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ScienceDirect
Food Chemistry 105 (2007) 950–958
Food Chemistry
www.elsevier.com/locate/foodchem

The antioxidant activity and free radical-scavenging capacity of dietary phenolic extracts from horse gram (*Macrotyloma uniflorum* (Lam.) Verdc.) seeds

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Received 30 January 2007; received in revised form 6 April 2007; accepted 18 April 2007

Abstract

Total phenolics and the antioxidative properties of two varieties of horse gram (*Macrotyloma uniflorum*) were studied. The raw and dry-heated seed samples were extracted successively with methanol and 70% acetone separately. After removing the solvents, the extracts were freeze-dried. The black seeds contained relatively high levels of total phenolics and tannins than the brown seeds with respect to the treatments and solvents extraction. The extracts were subjected to assess their potential antioxidant activities using systems such as α,α-diphenyl-β-picrylhydrazyl (DPPH$^{\cdot}$), ABTS$^{\cdot+}$, FRAP, linoleic acid emulsion, $O_2^{\cdot-}$ and OH$^{\cdot}$. The superoxide anion radical-scavenging activity was found to be higher in 70% acetone extract of the both raw and dry heated seeds of the respective varieties at the concentration of 600 μg in the reaction mixture. The DPPH radical and ABTS cation radical-scavenging activities were well proved and related with the ferric-reducing/antioxidant capacity of the extracts. Interestingly, among the various extracts, 70% acetone extracts of dry-heated samples of brown variety, and raw and dry-heated samples of black variety showed significantly ($P < 0.05$) higher hydroxyl radical-scavenging activity. In general, all extracts exhibited good antioxidant activity (53.3–73.1%) against the linoleic acid emulsion system but were significantly ($P < 0.05$) lower than the synthetic antioxidant, BHA (93.3%).

Keywords: *Macrotyloma uniflorum* seeds; Dry heating; Polyphenols; Antioxidant activity; NBT method; FRAP; ABTS$^{\cdot+}$

1. Introduction

Carotenoids, vitamins and phenolic compounds are present naturally in vegetables, fruits, grains and pulses and they possess the ability to reduce oxidative damage associated with many diseases, including cancer, cardiovascular diseases, cataracts, atherosclerosis, diabetes, arthritis, immune deficiency diseases and ageing (Lee, Koo, & Min, 2004; Lee, Mitchell, & Shibamoto, 2000; Middleton, Kandaswamy, & Theoharides, 2000; Pietta, Simonetti, & Mauri, 1998). Recently, the ability of phenolic substances including flavonoids, phenolic acids and lignans to act as potential antioxidants has been extensively investigated (Heim, Tagliaferro, & Bobilya, 2002; Suja, Jayalekshmy, & Arumughan, 2005). Grain legumes/pulses play an important role in the traditional diets of many parts of the world and they are low in fat; are excellent sources of protein, dietary fibre, a variety of micronutrients and phytochemicals (Anderson, Smith, & Washnock, 1999; Messina, 1999). The sauce prepared from the cooking liquors of coloured beans, contains mainly the seed coat pigments such as dietary tannins and non-tannin phenolics including phenolic acids, is consumed with cooked rice and other cereals and popular among the village people in certain parts of India. The consumption of such legumes has been linked to reduced risk of diabetes and obesity and found to have an inhibitory role in the reduction of coronary heart diseases (Bazzano et al., 2001). A nutraceutical is any substance that is a food, or part of a food, and provides medical or health benefits, including the prevention or

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doi:10.1016/j.foodchem.2007.04.040

treatment of disease. Nutraceuticals may be isolated nutrients, dietary supplements, specific diets, designer foods, herbal products, processed food or processed beverages (Morris, 2003). Researchers have studied polyphenolic constituents of various legume seeds and have reported that they contain potential medicinal/nutraceutical properties including antioxidant activities (Mazur, Duke, Wähälä, Rasku, & Adlercreutz, 1998; Shahidi, Chavan, Naczk, & Amarowicz, 2001; Siddhuraju, 2006; Tsuda, Ohshima, Kawakishi, & Osawa, 1994; Tsuda, Osawa, Nakayama, Kawakishi, & Ohshima, 1993a). Therefore the study of the importance and role of non-nutrient compounds particularly phenolic acids, flavonoids and high molecular tannins of legumes as natural antioxidants have greatly increased (Siddhuraju & Becker, 2007).

Horse gram (*Macrotyloma uniflorum* (Lam.) Verdc.) is largely cultivated, especially in dry areas of Australia, Burma, India, and Sri Lanka. It is used as a vegetable in India and is known as the poor man's pulse crop in southern India. Earlier studies showed that horse gram is a good source of protein (17.9–25.3%), carbohydrates (51.9–60.9%), essential amino acids, energy and a low content of lipid (0.58–2.06%) and is an excellent source of iron and molybdenum (Bravo, Siddhuraju, & Saura-Calixto, 1999; Kadam & Salunkhe, 1985; Sudha, Mushtari Begum, Shambulingappa, & Babu, 1995). It also possesses slow digestible starch, which is considered to have low postprandial glucose response when consumed by diabetic patients (Bravo, Siddhuraju, & Saura-Calixto, 1998). Moreover, the methanolic extract of whole plants of *Dolichos biflorus* Linn. (horse gram) possesses hypolipidemic activity in rats fed with high fat diet (Muthu, Sethupathy, Manavalan, & Karar, 2005). Administration of the extract to rabbits in which oxidative stress had been induced by being fed a high fat diet (HFD), led improvement in antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT), and increased in reduced glutathione (GSH) concentration (Muthu, Sethupathy, Manavalan, & Karar, 2006). A large population in rural areas of southern India consume the horse gram as a whole seed, sprouts, or whole meal. Nonetheless, the consumption of horse gram seeds, after processing such as soaking/dry heating followed by cooking, along with cooked rice; sorghum or pearl millet is a common practice among the rural people in India. In addition to that, the cooking liquor of the horse gram seeds with spices is considered to be a potential remedy for the common cold, throat infection and fever and the soup made from the seeds of this plant is said to generate heat and help dilute renal stones. It contains isoflavone diglycoside, 5-hydroxy-7,3′,4′-trimethoxy-8-methylisoflavone; 5-*O*-α-L-rhamnopyranosyl (1 → 2)-*O*-β-D-glucopyranoside (Mitra, Das, & Joshi, 1983). The whole seeds have been reported to contain about 1.6% tannins (Bravo et al., 1998; Reddy, Pierson, Sathe, & Salunkhe, 1985). However, only limited information is available on their antioxidant activity (Tsuda, Makino, Kato, Osawa, & Kawakishi, 1993b). Even though the processed horse gram seeds are increasingly consumed as human food, the beneficial effects of their bioactive compounds remain unexplored (Morris, 2003). Therefore, the present study has been aimed at evaluating the phenolic constituents, antioxidant potential and free radical-scavenging capacity of methanol and subsequently aqueous acetone extracts of the two varieties of raw and dry-heated horse gram seed samples.

2. Materials and methods

2.1. Chemicals

Butylated hydroxyanisole (BHA), potassium ferricyanide, α,α-diphenyl-β-picrylhydrazyl (DPPH•), nitro blue tetrazolium (NBT), thiobarbituric acid (TBA), trichloroacetic acid (TCA), 2-deoxy-D-ribose, methionine, linoleic acid, ethylenediamine tetraacetic acid (EDTA), ammonium thiocyanate, potassium persulfate, ferrous chloride, ascorbic acid, Tween 20, 2,4,6-tripyridyl-s-triazine, ferric chloride, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt were obtained from Himedia, Merck, Sigma or S.D. Fine-Chem Ltd. All other reagents were of analytical grade.

2.2. Seed samples and processing

The two varieties of horse gram (*M. uniflorum*), brown and black varieties were purchased (each 1 kg) from local market located at Salem, TN, India during July 2004. The samples were sun dried for one day and stored in a separate screw cap bottle at room temperature. A portion of the seeds (120 g) were dry heated in acid washed sea sand on an open hot plate at 135 ± 2 °C for 20 min. During the heat processing frequent (every 3 min) stirring of seeds, together with the sand, was done using a glass rod for uniform heating of the seeds. The seeds were separated by sieving and cleaned thoroughly. The raw and dry heated seed samples were ground to a fine powder (particle size of about 0.25 mm) and stored in a separate screw cap bottle at −20 °C before analysis.

2.3. Solvent extraction

Raw and dry-heated ground seed samples of both varieties (each 10 g) were defatted by using petroleum ether in the ratio of sample:solvent (1:10 w/v) with occasional shaking at room temperature for 24 h. Then the samples were filtered through Whatman No. 4 filter paper. The residues were air-dried and were extracted by stirring with 100 ml of methanol at 25 °C for 24 h and filtering through Whatman No. 4 filter paper. The residues were re-extracted with an additional 50 ml of methanol as described above, for 3 h. The residues, after air-drying, were re-extracted by occasional shaking with 100 ml of 70% acetone at room temperature for 24 h and filtering through Whatman No. 4 filter paper. The residues were re-extracted with same solvent

as mentioned above, for 3 h. The solvents of the respective combined extracts were evaporated under reduced pressure (52 kPa and 36 kPa, respectively) using a rotary vacuum-evaporator at 40 °C and the remaining water was removed by lyophilization and weighed to determine the yield of soluble components. The freeze-dried extracts thus obtained were used directly for estimation of total phenolics and tannins and also for the assessment of antioxidant capacity through various chemical assays.

2.4. Determination of total phenolic and tannin contents

The total phenolic content of the freeze-dried methanol and aqueous acetone extract of raw and processed horse gram seeds was determined according to the method described by Makkar (2003). Known aliquots of the extract (each 10 mg of extracts dissolved in 100 mL of respective solvent) were taken in a test tube and made up to a volume of 1 ml with distilled water. Then 0.5 ml of Folin-Ciocalteu reagent (1:1 with water) and 2.5 ml of sodium carbonate solution (20%; w/v) were added sequentially in each tube. Soon after vortexing the reaction mixture, the tubes were placed in the dark for 40 min and the absorbance was recorded at 725 nm against the reagent blank. The amount of total phenolics was calculated as pyrogallol equivalents (even though pyrogallol was used as a standard in this work, it is not a natural constituent of horse gram or legumes in general) from the calibration curve. Using the same methanol and aqueous acetone solutions, which contained known amount of freeze-dried extract, the tannins (Makkar, 2003) were estimated after treatment with polyvinyl polypyrrolidone (PVPP). Hundred millgrams of PVPP were weighed in a 100 × 12 mm test tube and to this 1.0 mL distilled water and then 1.0 mL of the tannin-containing phenolic extract were added. The content was vortexed and kept in the tube at 4 °C for 15 min. Then the sample was centrifuged (3000*g* for 10 min at room temperature) and the supernatant was collected. This supernatant has only simple phenolics other than tannins (the tannins would have been precipitated along with the PVPP). The phenolic content of the supernatant was measured as mentioned above and expressed as the content of non-tannin phenolics on a dry matter basis. From the above results, the tannin content of the sample was calculated as follows:

$$\text{Total phenolics (\%)} - \text{non-tannin phenolics (\%)} = \text{tannin (\%)}.$$

2.5. Antioxidant activity assays

2.5.1. Superoxide anion ($O_2^{\cdot-}$) radical-scavenging activity by NBT method

Superoxide radicals were generated by a modified method of Beauchamp and Fridovich (1971), described by Zhishen, Mengcheng, and Jianming (1999). All solutions were prepared in 0.05 M phosphate buffer (pH 7.8). The photo-induced reactions were performed using 20 W fluorescent lamps. The mass of extract in the reaction mixture was 200–600 μg. The total volume of the reactant mixture was 5 ml and the concentrations of the riboflavin, methionine and nitro blue tetrazolium (NBT) was 3×10^{-6}, 1×10^{-2} and 1×10^{-4} mol L^{-1}, respectively. The reactant was illuminated at 25 °C for 25 min. The photochemically reduced riboflavins generated $O_2^{\cdot-}$ which reduced NBT to form blue formazan. The unilluminated reaction mixture was used as a blank. The absorbance was measured at 560 nm. The sample extracts were added to the reaction mixture, in which $O_2^{\cdot-}$ radicals were scavenged, thereby inhibiting the NBT reduction. The net value of absorbance was calculated as the decrease in $O_2^{\cdot-}$ level which was represented by A (illum) − A (unillum), where A (illum) was the absorbance value of illuminated reaction mixture and A (unillum) was the absorbance value of unilluminated reaction mixture for control or test sample. The degree of the scavenging was calculated as scavenging (%) = [A (control) − A (test sample)]/A (control) × 100, where A (control) and A (test sample) were the net values of absorbance calculated for control and test sample, respectively.

2.5.2. Hydroxyl ($OH^{\cdot}$) radical-scavenging activity

The scavenging activity of the raw and dry-heated horse gram seed extracts on the hydroxyl radical ($OH^{\cdot}$) was measured by the deoxyribose method (Aruoma, 1994) modified by Hagerman et al. (1998). The reactions were performed in 10 mM phosphate buffer, pH 7.4, containing 2.8 mM deoxyribose, 2.8 mM H_2O_2, 25 μM $FeCl_3$, 100 μM EDTA, and the test sample (200 μg). The reaction was started by adding ascorbic acid to a final concentration of 100 μM and the reaction mixture was incubated for 1 h at 37 °C in a water bath. After incubation, the colour was developed by addition of 1% thiobarbituric acid followed by ice-cold 2.8% trichloroacetic acid and heating in a boiling water bath (95–100 °C) for 20 min. The sample was cooled, and the chromophore was extracted into *n*-butanol and the absorbance was measured at 532 nm against *n*-butanol (as blank). The reaction mixture not containing test sample was used as control. The scavenging activity on hydroxyl radicals (HRSA) was expressed as:

$$\text{HRSA (\%)} = [1 - (\text{OD at 532 nm in presence of sample} / \text{OD at 532 nm in absence of sample})] \times 100$$

2.5.3. Antioxidant activity in linoleic acid emulsion system

The antioxidant activity of un-processed and dry-heated seed extracts of two varieties of horse gram, ascorbic acid (AA) and BHA was determined using the thiocyanate method (Mitsuda, Yasumoto, & Iwami, 1966) as described by Yen and Hsieh (1998). Each sample (extract, 250 μg; AA, 250 μg and BHA, 250 μg) in 0.5 ml of absolute ethanol was mixed with linoleic acid emulsion (2.5 ml, 0.02 M, pH

7.0) in phosphate buffer (2 ml, 0.2 M, pH 7.0). The linoleic acid emulsion was prepared by mixing and homogenising 0.2804 g of linoleic acid, 0.2804 g of Tween 20 as emulsifier, and 50 ml phosphate buffer. The reaction mixture was incubated at 37 °C. Aliquots of 0.1 ml were taken at several intervals during incubation. The degree of oxidation was measured according to the thiocyanate method by sequentially adding ethanol (4.7 ml, 75%), ammonium thiocyanate (0.1 ml, 30%), sample solution (0.1 ml), and ferrous chloride (0.1 ml, 0.02 M in 3.5% HCl). After the mixture had rested for 3 min, the peroxide value was determined by monitoring absorbance at 500 nm using a spectrophotometer. A control was performed with linoleic acid but without the samples. The degree of oxidation was measured for every 24 h until a day after the absorbance of the control reached its maximum. The lipid peroxidation inhibition (LPI) % was calculated as

$$\text{LPI } (\%) = [1 - (\text{OD at 500 nm in presence of sample}_{72\text{ h}} / \text{OD at 500 nm in absence of sample}_{72\text{ h})}] \times 100$$

2.5.4. Free radical-scavenging activity on α,α-diphenyl-β-picrylhydrazyl (DPPH·)

The antioxidant activity of horse gram seed extracts and BHA was measured in terms of electron transfer/hydrogen donating ability, using the stable radical, DPPH· method (Brand-Williams, Cuvelier, & Berset, 1995) modified by Sánchez-Moreno, Larrauri, and Saura-Calixto (1998). A methanol solution (0.1 ml) of the sample extracts at various concentrations was added to a 3.9 ml (0.025 g l^{-1}) of DPPH· solution. The decrease in absorbance at 515 nm was determined continuously at every 1 min with a Hitachi UV–Visible model U-2000 Spectrophotometer until the reaction reached a plateau. The remaining concentration of DPPH· in the reaction medium was calculated from a calibration curve obtained with DPPH· at 515 nm. The percentage of remaining DPPH· ($DPPH^{\cdot}_R$) was calculated as follows:

$$DPPH^{\cdot}_R = [(DPPH^{\cdot})_T / (DPPH^{\cdot})_T = 0] \times 100$$

where $DPPH^{\cdot}_T$ was the concentration of DPPH· at the time of steady state and $DPPH^{\cdot}_T = 0$ was the concentration of DPPH· at the time of zero (initial concentration).

The percentage of remaining DPPH· against the sample/standard concentration was plotted to obtain the amount of antioxidant necessary to decrease the initial concentration of DPPH· by 50% (EC_{50}). Based on the parameter EC_{50}, the result was expressed in terms of mg dry matter of sample/standard equivalent g^{-1} DPPH· in the reaction medium.

2.5.5. Antioxidant activity by the $ABTS^{\cdot+}$ assay

The total antioxidant activity of seed extracts was measured by the $ABTS^{\cdot+}$ radical cation decolourization assay involving preformed $ABTS^{\cdot+}$ radical cation (Re et al., 1999). ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation ($ABTS^{\cdot+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. Oxidation of the ABTS commenced immediately, but the absorbance was not maximal and stable until more than 6 h had elapsed. The radical cation was stable in this form for more than 2 days in storage in the dark at room temperature. Prior to assay, the solution was diluted in ethanol (about 1:88 v/v) to give an absorbance at 734 nm of 0.700 ± 0.02 in a 1 cm cuvette and equilibrated to 30 °C, the temperature at which all the assays were performed. The stock solution of seed extracts, AA and BHA in ethanol were diluted such that, after introduction of a 10 μL aliquot of each dilution into the assay, they produced between 20% and 80% inhibition of the blank absorbance. After the addition of 1.0 mL of diluted $ABTS^{\cdot+}$ solution to 10 μL of antioxidant compounds or Trolox standards (final concentration 0–15 μM) prepared in ethanol was incubated at 30 °C exactly for 30 min after the initial mixing. Appropriate solvent blanks were also run in each assay. Triplicate determinations were made at each dilution of the standard, and the percentage inhibition was calculated of the blank absorbance at 734 nm and then was plotted as a function of Trolox concentration. The activity of seed extracts BHA and ascorbic acid was estimated at a minimum of three different concentrations within the range of dose–response curve, and the mean value was derived as the TEAC (Trolox equivalent antioxidant capacity) value. The unit of total antioxidant activity (TAA) was defined as the concentration of Trolox having the equivalent antioxidant activity expressed as mmol/kg seed extracts on dry matter basis.

2.5.6. Ferric-reducing/antioxidant power (FRAP) assay

The antioxidant capacity of phenolic extracts of raw and processed horse gram seed samples was estimated according to the procedure described by Benzie and Strain (1996) with slight modifications made by Pulido, Bravo, and Saura-Calixto (2000). FRAP reagent (900 μL), prepared freshly and incubated at 37 °C, was mixed with 90 μL of distilled water and 30 μL of test sample, BHA and Trolox or methanol (for the reagent blank). The test samples and reagent blank were incubated at 37 °C for 30 min in water bath. The final dilution of the test sample in the reaction mixture was 1/34. The FRAP reagent contained 2.5 mL of 20 mmol/L TPTZ solution in 40 mmol/L HCl plus 2.5 mL of 20 mmol/L $FeCl_3 \cdot 6H_2O$ and 25 mL of 0.3 mol/L acetate buffer, pH 3.6 (Benzie & Strain, 1996). At the end of incubation, the absorbance readings were taken immediately at 593 nm using a Spectrophotometer. Methanolic solutions of known Fe(II) concentration ranging from 100 to 2000 μmol/L ($FeSO_4 \cdot 7H_2O$) were used for the preparation of the calibration curve. The parameter Equivalent Concentration (EC_1) was defined as the concentration of antioxidant having a ferric-TPTZ reducing ability equivalent to that of 1 mmol/L $FeSO_4 \cdot$

$7H_2O$. EC_1 was calculated as the concentration of antioxidant giving an absorbance increase in the FRAP assay equivalent to the theoretical absorbance value of a 1 mmol/L concentration of Fe(II) solution determined using the corresponding regression equation.

2.5.7. Statistical analysis

The data were subjected to a one-way analysis of variance (ANOVA) and the significance of the difference between means was determined by Duncan's multiple range test ($P < 0.05$) using the Statistica (Statsoft Inc., Tulsa, USA). Values expressed are mean of three replicate determinations ± standard deviation.

3. Results and discussion

3.1. Recovery percent and phenolic content of extracts

The yield percent, total phenolics and tannins of extracts obtained from the raw and processed seed samples of two varieties of horse gram using methanol and aqueous acetone (70%) solvent are shown in Table 1. For both brown and black horse gram varieties, maximum yield was obtained for methanol extracts of dry-heated samples. The extractable total phenolics and tannins of the dry-heated samples were found to be higher than in raw samples for each solvent that could be due to the solubility of phenolics and other aroma compounds. However, the 70% acetone was found to be more efficient solvent for extracting the phenolic constituents in both raw and processed samples of brown and black varieties than that of absolute methanol. The presence of phenolic substances including tannins in horse gram was also reported in the study of Reddy et al. (1985) and Sudha et al. (1995).

3.2. Superoxide anion radical and hydroxyl radical-scavenging activities

The effects of phenolic extracts of the raw and dry-heated seed samples of brown and black varieties of horse gram on superoxide anion radical scavenging activity were estimated by the nitro blue tetrazolium (NBT) assay method and the results are compared to tannic acid and quercetin (Siddhuraju & Becker, 2007) (Table 3). All of the extracts had a scavenging activity on the superoxide radicals in a dose dependent manner (200–600 μg in the reaction mixture). Nonetheless, when compared to quercetin and tannic acid, the superoxide radical-scavenging activity of all the above-mentioned extracts was found to be low. This could be due to the presence of relative concentration of bioactive constituents and mixture of impurities/other nutrients in the extracts. The scavenging abilities of raw and processed horse gram seed extracts on hydroxyl radical inhibition are shown in Fig. 3. All the seed extracts showed hydroxyl radical-scavenging activities (38.7–72.7%) at a level of 200 μg in the reaction mixture. Methanol extracts of dry-heated seeds of both varieties showed relatively lower hydroxyl radical-scavenging activity. However, the acetone extract of the raw black variety had similar hydroxyl radical-scavenging activity to the acetone extracts of the dry-heated seeds of both varieties and also their values were significantly ($P < 0.05$) higher than all other sample extracts. Yen and Hsieh (1995) reported that xylose and lysine Maillard reaction products had scavenging activity on hydroxyl radical that depend on dose response manner and which might have been attributed to the combined effects of reducing power, donation of hydrogen atoms and scavenging of active oxygen. The study on antioxidant properties of faba bean tannins indicated that the antioxidant activity was accounted for by the direct interaction of tannin with hydroxyl radical rather than to a metal chelating activity (Carbonaro, Virgili, & Carnovale, 1996). These results show that the potential scavenging abilities of phenolic substances might be due to the active hydrogen donor ability of hydroxyl substitution. Hagerman et al. (1998) have also explained that high molecular weight and the proximity of many aromatic rings and hydroxyl groups are more important for the free radical-scavenging activity by tannins than their specific functional groups.

3.3. Antioxidant activity in linoleic acid emulsion system

The antioxidant effects of the extracts from un-processed and processed seed samples of two varieties of

Table 1
Recovery percent, total phenolics and tannins of two varieties of horse gram seed extracts (g 100 g^{-1} DM)

Sample	Extract recovery	SD	Total phenolics	SD	Tannins	SD
Brown variety						
RM	5.80	0.28	1.74	0.09	0.76	0.04
RA	4.45	0.35	6.09	1.41	2.65	0.61
HM	7.75	0.21	5.56	1.12	2.42	0.49
HA	5.35	0.35	9.67	2.18	4.52	1.21
Black variety						
RM	7.00	0.28	3.63	0.83	1.58	0.36
RA	5.45	0.35	8.93	0.08	3.89	0.03
HM	8.80	0.42	6.29	1.31	2.74	0.57
HA	5.95	0.21	12.63	1.41	5.80	0.61

Values are mean of three replicate determination; SD, standard deviation; RM, raw sample extracted with methanol; RA, raw sample extracted with 70% acetone; HM, dry heated sample extracted with methanol; HA, dry-heated sample extracted with 70% acetone.

horse gram, ascorbic acid and BHA on the peroxidation of linoleic acid were investigated and the results are presented in Fig. 1. At a level of 250 μg in the final reaction mixture, the raw and dry-heated seed samples of both varieties inhibited 53.3–72.7% peroxidation of linoleic acid after incubation for 72 h (3 days). However, those values were significantly lower and higher than those of the positive controls such as BHA (93.3%) and AA (43.0%), respectively. In summary, the results show that the inhibitory potential follows the order BHA > 1HA = 2RA > 2-HA > 1RA > 2RM > 1RM > 1HM > 2HM > AA. Tsuda et al. (1993b) also reported that methanol extracts of horse gram showed lipid peroxidation inhibiting activity in the linoleic acid. In general, seed coat may play an important role in chemical protection from oxidative damage by possessing endogenous antioxidants such as phenolic compounds. Similarly, the seed coat extracts containing phenolic substances, from red and black beans have been reported to have a strong antioxidant activity against the lipid peroxidation (Tsuda et al., 1994). The hydrolysable tannins from *Osbeckia chinensis* were found to have potential antioxidative efficiency in the linoleic acid-thiocyanate system (Su, Osawa, Kawakishi, & Namiki, 1998). The stability of antioxidant potential of dry-heated samples may be due to the formation of products from the Maillard reaction. Nicoli, Anese, Manzocco, and Lerici (1997) reported that medium dark roasted coffee brews had the highest antioxidant properties due to the development of products as a result of Maillard reaction. Similarly, extract of roasted followed by defatted legume, peanut kernels, displayed most remarkable antioxidative activity on linoleic acid emulsions system (Hwang, Shue, & Chang, 2001).

3.4. Radical-scavenging activities on α,α-diphenyl-β-picrylhydrazyl (DPPH·) and ABTS·+

The DPPH· radical-scavenging activities of methanol and aqueous acetone extracts of un-processed and processed seeds along with the reference standards ascorbic acid (AA) and BHA are shown in Fig. 2. A lower value of EC_{50} indicates a higher antioxidant activity. Extracts (by 70% acetone) obtained from raw and dry-heated seeds of black variety showed the highest DPPH radical-scavenging activity (2RA, black variety, raw, 70% acetone extracted; 0.26, 2HA, brown variety, heat treated, 70% acetone extracted; 0.44, 1HA brown variety, heat treated, 70% acetone extracted; 1.02 and 1RA, brown variety, raw, 70% acetone extracted; 1.44) and the values were significantly different ($P < 0.05$) when compared to the values of methanol extracts of the respective seed samples. However, the synthetic antioxidants, BHA and AA were found to be more potent for providing the hydrogen donating/electron transfer ability than all other seed samples. In a recent study Siddhuraju, Mohan, and Becker (2002) reported that a high concentration of tannins (proanthocyanidins) extracted from stem bark of *Cassia fistula* was found to have elevated DPPH radical quenching capacity. Similarly, Amarowicz, Naczk, and Shahidi (2000) reported that the tannins extracted from canola and rapeseed hulls exhibited a high scavenging efficiency toward DPPH radicals. The order of scavenging activity of seed extracts: 2HA > 2RA > 1HA > 1RA > 2RM > 2HM > 1HM > 1RM was found in the study. This antiradical-scavenging activity of untreated and treated seed extracts would be related to the nature of phenolics, thus contributing to their electron transfer/hydrogen donating ability (Brand-Williams et al., 1995).

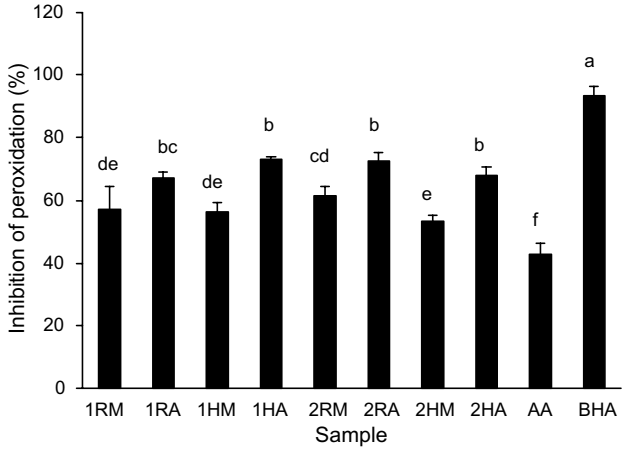

Fig. 1. Antioxidant activity of two varieties of raw and dry-heated horse gram seed extracts (the mass of extract in the reaction mixture was 250 μg): 1, Brown variety; 2, Black variety; RM, raw, methanol extracted; RA, raw, 70% acetone extracted; HM, dry heated, methanol extracted; HA, dry heated, 70% acetone extracted; AA, ascorbic acid (250 μg in the reaction mixture); BHA, butylated hydroxyanisole (250 μg in the reaction mixture). Values are mean of triplicate determinations ($n = 3$); ± standard deviation. Bars having different letters are significantly different ($P < 0.05$).

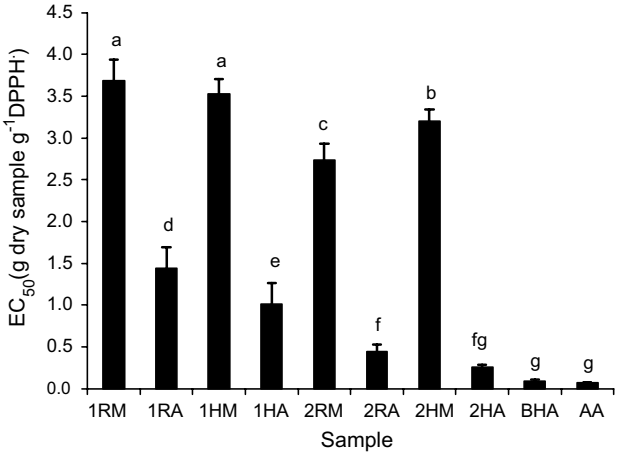

Fig. 2. DPPH free radical-scavenging activity of seed extracts of two varieties of horse gram. 1, Brown variety; 2, Black variety; RM, raw, methanol extracted; RA, raw, 70% acetone extracted; HM, dry heated, methanol extracted; HA, dry heated, 70% acetone extracted. Gram of sample necessary to decrease one g of the initial DPPH· concentration by 50%. Bars having different letters are significantly different ($P < 0.05$). Values are mean of three independent determinations ($n = 3$); ± standard deviation.

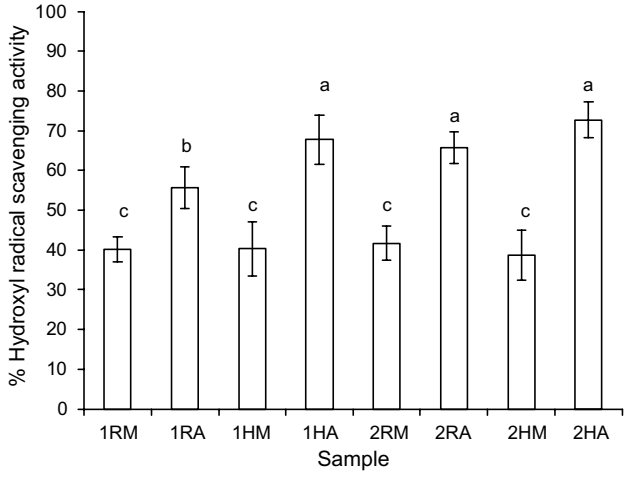

Fig. 3. Hydroxyl radical-scavenging activity of two varieties of hose gram seed extracts (the mass of extract in the reaction mixture was 200 μg): 1, Brown variety; 2, Black variety: RM, raw, methanol extracted; RA, raw, 70% acetone extracted; HM, dry heated, methanol extracted; HA, dry heated, 70% acetone extracted. Values are mean of three independent determination ($n = 3$); ± standard deviation. Bars having different letters are significantly different ($P < 0.05$).

Table 2
ABTS$^{\cdot+}$cation radical-scavenging activities and FRAP assay of un-processed and dry-heated seed extracts of two varieties of horse gram

Sample	TAA (mmol kg^{-1}DM)[A]		FRAP (mg DM mmol^{-1} Fe(II))[B]	
	Mean	SD	Mean	SD
Brown variety				
RM	1045.0^{b}	31.3	1724.3^{a}	72.4
RA	1351.4^{b}	46.7	1005.3^{e}	16.2
HM	902.3^{b}	120.2	1556.0^{b}	33.5
HA	1288.4^{b}	29.1	1176.0^{c}	16.5
Black variety				
RM	1056.5^{b}	15.6	1066.3^{d,e}	21.8
RA	1405.7^{b}	16.0	1003.5^{e}	29.9
HM	1150.4^{b}	18.1	1084.3^{d}	78.5
HA	1439.7^{b}	31.5	892.3^{f}	14.5
BHA	158022.9^{a}	8283.1	65.51^{g}	3.6
Trolox			87.34^{g}	4.3
AA	4368.7^{b}	209.1		

Values are mean of three independent determinations ($n = 3$); SD, standard deviation. RM, raw, methanol extracted; RA, raw, 70% acetone extracted; HM, dry heated, methanol extracted; HA, dry heated, 70% acetone extracted; AA, ascorbic acid; BHA, butylated hydroxyanisole. Values followed by different superscript in each column are significantly different ($P < 0.05$).

[A] Total antioxidant activity (mmol equivalent trolox performed by using ABTS$^{\cdot+}$).

[B] Ferric-reducing/antioxidant power assay (concentration of substance having ferric-TPTZ reducing ability equivalent to that of 1 mmol Fe(II)); a high FRAP value indicates low antioxidant activity.

On the other hand, the DPPH radical-scavenging efficiency of extracts from dry-heated seed samples might have also been partly attributed to Millard reaction products other than the phenolic constituents because they also effectively participate as a radical scavengers. However, when compared to standards, AA (EC_{50}; 0.07) and BHA (EC_{50}; 0.09), all the tested seed extracts showed significantly ($P < 0.05$) lower DPPH radical-scavenging activity.

In ABTS radical cation scavenging method, the activity of tested seed extracts was expressed as Trolox equivalent-the millimolar of Trolox solution having an antioxidant capacity equivalent to 1 kg dry matter of the substance under investigation. The total antioxidant activity of raw and processed seed sample (extracts) of two varieties of horse gram is presented in Table 2. Even though the raw and dry-heated seed samples exhibited good ABTS radical-scavenging activity, the 70% acetone extracts of all the samples showed insignificantly higher TAA (1288–1439 mmol kg^{-1}) than the methanol extract of samples (902–1150 mmol kg^{-1}). Though the total phenolic content of seed samples of both varieties were found to be relatively low (assumed to be non-harmful), the TAA of such samples seems to be sufficient for functioning as potential nutraceuticals when they are ingested along with nutrients. The extensive investigations on antiradical and antioxidant activities of small phenolics including flavonoids and phenolic acids have been reported (Heim et al., 2002). Apart from these, Hagerman et al. (1998) have reported that the high molecular weight phenolics (tannins) have more ability to quench free radicals (ABTS$^{\cdot+}$) and that effectiveness depends on the molecular weight, the number of aromatic rings and nature of hydroxyl groups' substitution than the specific functional groups. On the other hand, the formation of tannin–protein complexes, both in insoluble and soluble complexes, as the result of conventional food/seed processing have also been shown to be potential free radical scavenger and radical sinks. Moreover, such complexes can also be suggested as one of the nutraceutical contributors to prevent the free radical mediated diseases occurring in the gastrointestinal tract (Riedl & Hagerman, 2001).

3.5. Ferric-reducing antioxidant power

Antioxidants can be referred to as reductants, which inactivate oxidants. They are involved in redox reactions in which one reaction species (oxidant) is reduced at the expense of the oxidation of the antioxidant (reductant). The FRAP assay measures the antioxidant effect of any substance in the reaction medium as reducing ability. Antioxidant potential of the un-processed and processed seed extracts of horse gram was estimated from their ability to reduce TPTZ–Fe(III) complex to TPTZ–Fe(II) complex. The antioxidant capacities of raw and processed seed extracts of two varieties of horse gram varied significantly (high FRAP value indicates low antioxidant activity) (Table 2). The 70% acetone extracts of both varieties of raw and dry-heated samples showed highest FRAP antioxidant activity, as has been recorded in DPPH$^{\cdot}$ and ABTS$^{\cdot+}$ methods. However, black variety was found to have more ferric-reducing/antioxidant power than the brown variety. The order of FRAP activity of respective seed samples extract

Table 3
Superoxide anion ($O_2^{\cdot-}$) radical-scavenging activities of raw and processed seed extracts of two varieties of horse gram

Sample	Superoxide anion radical-scavenging activity (%)					
	200[A]	SD	400[A]	SD	600[A]	SD
Brown variety						
RM	37.23[a]	1.45	57.57[a]	1.49	65.05[a,b]	1.74
RA	41.76[a]	1.68	49.97[b]	2.11	70.46[a]	3.15
HM	22.78[c,d]	3.82	39.63[d]	1.33	56.42[c,d]	3.70
HA	18.20[d]	1.62	30.27[e]	1.89	61.21[b,c]	5.24
Black variety						
RM	23.43[b,c,d]	5.43	42.95[c,d]	1.83	54.91[c,d]	3.88
RA	28.71[b]	3.39	40.30[c,d]	4.32	64.43[a,b]	1.80
HM	22.78[c,d]	2.05	40.82[c,d]	4.44	52.94[d]	1.52
HA	26.21[b,c]	2.96	45.29[c]	2.12	65.99[a,b]	5.67
Tannic acid[B]	21.8	8.07	34.9	1.3	56.2	3.6
Quercetin[B]	20.3	1.4	32.1	2.6	41.2	0.9

Mean values followed by different superscript in a column are significantly different ($P < 0.05$). RM, raw, methanol extracted; RA, raw, 70% acetone extracted; HM, dry heated, methanol extracted; HA, dry heated, 70% acetone extracted.

[A] Mass of extract in the reaction mixture was 200–600 μg.

[B] Mass of compound in the reaction mixture was 20, 40 and 60 μg, respectively. Values are mean of three independent determinations ($n = 3$); SD, standard deviation.

is as follows: Brown variety; RA > HA > HM > RM and Black variety; HA > RA > HM > RM as in the case of DPPH$^{\cdot}$ and ABTS$^{\cdot+}$. Moreover, there was a noticeable correlation between extractable total phenolics and FRAP values in Brown ($r^2 = 0.5237$) and Black ($r^2 = 0.8534$) variety of horse gram. A high correlation has also been reported in guava fruit extracts (Jimènez-Escrig, Rincòn, Pulido, & Saura-Calixto, 2001) in a similar manner. Yen and Duh (1993) and Siddhuraju et al. (2002) have reported that the reducing power of bioactive compounds (mainly low and high molecular phenolics), which were extracted from peanut hulls and stem bark of Indian laburnum, respectively, depend on antioxidant activity which are specific for scavenging of free radicals. Nevertheless, recent *in vivo* research has revealed that dark chocolate containing phenolics, in particularly (−) epicatechin, had ferric-reducing antioxidant power (*in vitro*) and increased the total antioxidant capacity of blood plasma on consumption (Serafini et al., 2003).

This study suggests that not only the phenolic (tannins) substances from raw seeds but also the substances from the processed seeds of horse gram are potent antioxidant sources. Further, the research work on *in vitro* protein digestibility together with the assessment of antioxidant properties in the above said processed sample might be a fruitful approach for advocating them as nutraceuticals in addition to that of being a potential protein and carbohydrate suppliers. On the other hand, the presence of tannins–protein complexes and phenolics associated with dietary fibre and how these relate to the risk of oxidative injury during gastrointestinal digestion can be demonstrated through *in vivo* studies. After establishing such a balance between the antinutrient and the biological antioxidant effects of polyphenols (dietary tannins), the consumption of such a processed legume food would not only improve the nutrient utilization but also provide the potential nutraceuticals for human health. The isolation and preparation of bioactive compounds from the coloured seed coat of horse gram could serve as potential natural antioxidants for the food industries.

Acknowledgement

The author P.S. thank the Management and Administrative authorities of KASC, Coimbatore for providing necessary facilities to carry out this research work.

References

Amarowicz, R., Naczk, M., & Shahidi, F. (2000). Antioxidant activity of crude tannins of Canola and Rapeseed hulls. *Journal of American Oil Chemist's Society, 77*, 957–961.

Anderson, J. W., Smith, B. M., & Washnock, C. S. (1999). Cardiovascular and renal benefits of dry bean and soybean intake. *American Journal of Clinical Nutrition, 70*(suppl.), 464S–474S.

Aruoma, O. I. (1994). Deoxyribose assay for detecting hydroxyl radicals. *Methods in Enzymology, 233*, 57–66.

Bazzano, L., He, J., Ogden, L. G., Loria, C., Vuputuri, S., Myers, L., et al. (2001). Legume consumption and risk of coronary heart disease in US men and women. *Archives of Internal Medicine, 161*, 2573–2578.

Beauchamp, C., & Fridovich, I. (1971). Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. *Analytical Biochemistry, 44*, 276–287.

Benzie, I. F. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Analytical Biochemistry, 239*, 70–76.

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *Lebensmittel Wissenschaft-und Technologie, 28*, 25–30.

Bravo, L., Siddhuraju, P., & Saura-Calixto, F. (1998). Effect of various processing methods on the *in vitro* starch digestibility and resistant starch content of Indian pulses. *Journal of Agricultural and Food Chemistry, 46*, 4667–4674.

Bravo, L., Siddhuraju, P., & Saura-Calixto, F. (1999). Composition of underexploited Indian pulses. Comparison with common legumes. *Food Chemistry, 64*, 185–192.

Carbonaro, M., Virgili, F., & Carnovale, E. (1996). Evidence for protein–tannin interaction in legumes: Implications in the antioxidant properties of faba bean tannins. *Lebensmittel Wissenschaft-und Technologie, 29*, 743–750.

Hagerman, A. E., Riedl, K. M., Jones, G. A., Sovik, K. N., Ritchard, N. T., Hartzfeld, P. W., et al. (1998). High molecular weight plant polyphenolics (tannins) as biological antioxidants. *Journal of Agricultural and Food Chemistry, 46*, 1887–1892.

Heim, K. E., Tagliaferro, A. R., & Bobilya, D. J. (2002). Flavonoid antioxidants: Chemistry, metabolism and structure–activity relationships. *Journal of Nutritional Biochemistry, 13*, 572–584.

Hwang, J. Y., Shue, Y. S., & Chang, H. M. (2001). Antioxidant activity of roasted and defatted peanut kernels. *Food Research International, 34*, 639–647.

Jimènez-Escrig, A., Rincòn, M., Pulido, R., & Saura-Calixto, F. D. (2001). Guava fruit (*Psidium guajava* L.) as a new source of antioxidant dietary fiber. *Journal of Agricultural and Food Chemistry, 49*, 5489–5493.

Kadam, S. S., & Salunkhe, D. K. (1985). Nutritional composition, processing, and utilization of horse gram and moth bean. *Critical Reviews in Food Science and Nutrition, 22*, 1–26.

Lee, J., Koo, N., & Min, D. B. (2004). Reactive oxygen species, aging, and antioxidative nutraceuticals. *Comprehensive Reviews in Food Science and Food Safety, 3*, 21–33.

Lee, K. G., Mitchell, A. E., & Shibamoto, T. (2000). Determination of antioxidant properties of aroma extracts from various beans. *Journal of Agricultural and Food Chemistry, 48*, 4817–4820.

Makkar, H. P. S. (2003). *Quantification of tannins in tree and shrub foliage: A laboratory manual*. Dordrecht, The Netherlands: Kluwer Academic Publishers.

Mazur, W. M., Duke, J. A., Wähälä, K., Rasku, S., & Adlercreutz, H. (1998). Isoflavonoids and lignans in legumes: Nutritional and health aspects in humans. *Journal of Nutritional Biochemistry, 9*, 193–200.

Messina, M. J. (1999). Legume and soybeans: Overview of their nutritional profiles and health effects. *American Journal of Clinical Nutrition, 70*(suppl.), 439S–449S.

Middleton, E., Kandaswamy, C., & Theoharides, T. C. (2000). The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease, and cancer. *Pharmacological Reviews, 52*, 673–751.

Mitra, J., Das, A., & Joshi, T. (1983). An isoflavone diglycoside from the seeds of *Dolichos biflorus*. *Phytochemistry, 22*, 1063–1064.

Mitsuda, H., Yasumoto, K., & Iwami, K. (1966). Antioxidative action of indole compounds during the autoxidation of linoleic acid. *Eiyo to Shokuryo, 19*, 210–214.

Morris, B. (2003). Bio-functional legumes with nutraceutical, pharmaceutical and industrial uses. *Economic Botany, 57*, 254–261.

Muthu, A. K., Sethupathy, S., Manavalan, R., & Karar, P. K. (2005). Hypolipidemic effect of methanolic extract of *Dolichos biflorus* Linn. in high fat diet fed rats. *Indian Journal of Experimental Biology, 43*, 522–525.

Muthu, A. K., Sethupathy, S., Manavalan, R., & Karar, P. K. (2006). Antioxidant potential of methanolic extract of *Dolichos biflorus* Linn. in high fat diet fed for rabbits. *Indian Journal of Pharmacology, 38*, 131–132.

Nicoli, M. C., Anese, M., Manzocco, L., & Lerici, C. R. (1997). Antioxidant properties of coffee brews in relation to the roasting degree. *Lebensmittel Wisenschaft – und Technologie, 30*, 292–297.

Pietta, P., Simonetti, P., & Mauri, P. (1998). Antioxidant activity of selected medicinal plants. *Journal of Agricultural and Food Chemistry, 46*, 4487–4490.

Pulido, R., Bravo, L., & Saura-Calixto, F. (2000). Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. *Journal of Agricultural and Food Chemistry, 48*, 3396–3402.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology in Medicine, 26*, 1231–1237.

Reddy, N. R., Pierson, M. D., Sathe, S. K., & Salunkhe, D. K. (1985). Dry bean tannins: A review of nutritional implications. *Journal of American Oil Chemist's Society, 62*, 541–549.

Riedl, K., & Hagerman, A. E. (2001). Tannin–protein complexes as radical scavengers and radical sinks. *Journal of Agricultural and Food Chemistry, 49*, 4917–4923.

Sánchez-Moreno, C., Larrauri, J. A., & Saura-Calixto, F. (1998). A procedure to measure the antiradical efficiency of polyphenols. *Journal of the Science of Food Agriculture, 76*, 270–276.

Serafini, M., Bugianesi, R., Maiani, G., Valturna, S., Santis, S. D., & Crozier, A. (2003). Plasma antioxidants from chocolate. *Nature, 424*, 1013.

Shahidi, F., Chavan, U. D., Naczk, M., & Amarowicz, R. (2001). Nutrient distribution and phenolic antioxidants in air-classified fractions of Beach pea (*Lathyrus maritimus* L.). *Journal of Agricultural and Food Chemistry, 49*, 926–933.

Siddhuraju, P. (2006). The antioxidant activity and free radical scavenging capacity of phenolics of raw and dry heated moth bean (*Vigna aconitifolia*) (Jacq.) Marechal seed extracts. *Food Chemistry, 99*, 149–157.

Siddhuraju, P., & Becker, K. (2007). The antioxidant and free radical scavenging activities of processed cowpea (*Vigna unguiculata* (L.) Walp.) seed extracts. *Food Chemistry, 101*, 10–19.

Siddhuraju, P., Mohan, P. S., & Becker, K. (2002). Studies on the antioxidant activity of Indian Laburnum (*Cassia fistula* L.): A preliminary assessment of crude extracts from stem bark, leaves, flowers and fruit pulp. *Food Chemistry, 79*, 61–67.

Su, J. D., Osawa, T., Kawakishi, S., & Namiki, M. (1998). Tannin antioxidants from *Osbeckia chinensis*. *Phytochemistry, 27*, 1315–1319.

Sudha, N., Mushtari Begum, J., Shambulingappa, K. G., & Babu, C. K. (1995). Nutrients and some anti-nutrients in horse gram (*Macrotyloma uniflorum* (Lam.) Verdc.). *Food and Nutritional Bulletin, 16*, 81–83.

Suja, K. P., Jayalekshmy, A., & Arumughan, C. (2005). *In vitro* studies on antioxidant activity of lignans isolated from sesame cake extract. *Journal of the Science of Food and Agriculture, 85*, 1779–1783.

Tsuda, T., Makino, Y., Kato, H., Osawa, T., & Kawakishi, S. (1993b). Screening for antioxidative activity of edible pulses. *Bioscience, Biotechnology and Biochemistry, 57*, 1606–1608.

Tsuda, T., Ohshima, K., Kawakishi, S., & Osawa, T. (1994). Antioxidative pigments isolated from the seeds of *Phaseolus vulgaris* L.. *Journal of Agricultural and Food Chemistry, 42*, 248–251.

Tsuda, T., Osawa, T., Nakayama, T., Kawakishi, S., & Ohshima, K. (1993a). Antioxidant activity of pea bean (*Phaseolus vulgaris* L.) extract. *Journal of American Oil Chemist's Society, 70*, 909–913.

Yen, G. C., & Duh, P. D. (1993). Antioxidative properties of methanolic extracts from peanut hulls. *Journal of American Oil Chemist's Society, 70*, 383–386.

Yen, G. C., & Hsieh, P. P. (1995). Antioxidative activity and scavenging effects on active oxygen of xylose-lysine Maillard reaction products. *Journal of the Science of Food and Agriculture, 67*, 415–420.

Yen, G. C., & Hsieh, C. L. (1998). Antioxidant activity of extracts from Du-zhong (*Eucommia ulmoides*) toward various lipid peroxidation models *in vitro*. *Journal of Agricultural and Food Chemistry, 46*, 3952–3957.

Zhishen, J., Mengcheng, T., & Jianming, W. (1999). The determination of flavonoid contents on mulberry and their scavenging effects on superoxide radical. *Food Chemistry, 64*, 555–559.