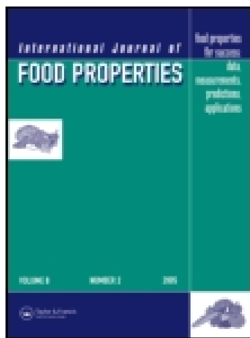

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ANTIOXIDANT ACTIVITY AND TOTAL PHENOLIC CONTENT IN SOME CEREALS AND LEGUMES

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The antioxidant activities and total phenolic content of 4 cereals (buckwheat, wheat germ, barley, and rye) and 4 legume seeds (lentils, mungo bean, red kidney bean, and soy bean) were determined. The total phenolic content (TPC), determined according to the Folin-Ciocalteu method, for cereal samples varied from 13.2 to 50.7 mg Gallic acid equivalent/g of dried extract, while for legume samples varied from 17.0 to 21.9 mg Gallic acid equivalent/g of dried extract. Antioxidant activities were comparatively assessed by 2,2-diphenyl-1- picrylhydrazyl (DPPH) scavenging capacity, ferric ion-reducing antioxidant power (FRAP) and the thiobarbituric acid (TBA) method. The tested plant extracts showed promising antioxidant and free radical scavenging activity, thus justifying their traditional use. Among examined cereals all the applied methods, except TBA method, have shown that buckwheat have the highest antioxidant activity, while among examined legumes results varied depending on the method used.

Keywords: ***Cereals, Legumes, TPC, Antioxidant activity, DPPH, FRAP, TBA.***

INTRODUCTION

Cereals and legumes are an important source of macronutrients. Legumes are rich and economical source of proteins, complex carbohydrates (dietary fiber), minerals, and vitamins,[1] while cereal grains provide significant quantities of carbohydrates (thus energy), proteins and selected micronutrients to the animal and human diet. Those plants, like many others (e.g., rosemary, ginkgo, mengkudu, and lettuce), also contain a wide range of chemical classes with antioxidant activity.[2–6] Cereal grains are rich in phenolic acids phytosterines, saponins, and phytoestrogens, and flavonoids are present in small quantities.[7] It has been suggested that these antioxidants may contribute to the health benefits of cereal-based foods in reducing the incidence of aging-related chronic diseases including heart diseases and some types of cancer.[8] Legume seeds are also rich in various active phytochemicals, e.g., isoflavones, coumestrol, phytate, saponins, lecithin, phytosterols and vitamin E.[9] Epidemiological studies have shown correlations between the consumption of legumes (and there isoflavones such as daidzein, genistein, daidzin and

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genistin from soybean), and decreasing incidence of several diseases, for example, cancer, aging, and cardiovascular diseases.[10] The chemical composition and bioavailability of nutrients varies between species and varieties of plants and may be affected by forms of processing as feed and food.[7]

Although synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and terc-butylhydroquinone (TBHQ), as well as propyl gallate (PG) have widely been used in retarding lipid oxidation, their safety has recently been questioned due to toxicity and possible carcinogenicity.[11] Thus, food manufacturers are considering the possibility to achieve antioxidative effect from natural sources[12] and development of safer natural antioxidants from extracts of plant materials may provide interest.[13] Food antioxidants such as amino acids, peptides, proteins, flavonoids and other phenolics compounds might play a significant role as physiological and dietary antioxidants, thereby augmenting the body's natural resistance to oxidative damage.[11]

The objective of this study was to determine and compare the four commercial cereals (buckwheat *Fagopyrum esculentum*, wheat *Triticum durum*, barley *Hordeum vulgare*, and rye *Secale cereale*) and four commercial legume seeds (lentils *Lens culinaris*, mungo bean *Vigna radiata*, red kidney bean *Phaseolus vulgaris* and soy bean *Glycine hispide*) for their free radical scavenging capacity against 2,2-diphenyl-1-picryhydrazyl radical (DPPH·), their ferric reducing antioxidant power (FRAP), ability for inhibition of lipid peroxidation (TBA test), and for their total phenolic contents (TPC). Nevertheless, these products were chosen because of their widespread availability and use in the Serbia. Studying such commercially prepared products may provide useful information to consumers and also incentive to food manufacturers to promote the consumption and production of value-added foods for improving human health.

MATERIALS AND METHODS

Materials

The cereal samples used in this study included buckwheat, unpeeled wheat, peeled rye and peeled barley were all manufactured by "KLAS" Sarajevo, and legume samples included green mungo bean, green lentils, yellow soybean and red kidney bean all manufactured by "Organic biopharm" China. The compounds 1,1-diphenyl-2-picrylhydrazyl (DPPH), thiobarbituric acid (TBA) and Gallic acid were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany), Folin-Ciocalteu reagents was purchased from Merck & Co., Inc. (New York, USA), and all other chemicals and solvents were the highest commercial grade purchased from Lachema Ltd. (Brno, Czech Republic) and Fluka Chemie GmbH (Buchs, Switzerland), and used without further purification.

Extraction and Sample Preparation

Cereals and legume grains (100 g) were sop up with distillate water for 24 h, then filtered and milled with 400 mL of distillate water. Plant mush was next sterilized in an autoclave for 1 hour and cooled out for 24 h. The sample was extracted with 70% (v/v) ethanol (700 mL) for three hours using a magnetic paddle, and then centrifuged 10 min on 4500 rpm. Extract was poured to a lab dish, and residue was extracted again with 70% (v/v) ethanol (300 mL) for another three hours using a magnetic paddle, following centrifugation for 10 min on 4500 rpm, the extract were combined. Volumes of extracts were approximately 1200 mL, and kept in a refrigerator until drying.

Before drying samples were concentrated to 100 mL with Büchi rotavapor (temperature 50°C, pressure 50–150 mbar). Concentrated extracts were dried on Büchi Mini Spray Dryer (Büchi Labortechnik AG, Flawil, Switzerland) after water dilution [working condition for this unit require ethanol concentration under 20% (v/v)]. Inlet temperature and pump were adjusted to 120–125°C and 15–20%, respectively, leading outlet temperature of 60–63°C. Dried samples were kept in hermetically closed dishes in a freezer (−22°C) until further analysis.

METHODS

Determination of the Total Phenolic Content

The content of total phenolics in extracts was determined according to a modified Folin-Ciocalteu method.[14] Briefly, 100 μl of each extract were shaken for 1 min with 500 μl of Folin-Ciocalteu reagent and 6 ml of distilled water. After the mixture was shaken, 2 ml of 15% Na_2CO_3 were added and the mixture was shaken once again for 0.5 min. Finally, the solution was brought up to 10 ml by adding distilled water. After 2 h, the absorbance at 750 nm (25°C) was read using glass cuvettes at 750 nm (25°C) with a UV/Visible spectrophotometer (Ultrospec 3300 pro, Amersham Bioscience, Sweden), against a blank (instead samples 100 μl of distilled water). The TPC was assessed by plotting the Gallic acid calibration curve (from 1 to 1500 μg/ml) and expressed as milligrams of garlic acid equivalents (GAE) per gram of dried extract. The equation of the Gallic acid calibration curve was $Y = 1.34577 \cdot X - 0.07823$ (where X was concentration of garlic acid equivalents (GAE) expressed as milligrams GAE per gram of dried extract and Y was measured absorbance), and the correlation coefficient was $R^2 = 0.9897$.

Determination of DPPH Radical Scavenging Activity

The antioxidant activity of the dried ethanol extract was measured on the basis of the scavenging activities of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (15). In lab dish with 50 μL of test samples of various concentrations were added 3.95 mL of methanol, and 1 mL 0.2 mmol methanol solution of DPPH. After 30 min of incubation period in the dark at room temperature, the absorbance was measured against a blank (methanol) at 517 nm. Inhibition of free radical DPPH in percent (%) was calculated using the formula:

$$\text{Percentage inhibition (\%)} = [(A_{control} - A_{sample})/A_{control})] \times 100, \quad (1)$$

where, $A_{control}$ is the absorbance of the control reaction (containing all reagents except test compound); and A_{sample} is the absorbance of the test compound. IC_{50} values (concentration of sample required to scavenge 50% of free radicals) were calculated from the regression equation, prepared from the concentration of the samples and percentage inhibition of free radical formation, percentage inhibition DPPH was assayed. Synthetic antioxidant L-ascorbic acid was used as a positive control and all tests were carried out in triplicate.

FRAP Method

In the FRAP method the yellow $Fe3^{+}$-TPTZ complex is reduced to the blue $Fe2^{+}$-TPTZ complex by electron-donating substances under acidic conditions. Any electron

donating substances with a half reaction of lower redox potential than $Fe3^{+}/Fe2^{+}$-TPTZ will drive the reaction and the formation of the blue complex forward. To prepare the FRAP reagent, a mixture of 300 mmol/L acetate buffer (pH 3.6), 10 mmol/L TPTZ (in 40 mmol/L HCl), and 20 mmol/L ferric chloride (10:1:1, v:v:v) was made. To 4.5 mL reagent 150 μL ethanol plant extract were added. The absorbance readings were started after 5 min and they were performed at 593 nm. The blank was consisted of FRAP reagent. The final absorbance of each sample was compared with those obtained from the standard curve made from ferric sulphate ($FeSO_4 \times 7H_2O$) (200–1000 μmol/L). Results were expressed in nmol $Fe2^{+}$/mg dried extract.[16]

Thiobarbituric Acid Test (TBA)

Thiobarbituric acid tests were performed according to the method of Afanas'ev et al. to determine the malonaldehyde formation from lipid peroxidation.[17] Lipid peroxidation was measured in liposome rimifon "Lipotech 10" (0.3 g lecithin/mL). The mixture was containing 20 μL $FeSO_4$ (0,075 *M*), 50 μL liposome, 10 μL of test samples of various concentrations (1–10% w/v), 20 μL L- ascorbic acid (0,1 *M*) and 3.9 mL phosphate buffer (pH 7.4). Mixture was left in thermostat on 37°C for one hour and then mixed with 0,2 mL ethylenediaminetetraacetic acid (EDTA) (0,1 *M*) and 1,5 mL TBA reagent (3 g thiobarbituric acid, 120 g trichloroacetic acid, and 10.4 mL perchloric acid in 800 mL trace element water). After heating on 100°C for 15 min, and centrifugation (10 min, 3000 rpm), the absorbance of the supernatant was measured at 532 nm, against blank (distilled water). Inhibition of lipid peroxidation in percent (%) was calculated by the formula:

$$\text{Percentage inhibition (\%)} = [(A_{control} - A_{sample})/A_{control})] \times 100, \qquad (2)$$

where, $A_{control}$ is the absorbance of the control reaction (containing all reagents and distilled water against test compound); and A_{sample} is the absorbance of the test compound.

Statistical Analyses

All analyse for antioxidative activity determination and also test for determination of the total phenolic content (TPC) were run in triplicate. The average value and standard deviation were calculated using the Microsoft Excel software (Microsoft Corporation, Redmond, WA).

RESULTS AND DISCUSSION

Total Phenolics and Antioxidant Activities of Plant Materials

The antioxidant activities and total phenolics of four examined cereals and 4 examined legumes are shown in Table 1. All plants showed a significant amount of total phenolics and more or less effective antioxidant activities (Table 1). Among cereals buckwheat had the highest amount of total phenolics, with highest DPPH radical scavenging activity and ability for $Fe3^{+}$ reduction, but it had the lowest lipid peroxidation inhibition. These data suggest that it might be more critical, in delaying the lipid peroxidation, to suppress the initiation of the radical chain reaction, than to terminate the radical chain reaction by quenching or removing the radicals generated during propagation of the radical chain

Table 1 Total phenolics and antioxidant activities of plant materials.

Sample name	TPC* (mg GAE/g d. e)	DPPH§ (IC50) (μg/ml)	FRAP‡ (nmol Fe2+/mg d.e.)	TBA# (%)
Lentils (Lens culinaris)	21.9 ± 0.03	143.7	22.38 ± 0.51	54.1 ± 0.55
Red kidney bean (Phaseolus vulgaris)	18.8 ± 0.05	138.0	19.83 ± 0.62	39.2 ± 0.75
Soybean (Glycine hispide)	18.7 ± 0.02	> 200	8.34 ± 0.54	49.2 ± 0.35
Mung bean (Vigna radiata)	17.0 ± 0.05	152.3	24.98 ± 0.58	47.2 ± 0.55
Buckwheat (Fagopyrum esculentum)	50.7 ± 0.04	76.7	49.43 ± 0.49	45.6 ± 0.55
Barley (Hordeum vulgare)	16.4 ± 0.04	> 200	15.56 ± 0.67	50.8 ± 0.75
Wheat (Triticum durum)	16.2 ± 0.07	> 200	12.15 ± 0.60	55.2 ± 0.35
Rye (Secale cereale)	13.2 ± 0.06	> 200	8.94 ± 0.86	57.6 ± 0.45

*TPC: Total Phenolic Content by Folin-Ciocalteu method; §DPPH Radical Scavenging Activity; ‡FRAP: Ferric Reducing Ability of Plasma; and #Tiobarbituric Acid method (TAB).

reaction.[18] Other cereals had similar TPC or AOA. Among legumes lentil had the highest amount of total phenolics and the highest lipid peroxidation inhibition ability (TBA), but mungo precede in ability for $Fe3+$ reduction (FRAP), while red kidney bean precede in DPPH radical scavenging activity.

Legumes generally precedes in TPC, DPPH scavenging ability, and FRAP comparing with cereals, with the exception of buckwheat, which had the highest phenolics content, DPPH radical inhibition activity, and ferric reducing power among all examined plants. Furthermore, soybean and rye had similar FRAP numbers, while cereals in generally precedes in lipid peroxidation inhibition ability comparing with legumes.

Total Phenolics Content

As shown in Table 1, total phenolic contents in examined cereals were the highest in buckwheat 50.7 mg GAE/g dry extract. Similar phenolic content in buckwheat was reported by Velioglu et al.[19] Lower total phenolic contents were found in wheat and barley (16.2 and 16.4 mg GAE/g d.e., respectively) and the lowest in rye 13.2 mg GAE/g dry extract. In the case of wheat and barley grain results reported by Zhou and Yu showed that the contents of TP were affected by the extraction solvents with the following order from high to low: acetone > ethanol > methanol, which can be used to explain lower amount of phenols in this plants.[20] According to Zielinski and Troszynska way and length of extraction has great affect on TPC in rye, so it is very difficult to compare results in our work with those given by this set of authors.[21] Generally it is difficult to compare our data with other data from literature, due to different methods of extraction, determination and plants results calculations applied by other authors.

Total phenolic content in examined legumes, as showed in Table 1, was the highest in lentils 21.9 mg GAE/g dry extract. Similar phenolic content of lentils was reported by other authors according to whom lentils have the highest amount of total phenols comparing with soybean, bean and peas.[22] Slightly lower total phenolic contents were in red bean and soybean (18.8 and 18.7 mg GAE/g d.e., respectively) and the lowest in mung bean (17.0 mg GAE/g d.e.). In the case of red beans, it is very difficult to compare results in our work with those given by other authors, due to different methods of extraction, determination, and plants results calculations applied by them. In many studies

TPC values were expressed as micromoles of catechin per gram of crude bean or bean seed.[23] According to Prakash et al. TPC for soybean showed wide variation from 6.4 to 81.7 mg GAE/g of seed extract from different varieties.[9] Also the biological activity of soybean poliphenoles may depend on the type of processing and storage conditions, knowing that degradation of the phenolics substances may be associated with the occurrence of oxidative reactions or decomposition of thermolabile compounds induced by heat, and also with the possibility of losses of volatile substances during spray drying.[24] For mung bean, similar phenolic content was reported by other authors,[25] while considerable variation in TP are present also among the mung bean cultivars, which may be attributed to factors such as natural chemical composition, maturity at harvest, soil state, and conditions of postharvest storage.[26]

DPPH Radical Scavenging Activity

DPPH radical has been widely used in assessment of radical scavenging activity because of its ease and convenience. The scavenging effect of cereal extracts on DPPH radical is shown in Fig. 1. The weakest effect was noted for the extract of wheat with only 31% of inhibition within the highest sample concentration (200 μg/mL), followed by barley and rye (36.6 and 45%, respectively). In other investigations those extracts also possessed a weak activity to scavenge DPPH radical with this sample concentration.[27] Much stronger scavenging effects on DPPH radical were found for buckwheat extract (82.5%), leading to IC50 76.7 μg/ml. The DPPH radical scavenging effect observed in this work is in agreement with literature data reported by Sun and Ho.[28]

As shown in Fig. 2 and Table 1, among examined legumes, soybean showed the weakest effect (35.4% within highest sample concentration, IC50 higher than the highest concentration), followed by mung bean (64.6%, IC50 152.3 μg/ml), lentil (67.0%, IC50 143.7 μg/ml), and red kidney bean (69.8%, IC50 138.0 μg/ml). The DPPH radical

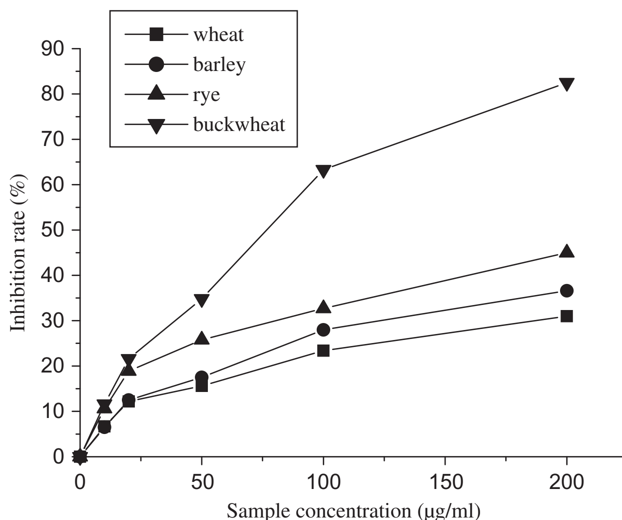

Figure 1 DPPH radical scavenging activity for cereal samples.

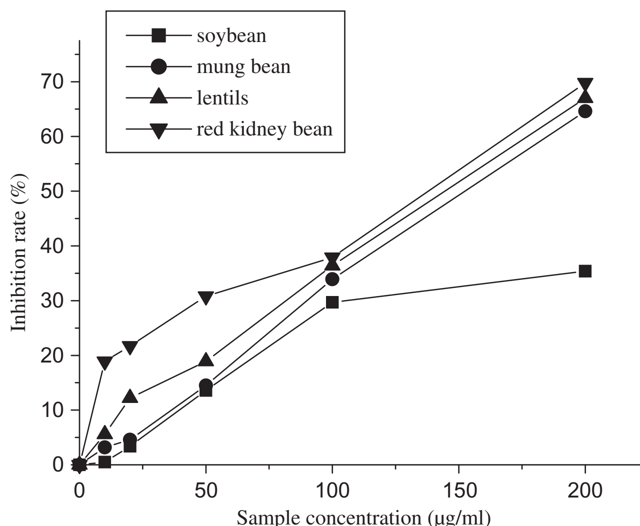

Figure 2 DPPH radical scavenging activity for legume samples.

scavenging effect observed in this work is in agreement with literature data applied by other authors.[9]

It is obvious from Table 1 that there was lack of correlation between TPC and DPPH radical scavenging activities of plants, because those cereals which had higher TPC values were not necessary better in DPPH inhibition (Table 1; Fig. 1). According to Brand-Williams, Cuvelier and Berset, ferulic acid, the main phenolic acids in cereal grains, showed a weak antiradical effect in experiments with DPPH radical, which can be used to explain this discordance among cereals.[29] Also, although red kidney bean and soybean among group of examined legumes showed similar TPC values (18.8 and 18.7 mg GAE/g d.e.), their DPPH radical scavenging activities different remarkably, in the way that red bean had the highest (IC50 143.7 μg/ml) while soybean showed the lowest DPPH inhibition (IC50 higher than the highest concentration of 200 μg/ml). This discordance can be explained by limitations of Folin-Ciocalteu method. Although the Folin-Ciocalteu method is widely used to determine the total phenolic contents in botanical and biological samples, it has limitations. Other reducing agents, such as L-ascorbic acid and sulphur dioxide, may also react with the Folin-Ciocalteu agent and contribute to the total absorbance, which generally results in overestimated levels of total phenolic contents. In addition, individual phenolics compounds may have different reactivity with the Folin-Ciocalteu reagent, which could result in potential errors in the total phenolic content measurements.[27]

Generally, there are several reasons to explain the ambiguous relationship between the antioxidant activity and total phenolics: (1) total phenolic content did not include all the antioxidants, such as ascorbic acid, carotenoid and tocopherol; (2) the synergism among the antioxidants in the mixture made the antioxidant activity, not only dependent on the concentration of antioxidant, but also on the structure and interaction among the antioxidants. That is why samples with similar concentrations of total phenolics may vary remarkably in their antioxidant activity. (3) Different methods to measure antioxidant activity with various mechanisms may lead to different observations.[28]

FRAP Method

Ferric reducing antioxidant power (FRAP) of examined cereals as showed in Table 1 was in correlation with total phenolic content. The highest FRAP number expressed in nmol of $Fe2^{+}$/mg dry extract was in buckwheat (49.43 nmol Fe2+/mg d. e.), followed by fairly lower FRAP in barley and wheat (15.56 and 12.15 nmol $Fe2^{+}$/mg d. e., respectively), and the lowest ferric reducing antioxidant ability in rye (8.94 nmol $Fe2^{+}$/mg d. e.).

Among examined legume samples ferric reducing antioxidant power (FRAP) was the highest in mung bean (24.98 nmol $Fe2^{+}$/mg d.e.), followed by lentils (22.38 nmol $Fe2^{+}$/mg d.e.), red kidney bean (19.83 nmol $Fe2^{+}$/mg d.e.), and considerably lower in soybean (8.34 nmol $Fe2^{+}$/mg d.e.) (Table 1). Difficulty in data comparing is even more obvious with FRAP method. In our work, results were expressed in nmol of $Fe2^{+}$/mg dry extract, but other authors reported their results in nmol or mmol $Fe2^{+}$ in mg or g grains or flour.[30,31] Besides, extraction solvents and methods of samples preparations in other works were different, and both is disproved to have influence on FRAP.

It is interesting that rye which had significant DPPH radical scavenging activity, showed the lowest ferric reducing power, and also legumes which had significant DPPH radical inhibitor activity, such as red kidney bean, showed lower ferric reducing power, comparing with legumes with lower DPPH scavenging activity and higher FRAP number, such as lentils. It appears that care should be taken when using free radicals as the basis for generating an antioxidant activity, because the activity is very dependent on the specific free radical used.[32] One should use different free radicals and calculate an antioxidant score as done by Cao, Sofic, and Prior or one should preferably use the FRAP assay, which is based on a much less selective reduction.[32]

Thiobarbituric Acid Test (TBA)

As shown in Table 1, there is lack of correlation between TPC and the ability of lipid peroxidation inhibition in cereals and legumes. Plants with higher TPC values were not necessary better in inhibition of lipid peroxidation. Wheat extracts, for instance, had very high ability to inhibit lipid peroxidation in liposome but showed the lowest ability to directly react with and quench radical DPPH, while although red kidney bean had the highest ability to directly react with and quench radical DPPH among examined legumes, it showed the lowest ability to inhibit lipid peroxidation in liposomes. This can be explained with complex mechanism of lipid peroxidation inhibition, which includes not only uncompounded phenols, but also high-molecular polyphenols and other nonphenolics antioxidants. In addition, these data may suggest that it might be more critical to suppress the initiation of the radical chain reaction, than to terminate the radical chain reaction by removing the radicals generated during propagation of the radical chain reaction.[18] According to results, rye had the greatest ability for inhibition of lipid peroxidation among examined cereals (57.6% within highest sample concentration (250 μg/mL)), followed by wheat and barley (55.2 and 50.8%, respectively), and the weakest result in TBA test was in buckwheat (45.6%) (Table 1). Among legumes lentils had the greatest ability for inhibition of lipid peroxidation (54.1% within the highest sample concentration), followed by soybean and mung bean (49.2 and 47.2%, respectively), and the weakest results in TBA test was in red kidney bean (39.2%) (Table 1).

CONCLUSION

This study indicated that cereals and legumes, used widely for human consumption, exhibited significant free radical scavenging activities, ferric reducing power, ability for inhibition of lipid peroxidation, and total phenolic contents. These factors suggest that cereal- and legume-based foods could contain important dietary antioxidants and therefore warrant further research to determine whether these dietary antioxidants could be beneficial to human health. There were several significant differences among cereals and legumes in these characteristics, which warrant further study, especially in regard to their effects on human health. More research is needed to adequately know the composition of the extracts, to identify the antioxidant compounds in the extracts, and to evaluate the potential use of cereal and legume products like natural antioxidants and therefore using for food supplements. In addition, identification of both biological (e.g., digestion) and food processing conditions that impact the distribution, stability and activity of wheat antioxidants is needed in order to be able to produce food products with maximum health benefits.

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