https://doi.org/10.17221/248/2018-CJFS

Antioxidant ability of polyphenols from black rice, buckwheat and oats: *In vitro* and *in vivo*

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Citation: Xie F., Lei Y., Han X., Zhao Y., Zhang S. (2020): Antioxidant ability of polyphenols from black rice, buckwheat and oats: *In vitro* and *in vivo*. Czech J. Food Sci., 242–247.

Abstract: Grains (black rice, buckwheat and oats) contain polyphenols and have stronger antioxidant capacity than staple foods. Their polyphenols were identified and investigated for their antioxidant capacity. The black rice and buckwheat polyphenols were mainly flavonoids; those in oats were phenolic acids. *In vitro*, their radical-scavenging capacities were determined as black rice > buckwheat > oats. Similarly, *in vivo*, the increase in total antioxidant capacities and decline in malondialdehyde indicated the enhancement of radical-scavenging and repair abilities of all polyphenol extracts. Differences in superoxide dismutase, catalase activities, glutathione peroxidase activities and oxidase activities suggested that polyphenols from black rice and buckwheat have higher antioxidant activities, indicating that their antioxidant ability is related to polyphenol composition which depends on a polyphenol source. Thus, a combination of diets will make a complementary mixture of polyphenols that can enhance absorption in the intestinal tract and defence ability against oxidative stress.

Keywords: coarse grain; polyphenolic compounds; HPLC/electrospray-ionization mass spectrometry

Grain, commonly grouped into five grains or cereals, refers to all grain crops except rice and wheat. Due to short growth period and strong growing adaptability of grains, polyphenols accumulate in large quantities, and have stronger antioxidant capacity than that of staple foods (Kamiya et al. 2014). The black rice polyphenols mainly comprise scabiolide-3-glucoside, paeoniflorin-3-glucoside, and other anthocyanins, which exist in the form of glycosides in the black rice seed coat (Asem et al. 2015; Ti et al. 2015). The polyphenols in buckwheat seeds are mainly rutin, 3-flavonols, phenolic acids, and their derivatives, providing the basis of the biological function of buckwheat (Giménez-Bastida et al. 2015). The oat alkaloids are a kind of polyphenol that is specific to oats, mainly existing in the bran and aleurone layer. There are fewer flavonoids in oats but many phenolic acids, like ferric acid and vanillic acid, which exist as complex compounds formed by combination with soluble esters, proteins, sugars, and other macromolecules (Chu et al. 2013). Cereal polyphenols in diet regulate the balance between the oxidation and antioxidant systems in the human body through free-radical scavenging, realising prevention against aging and diseases such as cancer (Luo et al. 2014; Ortiz-Martinez et al. 2014). Polyphenols have different forms and play antioxidant roles in different parts of the human body. Free polyphenolic compounds are mainly absorbed in the stomach and small intestine, whereas combined phenols, without decomposition by the intestinal enzymes, go through

Supported by the Natural Science Foundation of Heilongjiang Province, Project No. C2018015; the Young Talents Project of Northeast Agricultural University No. 17QC15; the Young Creative Talents Undergraduate Colleges of Heilongjiang Province, Project No. UNPYSCT-2016142; Postdoctoral Science Foundation of Heilongjiang Province, Project No. LBH-Z17012, and Postdoctoral Science Foundation of China, Project No. 2018M631901.

https://doi.org/10.17221/248/2018-CJFS

the gastrointestinal tract, arrive in the colon, and have antioxidant effects after fermentation by bacteria there (Bonoli et al. 2004; De et al. 2007).

In the present work, black rice, buckwheat, and oat extracts were investigated for main polyphenols, and their free-radical scavenging abilities were determined relative to a D-galactose induced aging animal model. Thus, a relationship between polyphenols from different grains and their antioxidant capacities was revealed and it provides a basis for the development of grain-based healthy foods with nutritional balance.

MATERIAL AND METHODS

Raw materials. The organic glutinous black rice, No. 2 flat common buckwheat, and oats for Naked Oat G4 used in this work were produced in the city of Mudanjiang in China. The above-mentioned coarse grains were crushed through 60 mesh sieves after comminution and were kept at 4 °C for further analysis.

Extraction of polyphenolic compounds from cereals. An aliquot of 10.0 g of grain powder dissolved in 100 mL of ethanol solution (70%, v/v) was extracted under stirring for 5 h at 37 °C. After centrifugation at 3 524 g for 15 min, the supernatant was concentrated at 55 °C using a rotary evaporator (RE201B; Shanghai Yarong, Ltd., China) to make a final volume of 25 mL, which is used as a grain polyphenol extract.

High-performance liquid chromatography/electrospray-ionization mass spectrometry (HPLC/ESI-MS). Polyphenol extracts (10 μL) from grains were passed through a 0.45 μm membrane and injected into an Agilent 1200 HPLC system (Angery Company, USA) equipped with Zorbax eclipse XDB-C_{18} column (150 mm × 4.6 mm, 5 μm). Acetonitrile (mobile phase A) and 2% aqueous acetic acid (mobile phase B) were used for gradient elution at 30 °C with a flow rate of 1 mL min^{-1}, as follows: 0–30 min, 100% A; 30–35 min, 40% A; 35–42 min, 1% A. The HPLC column effluent was directly injected into an ion trap mass spectrometer (G6320; Agilent, USA) operated with nebulizer pressure of 25 Psi, drying gas (N_2) at a flow rate of 9 L min^{-1}, and capillary voltage of 3.0 kV at 350 °C. The measurements were conducted in the spectral range of mass-to-charge ratio (m/z) 100–2 000, with positive/negative ion mode.

***In vitro* radical scavenging assays.** The hydroxyl radical (•OH) scavenging ability was determined as described by Smirnoff et al. (1989). The •OH clearance rate (%) was calculated as [A0 – (A1 – A2)] × 100/A0, where A0 is absorbance without scavenger (polyphenol extract), A1 is absorbance with scavenger, and A2 is absorbance of blank (without salicylic acid). The superoxide anion (O_2-•) free-radical scavenging activity was determined as described previously (He et al. 2013). The O_2-• clearance rate (%) was calculated as $(\Delta A1/\Delta t - \Delta A2/\Delta t) \times 100/\Delta A1/\Delta t$, where $\Delta A1/\Delta t$ is the self-oxidation reaction rate of pyrogallol, and $\Delta A2/\Delta t$ is the self-oxidation rate of pyrogallol after addition of rain extract. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) free-radical scavenging ability was measured according to Sahreen et al. (2010). The DPPH clearance rate (%) was calculated as (A0 – A1) × 100/A0, where A0 is absorbance of blank sample and A1 is absorbance of reaction solution with polyphenol extract.

Animals and diets. A group of 60 five-week-old, 14–18 g specific-pathogen-free (SPF) male Balb/c nude mice purchased from Experimental Animal Technology Co., Ltd. (Beijing, China) were kept in animal rooms (25 °C, relative humidity of 60%) in daylight, with standard feed (twice a day) and sterilised drinking water (once a day).

The mice were fed a basal diet for one week and then randomly divided into six groups (n = 10 in each). The normal control group was intraperitoneally injected with 0.1 mL physiological saline, whereas D-gal aging group, D-gal positive control, and test group were injected intraperitoneally with 0.1 mL D-galactose solution (100 mg kg^{-1}). The model rats were intragastrically administered with 50 mg kg^{-1} polyphenol extract; 50 mg kg^{-1} vitamin C daily gavage was given to positive control, with the same dose of physiological saline for normal control and aging group. The mice were weighed once a week, and gavage and D-galactose solution doses were adjusted according to mice weights.

Blood sampling and biochemical analysis. After 6 weeks of feeding, 24 h-fasted mice were slaughtered, and blood was collected by removing the eyeballs. The liver and other organs washed with cold physiological saline were dried off with filter paper. The total antioxidant capacity (T-AOC), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) and malondialdehyde (MDA) were determined by spectrophotometry using relative commercial enzymatic kits (Nanjing Institute of Biotechnology, Nanjing, China) according to the manufacturer's protocol.

Statistical analysis. All experiments were done in triplicate, and results were reported as mean ± standard deviation (SD). Data were analysed by one-way analysis of variance (ANOVA) test. Duncan's test ($P < 0.05$) was used to assess significant differences within groups using SPSS 17.0 software.

https://doi.org/10.17221/248/2018-CJFS

RESULTS AND DISCUSSION

Identification of phenolic compounds in grains. HPLC/ESI-MS was used for the analysis of grain polyphenols, and the main compounds are shown in Table 1. According to the retention times, molecular ion peaks and fragment ion peaks characteristic of these compounds (Verardo et al. 2010; Ren et al. 2013; Hao et al. 2015; Hitayezu et al. 2015), and the main polyphenols of grains were determined: main polyphenols in black rice are cyanidin-3-sophoroside, cyanidin-3-O-glucoside, peonidin-3-glucoside, and procyanidin glucoside; main polyphenols in buckwheat are (epi)afzelchine (epi)catechin isomers, caffeic acid hexose, procyanidin B2, glucosyl rutin sodium salt, epiafzelchine epicatechin-O-dimethylgallate, and epicatechin-O-3,4-dimethylgallate; and main polyphenols in oats are N-(3,4-dihydroxycinnamoyl)-5-hydroxyanthranilic acid, oat anthracene and its derivatives, two and twenty-six-1-alkyl alcohols, phenolic acid esters of twenty-eight alkyl acids, twenty-six hydroxyalkanoic acids, and twenty-eight hydroxyalkanoic acids. Polyphenol bioavailability is related to their degree of structural complexity and polymerisation, some low-molecular-weight polyphenols (such as monomeric and dimeric structures) may be readily absorbed in the small intestine or reach the colon almost unchanged (oligomeric and polymeric polyphenols ~40 kDa). Therefore, the differences in the composition of polyphenols from these three grains may affect their absorption in the intestinal tract and health benefit.

***In vitro* radical scavenging assays.** The antioxidant capacities of polyphenol extracts are shown in Table 2. The free-radical scavenging capacities of polyphenols from these three kinds of grain were significantly ($P < 0.01$) different form each other and higher than those of VC (0.2 mg mL^{-1}). The hydroxyl, superoxide anion, and DPPH radical scavenging capacities of polyphenol extracts from black rice and buckwheat were much higher than those of oat extract, which may be due to the difference in their main polyphenol. As shown in Table 1, polyphenols in black rice and buckwheat are mainly flavonoid compounds with many phenolic hydroxy groups. The structural frames of polyphenol in black rice are anthocyanins, 2-benzopyran with a large number of attached hydroxy groups can provide 3–4 hydrogen atoms when used as a hydrogen donor (Shao et al. 2014; Yoon et al. 2014). The B ring structure of polyphenols from buckwheat is a typical 3,4-catechol structure, which provides hydrogen and electrons to free radicals during the oxidation

Table 1. Phenolic compounds determined by HPLC/ESI-MS in black rice, buckwheat and oat extract

Peak	Remaining time (min)	Compound	Selected ion	*m/z* experimental	MS fragment
Black rice					
1	3.02	cyanidin-3-sophoroside	$[M + H]^+$	613.31	527.44, 326.21
2	12.78	cyanidin-3-O-glucoside	$[M + H]^+$	449.21	287.45
3	13.79	peonidin-3-glucoside	$[M + H]^+$	463.25	385.27, 301.40
4	16.38	procyanidin glucoside	$[M + H]^+$	895.24	733.16, 571.43
Buckwheat					
5	2.51	(epi)afzelchine(epi)catechin isomer	$[M - H]^-$	562.81	400.87, 340.97
6	2.73	caffeic acid hexose	$[M - H]^-$	341.03	195.09
7	13.43	procyanidin B2	$[M - H]^-$	575.06	383.02, 205.89
8	16.03	glucosyl rutin sodium salt	$[M - H]^-$	795.25	633.14, 301.28
9	18.88	epiafzelchineepicatechin-O-dimethylgallate	$[M - H]^-$	741.03	468.89, 271.10
10	20.18	epicatechin-O-3,4-dimethylgallate	$[M - H]^-$	468.93	331.13, 122.88
Oats					
11	2.11	n-3,4-two hydroxy cinnamoyl-5-hydroxy anthranilic acid	$[M + H]^+$	626.75	338.85, 316.82
12	2.53	oat anthracene	$[M + H]^+$	295.11	258.21, 125.06
13	2.74	oat anthracene derivatives	$[M + H]^+$	235.24	118.19, 118.19
14	17.73	phenolic esters	$[M + H]^+$	815.01	679.86, 114.05

m/z – mass-to-charge ratio; MS – mass spectrometry

https://doi.org/10.17221/248/2018-CJFS

Table 2. The antioxidant activities of three grain polyphenol extracts

	•OH clearance rate (%)	O2-• clearance rate (%)	DPPH clearance rate (%)
Black rice	58.66 ± 1.56[a]	51.35 ± 0.78[a]	79.29 ± 0.64[a]
Buckwheat	52.66 ± 0.27[b]	49.32 ± 3.51[b]	59.58 ± 1.55[b]
Oats	37.18 ± 1.35[c]	47.30 ± 2.87[c]	26.74 ± 0.88[c]
VC	20.75 ± 2.82[d]	10.7 ± 1.52[d]	23.72 ± 2.36[d]

The means in the same column with the different subscript significantly differ ($P < 0.05$); VC – ascorbic acid

Table 3. Effect of grain polyphenols on the total antioxidant capacity (T-AOC) of aging mice plasma and liver tissue

Group	Plasma (U mg prot^{-1})	Liver (U mg prot^{-1})
Blank control	209.42 ± 3.49[d]	6.30 ± 0.81[d]
D-gal positive control	131.72 ± 2.19[a]	5.63 ± 0.73[a]
Black rice	151.70 ± 2.53[b]	6.27 ± 0.57[d]
Buckwheat	167.49 ± 2.39[b]	5.79 ± 0.51[b]
Oats	147.48 ± 0.67[a]	5.71 ± 0.35[ab]
Positive control	165.49 ± 3.30[b]	6.06 ± 0.37[c]

The means in the same column with the different subscript significantly differ ($P < 0.05$); U mg prot^{-1} – unit number of enzyme activity per mg protein in liver tissue

process (Roleira et al. 2015). Since polyphenols in oats are mainly phenolic acids, and they exist in complexed form with soluble esters, proteins and sugars which is difficult to dissociate, leading to poor antioxidant activity. This is in an agreement with previous findings that the antioxidant capacity of phenolic acids and flavonoids is positively correlated with the number of hydroxy groups in rosmarinic acid and other molecular structures (Hitayezu et al. 2015).

Antioxidant capacity *in vivo*. The accumulation of active oxygen is one of the important physiological changes during the aging process in animals, which can be characterised by T-AOC (total antioxidant capacity) level (Lee et al. 2012). As shown in Table 3, the total antioxidant capacity in mice plasma and liver tissues of aging group is significantly ($P < 0.05$) lower than that of blank control, indicating that continual injection of D-galactose causes metabolic disorders and aging symptoms and the modelling was successful. For polyphenol and ascorbic acid (VC) positive control groups, plasma and liver T-AOC activities were significantly higher than in aging group, which indicates that the polyphenol extracts from grains and VC can improve the T-AOC and enhance their scavenging ability for free radicals and ability to repair the damaged liver tissues in aging mice.

The activity of SOD, GSH-Px and CAT in the plasma of aging mice is shown in Table 4. Plasma SOD and CAT activities in black rice and buckwheat groups are significantly ($P < 0.05$) higher than in control and aging group. GSH-Px activities in these groups were lower than in the control but significantly higher than in aging group ($P < 0.05$). This suggests that black rice and buckwheat polyphenol extracts can significantly improve the aging of mouse plasma by enhancing the body's ability to scavenge free radicals. Moreover, these data come to the same conclusion with the *in vitro* radical scavenging ability analysis, which can explain that main polyphenols in oats, phenolic acids existing in the form of large molecular complexes that cannot be decomposed by enzymes in the intestines, lead to lower levels of T-AOC, SOD, GSH-Px, and CAT activity in the plasma and liver tissues of oat group. The activities of three antioxidant enzymes in the plasma of oat group were lower than in control group, but higher than in aging group, indicating that the oat polyphenol extract also has free-radical scavenging ability in aging mice.

Table 4. Effect of grain polyphenols on the activity of antioxidant enzymes in the plasma of aging mice

Group	SOD (U mL^{-1})	GSH-Px (U)	CAT (U mL^{-1})
Blank control	120.28 ± 2.41[c]	313.86 ± 4.14[e]	11.85 ± 0.74[b]
D-gal positive control	105.16 ± 2.10[a]	216.98 ± 4.70[a]	10.48 ± 0.66[a]
Black rice	135.23 ± 2.13[e]	294.44 ± 4.08[c]	14.81 ± 0.69[c]
Buckwheat	126.00 ± 2.52[d]	254.55 ± 3.75[b]	15.09 ± 0.67[c]
Oats	107.23 ± 3.21[a]	222.24 ± 4.05[a]	12.47 ± 0.10[b]
Positive control	124.73 ± 2.49[cd]	297.14 ± 3.94[c]	16.53 ± 0.80[d]

The means in the same column with the different subscript significantly differ ($P < 0.05$); U mL^{-1} – unit number of enzyme activity per mL plasma

https://doi.org/10.17221/248/2018-CJFS

Table 5. Effect of grain polyphenols on MDA levels in the plasma and liver of aging mice

Group	Plasma (U mL^{-1})	Liver (U mg $prot^{-1}$)
Blank control	2.93 ± 0.36^{e}	3.31 ± 0.20^{f}
D-gal positive control	4.12 ± 0.14^{a}	6.83 ± 0.24^{h}
Black rice	3.26 ± 0.11^{d}	1.85 ± 0.12^{b}
Buckwheat	3.86 ± 0.27^{c}	2.15 ± 0.13^{d}
Oats	4.07 ± 0.33^{b}	2.04 ± 0.13^{cd}
Positive control	2.71 ± 0.33^{f}	2.50 ± 0.16^{e}

The means in the same column with the different subscript significantly differ ($P < 0.05$); U mL^{-1} – unit number of enzyme activity per mL plasma; U mg $prot^{-1}$ – unit number of enzyme activity per mg protein in liver tissue

Thus, these polyphenol extracts can improve the activity of antioxidant enzymes in the plasma of aging mice and reduce the degree of cell damage.

As shown in Table 5, the MDA levels in the plasma and liver tissues of aging group are significantly higher than in the control ($P < 0.05$), indicating a subacute aging model of mice. The plasma and liver MDA levels in mice were significantly different ($P < 0.05$) in test groups and aging group: Grain polyphenol extracts significantly decreased MDA levels in the plasma and liver tissues, which alleviated the damage caused by D-galactose by reducing the lipid peroxidation in the tissue cells and thus slowed down the aging process in mice.

CONCLUSION

The black rice and buckwheat polyphenols were mainly flavonoids; those in oats were phenolic acids. *In vitro*, antioxidant activities were evaluated as black rice > buckwheat > oats, from radical-scavenging capacities. Similarly, *in vivo*, the increase in total antioxidant capacities and decrease in malondialdehyde indicated the enhancement of radical-scavenging and repair abilities of all polyphenol extracts. Superoxide dismutase and catalase activities in black rice and buckwheat groups were significantly higher than in control and aging groups; glutathione peroxidase activities were lower than in the control but significantly higher than in aging group. Oxidase activities in oats were slightly higher than in aging group, which is consistent with the polyphenol difference, indicating that the antioxidant ability is related to the polyphenol composition. Thus, the combination of grains containing mixed multipolyphenols may improve intestinal tract absorption and the body's defences against oxidative stress by complementary antioxidant abilities.

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Received: August 31, 2018
Accepted: July 27, 2020