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The antioxidant activity and free radical-scavenging capacity of phenolics of raw and dry heated moth bean (*Vigna aconitifolia*) (Jacq.) Marechal seed extracts

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Abstract

The antioxidative properties and total phenolic contents of *Vigna aconitifolia* were examined. The raw and dry heated samples were extracted with 70% acetone and the extracts were freeze-dried. The raw seeds contained higher levels of total phenolics (6.54%) and tannins (1.91%) than the dry heated seeds. The extracts were screened for their potential antioxidant activities using $O_2^{\bullet -}$, $OH^{\bullet}$, α,α-diphenyl-β-picrylhydrazyl ($DPPH^{\bullet}$), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet +}$), Ferric reducing/antioxidant power (FRAP), linoleic acid emulsion and Fe^{2+} chelating systems. At 1 mg of extract in the reaction mixture, the superoxide anion radical-scavenging activity was found to be similar in raw and dry heated seed extracts. The DPPH radical and ABTS cation radical scavenging activities were well proved and correlated with the ferric reducing antioxidant capacity of the extracts. Interestingly, both raw and dry heated seed extracts showed the highest hydroxyl radical scavenging activity of 67.3% and 68.5%, respectively, at concentration of 1 mg/g extract. In addition, both extracts exhibited good peroxidation inhibiting activity (54.2% and 58.2%, respectively) against the linoleic acid emulsion system and the values were lower than BHA and Trolox. Fe^{2+} chelating activity was also detected in both raw and dry heated seed with EDTA equivalent of 0.61 mg and 0.45 mg/g extracts, respectively.

Keywords: *Vigna aconitifolia*; Seeds; Dry heating; Phenolics; Tannins; Antioxidant activity; $ABTS^{\bullet +}$; FRAP

1. Introduction

Ascorbic acid, vitamin E and phenolic compounds, which are present naturally in vegetables, fruits, grains and pulses, possess the ability to reduce oxidative damage that are believed to cause many diseases including cancer, cardiovascular diseases, cataracts, atherosclerosis, diabetes, arthritis, immune deficiency diseases and ageing (Lee, Mitchell, & Shibamoto, 2000; Middleton, Kandaswamy, & Theoharides, 2000; Pietta, Simonetti, & Mauri, 1998). Recently, the ability of phenolic substances including flavonoids and phenolic acids to act as antioxidants have been extensively investigated (Rice-Evans, Miller, & Paganda, 1996). Legumes play an important role in the traditional diets of many regions throughout the world and they are low in fat and are excellent sources of protein, dietary fibre, a variety of micronutrients and phytochemicals (Anderson, Smith, & Washnock, 1999; Messina, 1999). Coloured beans are preferred by most Latin American populations over white beans. Similarly, the sauces prepared from the cooking liquors of coloured beans, which contain mainly the seed coat pigments such as dietary tannins and non-tannin phenolics, are consumed in certain regions of India mixed with rice and cereals. The consumption of such legumes has been linked to reduced risk of diabetes and obesity and has a role in the reduction of coronary heart diseases (Bazzano et al., 2001). Recently,

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polyphenolic constituents of various legume seeds have been reported to have potential medicinal properties including antioxidant activities (Lou et al., 2004; Mazur, Duke, Wähälä, Rasku, & Adlercreutz, 1998; Nilsson, Stegmark, & Akesson, 2004; Shahidi, Chavan, Naczk, & Amarowicz, 2001; Siddhuraju & Becker, 2003; Tsuda, Ohshima, Kawakishi, & Osawa, 1994; Tsuda, Osawa, Nakayama, Kawakishi, & Ohshima, 1993a). Therefore, studies on the nutritional importance of legumes and the role of non-nutrient compound particularly phenolic acids, flavonoids and high molecular tannins of legumes as natural antioxidants have greatly increased.

Moth bean (*Vigna aconitifoia*) is recognised as a potential source of protein and other nutrients. It is cultivated for its immature pods and mature seeds and is consumed by people all around the world, especially in the developing nations (Adsule, 1996; Bravo, Siddhuraju, & Saura-Calixto, 1998; Kadam & Salunkhe, 1985). The consumption of moth bean 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. The whole seeds have been reported to contain about 1.3% tannins (Reddy, Pierson, Sathe, & Salunkhe, 1985) and 1.4% of total phenolics (Vijayakumari, Siddhuraju, Pugalenthi, & Janardhanan, 1998). However, only limited information is available on their antioxidant activity (Tsuda, Makino, Kato, Osawa, & Kawakishi, 1993b). Even though the processed moth bean seeds are increasingly consumed as food, the beneficial effects of their bioactive compounds remain unexplored. Therefore, the present study was aimed at evaluating the phenolic constituents, antioxidant potential and free radical-scavenging capacity of aqueous acetone extracts of raw and dry heated moth beans.

2. Materials and methods

2.1. Chemicals

Butylated hydroxyanisole (BHA), ascorbic acid, potassium ferricyanide, TPTZ (2,4,6-tripyridyl-s-triazine), α,α-diphenyl-β-picrylhydrazyl (DPPH$^{\bullet}$), nitro blue tetrazolium (NBT), thiobarbituric acid (TBA), trichloroacetic acid (TCA), 2-deoxy-D-ribose and linoleic acid were purchased from Himedia, India and ethylenediamine tetracetic acid (EDTA), Tween 20, ammonium thiocyanate, potassium persulfate, ferrous chloride and ferric chloride were purchased from Merck or SDS, India, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade.

2.2. Seed samples and processing

The matured seeds of *Vigna aconitifolia* were purchased from market located at Jalakandapuram, Salem, TN. The seeds (150 g) were dry heated along with acid washed sea sand on an open hot plate at 125 ± 2 °C for 25 min. During the heat processing frequent (every 3 min) clarification of seeds together with the sand was done using a glass rod for uniform heating. The seeds were separated by sieving and cleaned thoroughly. The raw and dry heated seed samples were ground to a fine powder and stored in a separate screw cap bottle at −20 °C before analysis.

2.3. Solvent extraction

Raw and processed ground seed samples (50 g) were extracted by stirring with 300 ml of 70% acetone at 25 °C for 24 h after extracted with petroleum ether and then filtered through Whatman No. 4 filter paper. The residues were re-extracted with an additional 150 ml of 70% acetone, as described above, for 3 h. The solvent of the combined extract was evaporated under reduced pressure using a rotary vacuum-evaporator at 45 °C and the remaining water was removed by lyophilisation. The freeze-dried extract thus obtained was used directly for total phenolics and tannins estimation and also for the assessment of antioxidant capacity through various chemical assays.

2.4. Determination of total phenolic content and tannins

The total phenolic content of the freeze-dried aqueous acetone extract of raw and dry heated moth bean seeds was determined according to the method described by Makkar, Becker, Abel, and Pawelzik (1997). Aliquots of the extract were taken in a test tube and made up to the 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%) 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 from a calibration curve. Using the same aqueous acetone solution, which contained a known amount of freeze-dried extract, the tannins (Makkar et al., 1997) were estimated after the treatment of PVPP.

2.5. Superoxide anion ($O_2^{\bullet -}$) radical scavenging activity

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 concentration of extracts in the reaction mixture was 200–1000 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 $O_2^{\bullet-}$. which reduced NBT to form blue formazan. The unilluminated reaction mixture was used as a blank. The absorbance (A) was measured at 560 nm. The extracts were added to the reaction mixture, in which $O_2^{\bullet-}$ was scavenged, thereby inhibiting the NBT reduction. Absorbance (A) was measured and the decrease in $O_2^{\bullet-}$ was represented by A_1–A_2. The degree of the scavenging was calculated by the following equation:

$$\text{Scavenging } (\%) = \frac{A - A_1}{A} \times 100\%.$$

2.6. Hydroxyl ($OH^{\bullet}$) radical-scavenging activity

The scavenging activity of the raw and dry heated moth bean seed extracts and trolox on the hydroxyl radical ($OH^{\bullet}$) 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 (250, 500 and 1000 μg). The reaction was started by adding ascorbic acid to a final concentration of 100 μM. 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, 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 the test sample was used as control. The scavenging activity on hydroxyl radicals (HRSA) was expressed as

$$\text{HRSA } (\%) = \left(1 - \frac{\text{absorbance at 532 nm in the presence of sample}}{\text{absorbance at 532 nm in the absence of sample}}\right) \times 100.$$

2.7. Antioxidant activity in linoleic acid emulsion system

The antioxidant activity of unprocessed and processed seed extracts of moth bean, BHA and Trolox was determined using the thiocyanate method (Mitsuda, Yasumoto, & Iwami, 1966) as described by Yen and Hsieh (1998). Each sample (1000 μg) in 0.5 ml of absolute ethanol was mixed with a 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 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

$$\text{LPI } (\%) = \left(1 - \frac{\text{absorbance at 500 nm in the presence of sample}_{72\text{ h}}}{\text{absorbance at 500 nm in the absence of sample}_{72\text{ h}}}\right) \times 100$$

2.8. Free radical-scavenging activity on α,α-diphenyl-β-picrylhydrazyl radical ($DPPH^{\bullet}$)

The antioxidant activity of moth bean seed extracts, ascorbic acid and BHA was measured in terms of hydrogen donating or radical-scavenging ability, using the $DPPH^{\bullet}$ 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 3.9 ml (0.025 g l^{-1}) of $DPPH^{\bullet}$ solution. The solution was incubated at room temperature for 60 min and the decrease in absorbance at 515 nm was determined at the end of incubation period with a Spectrophotometer. The remaining concentration of $DPPH^{\bullet}$ in the reaction medium was calculated from a calibration curve obtained

with DPPH$^{\bullet}$ at 515 nm. The percentage of remaining DPPH$^{\bullet}$ ($DPPH^{\bullet}_R$) was calculated as follows:

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

where $DPPH^{\bullet}_T$ was the concentration of DPPH$^{\bullet}$ at the time of 60 min and $DPPH^{\bullet}_T = 0$ was the concentration of DPPH$^{\bullet}$ at time zero (initial concentration).

The percentage of remaining DPPH$^{\bullet}$ against the sample/standard concentration was plotted to obtain the amount of antioxidant necessary to decrease the initial concentration of DPPH$^{\bullet}$ 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$^{\bullet}$ in the reaction medium.

2.9. Antioxidant activity by the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) assay

The total antioxidant activity of seed extracts was measured by the $ABTS^{\bullet+}$ radical cation decolourisation assay involving preformed $ABTS^{\bullet+}$ radical cation (Re et al., 1999). ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation ($ABTS^{\bullet+}$) 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 (1:89 v/v) and equilibrated to 30 °C, the temperature at which all the assays were performed to give an absorbance at 734 nm of 0.700 ± 0.02 in a 1 cm cuvette. The stock solution of seed extracts 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 $ABTS^{\bullet+}$ solution to 10 μl of antioxidant compounds or Trolox standards (final concentration 0–15 μM) in ethanol was taken at 30 °C exactly 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 of the blank absorbance 734 nm was calculated and then was plotted as a function of Trolox concentration. The activity of seed extracts was estimated at a minimum of three different concentrations within the range of the 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.

2.10. Ferric reducing/antioxidant power (FRAP) assay

The antioxidant capacity of phenolic extracts of raw and dry heated moth bean seed samples, BHA and Trolox was estimated according to the procedure described by Benzie and Strain (1996) as modified 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, or methanol (for the reagent blank). The test samples and reagent blank were incubated at 37 °C for 30 min in a 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.

2.11. Chelating capacity

Fe^{2+} chelating measured by 2,2″-bipyridyl competition assay (Yamaguchi, Ariga, Yoshimura, & Nakazawa, 2000). The reaction mixture contained 0.25 ml of 1 mM $FeSO_4$ solution, 0.25 ml of antioxidant solution, 1 ml of 0.2 M Tris–HCl buffer (pH 7.4), 1 ml of 2,2″-bipyridyl solution (0.1% in 0.2 M HCl), 0.4 ml of 10% hydroxylamine–HCl, and 2.5 ml of ethanol. The final volume was made up to 5 ml with pure water. The absorbance at 522 nm was determined and used to evaluate Fe^{2+} chelating activity using ethyelenediaminetetraacetate (EDTA) as a standard. The results were expressed as mg EDTA equivalent to g of seed extracts.

3. Results and discussion

3.1. Recovery percent and phenolic content of extracts

The yields, total phenolics and tannins of extracts obtained from the raw and processed seed samples of moth

bean using aqueous acetone (70%) solvent are shown in Table 1. Maximum yield was obtained for the extracts of dry-heated sample. However, the extractable total phenolics (6.54%) and tannins (1.91%) of the raw samples were higher than those of processed samples. Similarly, when drying red grape pomace peels at a temperature of 100 and 140 °C, a significant reduction in both the total extractable polyphenols (18.6% and 32.6%) and condensed tannins (11.1% and 16.6%), respectively, was found (Larrauri, Rupèrez, & Saura-Calixto, 1997). Makkar and Singh (1991) also reported that there was a decrease in the content of total proanthocyanidins in cassava and leucaena leaves (10.1% and 21.4%, respectively) when heated at 90 °C for 24 h. The tannins, which was observed in raw sample, were also reported previously by Reddy et al. (1985). Nonetheless, the dry heated seed sample had the lowest concentration of the respective phenolic fractions possibly due to the poor extractability of phenolics by the formation of insoluble tannin–protein and tannin–carbohydrate including cell wall polysaccharide complexes.

3.2. Superoxide anion radical and hydroxyl radical-scavenging activities

The effects of phenolic extracts of the raw and dry heated seed samples on superoxide radical generated by photochemical reaction were determined and the results are shown in Table 2. All of the extracts had a scavenging activity on the superoxide radicals in a dose-dependent manner (0.2–1.0 mg). However, the similar and highest scavenging ability was exhibited by both extracts, raw (61.8%) and dry heated (61.7%) samples, at concentration of 1 mg. The scavenging abilities of raw and dry heated seed extracts on hydroxyl radical inhibition are shown in Fig. 2. All the seed extracts showed good hydroxyl radical-scavenging activities at a concentration of 250, 500 and 1000 μg in the reaction mixture. However, among the samples, dry-heated seed extract was found to register the highest hydroxyl radical-scavenging activity. Similarly, Yen and Hsieh (1995) reported that xylose and lysine Maillard reaction products had dose dependant scavenging activity on hydroxyl radical which might be attributed to the combined effects of reducing power, donation of hydrogen atoms and scavenging of active oxygen. The 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). All together the potential scavenging abilities of phenolic substances might be due to the active hydrogen donor ability of hydroxyl substitution. Similarly, Hagerman et al. (1998) have also suggested that high molecular weight and the proximity of many aromatic rings and hydroxyl groups are more important for the free radical-scavenging by tannins than their specific functional groups.

Table 1
Yield percent of solvent extract and total phenolic content of raw and heat treated moth beans

Sample	Yield (%)	Total phenolics of extract (g 100 g^{-1})	Tannins (g 100 g^{-1})
Raw	12.42 ± 1.23	6.54 ± 6.54	1.91 ± 0.12
Dry heated	15.50 ± 0.88	5.81 ± 0.81	1.31 ± 0.11

Values are mean of triplicate determinations ± SD.

Table 2
Superoxide radical-scavenging activities of raw and dry heated moth bean extracts

Sample concentration (μg)	Superoxide radical-scavenging percent	
	Raw	Dry heated
200	19.73 ± 2.03	16.30 ± 1.05
400	35.40 ± 4.49	27.40 ± 1.18
600	45.87 ± 4.02	40.20 ± 2.10
800	55.23 ± 3.80	53.80 ± 2.36
1000	61.80 ± 1.42	61.73 ± 3.17

Values are mean of triplicate determinations ± SD.

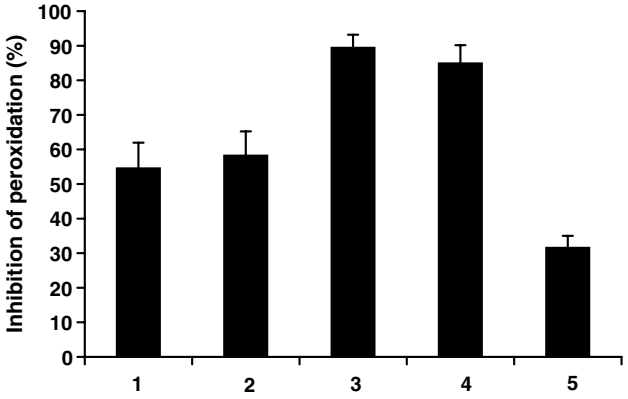

Fig. 1. Antioxidant activity of raw and dry heated moth bean extracts (1 mg/ml): (1) raw sample; (2) dry heated sample; (3) Trolox; (4) butylated hydroxyanisole; (5) ascorbic acid. Values are mean of triplicate determinations ± SD.

3.3. Antioxidant activity in linoleic acid emulsion system

The antioxidant effects of the extracts from un-processed and dry heated seed samples of moth bean, ascorbic acid, BHA and Trolox on the peroxidation of linoleic acid were investigated and the results are presented in Fig. 1. At a concentration of 1000 μg in the final reaction mixture, the raw (54.5%) and dry heated (58.2%) seed samples inhibited peroxidation of linoleic acid after incubation for 72 h (three days) and these values were higher than the ascorbic acid (31.5%).

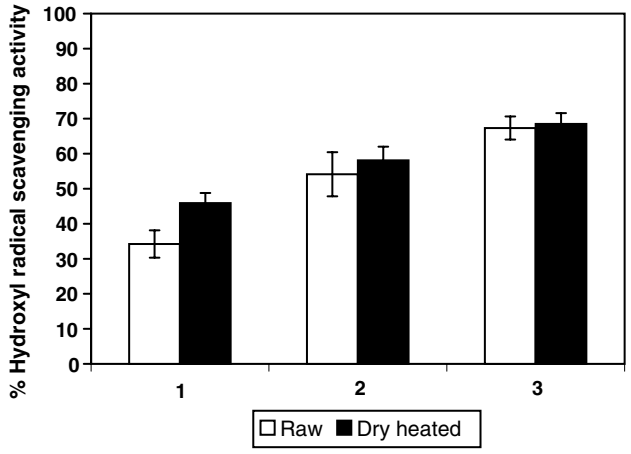

Fig. 2. Hydroxyl radical-scavenging activity of raw and dry heated moth bean extracts at a concentration of: (1) 250 μg; (2) 500 μg; (3) 1000 μg. Values are mean of triplicate determinations ± SD.

However, these values were lower than those of the positive controls such as BHA (84.9%) and Trolox (89.4%). In summary, the results show that the inhibitory potential follows the order Trolox > BHA > dry heated > raw > ascorbic acid. On the other hand, Tsuda et al. (1993b) have reported that methanol extracts of moth bean (*Vigna aconitifolia*) showed lipid peroxidation inhibiting activity in the linoleic acid system and this might be due to the relative colour intensity of the seed coats. 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, which contain phenolic substances, from red beans and black beans have been reported to have a strong antioxidant activity against lipid peroxidation (Tsuda et al., 1994). The stability of antioxidant potential of dry heated samples, even though the concentration of extractable phenolics was found to be relatively low, might be due to the formation of Maillard reaction products. Nicoli, Anese, Manzocco, and Lerici (1997) have previously reported that medium dark roasted coffee brews had the highest antioxidant properties due to the development of Maillard reaction products. Similarly, extracts 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 free radical-scavenging activities of aqueous acetone extracts of un-processed and dry heated moth bean seeds along with the reference standards such as ascorbic acid and BHA were determined by the DPPH· method and the results are shown in Table 3. A lower value of EC_{50} indicates a higher antioxidant activity. Extracts obtained from raw seeds registered the highest DPPH radical-scavenging activity (835) than the dry heated seed sample (1421) and the differences between them are directly proportional to the concentration of total phenolics including tannins of respective samples. Recently, Siddhuraju, Mohan, and Becker (2002) reported the elevated DPPH radical quenching capacity of tannins (proanthocyanidins) extracted from the stem bark of *Cassia fistula*. In terms of percentage the inhibiting activity at 16 min was 93% for the stem bark extract compared to 37.8% for butylated hydroxytoluene (BHT). Similarly, Amarowicz, Naczk, and Shahidi (2000) reported that the tannins extracted from canola and rapeseed hulls exhibited a high scavenging efficiency toward DPPH radicals. In the present study, the order of scavenging activity of seed extracts was as follows: raw > heated. Such a radical-scavenging activity of untreated and treated seed extracts would be related to substitution of hydroxyl groups in the aromatic rings of phenolics, thus contributing to their hydrogen donating ability (Brand-Williams et al., 1995). On the other hand, the DPPH radical-scavenging efficiency of extracts from dry heated seed sample might have also been partly contributed by the Maillard reaction products other than the phenolic constituents. However, when compared to the standards, ascorbic acid (EC_{50}; 75.1) and BHA (EC_{50}; 92.5), the

Table 3
DPPH radical and $ABTS^{\cdot+}$ cation radical scavenging activities and FRAP assay of un-processed and dry heated moth bean extracts

Sample	DPPH· EC_{50} (mg DM g^{-1} DPPH·)[a]		TAA (mmol kg^{-1} DM)[b]		FRAP (mg DM $mmol^{-1}$ Fe(II))[c]	
	Mean	SD	Mean	SD	Mean	SD
Raw	834.9	28.4	374.7	11.8	618.7	11.3
Dry heated	1421.2	47	244.4	8.69	1028.9	65.7
Ascorbic acid	75.1	3.6				
BHA	92.5	5.0			65.0	2.9
Trolox					86.4	8.5

Values are mean of three independent determinations; SD, standard deviation.
[a] Milligramme of sample required to decrease one g of the initial DPPH· concentration by 50%.
[b] Total antioxidant activity (mmol equivalent trolox performed by using $ABTS^{\cdot+}$).
[c] Ferric reducing/antioxidant power assay (concentration of substance having ferric-TPTZ reducing ability equivalent to that of 1 mmol Fe(II)).

tested seed extracts lower DPPH radical-scavenging activity.

In ABTS radical cation scavenging method, the activity of tested seed extracts was expressed as Trolox equivalent – the molarity of the Trolox solution having an antioxidant capacity equivalent to 1 kg dry matter of the substance under investigation. The total antioxidant activity of raw and dry heated seed sample (extracts) of moth bean is shown in Table 3. The raw seed samples exhibited the highest TAA (375 mmol/kg) compared to processed seed extracts (244 mmol/kg). Though the total phenolic content of processed seed sample was found to be relatively low, the TAA of such samples seems to be sufficient for functioning as potential nutraceuticals when they are ingested along with nutrients. Apart from these, Hagerman et al. (1998) have recently reported that the high molecular weight phenolics (tannins) have more ability to quench free radicals ($ABTS^{\bullet+}$) and that their effectiveness depends on the molecular weight, the number of aromatic rings and nature of hydroxyl group substitution than the specific functional groups. On the other hand, the formation of tannin–protein complexes, both insoluble and soluble, as the result of conventional food/seed processing have also been shown to be potential free radical-scavengers and radical sinks. Moreover, such complexes could also have been suggested to be 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 explained as reductants, and inactivation of oxidants by reductants can be described as redox reactions in which one reaction species (oxidant) is reduced at the expense of the oxidation of the other. The FRAP assay measures the antioxidant effect of any substance in the reaction medium as reducing ability. Antioxidant potential of the unprocessed and processed seed extracts of moth bean was estimated from their ability to reduce TPTZ-Fe(III) complex to TPTZ-Fe(II) complex. The antioxidant capacities of raw and dry heated seed extracts of moth bean are given in Table 3. The raw seed extracts showed highest FRAP antioxidant activity, as has been recorded in $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ methods. The order of FRAP activity of respective seed sample extracts and synthetic antioxidant is as follows: BHA > Trolox > raw > dry heated as in the case of $DPPH^{\bullet}$ and $ABTS^{\bullet+}$. Moreover, increasing concentration of extractable total phenolics exhibited relative efficiency of FRAP activity. Similar results have also been reported in guava fruit extracts (Jimènez-Escrig, Rincòn, Pulido, & Saura-Calixto, 2001). Yen and Duh (1993) and Siddhuraju et al. (2002) have reported that the reducing power of bioactive compounds (mainly low and high molecular phenolics) extracted from peanut hulls and stem bark of Indian laburnum, respectively, was associated with antioxidant activity, specifically scavenging of free radicals. Nevertheless, the recent in vitro research investigation has revealed that the consumption of dark chocolate, which contains phenolics, in particular (–) epicatechin, proved to be a potential antioxidants through ferric-reducing antioxidant power assay (in vitro), increased the total antioxidant capacity of blood plasma (Serafini et al., 2003).

3.6. Chelating capacity

It has been well recognised that formation of the first few radicals to initiate the radical chain reaction or the radical mediated lipid peroxidation must be catalysed (Nawar, 1996). Transition metals have been proposed to be the catalysts for the initial formation of radical. Chelating agents may stabilise transition metals in the living systems and inhibit radical generations, consequently reducing free radical damage. To better estimate the potential antioxidative properties of the extracts, chelating activity of each extract was evaluated against Fe^{2+} and expressed as EDTA equivalents on a dry weight basis. The EDTA equivalent of the raw seed extract was 0.61 mg/g followed by 0.45 mg/g dry heated extract. Similarly, certain commercial cereal products also exhibited good chelating capacity (Yu, Perret, Davy, Wilson, & Melby, 2002). However, BHA showed very poor (negligible) ferrous chelating ability due to the chemical structure properties.

The present study suggests that not only the phenolic (tannins) substances from raw seeds but also the substances from the processed seeds of moth bean are potent antioxidant sources. Nonetheless, evaluation of the occurrence of tannin–protein interactions in the above said processed samples by testing both the in vitro protein digestibility together with the assessment of antioxidant properties might be a fruitful approach for advocating them as nutraceuticals in addition to that of being potential protein and carbohydrate suppliers. Furthermore, such studies may also provide the insight information for the people who are claiming that the consumption of post cooking liquor of moth bean seeds as potential health promoters. On the other end, whether the presence of tannin–protein complexes and phenolics associated with dietary fibre in legume based food against the risk of oxidative injury during gastrointestinal digestion in vivo remains to be demonstrated. Once after establishing such a balance between the antinutrient and the biological antioxidant effects of phenolics (dietary tannins), the consumption of such a legume food would not only improve the nutrient utilisation but also provide the potential nutraceuticals for human health. Nonetheless, the isolation and preparation of bioactive compounds from the seed coat of moth

bean could serve as potent natural antioxidants for the industrial perspectives.

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