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Effect of in bio-processing on antioxidant activity of selected cereals

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ABSTRACT

The biological properties of antioxidants depend on their release from the food matrix during the digestion process. Cereals contain a wide range of phenolic compounds which are of great significance due to their antioxidant activity. In vitro digestion is a rapid and inexpensive method used to determine the availability of nutrients involved in the absorption studies with humans. Total phenol, flavonoids, flavonol and antioxidant activity of five cereals was evaluated by in vitro digestion and chemical extraction along with effect of cooking. All the raw and cooked in vitro digested cereal samples showed significantly higher phenolic value and flavonoid content compared to chemical extraction. Cooked in vitro digested cereal samples showed highest amount of flavonol content. Highest antioxidant activity was observed in raw in vitro digested wheat sample as measured by FRAP method. Cereal samples extracted using chemical approach showed highest % inhibition followed by raw in vitro digested samples, cooked in vitro digested samples as measured by DPPH method. In the TEAC (% inhibition) of all cereal samples measured by ABTS method, raw in vitro digested samples showed significantly higher values followed by cooked in vitro digested samples, control samples and chemically extracted cereal samples. Maximum TEAC was observed in raw in vitro digested pearl millet sample (79.39 %).

Keywords: Antioxidant, Chemical Extraction, Enzymatic Extraction, Phenolic compounds

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INTRODUCTION

There is growing scientific evidence associating diets rich in antioxidant compounds which occur particularly in plant foods with a lower risk of developing cardiovascular disease, certain kinds of cancer and age-related degenerative processes. (Onyeneho and Hettiarachchy 1992; Maillard et al. 1996; Bourne and Rice-Evans 1998; Deighton et al. 2000). Particular attention has been paid to their role as “free radical scavengers” and has provoked numerous studies into phenolic compounds in many plants, including cereals. But the study of each individual antioxidant compound is not cost effective due to the comxbplex nature of antioxidants in foods. Such studies will be of less significance due to the fact that synergistic interaction of antioxidant compounds is not considered. Due to these reasons, nowadays, antioxidant activity measurement assays are used widely (Serrano et al. 2007). Antioxidant capacity of different cereal products such as corn, wheat, rice, oats and ready to eat breakfast cereals has been reported previously (Adom and Liu 2002; Ruffian- Henares and Delgado-Andrade 2009, Singh et al. 2011). In these cereals, antioxidant activity of different extracts correlates with their total plant phenolic content (Zielinski and Kozlowska 2000). Cereals contain many phenolic compounds, having different chemical structures, of which phenolic acids are of great significance (Kahkonen et al.1999). But, the extraction of antioxidants from cereals can be partial, which can lead to misinterpret their actual biological availability and activity (Perez-Jimenez and Saura-Calixto 2005). The widely used solvent for phenolic extraction is ethanol, acetone alone or in combination with water (Yu et al. 2002a; Yu et al. 2002b; Adom et al. 2003; Liyana-Pathirana and Shahidi 2005; Nam et al. 2006,

Saura-Calixto and Goni 2006, Annegowda et al. 2011) which can be more improved by using methanol alone or in combination with water (Handelman et al. 1999). Also, antioxidants have to be present in some amount in the specific tissue or organ to employ their biological properties. Thus, release of antioxidants from complex food materials during digestion may decide the effect of their biological properties. This biological extraction of antioxidants within the digestive system might be different from the chemical extraction methods used in the studies. Thus, release of antioxidants from the food material during the digestion process affects their biological characteristics, which can be different than antioxidants extracted by chemical methods. It has also been suggested that antioxidant activity from the chemical extracts of the food material might misjudge the actual antioxidant capacity in the digestive tract (Serrano et al. 2007); hence measurement of actual antioxidant capacity of cereal becomes necessary, which depends up to a large extent on the method of extraction of antioxidants from foods (Perez-Jiminez et al. 2008). Long-term and short-term bioavailability studies of different compounds possessing antioxidant activity have been conducted in humans as well as appropriate animal models (Boileau et al. 1999, 2000; Bub et al. 2000; Bugianesi et al. 2002; Gomez Aracena et al. 2003). However, human and animal studies are time consuming and costly, and therefore there is a need to use *in vitro* digestion models that simulate the chemical and enzymatic reactions that occur during food digestion in human digestive system. Due to these reasons, nowadays, research work is increasing regarding *in vitro* digestion models in different food systems (Gawlik-Dziki et al. 2009; Toor et al. 2009, Bouayed et al. 2011;3 Cilla et al. 2011; Hur et al. 2011; Wootton-Beard et al. 2011). *In vitro* digestion method measures the bioavailability of the nutrient, which is the amount of the nutrient liberated from the food material during gastrointestinal digestion, which is available for absorption in the body (Hedren et al. 2002; Kulp et al. 2003). It can be used to evaluate a large number of food systems, which would be costly to analyze for different parameters using human or animal models. Effect of cooking on different foodstuffs is widely available in literature, like enhancement of aroma, test and flavor (Deol and Bains 2010). However, still little information is available regarding its effect on cereals. In cereals, it has been found to enhance its antioxidant activity (Fares et al. 2010). The present study was planned to compare two procedures; an enzymatic extraction (which is similar to *in vitro* physiological extraction) with chemical extraction for total phenol, flavonoid and antioxidant activity of five selected cereals, which are commonly consumed in India. urthermore, the effect of cooking was also analyzed on the total antioxidant capacity (TAC) of cereals.

MATERIALS AND METHODS

Procurements of cereals, chemicals and preparation of cooked cereals Five commonly consumed cereals namely Wheat (*Triticum aestivum* L.), Pearl millet (*Pennisetum glaucum* L.), Rice (*Oryza sativa)*, Maize *(Zea mays* L.), Sorghum (*Sorghum bicolor* L. Moench) were obtained from the local market. The local varieties procured were GW-273, GJ-39, Ganga-2, GHB-526 and GR-2 for Wheat, Sorghum, maize, Pearl millet and Rice, respectively. All the enzymes, Gallic acid, DPPH, TPTZ (2,4,6 -tripyridy-*s*-triazine), Catechin, Rutin, Trolox were purchased from Sigma, St. Louis, USA. Rest of the chemicals of highest purity grade was purchased locally. All five cereals were ground using a grinder (Maharaja Whiteline MX – 103, Maharaja Manufacturing, New Delhi) and obtained flours were sieved using 500 µ mesh sieve. To obtain cooked samples, each cereal was cooked traditionally, i.e., Chapatti was prepared from wheat; Rotlas were prepared from pearl millet, maize and sorghum. Rice was boiled to cook. After cooking, each sample was dried at 50°C in a hot air oven till removal of moisture and grounded further using mortar pestle to get the fine powder. Sample Extraction using Chemical and Enzymatic Approach The chemical extraction and enzymatic extraction were employed to determine the total content of antioxidant capacity in the cereals according to Serrano et al. (2007) with modifications. *Chemical Extraction* 300 mg of each of cereal flour were taken in 50 ml conical flask and 5 ml of Methanol: water (80:20) was added. The mixture was shaken for 30 minutes using a mechanical shaker at 150 rpm. The content of the flask was centrifuged at 5000 g for 10 minutes at 4°C and supernatants were collected. 5.0 ml of the same solvent was added again to each flask and process was repeated. Both supernatants were combined, filtered using Whatman filter paper No. 1 and used to determine total phenol, flavonoid, flavonol, Total Antioxidant Capacity by FRAP and DPPH methods. *Enzymatic Extraction* Enzymatic extraction or *in vitro* digestion was carried to make an effort to mimic the gastrointestinal conditions. Raw and cooked cereal samples (5 raw, 5 cooked, 5 control in which only buffers were added, and 1 blank in which only enzymes were added) were successively incubated with digestive enzymes. Briefly, 300 mg of flour of each cereal (cooked and raw) were incubated in a temperature controlled water bath with pepsin (0.2 ml of a 300 mg/ml solution in HCL – KCL buffer 0.2 M, pH 1.5, 37˚C, 1 hour), panceatin (1 ml of a 5 mg/ ml solution in phosphate buffer 0.1 M; pH 7.5, 37˚C, 6 hours), lipase (2 ml of a 7 mg/ml solution in phosphate buffer 0.1 M; pH 7.5, 37˚C, 6 hour), bile extract porcine (2 ml of a 17.5 mg/ml solution in phosphate buffer 0.1 M; pH 7.5, 37˚C, 6 hours) and α-amylase (1ml of a 120 mg/ml solution in tris-maleate buffer 0.1 M; pH 6.9, 37˚C, 16 hours). Then samples were

centrifuged (15 minutes at 5000 rpm) and supernatants were transferred to another tube. Residues were washed twice with 5 ml of distilled water and supernatants were combined. Each supernatant was incubated with 100 µl of amyloglucosidase for 45 minutes at 60˚C. Then all these supernatants were stored at -20˚C for various biochemical parameters' determination. Total Phenolic Compounds Estimation Total phenolic compounds were estimated according to the method described by Schwarz (2001). 0.05 ml aliquote from each extraction was taken in a test tube and volume was made up to 1 ml with distilled water. To this, 1 ml each of folin-ciocalteu reagent diluted with water (1:2) and 35 % Na2CO3 were added. The contents were incubated for 30 min at room temperature. 2 ml of distilled water was added and intensity of blue colour was recorded at 620 nm in UV visible double beam spectrophotometer (Hitachi 220S, Japan). Gallic acid of known concentration (5-20 mg) was used as standard.

Total Flavonoids Estimation

Flavonoid content was estimated by the method described by Zhishen et al. (1999). 0.1 ml of aliquot from each extraction was taken and volume was made up to 5 ml with distilled water. At 0 time, 5 % NaNO2 (0.3 ml) was added, after 5 min, 10 % AlCl3 (0.6 ml), and at the 6th min, 1 M NaOH (2 ml) solution was added and the total volume was made up to 10 ml with distilled water. The solution was mixed well and the absorbance was measured against prepared reagent blank at 510 nm. Standard series was prepared using known concentration of rutin, final volume as made up to 5 ml with distilled water and there after treated in similar way as for sample.

Total Flavonol Estimation

Total flavonol estimation was carried out according to Yermakov et al. (1987). 0.05 ml of aliquot of extract was taken and volume was made up to 1 ml with methanol. Then 0.5 ml of vanillin (1 % in methanol) and 0.5 ml 25% H2SO4 (in methanol) were added successively. The tubes were cyclomixed and allowed to react for 15 minutes at ambient temperature. The absorbance was read at 500 nm in a UV visible spectrophotometer (Hitachi 220S, Japan) against blank. Standard series of known concentration of catechine (10-40 µg) was taken and volume was made up to 1 ml with methanol. Thereafter all test tubes were treated in the same way as sample. For blank, 1.0 ml of methanol was taken and treated in the same way as sample.

Total Antioxidant Capacity

Ferric Reducing Antioxidant and Power assay (FRAP)

The FRAP (Ferric Reducing Antioxidant Power) method was used to evaluate the Total antioxidant capacity (TAC) of cereal extract according to Benzie and Strain (1996). For preparation of FRAP reagent, 20mM TPTZ solution (2.5 ml) was reacted with 40 mM HCl (containing 20 mM FeCl3.6 H2O, 2.5 ml; 0.3 M sodium acetate buffer pH 3.6; 25 ml). The reagent was prepared freshly and prior to use, was warmed at 37°C. 0.1 ml of cereal extract was taken in a test tube and volume was made up to 300 µl with distilled water. 1.8 ml of FRAP reagent was added and allowed to incubate at 37°C for 10 minute. The coloured complex was measured at 593 nm using double beam U.V. spectrophotometer (Hitachi 220S, Japan). For standard, known concentration of trolox (50-1000 µM) was taken and treated similar to sample. The FRAP values for the samples were then determined using the standard curve and the values were expressed as mg trolox equivalent/100 gm of sample. For blank, 300 µl of distilled water was taken and 1.8 ml FRAP reagent was added and treated similar to sample.

DPPH Radical Scavenging Activity

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity of cereal extract was measured according to McCue and Shetty (2003). 0.2 ml of cereal extract was taken and volume was made up to 1 ml with methanol. Then 3 ml of DPPH reagent (1 mM in methanol) were added. The content was mixed properly. It was incubated at 37˚C for 20 minutes. After incubation the absorbance was measured 517 nm in a UV visible double beam spectrophotometer (Hitachi 22OS, Japan). For control, 3 ml of DPPH was added to 1.0 ml of methanol. For standard, known concentration of trolox (10-40 µg) was taken and volume was made up to 1 ml with methanol. There after all test tubes were treated in the same way as sample. Methanol was used as a blank. Percent inhibition was calculated using the following formula: % inhibition = (Abs of control – Abs of sample)/Abs of control × 100

Statistical Analysis

Experiments were carried out in triplicate (n = 3) for each analysis and their means ± Standard deviation were reported. Differences between variable were tested for significance by using a one way analysis of variance procedure, Ducan, using level of significance $P \leq 0.05$ using SPSS 10.0 software for windows.

RESULTS AND DISCUSSION

Total Phenolic Content Plant phenolic compounds are nowadays getting increased attention in the diet due to their natural antioxidant potential. Increased consumption of phenolic compounds has been associated with the reduced risk of cardiovascular diseases and certain cancers (Liu 2004, 2007; Dykes and Rooney 2007). The mean value of total phenolic content of five commonly consumed cereals extracted using chemical and enzymatic approaches are shown in Table 1. In spite of cooking, the content of total phenolics was significantly ($p \leq 0.05$) lower in cooked *in vitro* digested (IVD) samples. All the raw and cooked IVD cereal samples showed significantly ($p \leq 0.05$) higher phenolic values than cereal samples treated with chemical approach. Thus, maximum amount of total phenolics were released during *in vitro* digestion process. The highest amount of total phenolics was found in the samples obtained from IVD Pearl millet. Extracts of wheat and maize obtained by chemical approach did not differ substantially than control samples. Thus, due to the effect of hydrolysis, phenolic compounds were liberated in the in vitro system. Among all the experimental samples, rice sample treated using chemical approach showed lowest value. Several reports mentioned that Rice phenolic compounds prevail in free, esterified and insoluble-bound forms, and insoluble-bound phenolics may be liberated by base, acid or enzymatic treatment of samples prior to extraction (Adom and Liu 2002; Zhou et al. 2004; Choi et al. 2007). Raw IVD rice samples showed significantly ($p \leq 0.05$) higher values than treated with chemical approach. The reduction in total phenolic compounds during cooking might be due to cooking treatment which may destroy some heat sensitive phenolic compounds. Recently, Fares et al (2010) reported decrease in free phenolic acid in cooked wheat pasta samples. They reported the decrease in the phenolics after heat treatment was mainly due to decrease in the p-hydroxybenzoic acid decrease, also, the increase in bound phenolic acids during *in vitro* digestion is mainly due to increase of ferulic acid. In the present study, IVD samples showed significantly higher ($p \leq 0.05$) amount of phenolic compounds compared to cereal samples treated by chemical approach. This is mainly due to release of bound phenolics from cereals. Ferulic acid is one of the important phenolic acids present in cereal cell walls (Sancho et al. 1999). Corn has 15 % whereas rice has 38 % free phenolics, while 62 % and 85 % bound phenolics are present in rice and corn, respectively (Adom and Liu 2002). Since in normal circumstances phenolics naturally occur in bound form, it is necessary to count the bound phenolic content to measure the total antioxidant activity of cereals.

Flavonoids Content

The mean values of flavonoids obtained by chemical approach were found to be the highest in pearl millet, followed by sorghum, wheat, maize and rice (Table 1). There was significant ($p \leq 0.05$) increase in the total flavonoid content of raw and cooked IVD cereal samples compared to control samples. Also, total flavonoids increased significantly ($p \leq 0.05$) in traditionally cooked IVD samples compared to control samples. Vallejo et al (2004) reported similar results in *in vitro* digestion of broccoli and mentioned that flavonoid, especially kaempferol and qercetin had no significant loss compared to their initial value. Among all the cereal samples, rice samples showed comparatively lower values. The results are in accordance with Adom and Liu (2002), who reported the flavonoid content of various grains and found out that the total flavonoid content of wheat and oat were similar, and both had levels higher than the rice. Shen et al (2009) reported that the flavonoid content in different varieties of rice ranged from 88.6 to 286.3 mg %. They also stated that the pigments present in the cereals contribute in total flavonoid content.

Flavonol Content

The flavonol content was increased significantly in all five raw as well as cooled cereal samples when enzymatic digestion was carried out and this increase was almost double than the flavonol content in the samples of chemical approach (Table 1). The flavonol content was found to be decreased significantly ($p \leq 0.05$) in control samples of all cereals compared to chemically approached samples. When raw and cooked IVD samples were compared with each other, the cooked samples showed a significant ($p \leq 0.05$) increase in the total flavonol content of wheat, pearlmillet and sorghum. The increase in the flavonol content might be an outcome of thermal effect during cooking. The formation of Maillard compounds during cooking may be responsible for it (Serpen et al. 2008). However, Tudela et al (2002) reported a partial loss of flavonols during domestic cooking of potatoes.

Table 1 Total phenolic, flavonoid and flavanol content of chemical extracts and enzymatic extracts of selected cereals (n =3)

Total phenolic content (mg %)				
Cereals	Chemical Extract (Methanol-water extract)	Enzymatic extract (*In vitro* digestion)		
		Control Raw IVD Cooked IVD	Control Raw IVD Cooked IVD	Control Raw IVD Cooked IVD
Wheat	346.7 ± 45.23a	381.8 ± 38.04a	2292.9 ± 59.35c	1920.1 ± 49.55b
Pearl millet	726.9 ± 41.33b	266.5 ± 27.99a	2438.3 ± 102.99d	1982.9 ± 225.26c
Rice	38.9 ± 4.98a	180.0 ± 11.71b	1900.8 ± 9.10d	1286.7 ± 66.30c
Maize	244.9 ± 43.28a	224.6 ± 14.73a	2112.3 ± 78.38c	1703.3 ± 45.94b
Sorghum	348.3 ± 18.21b	251.7 ± 26.41a	1973.3 ± 57.96d	1369.3 ± 74.42c
Total flavonoid (mg %)				
Wheat	279.4 ± 36.95b	135.8 ± 24.13a	407.5 ± 34.42c	482.9 ± 39.15d
Pearl millet	430.9 ± 18.69b	294.9 ± 59.78a	417.5 ± 37.16b	601.5 ± 66.63c
Rice	119.0 ± 16.59b	52.6 ± 5.77a	257.8 ± 37.55d	219.7 ± 26.96c
Maize	234.7 ± 23.17b	157.7 ± 14.20a	248.8 ± 31.28b	341.1 ± 12.70c
Sorghum	338.5 ± 25.97b	224.9 ± 19.96a	331.8 ± 33.15b	346.7 ± 20.23b
Total flavanol (µg %)				
Wheat	234.5 ± 14.34b	87.6 ± 3.49a	653.9 ± 39.24c	689.1 ± 50.71c
Pearl millet	503.3 ± 41.91b	245.0 ± 30.12a	805.2 ± 54.26c	1106.2 ± 39.07d
Rice	148.6 ± 5.17b	51.6 ± 4.92a	333.0 ± 27.94d	240.4 ± 4.74c
Maize	229.0 ± 18.91b	124.1 ± 12.74a	435.0 ± 15.85c	417.4 ± 11.70c
Sorghum	462.9 ± 24.04b	185.5 ± 11.52a	446.4 ± 40.13b	636.1 ± 12.52c

Values are the mean ±S.D; n = 3 for each observation; different letters in the same raw indicate significant difference (P ≤0.05), IVD = in vitro digested

Table 2 Total antioxidant capacity (mg equivalent Trolox) measured using FRAP method and DPPH radical scavenging assay (% inhibition) of chemical extracts and enzymatic extracts of selected cereals (n = 3)

Total antioxidant capacity (mg equivalent Trolox) by FRAP				
Cereals	Chemical Extract (Methanol-water extract)	Enzymatic extract (*In vitro* digestion)		
		Control Raw	Raw IVD	Cooked IVD
Wheat	121.5 ± 9.53b	45.2 ± 3.38a	608.7 ± 14.74d	
Pearl millet	404.2 ± 15.66c	69.5 ± 2.35a	463.1 ± 23.30d	
Rice	46.5 ± 3.83b	13.0 ± 1.63a	100.5 ± 4.24d	
Maize	210.0 ± 8.76d	63.9 ± 2.28a	95.3 ± 4.32b	
Sorghum	155.0 ± 6.71c	68.0 ± 2.00a	159.4 ± 13.83c	
DPPH radical scavenging assay (% inhibition)				
Wheat	79.1 ± 3.77b	25.1 ± 1.77a	80.5 ± 3.65c	72.9 ± 3.60c
Pearl millet	83.2 ± 2.28c	36.1 ± 3.10a	84.6 ± 1.92c	70.7 ± 3.75b
Rice	81.6 ± 4.06c	10.6 ± 2.10a	31.0 ± 2.03b	29.0 ± 1.11b
Maize	82.3 ± 0.74c	17.8 ± 1.54a	34.0 ± 2.50b	37.8 ± 1.41b
Sorghum	82.7 ± 0.86d	20.2 ± 0.85a	32.6 ± 1.74c	26.9 ± 2.24b

Values are the mean ±S.D; n = 3 for each observation; different letters in the same raw indicate significant difference (P ≤0.05), IVD = in vitro digested

Total Antioxidant Capacity using FRAP and DPPH Method

In FRAP method, ferric 2,4,6- tripyridyl-s-triazine complex gets reduced to its ferrous form. However FRAP assay does not respond quickly with some antioxidants like glutathione, it can be performed for cereals samples due to the fact that human body can absorb very few amount of plant glutathione (Schafer and Buettner 2001, Guo et al. 2003). In the chemical extracts, pearlmillet showed highest value, whereas rice showed the least (Table 2). In case of enzyme extracts of raw IVD, wheat showed the highest value, while maize showed the least. Except cooked IVD maize sample, all other cooked IVD samples showed less TAC as compared to their raw IVD samples. However, all the cooked IVD samples showed significantly higher TAC as compared to their respective control samples. Thus, domestic cooking was found to be effective in enhancing the TAC of cereals as measured by FRAP method. The higher TAC values among raw and cooked IVD cereals could be due to release of bound phenolic compounds from their conjugation with carbohydrate. The decrease in the TAC values of cooked cereals compared to their respective raw samples might be due to the heating process employed to cook the samples. Ranilla et al. (2009) mentioned that cooking time, temperature, soaking and draining can significantly affect the antioxidant activity. In the present study, Raw IVD Pearl millet exhibited highest DPPH radical scavenging activity (83.28 %) followed by the chemically extracted sorghum (82.72 %), maize (82.32 %), rice (81.63 %) and wheat (79.17 %) as represented in

Table 2. All the experimental cereal samples showed significantly ($p \leq 0.05$) higher values than their respective control samples. When chemically extracted and cooked IVD samples were compared, chemically extracted samples showed higher values. Maximum % inhibition was observed in the chemically extracted pearl millet sample. The results were found to be in accordance with the results obtained by Dykes and Rooney (2007), who reported highest antioxidant capacity of millet among their tested cereals. Boiling or cooking is generally considered as having detrimental effects on antioxidant compounds (Krishnaswamy and Raghuramulu 1998; Xu and Chang 2009). But in the present study after cooking, *in vitro* digested cereal samples showed an increase in antioxidant capacity compared to control samples in both the methods (FRAP and DPPH). Fares et al (2010) reported increased antioxidant activity in cooked wheat pasta and stated that the increase is due to the increase in bound phenolics, particularly bound ferulic acid, which get released from the cereal cell wall during cooking, might be due to the effect of boiling water. In the present study, observed variation of the TAC in all cooked cereal samples is mainly ascribable to the increase in the extraction of bound ferulic acid (Fares et al. 2010). However, other mechanisms must be involved, like production of Amadori compounds from the Maillard reaction during different steps of food processing like extrusion and drying. In the present study, rice samples showed lowest TAC. These nutritional deficiencies can be tackled by biofortification of rice, traditional breeding and also using genetic engineering.

CONCLUSION

From the present study, it can be concluded that in vitro digestion (enzymatic extract) of cereals showed higher values of total phenolics, flavonoid, flavonol as well as increased antioxidant activity measured by FRAP method compared to their chemical extracts. Cooking of cereals also resulted in increased TAC and enhanced total phenolic, flavonoid and flavonol content. In general, it can be concluded that antioxidant components like phenolic compounds, flavonoid and flavanol of selected cereals were clearly affected by *in vitro* digestion. *In vitro* digestion studies can be applied to analyze the dietary, processing factors on bioavailability of antioxidants. Measurement of *in vitro* physiological extracts of cereals could be use to analyze the effect of cereal antioxidants in different ailment conditions. Further research can be carried out to analyze the effect of each individual antioxidant compound from cereals in gastrointestinal digestion.

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