Effects of extraction solvents on antioxidant activities and total phenolic contents of four whole grains

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Abstract: The objectives of this study were to determine antioxidant activity and total phenolic contents (TPC) of the different solvent extractions (60% ethanol, 60% methanol, 60% acetone) from four whole grains, and to investigate relationships between antioxidant activities and TPC. The antioxidant activities of grain extractions were evaluated using the 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH), 2, 2’-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diaminonium Salt (ABTS⁺) and Ferrie-ion reducing antioxidant power (FRAP) methods and the TPC were determined by Folin-Ciocalteau reagent (FCR). Results showed that extraction solvent mixtures had different impacts on antioxidant activity and TPC of whole grain varieties. For 60% acetone extraction, TPC hierarchy was as follows: buckwheat>barley>rye>oat; from which, the highest DPPH and ABTS⁺ scavenging activities and reducing power were found in buckwheat. Furthermore, correlations analysis revealed that TPC, reducing power, DPPH and ABTS⁺ scavenging activities were positively correlated with each other. Buckwheat could be important source of natural antioxidants to improve health conditions and to reduce disease risks.

Key words: Antioxidant capacity; extraction; grains; FRAP; ABTS⁺; DPPH.

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Received: March 19 2016; Accepted: December 24 2016; Published: December 30 2016
doi: http://dx.doi.org/10.18088/ejbmr.2.4.2016.pp1-6

Introduction

Antioxidants are traditionally used in malting and brewing due to their ability to delay or prevent oxidation and oxygen free radical reactions (1, 2). More of more research evidences have shown that free radicals and oxidative stress are related to cancer, cardiovascular disease, autoimmune disorders, and neurological disorders (3-8).

Grains and their products are the most commonly consumed food and a staple in Chinese diet. Whole grains are rich in antioxidants, which are mainly in bound form, associated with fiber or the cell wall. They can pass from the stomach and small intestine to the colon, and then are released by bacterial fermentation (9). In addition, whole grain consumption has been consistently associated with reduced risk of developing chronic diseases, including cardiovascular disease (10), type2 diabetes (11, 12), obesity (13), and some cancers (7, 14-18). This is because they contain antioxidants that combat oxidative stress in the body and help maintain a balance between oxidants and antioxidants (19). Antioxidants can reduce oxidative damage to bio-molecules by modulating the effects of reactive oxidants (20-24).

ABTS⁺, DPPH, FRAP assays are widely used to determine the antioxidant capacity in plant extracts due to their simplicity, stability, and reproducibility. From a mechanical standpoint, with FRAP and ABTS⁺ , there is a single electron reaction (SET), whereas with DPPH it combines SET with a hydrogen atom transfer reaction. Because the antioxidant capacity of grains is derived from the cumulative synergistic actions of a wide variety of antioxidants such as phenols, vitamins, carotenoids, terpenoids, and trace mineral; single assay is insufficient to accurately reflect the antioxidative activities of all the individual antioxidants. Thus, it is necessary to combine more than one method to determine the total antioxidant capacity of food.

Researchers previously reported antioxidant activities of black rice, sorghum (25), barley (26), buckwheat (27), oat, wheat, corn, and cereal products including black rice bran (28), oat bran, oat meal, wheat meal, and ready-to-eat cold cereal. Various aqueous solutions of acetone, methanol, and ethanol have also been used to extract the free phenolic compounds from grains. However, most previous studies in the literature reported the phenols and antioxidant activity of individual or small groups of cereals, the diversity of extraction and analysis methods makes it difficult to directly compare the results from different investigations. The antioxidant activities of different extracts from whole grain were limited and restricted to a few varieties. Furthermore, the extraction capacity and specificity of different solvent mixtures for TPC have not been studied thoroughly in these four grains (barley, buckwheat, oat and rye). Correlations among grains antioxidant activity evaluation indices and the total phenolic contents have also not been fully elucidated.

Therefore, the main objectives of this study include: (1) determine the effects of different solvent (60% ethanol,
60% methanol, 60% acetone) on total phenolic contents extracted from four whole grains; (2) investigate antioxidant activities of four whole grains using three different \textit{in vitro} assays; (3) determine the relationship between antioxidant activity measured by different methods and total phenols of whole grains. Results from this preliminary study will provide a better understanding of antioxidant activities of these four whole grains.

**Material and Methods**

**Materials and chemicals**

6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox), 2, 2’-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium (ABTS’’), 2, 4, 6-tri (2-pyridyl)-s-triazine (TPTZ), 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) were purchased from Sigma-Aldrich (St. Louis, USA). Gallic acid was purchased from J&K (www.jkchemical.com). All other chemicals were used of analytical grade made in China.

**Sample preparation**

A total of four different whole grains, including barley, oat, buckwheat and rye, were collected from local markets in Wuxi, China. The collected samples were ground to fine powder by a high speed Chinese medicine grinder machine. The whole grains were ground to pass a 60-mesh sieve. The ground samples were freeze dried and then stored at −20°C until analyzed. One gram of sample was extracted twice with extraction solvent mixtures (10mL, each) at 60°C for 2h in a thermostat water bath shaker. After cooling the extract was centrifuged at 3000 g for 20 min and filtered to get the clear supernatant with two times. The two supernatants were collected with final volume of 25 mL using corresponding extraction solvent mixtures and stored at 4°C until use within 72 h.

**Determination of total phenolic content**

The total phenolic content of each extract was determined using methods previously described by Singleton et al (29) and Ainsworth EA (30). Briefly, the appropriate dilutions of extracts were oxidized with Folin-Ciocalteu reagent, and the reaction was neutralized with sodium carbonate. The absorbance of the resulting blue color was measured at 765 nm after 30 min. Gallic acid was used as a reference standard and the result were expressed as milligram gallic acid equivalents (mg GAE)/100g DW of cereal grains. Data are reported as mean ± SD for at least three replications.

**Free radical scavenging by using DPPH radical**

Antioxidant activity of the obtained extracts was also tested using the 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) as previously described (31) with some modifications. Briefly, the DPPH free radical scavenging activity of grain extracts was determined using a 2×10⁻²⁴M DPPH solution. Each sample of whole grains extracts (0.5ml) was mixed with 4 ml 2×10⁻¹⁰M DPPH in ethanol. The mixture was shaken, and then left to stand for 60 min in the dark. The absorbance was measured at 517 nm in a spectrophotometer. The absorbance of the control was obtained by replacing the sample with methanol. The DPPH radical scavenging activity of the sample was calculated as follows:

\[
\text{DPPH radical scavenging activity (\%) = (1- absorbance of sample/absorbance of control) x 100.}
\]

Data are reported as mean ±SD for at least three replications.

**ABTS’’ radical Scavenging Activity**

The ABTS’’ radical scavenging assay was done according to Ke et al. (32) and Re et al. (26) with some slight modifications. The ABTS’’ radical was generated by the oxidation of ABTS’’ with potassium persulphate. The ABTS’’ radical cation solution was obtained as follows: 25ml of ABTS’’ (7mM) was mixed with 440µl of potassium persulphate (140 mM) and then incubated in the dark at room temperature for 12-16 h.

The working solution was pre-pared by diluting the previous solution with ethanol until the absorbance at 734 nm was 0.70 ± 0.02. The solution was kept for 30min in the dark before being used 0.1ml of each sample was mixed with 4 ml of the working solution, shaken vigorously, and left to stand for 10 min at room temperature. The absorbance of the reaction mixture was determined at 734nm. The controls contained the extraction solvent instead of the antioxidant solution. The Trolox calibration curve was plotted as a function of the percentage of ABTS’’ scavenging activity. The final results were expressed as millimoles of Trolox equivalents antioxidant activity (TEAC) per gram of dry whole grains (mmolTEAC/100g DW). Data are reported as mean ± SD for at least three replications.

**Ferric reducing antioxidant power (FRAP) assay**

This study employed the FRAP assay developed in a previous study (33). Briefly, freshly prepared FRAP reagent (2.5 ml of a 10 mmol/l TPTZ solution in 40 mmol/l HCl, 2.5 ml of 20 mmol/l FeCl₃, and 25 ml of 0.1 mol/l acetate buffer, pH 3.6) was incubated at 37°C for 10 min. Then, 0.05 ml of grain extracts and 2 ml of FRAP reagent were transferred into a 10-ml volumetric flask and made up to volume with redistilled water. The obtained blue solutions were kept at room temperature for 20 min. The absorbance was measured at 593 nm against a reagent blank (2 ml of FRAP reagent made up to 10ml with redistilled water) using a UV spectrophotometer in a 1cm quartz cell. All solutions were used within the day for preparation. A standard curve was made with Trolox and the results were expressed as mmol Trolox equivalents (TEAC) per 100g dry weight (DW) of the grains powders. Data are reported as mean ± SD for at least three replications.

**Statistical analysis**

The experiments and analyses were performed at least in triplicate. Data were expressed as means ±SD (standard deviation). Analysis of variance (ANOVA) was carried out to determine any significant differences of measurements by the SPSS statistical software (version 17.0, SPSS Inc., USA). The significance of the difference was checked by the Duncan test and the differences were considered as significant with \( p<0.05 \) or very significant with \( p<0.01 \). The Pearson correlation test was employed to determine the correlations among means.

**Results and Discussion**

**Total phenolic contents of different whole grains**

Phenolic compounds were considered as a major group...
of compounds that contributed to the antioxidant activity of grains (34, 35). There are various classes of phenolic compounds in grains including derivatives of benzoic and cinnamic acids, anthocyanidins, quinones, flavonols, chalcones, flavones, flavanones, and amino phenolic compounds. Phenolic compounds present in grains have antioxidant properties associated with reducing risk of chronic diseases (36). They also have anti-inflammatory and anti-carcinogenic properties reported by many literatures (37, 38). To better understand the relationship between the antioxidant activity and TPC, the TPC of different grains extracts were determined using the Folin-Ciocalteu phenol reagent.

The recovery of total phenolic contents in different grains is influenced by the polarity of extracting solvents and the solubility of this compound in the solvent. Therefore, it is difficult to select an appropriate solvent for the extraction of total phenolic contents from all samples (39). In this study, different solvents such as 60% ethanol, 60% methanol, 60% acetone have been used for the extraction of total phenolic compounds from different grains. The results are expressed as milligrams of gallic acid equivalents per 100 g of grain on a DW basis and are presented in Figure 1. These results showed that the total phenolic contents varied greatly among different solvents. This indicated the possible influence of extracting solvent on total phenolic contents. For 60% ethanol extraction and 60% methanol extraction, buckwheat has the highest TPC values, followed by barley, rye and oat. Among all extractions from grains varieties, the TPC of barley, oat, buckwheat, rye are higher in 60% acetone than other corresponding solvent extractions. Furthermore, for 60% acetone extraction, buckwheat (210.17 mgGAE/100g DW) and barley (195.37mgGAE/100g DW) were found with the highest TPC, followed by rye (164.99mgGAE/100g DW) and oat (103.41mgGAE/100g DW). There were no significant difference between buckwheat and barley. These results indicated that 60% acetone is best extraction solvent for TPC of selected grains.

Several earlier studies have measured the effect of different solvents in total phenolic compounds and antioxidant activities. For example, 70% acetone is the efficient solvent, and distilled water was found as inefficient solvent for extracting phenolic compounds from different vegetables species (40). 50% acetone contained the highest level of total phenolic contents for two varieties of wheat bran samples (41). These results are partly in agreement with our results and suggest that acetone/water mixtures are good solvents for the extraction of total phenolic contents. Thus, 60% acetone could be used as the solvents for extracting phenolic compounds from selected grains.

**DPPH free-radical scavenging activity**

The DPPH is a stable free radical widely used to determine the antioxidant properties or radical scavenging activity of pure antioxidant compounds as well as different grain extracts. Furthermore, free-radicals are highly reactive species made of molecules or atoms that are unstable owing to single or unbalanced electrons. Although free-radicals at physiological concentration may be required for normal cell function, excessive amount of free-radicals can damage cellular components such as lipids, protein, and DNA (42). Thus, the free-radical scavenging activities of antioxidants can protect the human body from serious cellular or molecular damage by free radicals and retard the progress of many chronic diseases, as well as lipid peroxidation in food.

In the present study, free radical scavenging activities of the various extracts are presented in Figure 2. These results showed that the DPPH radical scavenging activity varied greatly among different solvent extractions of different whole grains. The higher DPPH radical scavenging activity was found in 60% ethanol extraction of selected grains than 60% methanol and acetone extraction of selected grains. For 60% methanol extraction, buckwheat has the highest DPPH radical scavenging activity followed by barley, rye and oat. For 60% acetone extraction, buckwheat has the highest DPPH radical scavenging activity followed by barley. There was no significant difference between oat and rye. For 60% ethanol extraction, buckwheat had the highest DPPH radical scavenging activity (93.41%); however, rye had the lowest DPPH radical scavenging activity (74.5%). These results indicated that 60% ethanol might be a better antioxidant extraction solvent for our selected whole grains than other solvents, which is partly in accordance with the report from Hai Feng Zhao (43).

**ABTS⁺ radical cation scavenging activity**

The ABTS⁺ method is widely employed for measuring the relative radical scavenging activity of hydrogen donating and chain breaking antioxidants in many plant extracts (44, 45). The ABTS⁺ radical cation scavenging activity was found in 60% acetone extraction.
activities of grain extracts are expressed as mill moles of Trolox equivalents per gram of dry grains (mmol TEAC/100g DW) and are presented in Figure 3. These results showed that the ABTS⁺ radical cation scavenging activities varied greatly among different solvent extractions of different whole grains. This is similar with results of the DPPH scavenging ability of different extractions from whole grains. For 60% methanol extraction, buckwheat has the highest ABTS⁺ radical cation scavenging activity, followed by barley, rye and oat. There was no difference between barley and rye. For 60% acetone extraction, buckwheat has the highest ABTS⁺ radical cation scavenging activity, followed by oat, barley and rye. There was no difference between barley and rye. 60% ethanol was the best solvent for extracting ABTS⁺ radical cation scavenging activity of selected whole grains. Furthermore, for 60% ethanol extraction, buckwheat was found the highest ABTS⁺ radical cation scavenging activity (2.79 mmol TEAC/100g DW), followed by barley (1.71 mmol TEAC/100g DW), rye (1.24 mmol TEAC/100g DW) and oat (0.91 mmol TEAC/100g DW). Our results were partly similar with the previous findings on the ABTS⁺ radical cation scavenging activity of water methanol mixture extractions from wheat germ (46).

Ferric-ion reducing antioxidant power
FRAP assay is based on the ability of antioxidant to reduce Fe³⁺ to Fe²⁺ in the presence of TPTZ, which forms an intense blue Fe²⁺-TPTZ complex with an absorption peak at 593nm. The absorbance increase is proportional to the antioxidant content (47). The results of reducing power for extractions of different grain samples are shown in Figure 4 using FRAP method. These results showed that the reducing power varied greatly among different solvent extractions of different whole grains. For 60% methanol extraction, buckwheat has the highest reducing antioxidant power, followed by barley, rye and oat. For 60% acetone extraction, buckwheat has the highest reducing antioxidant power, followed by rye, buckwheat and oat. The reducing power of selected grains were found the highest in 60% ethanol extraction, followed by 60% methanol and 60% acetone extractions. Furthermore, for 60% ethanol extraction, barley and buckwheat had the highest reducing power, followed by rye and oat. These results were similar with previously studies (34). There was no significant difference of reducing power between barley and buckwheat. These results indicated that 60% ethanol is the best solvent for extracting reducing antioxidant power of selected whole grains.

Correlation among antioxidant activity and total phenolic content
In general, the different methods used to determine the antioxidant activity are based on different reaction mechanisms, thus they often give different results. Moreover, the response of phenols for antioxidant activity estimated by various methods also depends on their chemical structures. Phenolic compounds were considered as a major group of compounds that contributed to the antioxidant activity of grains (35). The correlation between total antioxidant capacities and total phenolic contents of four cereal grains is shown in Table 1. The results showed a positive linear correlation between total antioxidant capacities and total phenolic contents (r² = 0.71, 0.72 and 0.62 for the results from DPPH, ABTS⁺ and FRAP, respectively), which indicated that the phenolic compounds could be main contributor of antioxidant capacities of different extractions from grains. There were significant linear correlations among antioxidant activity methods (p<0.01, r² = 0.85, 0.74 and 0.81 for DPPH and ABTS⁺, DPPH and FRAP, ABTS⁺ and FRAP, respectively). This result was partly in agreement with the previous study on different grains (34). Zhou (48) reported good correlations for the wheat grain and fractions when DPPH radical scavenging activity and ABTS radical scavenging activity were compared with TPC. This finding was also supported by the observation that DPPH radical scavenging activity was positively correlated with phenolic contents in black soybean varieties (49).

Conclusions
In the present study, it clearly demonstrated that extracting solvents on reducing antioxidant power of four whole grains.

Table 1. Correlation between antioxidant capacity and total phenolic content.

<table>
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<tr>
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<th>TPC</th>
<th>DPPH</th>
<th>ABTS</th>
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<tbody>
<tr>
<td>TPC</td>
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<td></td>
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<tr>
<td>DPPH</td>
<td>0.71*</td>
<td></td>
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<tr>
<td>ABTS</td>
<td>0.72**</td>
<td>0.85**</td>
<td></td>
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<tr>
<td>FRAP</td>
<td>0.62*</td>
<td>0.74**</td>
<td>0.81**</td>
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</tbody>
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* Correlation is significant at the 0.05 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed)

Correlation among antioxidant activity and total phenolic content
Figure 3. Effects of extraction solvents on ABTS⁺ radical cation scavenging activity of four whole grains.

Figure 4. Effects of extraction solvents on reducing antioxidant power of four whole grains.

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traction solvents greatly affected TPC of grains and their antioxidant activity evaluation including DPPH radical, ABTS radical cation reducing power. Buckwheat had highest TPC contents among our selected grains. 60% acetone could be better to extract TPC of selected whole grains. Buckwheat was found the highest DPPH, ABTS scavenging ability and reducing power using the same solvent for extraction. This study provided new information on the antioxidant activity of different extraction solvents of grains, which may have a direct impact on grain consumption by increasing consumer awareness of the health benefits of grains.

Acknowledgements
This work was financially supported by the Project of China National Key Technology Research and Development Program for the 12th Five-year Plan (No. 2012BAD37B08-3), National High Technology Research and Development Program 863 (No.2013AA102203-7), and National Natural Science Foundation of China (No. 31471617). This work was also financially supported by the Project of National science foundation of jiangsu province(BK20130410).

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