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The antioxidant and free radical scavenging activities of processed cowpea (*Vigna unguiculata* (L.) Walp.) seed extracts

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Abstract

The antioxidative properties and total phenolic contents of two varieties of cowpea (*Vigna unguiculata*) were examined. The raw, dry heated and hydrothermal treated samples were extracted with 70% acetone and the extracts were freeze-dried. The unprocessed light brown seeds (LB) contained significantly higher level of total phenolics and tannins than the dark brown seeds (DB). The extracts were screened for their potential antioxidant activities using tests such as $O_2^{\bullet-}$, $OH^{\bullet}$, α,α-diphenyl-β-picrylhydrazyl (DPPH$^{\bullet}$), ABTS$^{\bullet+}$, FRAP, linoleic acid emulsion and β-carotene–linoleic acid in vitro model systems. At 800 μg of extract in the reaction mixture, the superoxide anion radical scavenging activity was found to be significantly higher in the raw and dry heated seed extracts than the hydrothermally processed seed samples of the respective varieties. The DPPH radical and ABTS cation radical scavenging activities were well proved and correlated with the ferric reducing antioxidant capacity of the extracts. Interestingly, among the various extracts, dry heated samples of LB and DB showed the highest hydroxyl radical scavenging activity of 83.6% and 68.2%, respectively. All extracts exhibited good antioxidant activity (74.3–84.6%) against the linoleic acid emulsion system. Using the β-carotene method, the values were significantly lower than BHT, BHA and Trolox. Owing to this property, the studies can be further extended to exploit not only the phenolic extracts but also the residual phenolic constituents associated with processed seed samples as health supplements and nutraceuticals.

Keywords: *Vigna unguiculata*; Seeds; Heat process; Phenolics; Tannins; Antioxidant activity; ABTS$^{\bullet+}$

1. Introduction

A nutraceutical is any substance that is a food, or part of a food, and provides medical or health benefits, including the prevention or treatment of disease. Nutraceuticals may be isolated nutrients, dietary supplements, specific diets, designer foods, herbal products, processed foods, or processed beverages (Morris, 2003). Vitamin C, α-tocopherol and phenolic compounds, which are present naturally in vegetables, fruits, grains and pulses, possess the ability to reduce oxidative damage associated with many diseases, including cancer, cardiovascular diseases, cataracts, atherosclerosis, diabetes, asthma, hepatitis, liver injury, 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 has been extensively investigated (Rice-Evans, Miller, & Paganda, 1996).

Legumes play an important role in the traditional diets of many regions throughout the world. 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). Most Latin American populations prefer coloured beans to white beans. Similarly, the typical taste of the sauce prepared from the cooking liquors of coloured beans, which contains mainly the seed coat pigments such as dietary tannins and

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non-tannin phenolics, with rice and other cereals, are also popular among the village people in certain regions of India. The consumption of such legumes has been linked to reduced risk of diabetes and obesity and have an inhibitory role in the reduction of coronary heart diseases, which has been noted and confirmed (Bazzano et al., 2001). Recently, polyphenolic constituents of various legume seeds have been reported to contain potential medicinal properties, including antioxidant activities (Cardador-Martìnez, Loarca-Pina, & Oomah, 2002; Mazur, Duke, Wähälä, Rasku, & Adlercreutz, 1998; Shahidi, Chavan, Naczk, & Amarowicz, 2001; Tsuda, Ohshima, Kawakishi, & Osawa, 1994; Tsuda, Osawa, Nakayama, Kawakishi, & Ohshima, 1993a). Therefore, the study on the importance and role of non-nutrient compounds particularly phenolic acids, flavonoids and high molecular tannins of legumes, as natural antioxidants and nutraceuticals has greatly increased.

Cowpea (*Vigna unguiculata* (L.) Walp.) 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 (Phillips & McWatters, 1991; Uzogara & Ofuya, 1992). The worldwide production of dry cowpea for 2002 was estimated at 7.4 billion pounds from 20 million acres and the major producing countries are of Africa, Asia and Latin America. The consumption of cowpea 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 seeds with spices is considered to be a potential remedy for the common cold. Sometimes cowpea seeds are used as a coffee substitute. The whole seeds have been reported to contain about 0.18–0.59% tannins (Reddy, Pierson, Sathe, & Salunkhe, 1985) phenolic acids, such as *p*-hydroxybenzoic acid, protocatechuic acid, 2,4-dimethoxybenzoic acid, and cinnamic acid derivatives, such as *p*-coumaric acid, caffeic acid, cinnamic acid and ferulic acid (Cai, Hettiarachchy, & Jalaluddin, 2003; Sosulski & Dabrowski, 1984). However, limited information is available on their antioxidant activity (Tsuda, Makino, Kato, Osawa, & Kawakishi, 1993b). Even though processed cowpea seeds are increasingly consumed as human food, the beneficial effects of their bioactive compounds remain unexplored. Therefore, the present study was aimed at evaluating their phenolic constituents, antioxidant potential and free radical scavenging capacity of aqueous acetone extracts of two varieties of raw, dry heated and pressure cooked (autoclaved) cowpea samples.

2. Materials and methods

2.1. Chemicals

Quercetin, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), ascorbic acid, potassium ferricyanide, α,α-diphenyl-β-picrylhydrazyl (DPPH·), nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), β-dihydronicotinamide adenine dinucleotide (β-NADH), thiobarbituric acid (TBA), trichloroacetic acid (TCA), 2-deoxy-D-ribose, linoleic acid, ethylenediamine tetraacetic acid (EDTA), ammonium thiocyanate, potassium persulfate, ferrous chloride and ferric chloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tannic acid, 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–Aldrich Chemical Co. (Steinheim, Germany). β-Carotene, TPTZ (2,4,6-tripyridyl-*s*-triazine) and Tween 40 were obtained from Fluka Chemical Co. (Buchs, Switzerland). All other reagents were of analytical grade.

2.2. Seed samples and processing

The two varieties of cowpea (*Vigna ungiculata*), light and dark brown seeds, were purchased from an Asian shop located in Stuttgart, Germany. The seeds (150 g) were dry heated along with acid-washed sea sand on an open hot plate (Tecator 1022, Sweden) at 135 ± 2 °C for 25 min. During the heat processing frequent (every 3 min) agitation of the 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; for the pressure-cooking treatment, the seeds (100 g) were soaked in distilled water in the ratio of 1:10 (seed:water, w/v) for 24 h at room temperature (25 °C). After decanting the water, the soaked seeds (seed:water 1:5 w/v) were subjected to autoclaving for 20 min at 120 °C. Soon after decanting the liquid, the autoclaved seeds were freeze-dried. The raw and autoclaved seed samples were ground to a fine powder (particle size of about 0.25 mm) and stored in separate screw cap bottles at −20 °C before analysis.

2.3. Solvent extraction

Raw and processed ground seed samples (10 g) were extracted by stirring with 100 ml of 70% acetone 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 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 40 °C and the remaining water was removed by lyophilization. The freeze-dried extract thus obtained was used directly for total phenolics, tannins, and condensed tannins estimation and also for the assessment of antioxidant capacity through various chemical assays.

2.4. Determination of total phenolic content, tannins and condensed tannins

The total phenolic content of the freeze-dried aqueous acetone extract of raw and processed cowpea 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 to 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 tannic acid equivalents from the calibration curve. Using the same aqueous acetone solution, which contained known amount of freeze-dried extract, the tannins (Makkar et al., 1997) and condensed tannins (Porter, Hrstich, & Chan, 1986) were estimated after the treatment of polyvinyl polypyrrolidone (PVPP) and acidic butanol–ferric ammonium sulfate reagent, respectively.

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

The superoxide anion scavenging activity of samples (cowpea seed extracts, tannic acid and quercetin) was measured by the method of Robak and Gryglewski (1988). An aliquot of 1 ml of each of the following solutions prepared in 0.1 M phosphate buffer at pH 7.4: 150 μM nitroblue tetrazolium (NBT), 60 μM phenazine methosulphate and 468 μM NADH were added, respectively, to 1 ml, which contained 0.2–0.8 mg of seed extracts, 0.02–0.08 mg of quercetin and tannic acid, respectively. The scavenging activity on superoxide anion (SASA) radicals was expressed as

$$\text{SASA}\ (\%) = \left(1 - \frac{\text{Absorbance at 560 nm in the presence of sample}}{\text{Absorbance at 560 nm in the absence of sample}}\right) \times 100$$

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

The scavenging activity of the raw and processed cowpea seed extracts and tannic acid 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 (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

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

2.7. Antioxidant activity in linoleic acid emulsion system

The antioxidant activity of unprocessed and processed seed extracts of two varieties of cowpea, BHT, BHA and Trolox was determined using the thiocyanate method (Mitsuda, Yasumoto, & Iwami, 1966) as described by Yen and Hsieh (1998). Each sample (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 40 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 (Hitachi U-2000). 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

$$\text{LPI}\ (\%) = \left(1 - \frac{\text{Absorbance at 500 nm in the presence of sample}_{48\text{h}}}{\text{Absorbance at 500 nm in the absence of sample}_{48\text{h}}}\right) \times 100$$

2.8. The β-carotene/linoleic acid antioxidant activity

One millilitre of a β-carotene solution in chloroform (1 mg 10^{-1} ml) was pipetted into a flask containing 20 mg of linoleic acid and 200 mg of Tween 40. The chloroform was removed by rotary vacuum evaporator at 45 °C for 4 min and, 50 ml of oxygenated distilled water was added slowly to the semi-solid residue with vigorous agitation, to form an emulsion. A 5 ml aliquot of the emulsion was added to a tube containing 0.2 ml of the antioxidant (cowpea seed extracts, BHA or Trolox) solution at 1000 mg l^{-1} and the absorbance was measured at 470 nm, immediately, against a blank, consisting of the emulsion without β-carotene (Taga, Miller, & Pratt, 1984). The tubes were placed in a water bath at 50 °C and the absorbance was monitored at 15 min intervals until 180 min. All determinations were carried out in triplicate. The antioxidant activity of the seed extracts and pure compounds was evaluated in terms of

bleaching of β-carotene using the following formula: $AA = [1 - (A_0 - A_t)/(A'_0 - A'_t)] \times 100$ where A_0 and A'_0 are the absorbance measured at zero time of incubation for the test sample and control, respectively, and A_t and A'_t are the absorbances measured in the test sample and control, respectively, after incubation for 180 min.

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

The antioxidant activity of cowpea seed extracts, quercetin and BHA was measured in terms of hydrogen donating or radical scavenging 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 3.9 ml ($0.025\ g\ l^{-1}$) of DPPH· solution. The decrease in absorbance at 515 nm was determined continuously at every minute with a Hitachi UV–Vis 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· ($DPPH^{\bullet}_R$) 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· at the time of steady-state and $DPPH^{\bullet}_{T=0}$ was the concentration of DPPH· at time 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.

2.10. Antioxidant activity by the ABTS·+ 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 dark at room temperature for 12–16 h before use. Oxidation of 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 at 30 °C, the temperature at which all of the assays were performed. The stock solution of seed extracts and quercetin 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 samples were taken at 30 °C exactly 30 min after 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 at an absorbance 734 nm and then was plotted as a function of Trolox concentration. The activity of seed extracts and quercetin 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 a dry matter basis.

2.11. Ferric reducing/antioxidant power (FRAP) assay

The antioxidant capacity of phenolic extracts of raw and processed cowpea 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 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 (Hitachi U-2000), 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.

2.12. Statistical analysis

The data were subjected to a one way analysis of variance and the significance of the difference between means was determined by Duncan's multiple range test ($P < 0.05$) using the Statistica for Windows H'97, Version 5.1 (Statsoft Inc., Tulsa, USA). Values expressed are mean of triplicate determination ± SD.

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 two varieties of cowpea using aqueous acetone (70%) solvent are shown in Table 1. Maximum yield was obtained for the extracts of dry-heated samples. The extractable total phenolics, tannins and condensed tannins of the raw samples were significantly 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 tannin levels, which was observed in both LB (0.6%) and DB (0.4%) raw samples, were comparable to those reported by Price, Hagerman, and Butler (1980) and Reddy et al. (1985). The hydrothermally treated seed sample had the lowest concentration of phenolic fractions, possibly due to (1) the dissolution of phenolics in the soaking medium as well as in the autoclaving medium and the subsequent discarding of both of them, and (2) phenolics not extracted by solvent, due to the formation of insoluble tannin–protein and tannin–carbohydrate, including cell wall polysaccharide complexes.

3.2. Superoxide anion radical and hydroxyl radical scavenging activities

Superoxide anion is a reduced form of molecular oxygen created by receiving one electron. Superoxide anion is an initial free radical formed from mitochondrial electron transport systems. Mitochondria generate energy using a 4-electron chain reactions, reducing oxygen to water. Some of the electrons escaping from the chain reaction of mitochondria directly react with oxygen and form superoxide anion. It plays an important role in the formation of other reactive oxygen species, such as hydrogen peroxide, hydroxyl radical, or singlet oxygen in living systems (Lee, Koo, & Min, 2004).

The effects of phenolic extracts of the raw and processed seed samples, LB and DB of cowpeas, tannic acid and quercetin on superoxide radical were determined by the PMS-NADH superoxide generating system and the results are shown in Table 2. All of the extracts had a scavenging

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

Sample	Extract recovery	SD	Total phenolics	SD	Tannins	SD	Condensed tannins	SD
LB	4.92^{de}	0.27	16.36^{a}	1.06	12.31^{a}	0.75	4.75^{b}	0.85
DB	4.00^{e}	0.14	13.27^{b}	1.37	9.52^{b}	1.31	6.08^{a}	0.89
LBD	9.73^{a}	0.17	8.42^{d}	0.39	5.61^{d}	0.48	2.78^{c}	0.09
DBD	8.45^{b}	0.49	11.14^{c}	0.96	7.96^{c}	1.00	3.45^{bc}	0.39
LBA	7.34^{c}	0.65	6.45^{e}	0.21	3.35^{e}	0.77	1.70^{d}	0.01
DBA	5.49^{d}	0.31	9.53^{d}	0.46	5.96^{d}	0.52	2.24^{cd}	0.09

Values are means of three replicate determinations; SD, standard deviation.
Mean values followed by different superscript in the same column are significantly ($P < 0.05$) different.
LB, light brown, DB, dark brown; LBD, light brown, dry heated; DBD, dark brown, dry heated; LBA, light brown, soaking followed by autoclaving; DBA, dark brown, soaking followed by autoclaving.

Table 2
Superoxide anion ($O_2^{\bullet -}$) radical scavenging activities of raw and processed seed extracts of cowpea, tannic acid and quercetin

Sample	Superoxide anion scavenging activity (%)							
	200[A]	SD	400[A]	SD	600[A]	SD	800[A]	SD
LB	32.0	1.8	52.8	2.1	64.1	0.9	69.7^{a}	1.5
DB	32.8	1.4	36.4	0.8	44.2	1.0	52.0^{b}	0.8
LBD	22.8	3.1	40.3	1.9	44.4	2.5	51.2^{b}	3.5
DBD	14.4	1.7	22.1	2.5	36.7	1.2	49.5^{b}	4.2
LBA	7.7	1.4	14.6	1.2	18.6	3.1	27.0^{d}	2.0
DBA	12.6	1.2	19.0	0.7	28.3	1.4	38.2^{c}	2.0
Tannic acid[B]	21.8	1.8	34.9	1.3	56.2	3.6	72.3	1.0
Quercetin[B]	20.3	1.4	32.1	2.6	41.2	0.9	55.7	2.5

Values are means of three independent determinations; SD, standard deviation.
Values followed by different superscript in a column are significantly different ($P < 0.05$).
LB, light brown, DB, dark brown; LBD, light brown, dry heated; DBD, dark brown, dry heated; LBA, light brown, soaking followed by autoclaving; DBA, dark brown, soaking followed by autoclaving.
[A] Concentration in μg.
[B] Concentration was 20, 40, 60 and 80 μg, respectively.

activity on the superoxide radicals in a dose dependent manner (0.2–0.8 mg ml^{-1}). However, the highest scavenging ability was exhibited by extracts from raw and dry heated samples rather than the autoclaved samples. The lower activity of hydrothermally processed samples may be attributed to the partial loss of active phytocompounds in the seeds during the soaking as well as decanting of autoclaved liquid. Nonetheless, when compared to the flavonoid, quercetin, considered to be a potent superoxide radical scavenger, and tannic acid, the scavenging activity of all the above mentioned extracts was found to be low.

Hydroxyl radical is the most reactive free radical and can be formed from superoxide anion and hydrogen peroxide, in the presence of metal ions, such as copper or iron. Hydroxyl radicals have the highest 1-electron reduction potential (2310 mV) and can react with everything in living organisms at the second-order rate constants of 10^9–10^{10} mol/s. Hydroxyl radicals react with lipid, polypeptides, proteins, and DNA, especially thiamine and guanosine. When a hydroxyl radical reacts with aromatic compounds, it can add across a double bond, resulting in hydroxycyclohexadienyl radical. The resulting radical can undergo further reactions, such as reaction with oxygen, to give peroxyl radical, or decompose to phenoxyl-type radicals by water elimination (Lee et al., 2004). The scavenging abilities of tannic acid, raw and processed cowpea seed extracts on hydroxyl radical inhibition are shown in Fig. 1. All the seed extracts showed good hydroxyl radical scavenging activities (25.2–83.6%) at a concentration of 200 μg in the reaction mixture when compared to tannic acid (7.5%). However, among the various processed samples, dry-heated seed extracts of both varieties were found to register the highest ($P < 0.05$) hydroxyl radical scavenging activity. Yen and Hsieh (1995) reported that xylose and lysine Maillard reaction products had scavenging activity on hydroxyl radical that depended 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 antioxidant properties of faba bean tannins indicated that the antioxidant activity was accounted for 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. Nonetheless, the antioxidant potency of tannic acid is inversely proportional to Fe(II) concentration, demonstrating a competition between H_2O_2 and tannic acid for reaction with Fe(II). Moreover, the efficiency of tannic acid is nearly unchanged with increasing concentrations of the $^{\bullet}OH$ detector molecule, 2-deoxyribose and these results indicate that the antioxidant activity of tannic acid is due to iron chelation rather than $^{\bullet}OH$ scavenging (Lopes, Schulman, & Hermes-Lima, 1999).

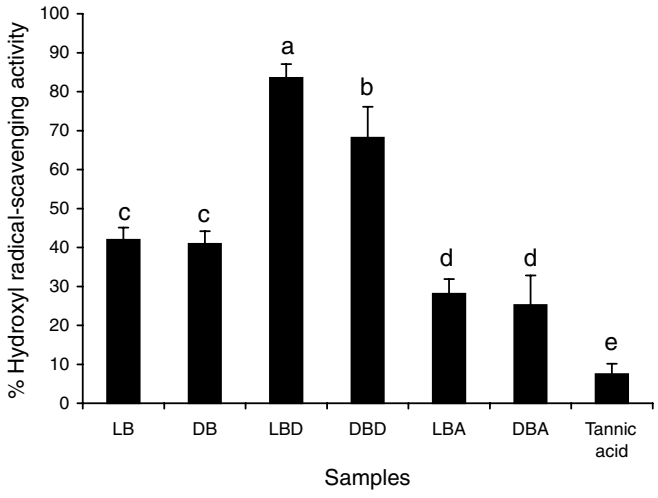

Fig. 1. Hydroxyl radical scavenging activity of two varieties of cowpea seed extracts and tannic acid at a concentration of 200 μg in the reaction mixture. LB, light brown; DB, dark brown; LBD, light brown, dry heated; DBD, dark brown, dry heated; LBA; light brown, soaking followed by autoclaving; DBA, dark brown, soaking followed by autoclaving. Bars having different letters are significantly different ($P < 0.05$).

3.3. Antioxidant activity in linoleic acid emulsion system

Peroxyl radicals are formed by a direct reaction of oxygen with alkyl radicals. Decomposition of alkyl peroxides also results in peroxyl radicals. Peroxyl radicals are good oxidising agents, having more than 1000 mV of standard reduction potential (Decker, 1998). They can abstract hydrogen from other molecules with lower standard reduction potential. This reaction is frequently observed in the propagation stage of lipid peroxidation. Cell membranes are phospholipid bilayers with extrinsic proteins and are the direct target of lipid oxidation (Girotti, 1998). As lipid oxidation of cell membranes increases, the polarity of lipid-phase surface charge and formation of protein oligomers increase; and molecular mobility of lipids, number of SH groups, and resistance to thermal denaturation decrease. Malonaldehyde, one of the lipid oxidation products, can react with free amino group of proteins, phospholipid, and nucleic acids leading to structural modification, which induce dysfunction of immune systems.

The antioxidant effects of the extracts from unprocessed and processed seed samples of two varieties of cowpea, BHT, BHA and Trolox on the peroxidation of linoleic acid were investigated and the results are presented in Fig. 2. At a concentration of 250 μg in the final reaction mixture, the raw and dry heated seed samples of both varieties inhibited 78.3–84.6% peroxidation of linoleic acid after incubation for 48 h (two days) and these values were higher than the extracts of hydrothermally treated seeds of respective varieties. However, those values were significantly lower ($P < 0.05$) than those of the positive controls BHT (99.0%), BHA (96.0%) and Trolox (99.0%). In summary,

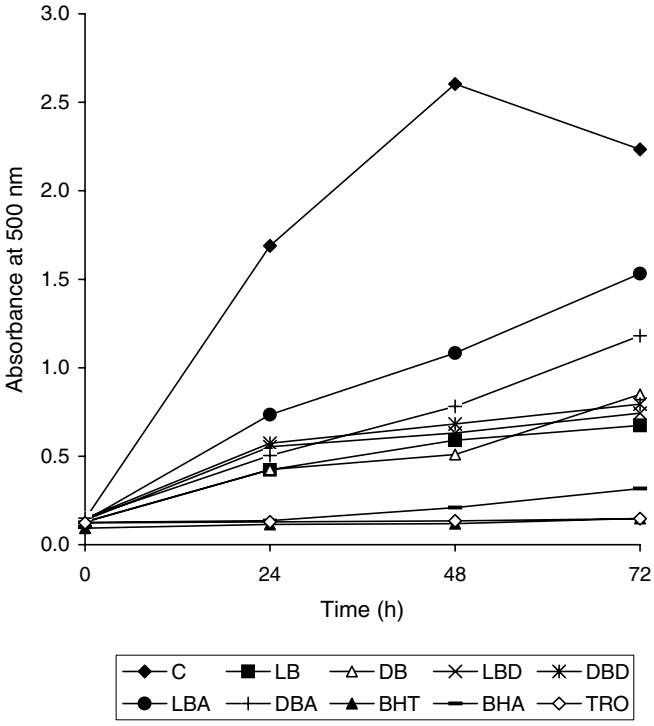

Fig. 2. Antioxidant activity of two varieties of cowpea seed extracts and other antioxidants as measured by thiocyanate method. Higher absorbance at 500 nm indicates low antioxidant activity. C, control; LB, light brown; DB, dark brown; LBD, light brown, dry heated; DBD, dark brown, dry heated; LBA; light brown, soaking followed by autoclaving; DBA, dark brown, soaking followed by autoclaving. BHT, butylated hydroxytoluene; BHA, butylated hydroxyanisole; TRO, Trolox.

the results show that the inhibitory potential follows the order BHT = Trolox > BHA > DB > LB > LBD > DBD > DBA > LBA. On the other hand, Tsuda et al. (1993b) have reported that methanol extracts of two different seed samples of cowpea (*Vigna unguiculata*) did not show lipid peroxidation inhibiting activity in the linoleic acid system and this might be due to the relative colour intensity of the seed coats. In generally, 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). Many hydrolysable tannins from *Osbeckia chinensis* were found to have potential antioxidative efficiency in a linoleic acid–thiocyanate system (Su, Osawa, Kawakishi, & Namiki, 1988). The antioxidant potential of dry heated samples might be due to the formation of Maillard reaction products. Nicoli, Anese, Manzocco, and Lerici (1997) reported that medium dark roasted coffee brews had the highest antioxidant properties, due to the development of Maillard reaction products. Similarly, extract of roasted followed by defatted legume, peanut kernels, displayed a most remarkable antioxidative activity on a linoleic acid emulsion system (Hwang, Shue, & Chang, 2001).

3.4. Antioxidant activity in the β-carotene bleaching assay

The antioxidant activity of unprocessed and processed seed extracts as measured by bleaching of β-carotene is presented in Fig. 3. Raw seed extracts of both varieties showed significantly higher antioxidant activity (LB and DB, 10.5% and 13.3%, respectively) than both the dry heated and hydrothermally treated seed samples at 200 μg in the final reaction mixture. The antioxidant activity was related to phenolic concentration dependency, including high molecular phenolics, tannins and condensed tannins. However, the inhibition of β-carotene bleaching by cowpea seed extracts was lower than those of standards BHA and Trolox. Different solvent extracts of various fractions of common beans have also been reported to have potential inhibiting activity against the oxidation of β-carotene molecules (Cardador-Martìnez et al., 2002).

3.5. Radical-scavenging activities on α,α-diphenyl-β-picrylhydrazyl (DPPH$^{\cdot}$) and ABTS$^{\cdot+}$

The free radical-scavenging activities of aqueous acetone extracts of unprocessed and processed cowpea seeds along with reference standards, such as quercetin and BHA, were determined by the DPPH$^{\cdot}$, and the results are shown in Table 3. The decrease in absorbance of the DPPH radical caused by antioxidant was due to the scavenging of the radial by hydrogen donation. It is visually noticeable as a colour change from purple to yellow. A lower value of EC_{50} indicates a higher antioxidant activity. Extracts obtained from raw seeds registered the

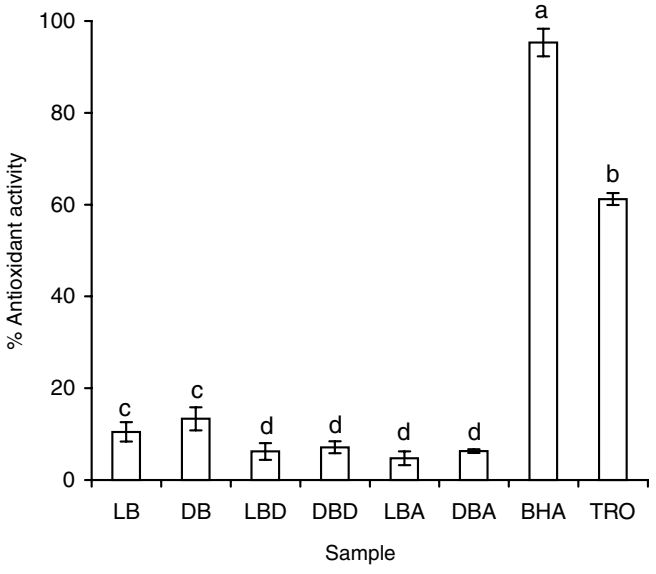

Fig. 3. Antioxidant activity of two varieties of cowpea seed extracts and other antioxidants in β-carotene/linoleic acid system. LB, light brown; DB, dark brown; LBD, light brown, dry heated; DBD, dark brown, dry heated; LBA; light brown, soaking followed by autoclaving; DBA, dark brown, soaking followed by autoclaving. BHA, butylated hydroxyanisole; TRO, Trolox. Values are means of triplicate determination ± standard deviation. Bars having different letters are significantly different ($P < 0.05$).

Table 3
DPPH· radical and $ABTS^{\cdot+}$ cation radical scavenging activities, and FRAP assay of unprocessed and processed cowpea seed 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
LB	705[d]	12	591[b]	7.5	545[d]	34.7
DB	618[e]	18	662[a]	4.4	487[e]	4.6
LBD	1380[b]	24	431[d]	7.3	1180[b]	16.5
DBD	1210[c]	126	500[c]	14.9	884[c]	21.8
LBA	1922[a]	18	285[e]	14.9	1560[a]	33.5
DBA	1238[c]	21	487[c]	17.5	918[c]	29.8
Quercetin	84.7[f]	12				
BHA	103[f]	9			60.4[f]	3.1
Trolox					90.3[f]	5.0

Values are means of three independent determinations; SD, standard deviation.
Values followed by different superscript in each column are significantly different ($P < 0.05$).
LB, light brown, DB, dark brown; LBD, light brown, dry heated; DBD, dark brown, dry heated; LBA, light brown, soaking followed by autoclaving; DBA, dark brown, soaking followed by autoclaving.
[A] mg of sample required to decrease one g of the initial DPPH· concentration by 50%.
[B] Total antioxidant activity.
[C] Ferric reducing/antioxidant power assay (concentration of substance having ferric-TPTZ reducing ability equivalent to that of 1 mmol Fe(II)).

highest DPPH radical scavenging activity (LB: 705 and DB: 618) and the significant differences ($P < 0.05$) between them and each other of other samples are directly proportional to the concentration of total phenolics including tannins of respective samples. Recently Siddhuraju, Mohan, and Becker (2002) reported that high concentration of tannins (proanthocyanidins) extracted from stem bark of *Cassia fistula* possessed 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. In the present study, the order of scavenging activity of the seed extracts was as follows: DB > LB > DBD > DBA > LBD > LBA. Such antiradical scavenging activity of untreated and treated seed extracts would be related to substitution of hydroxyl groups in the aromatic rings of phenolics, particularly the presence of high concentration of protocatechuic acid, thus contributing to their hydrogen donating ability (Brand-Williams et al., 1995; Yen, Chang, & Duh, 2005). On the other hand, the DPPH radical scavenging efficiency of extracts from both dry heated and hydrothermally processed seed samples might have also been partly contributed to by the Maillard reaction products, other than the phenolic constituents, because they are also effectively participating as radical scavengers. However, when compared to standards, quercetin (EC_{50}: 84.7) and BHA (EC_{50}: 103), 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 concentration of Trolox solution having an antioxidant capacity equivalent to 1 kg dry matter of the substance under investigation. The total antioxidant activity (TAA) of raw and processed seed sample extracts of cowpea are shown in Table 3. The raw seed samples exhibited the highest TAA compared to processed seed extracts. Nonetheless, the dark-brown (DB) seed extracts that contained the highest total phenolic substances (including tannins) showed significantly higher TAA (662 mmol kg^{-1}) than the light-brown seed extracts (LB) (591 mmol kg^{-1}). Though the total phenolic content of processed seed samples of respective 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 (Rice-Evans et al., 1996). Apart from these, Hagerman et al. (1998) have recently reported that the high molecular weight phenolics (tannins) have more ability to quench free radicals ($ABTS^{\cdot+}$) and that effectiveness depends on the molecular weight, the number of aromatic rings and nature of hydroxyl groups' substitution, rather 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 could also have been suggested to be one of the nutraceutical contributors, that prevent free radical mediated diseases occurring in the gastrointestinal tract (Riedl & Hagerman, 2001).

3.6. 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 another antioxidant. 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 cowpea was estimated from their ability to reduce TPTZ-Fe(III) complex to TPTZ-Fe(II) complex. The antioxidant capacities of raw and differentially

processed seed extracts of two varieties of cowpea varied significantly (Table 3). The raw seed extracts of LB and DB showed highest FRAP antioxidant activity, as has been recorded in DPPH$^{\cdot}$ and ABTS$^{\cdot+}$ methods. Nonetheless, the order of FRAP activity of respective seed samples extract is as follows: raw > dry heated > soaking, followed by autoclaving, as in the case of DPPH$^{\cdot}$ and ABTS$^{\cdot+}$. Moreover, there was a noticeable correlation between extractable total phenolics and FRAP values in LB ($r^2 = 0.9635$) and DB ($r^2 = 0.8666$) seeds of cowpea. Similarly, high correlation has 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, was associated with antioxidant activity, specifically scavenging of free radicals.

This study suggests that not only the phenolic (tannins) substances from raw seeds but also the substances from the processed seeds of cowpea 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 them being potential protein and carbohydrate suppliers. Furthermore, such studies may also provide the information for the people who claim that the consumption of post cooking liquor of cowpea seeds is a potential health promoter. On the other hand, whether the presence of tannins–protein complexes and phenolics associated with dietary fibre in legume-based food protects against the risk of oxidative injury during gastrointestinal digestion in vivo remains to be demonstrated. After establishing a balance between the antinutrient and the biological antioxidant effects of phenolics, the consumption of a processed legume food would not only improve nutrient utilisation but also provide potential nutraceuticals for human health. In addition, the isolation and preparation of bioactive compounds (benzoic acid and cinnamic acid derivatives) from the coloured seed coat of cowpeas, which is considered to be a waste material, could serve as potent natural antioxidants from an industrial perspective.

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