of oat-based food.

KEYWORDS: *Solid-state fermentation, TPC, flavonoids, antioxidant activity, fungi*

1. INTRODUCTION

In normal human activities, it is unavoidable to generate reactive oxygen species (ROS), such as superoxide anion radicals, hydroxyl radicals, singlet oxygen, etc. The body can eliminate most ROS through antioxidant defense systems, such as glutathione, catalase, and superoxide dismutase, to prevent ROS damage under normal conditions.[1] However, when severe oxidative stress is caused by pollution, smoking, and other stresses, these innate defenses become inadequate, resulting in ROS-mediated tissue damage.[2] The overproduction of ROS is believed to contribute to coronary heart disease, cancer, age-related degenerative processes, and other chronic diseases.[3] Therefore, the body requires additional strategies to deal with excess ROS. Diet-supplement antioxidants can potentially meet this requirement and act as a nonenzymatic antioxidant defense system to scavenge excess ROS.[4]

Many synthetic antioxidants, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), *tert*-butylhydroquinone (TBHQ), etc., are widely used in foods. However, recently, their application has been restricted because these synthetic antioxidants are suspected of being highly carcinogenic.[5] Thus, natural antioxidants from plants, such as vitamin C, vitamin E, flavonoids, polyphenolic compounds, etc., have received increasing attention as replacements for synthetic antioxidants, and these natural antioxidants in food may play important roles in enhancing the natural resistance of the body to ROS damage.[6]

Cereals, as one of the main food sources for humans, provide energy, protein, and other nutrition elements. In many studies, cereals were also found to contain a wide range of chemical substances having good antioxidant activity.[7] Madhujith and Shahidi[8] found that barley contains a large number of phenolic antioxidants that could effectively scavenge peroxyl and hydroxyl radicals and 1,1-diphenyl-2-picrylhydrazyl radical 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH). Previous studies also found that the wheat extracts have potential antioxidant properties and the wheat phenolics appear to serve as powerful antioxidants.[9,10]

The process of fermentation by microorganisms has been used for centuries to improve the nutritional value of foods. Lee et al.[11] have shown that the total phenolics and anthocyanin contents and antioxidative activity of black bean enhance remarkably after fermentation by filamentous fungi. Studies also found that the constituents and bioactivity properties of cereal can be distinctly changed during fermentation.[12] Recently, Dordević et al.[13] studied the influence of fermentation on antioxidant properties of buckwheat, wheat germ, barley, and rye. They found that the total phenolics content (TPC) increased significantly and the antioxidant activities were also enhanced through fermentation by lactic acid bacteria and yeast.

In comparison to wheat (*Triticum aestivum* L. and *Triticum durum* L.), oat (*Avena sativa* L.) is consumed in lower quantities worldwide. However, oat has the advantage that it has been recognized as a kind of healthy food for some time. The bran of oat is rich in antioxidants, such as avenanthramides, vitamin E, and other phenolic compounds having strong antioxidant activity *in vitro*.[14] The effect of fermentation on the biochemical and physicochemical properties of oat by *Lactobacillus* had been

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investigated previously.[15] However, the influence of fermentation on the antioxidant activity and polyphenol compounds of whole oat has received little attention. Therefore, the objective of the present work is to study the influence of solid-state fermentation by a variety of fungi (generally recognized as safe) on the TPC, flavonoids, and antioxidant activities of four subfractions from oat (*A. sativa* L.) ethanol extract.

2. MATERIALS AND METHODS

2.1. Chemicals. 2,2-Azobis-2-methylpropion-amidine dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), fluorescein sodium salt, *p*-coumaric acid, protocatechuic acid, gallic acid, chlorogenic acid, cinnamic acid, caffeic acid, ferulic acid, and rutin were purchased from Sigma-Aldrich. Avenanthramide standards, *N*-(3,4-dihydroxy-(*E*)-cinnamoyl)-5-hydroxyanthranilic acid (**2c**), *N*-(4-hydroxy-(*E*)-cinnamoyl)-5-hydroxyanthranilic acid (**2p**), and *N*-(4-hydroxy-3-methoxy-(*E*)-cinnamoyl)-5-hydroxyanthranilic acid (**2f**), were kindly provided by Dr. Mitchell L. Wise, United States Department of Agriculture (USDA). The Folin−Ciocalteu reagent was purchased from Merck and Co., Inc.

2.2. Microorganism. *Aspergillus oryzae* var. *effuses*, *Aspergillus oryzae*, and *Aspergillus niger* employed for this study were purchased from the Institute of Microbiology, Chinese Academy of Sciences, which were kept in lyophilized form and activated in potato dextrose agar (PDA). After three passages in PDA media, all fungi were cultivated at 25 °C for 7 days to produce spores. Spore suspensions were prepared by suspending the spores from a PDA culture in 20 mL of normal saline (0.9% NaCl). The spore suspensions were maintained at 4 °C for further use.

2.3. Fermentation of Oat. Oat (harvested in 2010) was purchased from the Chinese Academy of Agricultural Sciences (genotype, G4, Beijing, China) in November 2010 and was stored at 4 °C prior to use. Oat was soaked in water for 8 h and then smashed using the HK-06A high-speed grinder (Changsha, Hunan, China), sterilized at 121 °C for 15 min. The solid-state fermentation culture was prepared by inoculating with a conidiospore suspension (10^6 spores/g of oat) in a 300 mL Erlenmeyer flask containing 50 g of oat smash and was incubated at 25 °C for 3 days under static conditions. During the cultivation period, the oat was stirred after 24, 48, and 60 h of cultivation. The oat with treated the same, except that inoculating conidiospore suspension was used as the control.

2.4. Preparation of Extracts. Native and fermented oat materials were ultrasonically extracted with 80% ethanol at 45 °C for 30 min. After cooling to room temperature, the slurries were centrifuged at 2862*g* for 15 min and the supernatant was collected. The residue was extracted once more under the same conditions, and the supernatants were combined. The solution was then evaporated at 45 °C under reduced pressure to obtain the ethanol extract. The ethanol extract was then dissolved in 80% methanol and sequentially extracted with *n*-hexane, ethyl acetate (EA), and water-saturated *n*-butanol, using liquid−liquid partition. After removal of the solvents, four subfractions were obtained, namely, *n*-hexane, EA, *n*-butanol, and water.

2.5. Determination of TPC. The TPC in all samples was determined according to the Folin−Ciocalteu method described by Singleton and Rossi.[16] Briefly, 1 mL of sample solutions was mixed with 1 mL of Folin−Ciocalteu reagent. After 1 min of incubation at room temperature, 1.5 mL of Na_2CO_3 solution (20% aqueous solution) was added to the mixtures, followed by the addition of 7.5 mL of distilled water. The mixtures were then kept in a constant-temperature water bath at 70 °C for 10 min. After cooling to room temperature, the absorbances were measured at 765 nm. The TPC was expressed as gallic acid equivalent (GAE) from the calibration curve of a gallic acid standard solution and expressed as milligrams of GAE/100 g. All samples were performed in triplicate.

2.6. Determination of Flavonoids. The total flavonoids in all samples were determined by a colorimetric method, with minor modification.[17] In brief, 1 mL of solved samples was placed in a 10 mL volumetric flask, distilled water was added to make 5 mL, and 0.3 mL of $NaNO_2$ was added. A total of 0.3 mL of $AlCl_3$ was added 5 min later, and the mixture was allowed to stand for another 6 min. A total of 2 mL of 1 M NaOH was added, and the total was made up to 10 mL with distilled water. The solution was mixed well again and allowed to stands for 30 min. The absorbance was measured against a blank at 510 nm, and the flavonoid contents was determined as rutin equivalent from the calibration curve of rutin standard solutions and expressed as milligrams of rutin/100 g of DW. All measurements were performed in triplicate.

2.7. Determination of Antioxidant Activity *in Vitro*. *2.7.1. Oxygen Radical Absorbance Capacity (ORAC) Method.* A fluorescein stock solution was prepared (7.98×10^{-4} mM) in phosphate buffer (75 mM, pH 7.4). AAPH was dissolved in phosphate buffer to a final concentration of 173 mM. The assay was performed using black 96-well microtiter plates (Nunc, Thermo Fisher Scientific, Roskilde, Denmark). The fluorescein solution (100 μL) mixed with sample extract (25 μL) was incubated at 37 °C for 10 min. The assay was initiated by the addition of 75 μL of AAPH solution. The fluorescence was read every minute for 120 min using a fluorescence filter with an excitation wavelength of 485 nm and an emission wavelength of 515 nm using a multidetection microplate reader, SpetraMax M2^e. The Trolox equivalents were calculated from a standard curve.

2.7.2. Cyclic Voltammetry (CV) Assay. The CV assay were carried out on a model CHI 620 C electrochemical analyzer (Chenhua, Shanghai, China), using a three-electrode system. The working electrode was a glassy carbon electrode. An Ag/AgCl electrode was used as the reference, and a platinum foil served as the auxiliary electrode. Before each scanning, the glassy carbon electrode was polished on alumina powder. The supporting electrolytes were the 0.2 M Britton−Robinson buffered solutions (BRS), containing 0.3 M KCl, at pH 7.24. All samples were prepared with methanol as the solvent and diluted into an appropriate concentration by BRS. Prior to each run, sample solutions were bubbled with N_2 for about 15 min to remove dissolved oxygen. Voltammetric scans were carried out from −0.4 V versus Ag/AgCl to 1.2 V with a scan rate of 400 mV/s at room temperature. The area under the anodic current wave (Q) was obtained by EC Application Software.

2.8. High-Performance Liquid Chromatography (HPLC) Analysis of EA Subfractions. The main phenolic compounds of all EA subfractions were analyzed as previously reported, with minor modification.[18] A HPLC system consisting of a Shimadzu HPLC (model LC-10ATvp Pumps and DGU-12A Degasser) equipped with a diode array detector (model SPD-M10Avp, Shimadzu, Kyoto, Japan) was employed in the present assay. The separation was performed on a Shim-Pack VP-ODS column (250 × 4.6 mm inner diameter, with a particle size of 5 μm) with a guard column (Shim-pack G VP-ODS, 10 × 4.6 mm inner diameter, with a particle size of 5 μm) (Shimadzu, Kyoto, Japan). Two solvents were used for the mobile phase: (A) acetonitrile and (B) 0.1% formic acid. The gradient elution used for the EA subfractions was 0−5 min, 10% A; 5−45 min, 10−60% A; 45−55 min, 60% A; 55−56 min, 60−10% A; and 56−70 min, 10% A. Each EA subfraction was injected to the column, using a 20 μL loop valve. The flow rate was 1.0 mL/min, and the detection was performed at 280 and 330 nm. Components were tentatively identified by a comparison of their retention times to those of authentic standards under identical analysis conditions at 280 and 330 nm. All standard and sample solutions were injected in triplicate.

2.9. Statistical Analysis. Data were expressed as the mean values ± standard deviation (SD) for each measurement. The data were also analyzed by one-way analysis of variance (one-way ANOVA). Tukey's procedure was used for significance of difference ($p < 0.05$). Analysis was performed with SPSS 13.0 (SPSS, Inc., Chicago, IL).

3. RESULTS AND DISCUSSION

3.1. TPC. The TPC of four subfractions from native oat ethanol extract is summarized in Table 1. In these four subfractions, the EA subfraction had the highest value of TPC ($p < 0.05$), followed by the *n*-butanol and water subfractions, while the *n*-hexane fraction had the lowest value ($p < 0.05$). The results indicate that

Table 1. Results of TPC of the Subfractions from Native and Fermented Oat (*A. sativa* L.) Ethanol Extract[a]

	n-hexane	EA	*n*-butanol	water
oat	1580.1 ± 62.6 a	3654.9 ± 41.8 a	2926.6 ± 80.9 a	2296.2 ± 56.1 a
fermented with *A. oryzae* var. *effuses*	1599.5 ± 60.8 a	10911.8 ± 197.4 b	5062.2 ± 131.8 b	3632.7 ± 73.1 b
fermented with *A. oryzae*	1428.8 ± 21.8 a	9912.7 ± 229.5 c	4067.8 ± 146.2 c	3228.3 ± 268.3 c
fermented with *A. niger*	1665.4 ± 67.9 a	8246.5 ± 151.0 d	3189.5 ± 106.6 a	2409.1 ± 78.8 a

[a]All measurements were expressed as the mean ± SD (n = 3). TPC was expressed as milligrams of GAE/100 g of DW. Means in columns without letters in common differ significantly ($p < 0.05$).

phenolic compounds in the oat ethanol extract were much more soluble in weak polar organic solvents (especially EA) than in water and nonpolar organic solvents, e.g., *n*-hexane.

The fermentation process markedly increased the TPC of all fermented oat subfractions ($p < 0.05$), except that of the *n*-hexane subfractions ($p > 0.05$) (Table 1). In *A. oryzae* var. *effuses* and *A. oryzae* fermented oats, the EA subfraction had the highest value of TPC ($p < 0.05$), followed by the *n*-butanol and water subfractions. The TPC of the EA subfraction of *A. niger* fermented oat was significantly higher than that of native oat ($p < 0.05$), while the TPC of the other three subfractions had no statistically significant difference with that of native oat ($p > 0.05$). The three fungi employed in the present study are commonly used in the preparation of many Asian foods and generally recognized as safe. All of these fungi produce many different types of enzymes during the fermentation process, e.g., glycoside hydrolase, cellulose- or xylan-degrading enzymes, and esterase, that can soften the kernel structure, break down the cereal cell walls, and release esterified and insoluble-bound nutrient substances.[19,20] Because most oat phenols are esterified and insoluble-bound in oat bran,[21] the increase of TPC in fermented oat subfractions may be attributed to the effects of these enzymes, which could potentially release bound phenols during fermentation. While the amounts and/or activities of enzymes vary with the strains, those variances will eventually influence the increase ratios of TPC of different fungi-fermented oats. From Table 1, the data clearly indicate that the increased TPC ratios were dependent upon fungi types. Dordević et al.[13] also reported that increased TPC and enhanced antioxidant activity of cereals resulting from fermentation are highly dependent upon the species employed.

3.2. Flavonoids of the Four Subfractions of Native and Fermented Oats. Flavonoids have been proposed to act as beneficial agents in a multitude of disease states, including cancer, cardiovascular disease, and neurodegenerative disorders.[22] Dependent upon flavonoids content of glycosides, isoprenoids, and aliphatic ethers, the flavonoids have almost any polarity and, thus, are soluble in a range of solvents from water to diethyl ether.[23] In oat, three major flavonoids have been identified, namely, apigenin, luteolin, and tricin.[24] As shown in Table 2, the EA subfraction of native oat had the highest value of flavonoids ($p < 0.05$), followed by the *n*-hexane subfraction, while the flavonoids were not detected in the *n*-butanol and water sufbfractions (Table 2). In comparison to native oat, the flavonoid values of the EA subfractions of oat fermented with *A. oryzae* var. *effuses*, *A. oryzae*, and *A. niger* increased remarkably ($p < 0.05$). While there was no significant difference between *n*-hexane subfraction flavonoids of native and fermented oats. The flavonoids were also not detected in *n*-butanol and water subfractions of fermented oat.

These results indicate that the flavonoids in oat have a relatively weak polarity and fermentation by the three fungi markedly increased the amount of flavonoids in the EA subfraction.

Table 2. Results of Flavonoids of the Subfractions from Native and Fermented Oat (*A. sativa* L.) Ethanol Extract[a]

	n-hexane	EA	*n*-butanol	water
oat	381.6 ± 45.3 a	3714.8 ± 94.3 a	ND	ND
fermented with *A. oryzae* var. *effuses*	407.7 ± 13.2 a	7893.1 ± 397.3 b	ND	ND
fermented with *A. oryzae*	329.0 ± 40.7 a	5749.9 ± 158.4 c	ND	ND
fermented with *A. niger*	342.1 ± 37.1 a	5285.3 ± 153.7 d	ND	ND

[a]All measurements were expressed as the mean ± SD (n = 3). Flavonoids were expressed as milligrams of rutin equivalents/100 g of DW. Means in columns without letters in common differ significantly ($p < 0.05$). ND means not detected.

Subsequent to fermentation by the different fungi, the flavonoids were still not detected in *n*-butanol and water subfractions, indicating that fermentation has no effect on the polarity of flavonoids in oat.

3.3. Comparing the Antioxidant Activity of the Four Subfractions of Native and Fermented Oats. Antioxidant activity is an important property of the phenolic compounds. Numerous studies demonstrate a clear correlation between phenolic contents and antioxidant activities in plant extracts.[25] The Trolox equivalent antioxidant capacity (TEAC), ORAC, ferric reducing activity of plasma (FRAP), and DPPH assays are the most popular and accepted methods to study the antioxidant activity.[26] CV has also become a widely accepted method because it is easier, convenient, and less time-consuming.[27] In this study, ORAC and CV assays were used to measure the total antioxidant capacity of different subfractions from native and fermented oats.

3.3.1. ORAC Method. The ORAC assay is widely employed to determine the antioxidant content of foods. This method, allowing for the reaction to complete, measures the total antioxidant capacity of the samples.[28] The antioxidant activity of the different subfractions from native and fermented oats measured by the ORAC procedure is shown in Table 3 and expressed as micromoles of Trolox per gram of DW. Among the subfractions of native oat, the EA subfraction had the highest ORAC value ($p < 0.05$). Hence, the EA subfraction had the strongest antioxidant activity, followed by the *n*-butanol and water subfractions. The *n*-hexane subfraction had the lowest ORAC value ($p < 0.05$). The antioxidant activity order of native oat subfractions was highly correlated with the TPC of native oat ($r = 0.965$, $p < 0.05$), thus indicating that phenols are the major antioxidant compounds in native oat. Liyana-Pathirana et al.[10] reached a similar conclusion in their study on phenolic antioxidants of wheat.

Just as the fermentation significantly improved the TPC of the subfractions, the ORAC values of the subfractions were also increased dramatically after fermentation with each of the three

Table 3. Results of ORAC of the Subfractions from Native and Fermented Oat (*A. sativa* L.) Ethanol Extract[a]

	n-hexane	EA	*n*-butanol	water
oat	149.0 ± 11.6 a	747.5 ± 14.6 a	466.7 ± 21.1 ac	448.6 ± 34.2 a
fermented with *A. oryzae* var. *effuses*	332.8 ± 28.6 b	1687.9 ± 40.7 b	435.5 ± 15.9 a	355.6 ± 19.2 a
fermented with *A. oryzae*	191.0 ± 4.5 a	1413.9 ± 76.2 c	599.7 ± 28.1 b	468.0 ± 20.5 a
fermented with *A. niger*	246.6 ± 22.7 ab	1378.9 ± 17.2 c	576.5 ± 43.3 b	577.8 ± 12.2 b

[a]All measurements were expressed as the mean ± SD (n = 3). Results were expressed as micromoles of Trolox per gram of DW. Means in columns without letters in common differ significantly ($p < 0.05$).

Table 4. Areas under Anodic Current Waves (Q_{1200}, μC) of the Subfractions from Native and Fermented Oat (*A. sativa* L.) Ethanol Extract[a]

	n-hexane	EA	*n*-butanol	water
oat	0.4 ± 0.1 a	7.2 ± 0.7 a	4.7 ± 0.1 a	3.8 ± 0.3 a
fermented with *A. oryzae* var. *effuses*	1.2 ± 0.1 a	31.8 ± 0.6 b	13.9 ± 0.4 b	9.9 ± 0.4 b
fermented with *A. oryzae*	0.5 ± 0.1 a	21.4 ± 0.6 c	14.2 ± 0.5 b	7.6 ± 0.8 c
fermented with *A. niger*	1.0 ± 0.1 a	29.3 ± 0.5 d	14.9 ± 0.4 b	11.5 ± 0.7 b

[a]All measurements were expressed as the mean ± SD (n = 3). Means in columns without letters in common differ significantly ($p < 0.05$).

fungi employed in these experiments, especially those of the EA subfractions ($p < 0.05$) (Table 3). In the *A. oryzae* var. *effuses* fermented oat, the ORAC values of *n*-hexane and EA subfractions increased remarkably ($p < 0.05$) but the values of the *n*-butanol and water subfractions had no signicant difference with the values of their native oat counterparts ($p > 0.05$). In contrast, the ORAC values of the EA and *n*-butanol subfractions of the *A. oryzae* fermented oat increased significantly ($p < 0.05$), while the values of the *n*-hexane and water subfractions had no significant difference compared to those of the corresponding parts of native oat. In the EA, *n*-butanol, and water subfractions of the *A. niger* fermented oat, the ORAC values increased dramatically and significantly higher than those of native oat ($p < 0.05$). Although the change of ORAC values of the fermented subfractions had some differences with each other, the fermentation process, as a whole, effectively increased the total antioxidant capacity of oat. Dordević et al.[13] also found that the antioxidant activities of cereals and pseudocereals were more or less enhanced through the fermentation by lactic acid bacteria (*Lactobacillus rhamnosus*) and yeast (*Saccharomyces cerevisiae*).

As shown in Table 3, the EA subfraction of native and fermented oats had the highest antioxidant activity compared to the other three subfractions. These subfractions also had the highest TPC (Table 1). A good correlation between TPC and ORAC values in four subfractions of each fermented oat was also found (r = 0.955, $p < 0.05$; r = 1.000, $p < 0.01$; and r = 0.983, $p < 0.05$ for *A. oryzae* var. *effuses*, *A. oryzae*, and *A. niger*, respectively). Taking all subfractions of native and fermented oats into account, there was also a clear correlation between TPC and ORAC values (r = 0.953, $p < 0.01$) (Tables 1 and 3). Rusak et al.[29] also found that the antioxidative capacity of tea extracts correlated with their phenolic content, while Dordević et al.[13] found that there was no correlation between TPC and DPPH radical scavenging activity in different fermented cereals. This may be attributed to the difference of materials and microorganisms, which could have significant effect on the TPC and antioxidant activity. The methods used to detect antioxidant activity could also influence the results. Furthermore, the discrepancies between the antioxidant activity and TPC among some subfractions in the present work could be explained as follows: (1) The oat had lots of starch, which could be hydrolyzed as a reducing sugar during fermentation. The resulting reducing sugar could react with the Folin–Ciocalteu reagent and contribute to total absorbance, ultimately resulting in overestimated levels of TPC. The yield of reducing sugar likely varied with the fungi strains. (2) There are many other antioxidants that are not included in TPC that could be released by fungal fermentation, such as ascorbic acid. The yield also depended upon the fungi strains. (3) Fungi may produce some secondary metabolites that could be responsible for the ambiguous relationship between the antioxidant activity and total phenolics during fermentation.

3.3.2. CV Assay. In CV, the working electrode potential is scanned in two inverse scans to form an area in a current–potential plot. When used to study the antioxidant capacity of antioxidant compounds, CV actually measures the ability of these compounds to donate electron(s) and the area under the anodic current wave represents the amount of donated electrons. In this study, the values of areas under anodic current waves (Q) were taken into consideration to rank the antioxidant capacity of different subfractions of native and fermented oats. The larger the value of the area (Q), the more electrons that can be donated, and therefore, the subfractions have a stronger antioxidant activity.

As shown in Table 4, the Q_{1200} values of different subfractions of native and fermented oat in the present work were obtained by CV and the range of potential varies from −0.4 to 1.2 V versus Ag/AgCl. The EA subfraction had the highest Q_{1200} value among the subfractions of native oat, followed by the Q_{1200} values of the *n*-butanol and water subfractions. The *n*-hexane subfraction had the lowest Q value ($p < 0.05$). There was also a significant correlation between TPC and Q_{1200} values of all four subfractions of native oat (r = 0.982, $p < 0.05$).

Fermentation with the fungi used in the present study was also found to have a significant effect on Q_{1200} values of the subfractions examined (Table 4). The Q_{1200} values of all subfractions of the three fungi-fermented oat increased significantly ($p < 0.05$), except those of the *n*-hexane subfractions, which showed no difference from those of their native oat counterpart ($p > 0.05$). Among the subfractions of fermented oats, the EA subfraction of *A. oryzae* var. *effuses* fermented oat had the highest Q_{1200} values ($p < 0.05$). The antioxidant activity order of the four subfractions of each fermented oat, as determined by CA, was as follows: EA > *n*-butanol > water > *n*-hexane, which is consistent with the order observed for TPC and ORAC. The correlation between TPC and Q_{1200} of all samples was also found (r = 0.923, $p < 0.01$) (Tables 1 and 4), providing

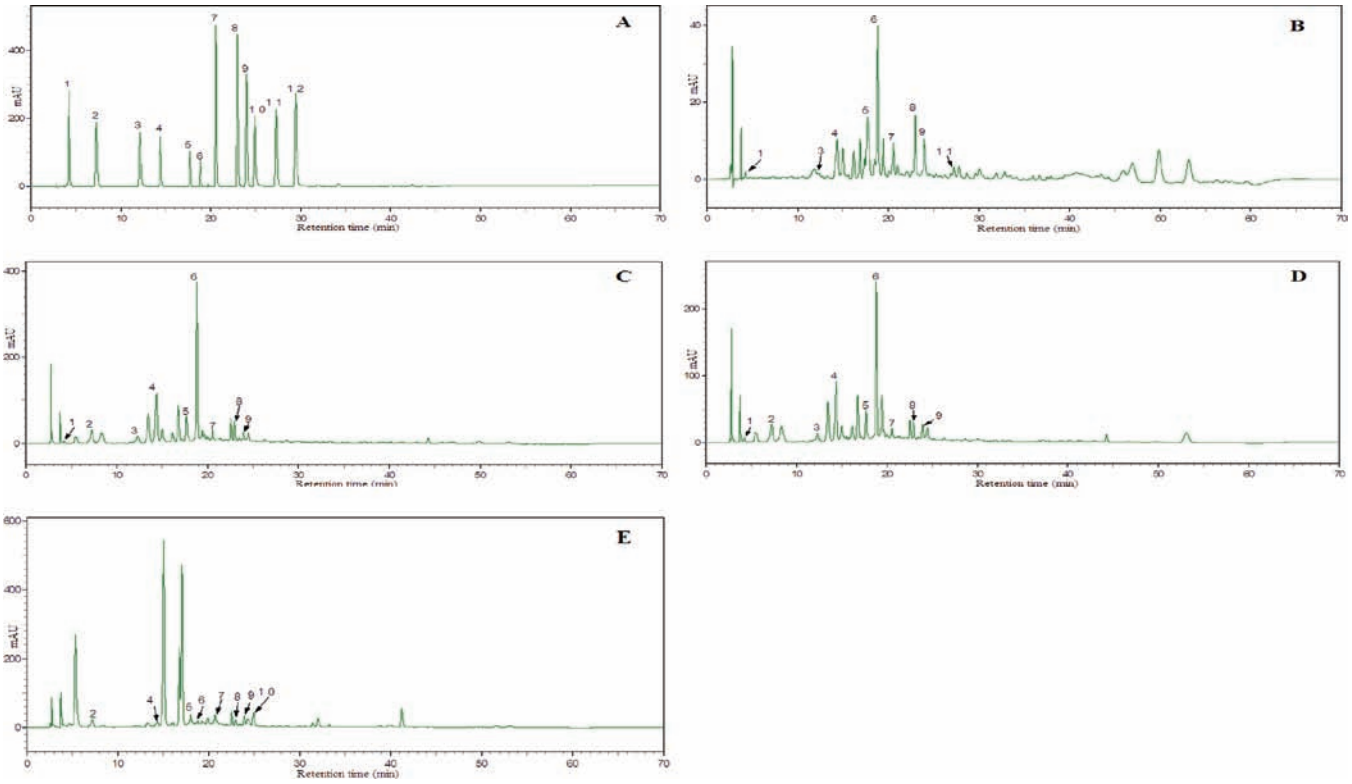

Figure 1. HPLC chromatograms of (A) mixture of 12 standards that were investigated, (B) representative EA subfractions of native oat, (C) *A. oryzae* var. *effuse* fermented oat, (D) *A. oryzae* fermented oat, and (E) *A. niger* fermented oat obtained using a gradient elution system (see section 2.8) by the diode array detector at 280 nm: 1, gallic acid; 2, protocatechuic acid; 3, chlorogenic acid; 4, caffeic acid; 5, *p*-coumaric acid; 6, ferulic acid; 7, avenanthramide **2c**; 8, avenanthramide **2p**; 9, avenanthramide **2f**; 10, luteolin; 11, cinnamic acid; and 12, apigenin.

additional evidence that TPC is the main source for the antioxidant activity of subfractions of native and fermented oats. Moreover, there was also a good correlation between Q_{1200} and ORAC values ($r = 0.917$, $p < 0.01$), which indicated that these two methods are comparable for evaluating the antioxidant activity of oat and oat products.

3.4. HPLC Analysis of EA Subfractions. There have been numerous studies to identify the compounds responsible for the antioxidant property of oat. Caffeic and ferulic acids are typically the main phenolic acids in oat.[21] Subsequent works have also found that vanillic, sinapic, *p*-coumaric, protocatechuic, and gallic acids are included in oat.[30] Another important class of antioxidant compounds that has been reported only in oats among food crops is avenanthramides.[31] The most abundant avenanthramides, which are constitutive in oat seeds, are **2p**, **2c**, and **2f**.[32] Because the EA subfractions showed the highest TPC and flavonoids and the strongest antioxidant activity, this subfraction was analyzed by HPLC. The chromatographic profile of this subfraction was compared to 12 standard samples, including 7 phenolic acids (*p*-coumaric, protocatechuic, gallic, chlorogenic, cinnamic, caffeic, and ferulic acids), 2 flavonoids (apigenin and luteolin), and 3 avenanthramides (**2p**, **2c**, and **2f**). The elution positions of a mixture of the 12 standards and chromatograms of representative EA subfractions of the native and three fermented oat samples are shown in panels A–E of Figure 1.

Figure 1A shows that all 12 standards were successfully separated by the HPLC method. A total of 9 of the 12 standards were detected in the native oat (5 phenolic acids: gallic, chlorogenic, caffeic, *p*-coumaric, and ferulic acids; 1 flavonoid: luteolin; and 3 avenanthramides: **2p**, **2c**, and **2f**) (Figure 1B). For the EA subfraction of *A. oryzae* var. *effuse* fermented oat, again, 9 compounds were detected. These are consistent with that of the EA subfraction of native oat, except that the protocatechuic acid instead of luteolin was detected. Figure 1C also shows that these 9 compounds were increased significantly in comparison to that of the native oat sample, especially the ferulic and caffeic acids. Most of these two phenolic acids in oat may be esterified through hydroxyl substitutions in the benzene ring or carboxyl group of their molecular structures (Figure 2) and become

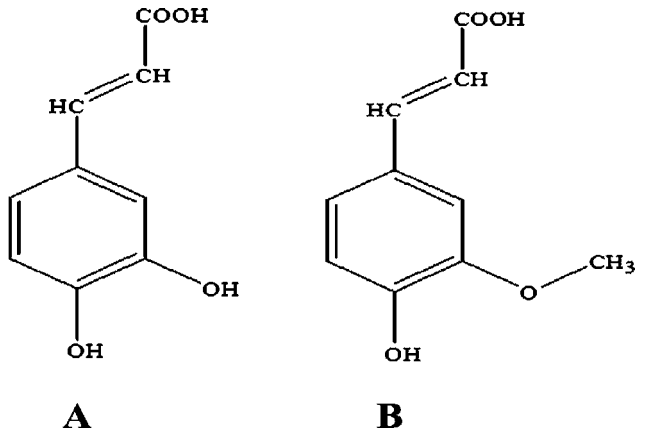

Figure 2. Structures of (A) caffeic and (B) ferulic acids.

insoluble-bound forms. When these insoluble-bound forms are hydrolyzed with enzymes produced by fungi, ferulic and caffeic acids will be released. Chandrasekara et al.[33] also found that ferulic acid is a major phenolic acid, and most of this phenolic

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Table 5. Major Identified Phenolic Acid Contents of the EA Subfractions from Native and Fermented Oat (*A. sativa* L.) Ethanol Extract[a]

	chlorogenic acid	caffeic acid	*p*-coumaric acid	ferulic acid
oat	63.9 ± 5.34 a	116.6 ± 2.34 a	55.1 ± 1.23 a	89.0 ± 0.84 a
fermented with *A. oryzae* var. *effuses*	163.8 ± 2.72 b	385.7 ± 4.57 b	119.0 ± 5.69 b	793.8 ± 6.85 b
fermented with *A. oryzae*	138.4 ± 0.76 c	319.6 ± 0.72 c	98.5 ± 3.35 c	493.1 ± 5.36 c
fermented with *A. niger*	ND	160.6 ± 3.21 d	104.9 ± 4.78 c	87.2 ± 4.12 a

[a]All measurements were expressed as the mean ± SD (n = 3). Results were expressed as micrograms/100 mg of DW. Means in columns without letters in common differ significantly (p < 0.05).

acid is insoluble-bound in millet grains. The chromatograms of the EA subfraction of *A. oryzae* were very similar to that of the EA subfraction of *A. oryzae* var. *effuse*, except the absorption peaks of the *A. oryzae* fermented sample were lower than that of the *A. oryzae* var. *effuse* fermented sample (panels C and D of Figure 1). This result indicated that *A. oryzae* var. *effuse* and *A. oryzae* might provide the same suite of enzymes during fermentation, while the contents and activities of enzymes produced by *A. oryzae* var. *effuse* were higher than the corresponding enzymes produced by *A. oryzae*. The chromatograms of the *A. niger* fermented sample were different from those of the *A. oryzae* var. *effuse* and *A. oryzae* fermented samples, which revealed that the types of enzymes produced by *A. niger* were likely different from those produced by *A. oryzae* var. *effuse* and *A. oryzae* (panels C–E of Figure 1). The most significant unknown peaks in the *A. niger* fermented sample are needed for further analysis (Figure 1E).

The contents of major identified phenolic acids are summarized in Table 5. In the EA subfraction of *A. oryzae* var. *effuses* fermented oat, the caffeic and ferulic acid contents were about 3 and 9 times higher, respectively, when compared to the corresponding phenolic acid in native oat. In addition, both chlorogenic and *p*-coumaric acids increased more than 2 times higher (Table 5). The EA subfraction of *A. oryzae* fermented oat contained about 2.7- and 5.5-fold more caffeic and ferulic acids, respectively, compared to the counterparts in native oat. Chlorogenic and *p*-coumaric acids in the EA subfraction of *A. oryzae* fermented oat also increased about 2 times higher (Table 5). However, only two of those four phenolic acids, namely, caffeic and *p*-coumaric acids, increased significantly in the EA subfraction of *A. niger* fermented oat.

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REFERENCES

(1) Sonia, R. Cancer chemoprevention and chemotherapy: Dietary polyphenols and signaling pathways. *Mol. Nutr. Food Res.* **2008**, *52*, 507–526.

(2) Blokhina, O.; Virolainen, E.; Fagerstedt, K. V. Antioxidants, oxidative damage and oxygen deprivation stress: A review. *Ann. Bot.* **2003**, *91*, 179–194.

(3) Valko, M.; Leibfritz, D.; Moncol, J.; Cronin, M. T. D.; Mazur, M.; Telser, J. Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.* **2007**, *39*, 44–84.

(4) Aruoma, O. I. Free radicals, oxidative stress, and antioxidants in human health and disease. *J. Am. Oil Chem. Soc.* **1998**, *75*, 199–212.

(5) Valentão, P.; Fernandes, E.; Carvalho, F.; Andrade, P. B.; Seabra, R. M.; Bastos, M. L. Antioxidant properties of cardoon (*Cynara carduncul*us L.) infusion against superoxide radical, hydroxyl radical and hypochlorous acid. *J. Agric. Food Chem.* **2002**, *50*, 4989–4993.

(6) Shahidi, F. Antioxidants in food and food antioxidants. *Nahrung* **2000**, *44*, 158–163.

(7) Baublis, A.; Decker, E. A.; Clydesdale, F. M. Antioxidant effects of aqueous extracts from wheat-based ready-to-eat breakfast cereals. *Food Chem.* **2000**, *68*, 1–6.

(8) Madhujith, T.; Shahidi, F. Optimization of the extraction of antioxidative constituents of six barley cultivars and their antioxidant properties. *J. Agric. Food Chem.* **2006**, *54*, 8048–8057.

(9) Liyana-Pathirana, C.; Dexter, J.; Shahidi, F. Antioxidant properties of wheat as affected by pearling. *J. Agric. Food Chem.* **2006**, *54*, 6177–6184.

(10) Liyana-Pathirana, C. M.; Shahidi, F. Antioxidant properties of commercial soft and hard winter wheats (*Triticum aestivum* L.) and their milling fractions. *J. Sci. Food Agric.* **2006**, *86*, 477–485.

(11) Lee, I. H.; Hung, Y. H.; Chou, C. C. Solid-state fermentation with fungi to enhance the antioxidative activity, total phenolic and anthocyanin contents of black bean. *Int. J. Food Microbiol.* **2008**, *121*, 150–156.

(12) Katina, K.; Laitila, A.; Juvonen, R.; Liukkonen, K. H.; Kariluoto, S.; Piironen, V.; Landberg, R.; Åman, P.; Poutanen, K. Bran fermentation as a means to enhance technological properties and bioactivity of rye. *Food Microbiol.* **2007**, *24*, 175–186.

(13) Dordević, T. M.; Šiler-Marinković, S. S.; Dimitrijević-Branković, S. I. Effect of fermentation on antioxidant properties of some cereals and pseudocereals. *Food Chem.* **2010**, *119*, 957–963.

(14) Peterson, D. M.; Hahn, M. J.; Emmons, C. L. Oat avenanthramides exhibit antioxidant activities in vitro. *Food Chem.* **2002**, *79*, 473–478.

(15) Kedia, G.; Vázquez, J. A.; Pandiella, S. S. Fermentability of whole oat flour, PeriTec flour and bran by *Lactobacillus plantarum*. *J. Food Eng.* **2008**, *89*, 246–249.

(16) Singleton, V. L.; Rossi, J. A. Colorimetry of total phenolics with phosphomolybdic phosphotungstic acid reagent. *Am. J. Enol. Vitic.* **1956**, *16*, 144–158.

(17) Dewanto, V.; Wu, X.; Adom, K. K.; Liu, R. H. Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. *J. Agric. Food Chem.* **2002**, *50*, 3010–3014.

(18) Cai, S.; Huang, C.; Ji, B.; Zhou, F.; Wise, M. L.; Zhang, D.; Yang, P. In vitro antioxidant activity and inhibitory effect, on oleic acid-induced hepatic steatosis, of fractions and subfractions from oat (*Avena sativa* L.) ethanol extract. *Food Chem.* **2011**, *124*, 900–905.

(19) Yokoyama, S.; Hayakawa, K. Production of cellulose- and xylan-degrading enzymes by a koji mold, *Aspergillus oryzae*, and their contribution to the maceration of rice endosperm cell wall. *J. Biosci. Bioeng.* **2002**, *93*, 9–14.

(20) Koseki, T.; Mihara, K.; Murayama, T.; Shion, Y. A novel *Aspergillus oryzae* esterase that hydrolyzes 4-hydroxybenzoic acid esters. *FEBS Lett.* **2010**, *584*, 4032–4036.

(21) Daniels, D. G. H.; Martin, H. F. Antioxidants in oats: Glyceryl esters of caffeic and ferulic acids. *J. Sci. Food. Agric.* **1968**, *19*, 710–712.

(22) Spencer, J. P. E.; Mohsen, M. M. A. E.; Rice-Evans, C. Cellular uptake and metabolism of flavonoids and their metabolites: Implications for their bioactivity. *Arch. Biochem. Biophys.* **2004**, *423*, 148–161.

(23) Havsteen, B. H. The biochemistry and medical significance of the flavonoids. *Pharmacol. Ther.* **2002**, *96* (2−3), 67−202.
(24) Collins, F. W. Oat phenolics: Structure, occurrence, and function. In *Oats Chemistry and Technology*; Webster, F. H., Ed.; American Association of Cereal Chemists: St. Paul, MN, 1986; pp 227−295.
(25) Cao, G.; Sofic, E.; Prior, R. L. Antioxidant capacity of tea and common vegetables. *J. Agric. Food Chem.* **1996**, *44*, 3426−3431.
(26) Ryan, L.; Prescott, S. L. Stability of the antioxidant capacity of twenty-five commercially available fruit juices subjected to an in vitro digestion. *Int. J. Food Sci. Technol.* **2010**, *45*, 1191−1197.
(27) Keyrouz, R.; Abasq, M. L.; Bourvellec, C. L.; Blanc, N.; Audibert, L.; ArGall, E.; Hauchard, D. Total phenolic contents, radical scavenging and cyclic voltammetry of seaweeds from Brittany. *Food Chem.* **2011**, *126*, 831−836.
(28) Ou, B.; Hampsch-Woodill, M.; Prior, R. L. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J. Agric. Food Chem.* **2001**, *49*, 4619−4626.
(29) Rusak, G.; Komes, D.; Likić, S.; Horzic, D.; Kovac, M. Phenolic content and antioxidative capacity of green and white tea extracts depending on extraction conditions and the solvent used. *Food Chem.* **2008**, *110*, 852−858.
(30) Durkee, A. B.; Thivierge, P. A. Ferulic acid and other phenolics in oat seeds (*Avena sativa* L. var. Hinoat). *J. Food Sci.* **1977**, *42* (2), 551−552.
(31) Collins, F. W. Oat phenolics: Avenanthramides, novel substituted *N*-cinnamoylanthranilate alkaloids from oat groats and hulls. *J. Agric. Food Chem.* **1989**, *37*, 60−66.
(32) Skoglund, M.; Peterson, D. M.; Andersson, R.; Nilsson, J.; Dimberg, L. H. Avenanthramide content and related enzyme activities in oats as affected by steeping and germination. *J. Cereal Sci.* **2008**, *48*, 294−303.
(33) Chandrasekara, A.; Shahidi, F. Bioactivities and antiradical properties of millet grains and hulls. *J. Agric. Food Chem.* **2011**, *59*, 9563−9571.