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Full Length Research Paper

Anti-oxidative activities of sorghum, foxtail millet and proso millet extracts

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In this study, sorghum, foxtail millet and proso millet extracts were evaluated by various *in vitro* anti-oxidant assays, including 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging activity, reducing power by Fe^{3+}- Fe^{2+} transformation, and anti-lipid peroxidative activity by ferric thiocyanate. The sorghum extract contained high amount of phenolic compounds as well as a high level of anti-oxidant activity compared with foxtail millet and proso millet extracts. In addition, among the sorghum cultivar, me-susu (*Sorghum dochna* var. *technicum*, Snowden) extracts exhibited high levels of free radical scavenging activity, anti-oxidant capacity and anti-lipid peroxidative activity compared with α-tocopherol. Taken together, these findings suggest that me-susu extracts can be considered good sources of natural anti-oxidants.

Key words: Anti-oxidant activity, foxtail millet, proso millet, sorghum.

INTRODUCTION

Free radicals are defined as atoms or molecular fragments, easily generated during normal cellular metabolism (Valko et al., 2006) that contain a number of unpaired electrons in their respective atomic or molecular orbitals (Valko et al., 2007). Although reactive oxygen species (ROS) function in cellular signaling systems, an excessive amount of ROS caused by an imbalance between their cellular production and anti-oxidative mechanisms result in oxidative damage to cellular components such as lipids, membranes, proteins and nucleic acids and a variety of neurological diseases including stroke (Alexandrova and Bochev, 2005), Parkinson's (Everse and Coates, 2005) and Alzheimer's disease (Markesbery, 1997). Excessive ROS are also responsible for other pathologies such as atherosclerosis, hypertension, cardiac disease, diabetic complication, autoimmune rheumatic disease, cancer and aging (Gutteridge, 1995; Halliwell, 1996; Valko et al., 2006, 2007). Therefore, anti-oxidant therapy using free radical scavengers has received increasing attention in clinical setting, and consequently, several anti-oxidants have been developed and used in primary and/or complementary therapies (Delanty and Dichter, 2000). Folk plants and other sources of natural anti-oxidants such as spices and herbs have been increasingly used due to growing concerns over the long-term safety of synthetic anti-oxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tertiary butylhydroquinone (TBHQ) (Atawodi, 2005). The long-term safety of natural anti-oxidants may play a pivotal role in the chemoprevention of diseases as well as additional health benefits.

Sorghum (*Sorghum bicolor*), foxtail millet (*Setaria italica*) and proso millet (*Panicum miliaceum*) are commonly cultivated staple cereal crops in Africa and Asia, and form a substantial part of the farming system for people living in these ecologies (Soh et al., 2002). In East Asia, these crops are cultivated for emergency purposes and are widely consumed due to their ability to compensate for nutrient deficiencies in rice, as in vitamins and minerals (Soh et al., 2002). The vast array of biologically active compounds in these crops, including tannins, phenols, anthocyanins, flavonoids and phytates, may indicate their

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Abbreviations: BHA, Butylated hydroxyanisole; **BHT**, butylated hydroxytoluene; **DPPH,** 1,1-diphenyl-2-picrylhydrazyl; **FRAP,** ferric-reducing ability power; **TBHQ,** tertiary butylhydroquinone; **ROS,** reactive oxygen species.

potential as therapeutic agents (Awika and Rooney, 2004; Kayode et al., 2007). In fact, there are some reports on the anti-microbial (Soetan et al., 2006; Kil et al., 2009) and anti-carcinogenic (Awika and Rooney, 2004; Kwak et al., 2004) effects of sorghum, whereas studies on millets have mainly focused on the treatment of diabetes by improving cholesterol-metabolism (Nishizawa and Fudamoto, 1995; Choi et al., 2005). Wheat and wheat products (Baublis et al., 2000; Zielinski and Kozlowska, 2000), oats (Peterson, 2001) and rice (Kejian et al., 1994) have all been investigated for anti-oxidant properties, and likewise have sorghum, foxtail millet and proso millet (Awika and Rooney, 2004; Kamath et al., 2004; Choi et al., 2007; Kil et al., 2009). Although the presence of tannins, phenolic acids and anthocyanins in sorghum has been linked with potential anti-oxidant activity, foxtail millet and proso millet have been investigated only to a limited extent (Awika and Rooney, 2004). Therefore, additional analysis using various assay methods is required to better understand the anti-oxidant properties of these crops.

In this present study, we report the anti-oxidant properties of different cultivars of sorghum, foxtail millet and proso millet. To obtain a better understanding of the potent anti-oxidant extracts, we determined the amount of phenols and flavonoids in these crops, and analyzed anti-oxidant properties using various assays, including DPPH radical scavenging, ferric ion reducing anti-oxidant power assay and anti-lipid peroxidative effect.

MATERIALS AND METHODS

Chemicals

α-Tocopherols, BHA, BHT, and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade or higher.

Plant materials and extraction

Sorghum (Korean name; susu), foxtail millet (Jo) and proso millet (Gijang) cultivar were provided by the Shinlim Agricultural Co-operative, Wonju-si, Gangwon-do, Korea. Jo cultivars used in this study were Gaebalcha-jo, Kkojang-jo, Nuc-jo, Mongdang-jo, Sangjung-jo, Shinnalgeori, Oruncha-jo and All-jo. Gijang cultivars were Bulgun-gijang, Byolook-gijang, Chal-gijang, Hwang-gijang and Heuin-gijang. Susu cultivars were Mongdang-susu, Me-susu, Susongsaengi-susu, Sikyung-susu, Jangsu-susu and Heuin-susu. Before the experiment, dried grain of sorghum, foxtail millet and proso millet were ground into powder using a blender. Fifty gram samples were soaked with 70% ethanol for 2 h, placed in an ultrasonic bath (Power sonic 420, Hwashin co., Korea) and sonicated at 55°C. This was followed by filtration, evaporation under reduced pressure and lyophilization to produce dried powder extract. The freeze-dried extract was re-dissolved in 70% ethanol (v/v).

DPPH radical scavenging assay

Radical scavenging activity was measured using DPPH. The sorghum, foxtail millet and proso millet extracts were re-dissolved in 70% ethanol. The 5 ml assay mixture contained 3.98 ml methanol, 20 µl extract (10, 100, 1000, 10000, 20000 ppm), and 1 ml DPPH (0.15 mM in methanol). After incubation at room temperature for 30 min, the decrease in absorbance was measured at 517 nm using a spectrophotometer (V-530, Jasco Co., Japan). Ascorbic acid, BHA, BHT and α-tocopherol were used as references. The RC50 value indicates the concentration of tested sample required to reduce the free radical concentration by 50%. The experiment was performed in triplicate.

Determination of reducing power activity

The reducing power of the sample was determined by the Oyaizu (1986) method with some modifications. Reducing power activity is based on the reduction of ferricyanide (Fe^{3+}) in stoichiometric excess relative to the amount of anti-oxidants (Benzie and Strain, 1996). Samples with different concentrations were mixed with 0.5 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 0.5 ml of 1% potassium ferricyanide (w/v) and incubated at 50°C for 20 min. After incubation, 2 ml of 10% trichloroacetic acid (w/v) were added to the mixture, followed by centrifugation at 650 rpm for 10 min. The upper layer (0.5 ml) was mixed with 0.5 ml of deionized water and 0.1 ml of 0.1% ferric chloride (w/v), and the absorbance of the resultant solution was measured at 700 nm. Ascorbic acid, α-tocopherols, BHA and BHT were used as references.

Total phenol and flavonoid analysis

The total phenolic content was determined using Folin-Ciocalteu reagent according to the method described by Singleton and Rossi (1965). Briefly, 0.1 ml of sample and 50 µl of 2 N Folin-Ciocalteu reagent was added to a 5 ml volumetric flask. The solution was mixed and allowed to stand for 3 - 5 min at room temperature. Next, 0.3 ml of 20% sodium carbonate solution (w/v) was added, and the solution was mixed and kept aside for 15 min. Finally, 1 ml of distilled water was added. The blue color was measured against reagent blank at 725 nm using a UV-spectrophotometer. The total phenolic content of the sample was determined by comparison with the optical density values of different concentrations of the standard phenolic compound gallic acid. Each sample was analyzed in triplicate, and a calibration curve of gallic acid was constructed by plotting absorbance versus concentration.

The total flavonoid content of the extract was determined according to the calorimetric method as described by Park et al. (1997). 0.2 ml aliquot was added to test tubes containing 0.1 ml of 10% aluminum nitrate (w/v), 0.1 ml of 1 M potassium acetate and 4.6 ml of 80% ethanol. After incubation for 40 min at room temperature, the absorbance was determined at 415 nm. The total flavonoid content was expressed in milligrams of quercetin equivalents (QE) per gram of samples.

Ferric thiocyanate (FTC) test

The test was conducted via the FTC method in a linoleic acid emulsion with some modifications (Haraguchi et al., 1992). The reaction medium contained 0.02 ml of sample (10,000 ppm), 0.2 ml of 2.51% linoleic acid in ethanol, 0.4 ml of 0.04 M potassium phosphate buffer (pH 7.0) and 0.38 ml of distilled water. The solution (1 ml) was mixed and incubated at 70°C in the dark. A sample of the reaction medium without any additive was used as control sample. Synthetic anti-oxidants (BHA and α-tocopherol) were added until the same concentration was reached and then used for comparison. At regular intervals during incubation, 0.05 ml aliquot of the mixture was diluted with 2.85 ml of 75%

Table 1. Total phenolic content, total flavonoid content and DPPH free radical scavenging activity of sorghum, foxtail millet and proso millet extracts.

Plant	Cultivar	Remark	RC_{50}[a]	TPC[b]	TFC[c]
Foxtail millet (Jo)	Gaebalcha-jo	JO-1	254 ± 10	20.1 ± 0.2	6.7 ± 1.6
	Kkojang-jo	JO-2	259 ± 14	19.4 ± 0.2	9.1 ± 0.8
	Nuzz-jo	JO-3	270 ± 11	18.0 ± 2.3	5.7 ± 0.4
	Mongdang-jo	JO-4	230 ± 4.6	20.5 ± 0.4	3.4 ± 0.2
	Sangjung-jo	JO-5	267 ± 12	17.7 ± 1.1	5.3 ± 0.0
	Shinnalgeori	JO-6	196 ± 6.4	26.5 ± 0.8	11.5 ± 0.6
	Oruncha-jo	JO-7	318 ± 14	18.4 ± 0.7	5.9 ± 0.0
	All-jo	JO-8	250 ± 7.5	20.5 ± 0.1	6.4 ± 0.1
Proso millet(Gijang)	Bulgun-gijang	GJ-1	122 ± 5.1	21.6 ± 0.6	8.0 ± 0.2
	Byolook-gijang	GJ-2	58 ± 2.2	26.7 ± 0.6	8.1 ± 0.2
	Chal-gijang	GJ-3	297 ± 6.0	12.0 ± 0.4	4.0 ± 0.1
	Hwang-gijang	GJ-4	192 ± 2.9	16.0 ± 0.4	5.5 ± 0.1
	Heuin-gijang	GJ-5	205 ± 1.7	14.5 ± 0.3	6.2 ± 0.3
Sorghum(Susu)	Mongdang-susu	SS-1	3.1 ± 0.1	181.5 ± 2.0	29.0 ± 0.2
	Me-susu	SS-2	1.9 ± 0.1	263.3 ± 16.0	33.7 ± 0.6
	Susongsaengi-susu	SS-3	6.4 ± 0.1	105.7 ± 5.9	31.5 ± 0.8
	Sikyung-susu	SS-4	4.3 ± 0.0	153.6 ± 10.6	38.1 ± 0.4
	Jangsu-susu	SS-5	8.4 ± 0.2	72.8 ± 0.3	23.0 ± 4.7
	Heuin-susu	SS-6	31 ± 1.4	28.5 ± 0.6	14.5 ± 0.0
Positive control	Ascorbic acid		1.1 ± 0.0		
	α-Tocopherol		3.3 ± 0.1		
	BHA		5.0 ± 0.2		
	BHT		56 ± 4.3		

[a]RC_{50}: Amount required for the 50% reduction of DPPH after 30 min. Each value is mean ± standard derivation of triplicate experiments. [b]Total phenol content analyzed as gallic acid equivalent (GAE) mg/g of extract; values are the average of triplicate experiments. [c]Total flavonoid content analyzed as quercetin equivalent (QE) mg/g of extract; values are the average of triplicate experiments.

ethanol, followed by the addition of 0.05 ml of 30% ammonium thiocyanate (w/v) and 0.05 ml of 20 mM of ferrous chloride in 3.5% HCl. Absorbance for the red color was measured at 500 nm. These steps were repeated every 3 h until the control reached its maximum absorbance value.

RESULTS AND DISCUSSION

To investigate the anti-oxidant activities of different cultivars of sorghum, foxtail millet and proso millet, we prepared the extract using 70% ethanol, and analyzed the free radical scavenging activity. As shown in Table 1, the 70% ethanol extracts of sorghum, foxtail millet and proso millet displayed varying free radical scavenging activities. Sorghum extracts showed the highest anti-oxidant activity (RC_{50} = 1.9 to 31 µg/ ml) compared with foxtail millet or proso millet extracts, supporting the idea that the polyphenolic constituents are responsible for the free radical scavenging activities of the crops. Interestingly, me-susu, which is the industrial sorghum, showed a free radical scavenging activity about 29.5 fold more than that of the positive control, BHT (Table 1). It has been shown that phenolic compounds act mainly as reducing agents, hydrogen donors and singlet-oxygen quenchers during anti-oxidant mechanisms (Kang et al., 2006), indicating that the high level of free radical scavenging activity in the extract of sorghum including me-susu might be medicated by the presence of high amount of phenolic compounds. In order to ensure the relationship between the level of phenolic compounds and free radical scavenging activity, we analyzed the total phenol and flavonoid content from 70% ethanol extract of sorghum, foxtail millet and proso millet. As shown in Table 1, sorghum extract contained the highest amount of phenolic and flavonoid compounds. The total phenolic content ranged from 18.0 to 26.5 mg GAE/g in proso millet, from 12.0 to 26.7 mg GAE/g in foxtail millet, and from 28.5 to 263.3 mg GAE/g in sorghum. Me-susu extract contained a relatively higher level of phenolic compounds compared to that of other samples. Chal-gijang extract was found to possess the lowest total phenolic content. In addition, total flavonoid levels proceeded in the following order:

sorghum (14.5 - 38.1 mg QE/g), proso millet (3.4 - 11.5 mg QE/g), foxtail millet (4.0-8.1mg QE/g). Although, these findings indicated that the high amount of phenolic compounds in sorghum should be responsible for its free radical scavenging properties, it has been shown that most phenolic compounds react slowly with DPPH, reaching a steady state in 1 - 6 h (Bondet et al., 1997). In addition, the color interference of DPPH with sample which contained anthocyanins results in the under-estimation of antioxidant activity (Arnao, 2000), indicating that at least two methods due to the differences between the test systems should be required for the comparison of the antioxidant activities and phenolic compounds (Schlesier et al., 2002).

To further characterize the anti-oxidant properties in the extracts, we tested the anti-oxidant capacity from different samples using the ferric-reducing ability power (FRAP) assay. FRAP assay is known as a robust and useful method for measuring a wide concentration range of anti-oxidant activities and capacities (Benzie and Strain, 1996), and has been employed to provide experimental evidence of anti-oxidant activity (Huang et al., 2005; Hajimahmoodi et al., 2008). We incubated extracts from samples in sodium phosphate buffer (pH 6.6) and potassium ferricyanide. After incubation, trichloroacetic acid was added into the mixture, and the anti-oxidant capacity was measured by UV-spectrophotometer. As shown in Figure 1, 100 µg/ml sorghum extracts exhibited OD_{700} values ranging from 0.17 to 0.88, while 500 µg/ml foxtail millet or proso millet extracts demonstrated OD_{700} values from 0.26 to 0.34 and from 0.27 to 0.46, respectively. In addition, me-susu extracts (0.88) showed stronger activity than α-tocopherol (0.51) or BHT (0.70).

In order to determine how anti-oxidant activity is related to the phenol or flavonoid content of different cultivars of sorghum, foxtail millet and proso millet, we established a positive linear correlation between phenol content and DPPH free radical scavenging activity (linear correlation coefficient $r^2 = 0.990$) (Figure 2A). A similar situation was observed in our examination of total phenol content and reducing power ($r^2 = 0.985$) (Figure 2A). This positive linear correlation shows that the sample with the highest total phenol content has the highest absorbance values. Correlations were also established between flavonoid content and DPPH free radical scavenging activity ($r^2 = 0.736$), as well as between total flavonoid content and reducing power ($r^2 = 0.821$) (Figure 2B). It has been suggested that there is high correlation between total phenolic content and anti-oxidant activity, such as reducing power and radical scavenging effect on DPPH radicals (Sousa et al., 2008). Similarly, linear relationships between these parameters have been found for extracts of several plant materials, indicating that the correlation coefficient could be used as an indicator of anti-oxidant capacity (Hajimahmoodi et al., 2008; Vásquez et al., 2008). Taken together, the high correlation between phenolic compounds and flavonoids from extracts of sorghum, foxtail millet and proso millet and their anti-oxidant activities suggest that phenolic compounds, among other compounds, act as anti-oxidants in these crops.

The anti-oxidant activities of extracts as determined by the ferric thiocyanate method, which measures the amount of peroxide produced by linoleic acid emulsion during incubation were compared with two commercial anti-oxidants, α-tocopherol and BHT. Low absorbance values in the FTC method indicate a high level of anti-oxidant activity. Figure 3 shows the changes in absorbance for each sample during 30 h of incubation at 70°C. The rapidly increased amount of peroxide product was observed for the treated control, whereas BHT inhibited the peroxidation of linoleic acid. In the case of α-tocopherol, the inhibitory effect on the peroxidation of linoleic acid was only shown during a short incubation. This may indicate that oxidation of α-tocopherol results in increased levels of peroxide product. An interesting order of antioxidant activity, different from that determined by DPPH and FRAP assays seen in Table 1 and Figure 1, was observed by the FTC test seen in Figure 3. In foxtail millet extract, JO-4 (Mongdang-jo), JO-5 (Sangjung-jo) and JO-6 (Shinnalgeori-jo) showed lower anti-oxidant activities compared with other cultivars (Figure 3A), whereas most cultivars displayed similar level of anti-oxidant activities and capacities via DPPH and FRAP assays (Table 1 and Figure 1A). Although SS-5 (Jangsu-susu) and -6 (Heuin-susu) exhibited low level of free radical scavenging activity and anti-oxidant capacity compared with SS-1 (Mongdang-susu) and -2 (Me-susu) (Table 1 and Figure 1C), the incubation with linoleic acid and SS-5 and -6 resulted in the inhibition of peroxide product (Figure 3C). These differential aspects may be explained on the basis of hydrophobicity of extracts and thus their solubility in linoleic acid emulsions as reported by Frankel and Meyer (2000); whereas the solubility or hydrophobicity is not the factor in the previous tests of DPPH and FRAP. The lower level of antioxidant activity in the FTC test may again be attributed to the presumably higher hydrophobicity of SS-1 and -2 extracts, with higher phenolic content, compared to SS-5 and -6 extracts.

In this study, we analyzed the anti-oxidant activities of miscellaneous grain crop extracts using *in vitro* anti-oxidant assays, including DPPH free radical scavenging, reducing power and anti-lipid peroxidative effect. As a result, we found that the anti-oxidant potential of sorghum is higher than that of other miscellaneous grain crops. Interestingly, me-susu extract showed the strongest free radical scavenging activity, reducing power and anti-lipid peroxidative activity compared with two commercial anti-oxidants, α-tocopherol and BHT, suggesting it as a possible candidate for the production of anti-oxidants. To clarify the anti-oxidant properties of me-susu and to develop natural anti-oxidant agents, the purification and identification of active compounds will be necessary. Our study provides basic information about the relationship between

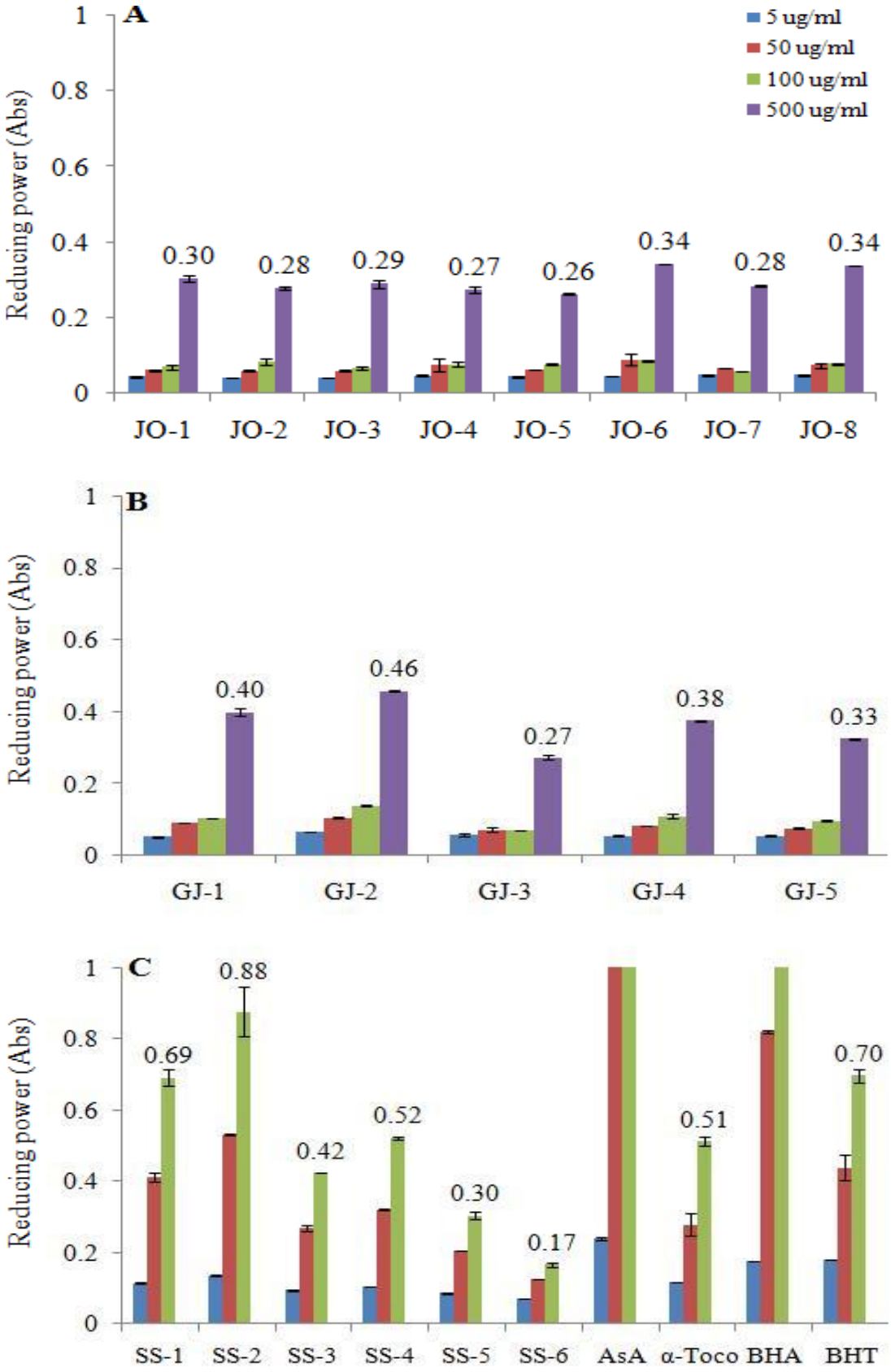

Figure 1. Reducing power of sorghum, foxtail millet and proso millet extracts. (A) Foxtail millet (B) proso millet and (C) sorghum.

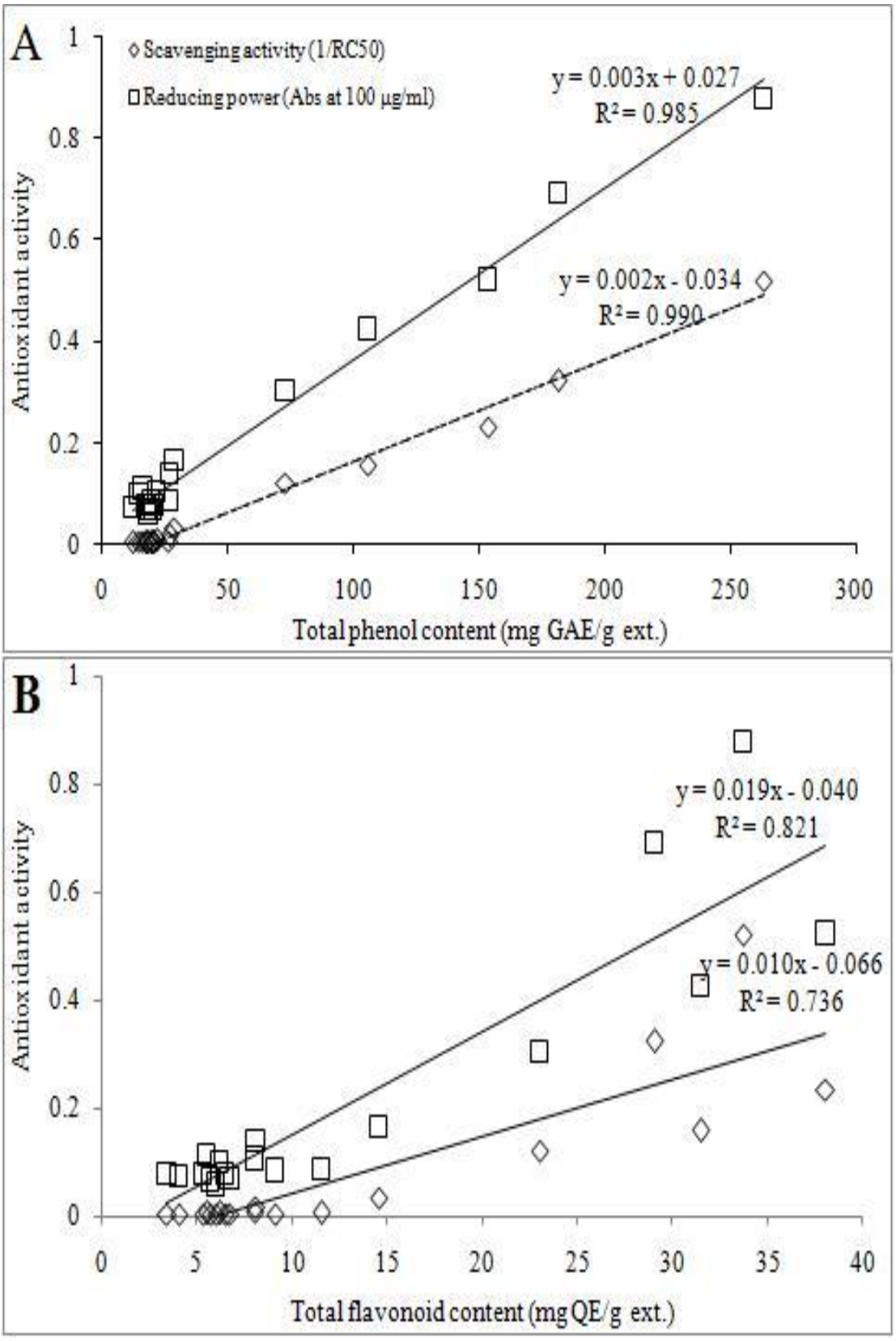

Figure 2. Correlation established between the anti-oxidant activity and total phenolic content of sorghum, foxtail millet and proso millet extracts. A: correlation between anti-oxidant activity and total phenol content; B: correlation between anti-oxidant activity and total flavonoid content.

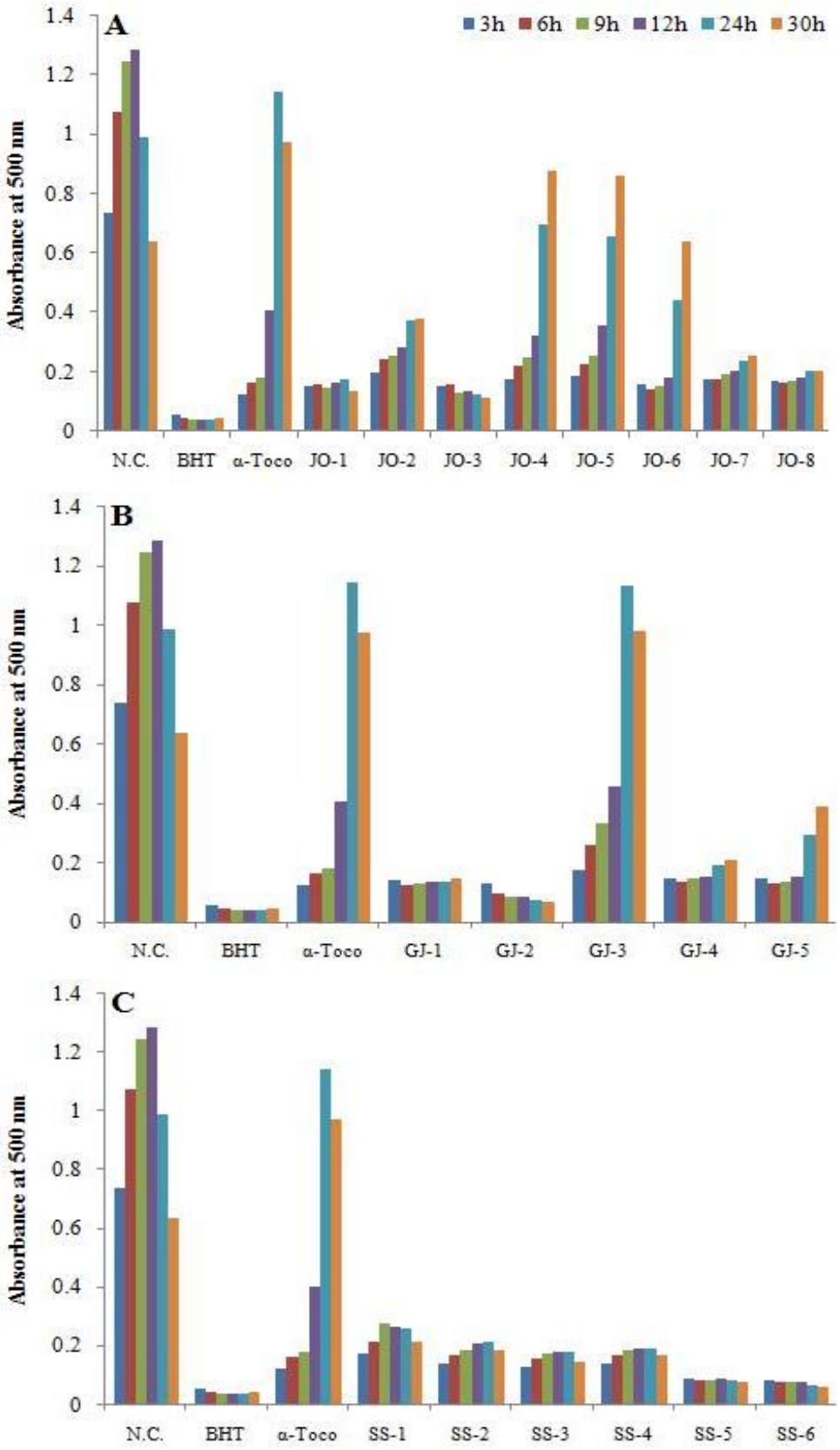

Figure 3. Anti-oxidant activities of sorghum, foxtail millet and proso millet extracts based on ferric thiocyanate method. (A) Foxtail millet, (B) proso millet and (C) sorghum.

phenolic compounds from different cultivars of miscellaneous grain crops and anti-oxidant properties.

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