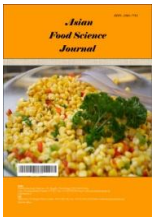

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A Comparative Study on Potential Antioxidants and Antioxidant Activity in Raw and Cooked Selected Locally Grown Legumes in Sri Lanka

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Authors' contributions

This work was carried out in collaboration among all authors. Authors LLPSL, MDWS and SLL managed the literature searches, the basic laboratory work, data analysis, wrote the protocol and wrote the first draft of the manuscript. Author HMTH managed the literature searches, designed the study, assisted financial support and reviewing manuscript draft. Author RHMKR managed the supervision of students. Author WKSMA managed the analyses of the study and trained students for bio assays. All authors' read and approved the final manuscript.

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ABSTRACT

Legumes are important crop species belonging to the family Fabaceae and constitute a significant part in the diet of Sri Lankans, as a meat substitute. The present study evaluated antioxidants and their activity in locally grown legume varieties for potential utilization as health foods. Twelve legume varieties grown in Complete Randomized Block Design (CRBD) were used for screening purpose. Results were statistically analysed using Univariate General Linear Model (SPSS Version 20) followed by mean separation Tukey HSD test. The Total Phenolics Content (TPC) of raw and

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cooked form of legume varieties ranged from 0.84 ± 0.04 (MICP 1) to 4.34 ± 0.15 mg (GAE)/g (ANKCP 2) and 0.30 ± 0.04 (MICP 1) to 3.71 ± 0.12 mg (GAE) /g (Dhawala) whereas Total Flavonoids Content (TFC) ranged from 0.88 ± 0.03 (ANK-Black) to 2.19 ± 0.04 mg (QE)/g (Waruni) and 0.62 ± 0.04 (Waruni) to 8.33 ± 0.16 mg (QE)/g (Dhawala) respectively. TPC and TFC were significantly differed ($p < 0.05$) among the varieties as well as raw and cooked form in each variety. The significant highest ($p<0.05$) antioxidant activity in terms of DPPH was shown in both forms of raw and cooked in dark seed coat coloured varieties of ANK-Brown (4.95± 0.42 and 2.18± 0.45 (TE) / g ; dark brown) ANK-Black (4.11± 0.41and 3.17± 0.60 (TE) / g ; black) and Waruni (3.38± 0.18 and 1.51± 0.13 (TE) / g ; purple) respectively while the significant ($p<0.05$) highest ABTS and FRAP were shown in the same varieties in raw form only. Similarly, the highest results for activity for ABTS (11.74 ± 0.26 (TE) / g) and FRAP (0.32 ± 0.02 (TE) / g) were found in cooked form of variety Dhawala. Results demonstrated the varietal identification of ANK-Brown, ANK-Black, Waruni and Dhawala with a high potential in developing functional foods.

Keywords: Antioxidant activity; antioxidant potential; phenolic acids; flavonoids; legumes.

1. INTRODUCTION

Legumes are very important crop species coming under the family Fabaceae and consumed widely in the world [1]. They are the edible seeds of certain leguminous plants which generally include locally grown varieties of *Glycine max, Macrotyloma uniflorum, Vigna radiata* and *Vigna unguiculata* in Sri Lanka. The land extent of legume cultivation was 34,546 ha and the annual production was approximately 39128 MT in the country [2]. Grain legumes constitute a significant part in the diet of Sri Lankans, both in simple boiled form and as value added products. Specially, they are very popular diet among Sri Lankans as a substitute for meat by vegetarians.

Legume seeds are rich in macro- and micronutrients; consisting starch, protein including polypeptides and amino acids, dietary fiber and significant amount of vitamins and minerals [3]. They contain high amount of minerals such as iron, zinc, calcium and magnesium and a vitamin B-group (especially folate) [4]. Generally, legume posses low content of fat consisting majority of saturated fats.

A regular consumption of legumes is reported to be health beneficial effects to the human body since they contain bio active compounds [5]. Some of those non nutritive bioactive components are the protease inhibitors, phytates and phenolic acids and flavonoids. Phenolic acids and flavonoids of legume seeds are class of plant photo-chemicals which have been well reported to be contained potential medicinal property of antioxidant activity [6,7]. They demonstrate their antioxidant activity by removing free radicals, chelating metal catalysts, activating antioxidant enzymes and inhibiting oxidases. Specially, in phenolic acids have an ability to form stable radical intermediates by donating hydrogen atom [8]. Further, previous studies on a positive correlation of antioxidant activity and phenolic compounds in various common beans were reported in China [9].

Antioxidants are the compounds which have a capability to reduce the oxidative stress which initiates from the excessive generation of free radicals resulted from human biological reactions [10]. Eventually, those radicals lead to progression of several non communicable diseases such as cancer, diabetes, heart diseases, neurological diseases, inflammatory diseases and aging.

An incorporation of antioxidants rich legumes in daily diets had proven the potential health benefits in prevention and management of non communicable diseases such as coronary heart diseases, diabetes mellitus and obesity due to the presence of their low content of fat and high contents of fibre, protein and phyto-chemicals [11-13]. A correlation between consumption of legumes is in relation to the reduction of non communicable diseases have been reported by the number of epidemiological studies [14]. Therefore legumes are significant source for developing functional foods and other medicinal/nutraceutical applications [15-17].

The interest on studies of effect of processing on natural antioxidants, especially phenolic acid and flavonoids in legumes and their activity have drastically increased with the food applications [16] [18]. However, the local grain legume varieties have not been systematically studied for their antioxidant properties in Sri Lanka and there was no available reported data on knowhow of

cooking process and how it affects to those bioactive compounds in grain legumes. Therefore, this is the first study which was conducted to determine the antioxidants and antioxidant activity of grain legume varieties in Sri Lanka under raw and processed (pressure-cooked) forms.

2. MATERIALS AND METHODS

2.1 Materials

Mature seeds of legume crops were collected from the Grain Legumes and Oil Crops Research and Development Centre (GLOCRDC), Angunakolapelessa, Sri Lanka. Six cowpea (*Vigna unguiculata*) varieties (Bombay, Dhawala, Waruni, MICP1, ANKCP1, ANKCP2), two mung bean (*Vigna radiata*) varieties (MI5, MI6), two soya bean (*Glycine max*) varieties (Pb1, MISB1) and two horse gram (*Macrotyloma uniflorum*) varieties (ANK-Black, ANK-Brown) were used (Table 1). Those varieties were grown and harvested in experimental field conditions at GLOCRDC.

2.1.1 Chemicals

2,2'-Azino-bis (3-ethylbenzothiazoline-6- sulfonic acid) diammonium salt (ABTS), 1,1-diphenyl-2-picrylhydrazine (DPPH), 6-hydroxy2,5,7,8-tetramethylchroman-2carboxylic acid (Trolox), gallic acid, 2,4,6-tripyridyl-s-triazine (TPTZ), potassium persulfate, ferric chloride, quercetin and Folin Ciocalteu phenol reagent were purchased from Sigma-Aldrich, USA. Chemicals for the purpose of preparation of buffers and solvents were of analytical grade unless otherwise specified.

2.2 Methods

2.2.1 Sample preparation

For the preparation of raw legumes samples, the seeds were milled and passed through a 0.5 mm sieve.

For the preparation of cooked samples autoclaving treatment was carried out as procedure described by Siddhuraju & Becker, 2007[16]. The seeds (25 g) were soaked 12 h (approx. overnight) in distilled water in ratio of 1:10 (seed: water; w/v) at room temperature (25 ± 2°C). After decanting water, the soaked seeds (seeds: water; 1:5 w/v) were subject to autoclaving for 10 min at 120°C. Soon after decanting the liquid, the autoclaved seeds were freeze-dried at -40^{0}C, 18 h, 0.1 mbar. The freeze-dried cooked seed samples were ground to fine powder to pass through a 0.5 mm sieve and stored in refrigerator until analysis.

2.2.2 Sample extraction

The ground grain powder (2.5 g) and 20 times the sample weight of volume of 70% ethanol were shaken overnight at room temperature (25 ± 2°C). Extracts were then centrifuged at 3000 g for 15 min, supernatant filtered through syringe filters, evaporated under vacuum in a rotary evaporator and resulted solution was freeze dried at -40^{0}C, 18 h, 0.1 mbar [19].

2.3 Determination of Antioxidants

2.3.1 Total Polyphenolic Content (TPC)

The TPC of raw and cooked samples of legumes was carried out as method described by Singleton *et al.*, 1999 [20].

Method in briefly, each extract was diluted in distilled water (2 mg/ml). 20 µL of sample, 110 µL of 10 times diluted Folin-Ciocalteu reagent and 70 µL of 10% sodium carbonate (Na_2CO_3) solution were mixed in a well of 96-well micro plate. After incubating 30 min at 25 ± 2°C, absorbance was measured at 765 nm using a 96-well micro plate reader (SpectraMax Plus ^{384}, Molecular Devices, USA) using gallic acid as the standard.

TPC was expressed as mg gallic acid equivalents (GAE) / g of the whole grain legume flour in dry weight basis.

2.3.2 Total Flavonoids of Content (TFC)

The TFC of raw and cooked legumes was carried out as described by Pourmorad, 2006 [21].

Method in briefly, each extract was diluted in methanol (2 mg/ml). 100 µL diluted sample and 100 µL of 2% aluminium chloride were added into a well of 96-well micro plate. After incubating 10 min at 25 ± 2^{0}C, the absorbance was measured at 415 nm using quercetin as the standard using micro plate reader (SpectraMax Plus ^{384}, Molecular Devices, USA).

The results were expressed as mg quercetin equivalents (QE) /g of the whole grain legume flour in dry weight basis.

Table 1. Morphological characteristics of grain legume varieties

Common name	Species	Variety	Seed Picture	Seed coat colour	Seed size	Seed shape
Horse Gram	*Macrotyloma uniflorum*	ANK-Brown		Brown	Small	Rhomboid
	Macrotyloma uniflorum	ANK-Black		Jet black	Small	Rhomboid
Soya Bean	*Glycine max*	MISB1		Cream colour	Small	Spherical
	Glycine max	Pb 1		Cream colour	Small	Spherical
Mung Bean	*Vigna radiata*	MI 6		Green	Small	Oblong
	Vigna radiata	MI 5		Green	Small	Oblong
Cowpea	*Vigna unguiculata*	ANKCP2		Bicoloured white-brown	Medium	Rhomboid
	Vigna unguiculata	ANKCP1		Pale brown colour	Small	Rhomboid
	Vigna unguiculata	MICP1		Cream colour	Small	kidney
	Vigna unguiculata	Waruni		Reddish brown	Small	Rhomboid
	Vigna unguiculata	Dhawala		Cream colour	Medium	Rhomboid
	Vigna unguiculata	Bombay		Grey brown	Medium	kidney

Seed Size; Varieties with 100 seeds weight less than 15 g – small size; 15.1-20 g medium size; 20.1-25 g -large size and over 25 g very large

2.4 Determination of Antioxidant Potential

2.4.1 DPPH assay

The DPPH radical scavenging activity of r aw and cooked legume samples was carried out as method described by Blois, 1958 [22].

Method in briefly, 125 µL of DPPH radical (20 mg/100 ml) and 50 µL(2 mg/ml) of sample were mixed in a well and incubated at 25 ± 2^{o}C for 10 min. Absorbance was measured 517 nm.

Activity in term of Trolox equivalents (TE) / g for each legume flour was calculated using Trolox standard curve.

Selected high activity extracts were run for the dose response studies at concentrations of 9.375, 18.75, 37.5, 75, 150, 300 µg/mL and IC_{50} values for extracts were calculated using a graph (activity *Vs.* extract concentration).

DPPH radical scavenging activity (%) = [(Ac - As) /Ac]*100

where,

Ac is the absorbance of the control and as is the absorbance of the sample.

2.4.2 ABTS assay

The ABTS radical scavenging activity of raw and cooked legumes was carried out as method described by Re *et al.*, 1999 [23].

40 µL of seven times diluted ABTS stock solution (10 mg of ABTS in 2.5 ml of 2.5 mM potassium persulphate solution incubating at 37^0C for 16 h in dark), 110 µL phosphate buffer and 50 µL (2 mg/ml) of sample was incubated at 25 ± 2^oC for 10 min. Absorbance was recorded at 734 nm using 96-well micro plate reader (SpectraMax Plus^{384}, Molecular Devices, USA). Activity in term of Trolox equivalents (TE) / g for each legume flour was calculated using Trolox standard curve.

Selected high activity extracts were run for the dose response studies at concentrations of 7.81, 15.62, 31.25, 62.5, 125, 250 µg/mL and IC_{50} values for extracts were calculated using a graph (% activity *Vs.* extract concentration).

ABTS radical scavenging activity (%) = [(Ac - As) /Ac]*100 where, Ac is the absorbance of the control and As is the absorbance of the sample.

2.4.3 Ferric Reducing Antioxidant Power (FRAP) assay

Ferric reducing antioxidant power (FRAP) of raw and cooked legumes was carried out as method described by Benzie & Szeto, 1999 [24].

Method in briefly, 150 µL of FRAP reagent (mixture containing 300 mM of acetate buffer at pH 3.6, 10mM 2,4,6-tripyridyl-s-triaine (TPTZ) in 40mM HCl solution and 20mM $FeCl_3.6H_2O$ in a ratio of 10:1:1 followed by incubation at 37°C for 10min), 30 µL acetate buffer and 10 µL (2 mg/ml) were transferred to a micro well. After incubating at 25 ± 2°C for 10 min, absorbance was measured at 600 nm via 96-well micro plate reader (SpectraMax Plus ^{384}, Molecular Devices, USA).

Results were expressed as mg Trolox equivalents (TE)/ g of the each legume variety on dry weight basis.

2.5 Experimental Design and Data Analysis

Three replicate samples from each grain legume variety in forms of raw and cooked were used for testing. Data were statistically analysed using SPSS (Version 20). Univariate General Linear Model was carried out to detect statistical significant difference between treatments and mean separation was performed by Tukey HSD test. Two –way ANOVA was used for the detection of interactions. Paired t-test was performed for the detection of significant difference between raw and cooked forms of each variety.

3. RESULTS AND DISCUSSION

Present study demonstrated the antioxidants and antioxidant activity of locally grown grain legume varieties in Sri Lanka with reference to raw and cooked, the most commonly consumption method of boiled form.

3.1 Antioxidants of Raw and Cooked Form of Grain Legume

3.1.1 Total Phenolic Content (TPC)

Total Phenolic Content (TPC) of ethanolic extracts of raw and cooked form of whole grain legumes are given in Table 2.

The TPC of raw and cooked form of different legume varieties ranged from 0.84 ± 0.04 (MICP1) to 4.34 ± 0.15 mg (GAE) /g (ANKCP2) and 0.30 ± 0.04 (MICP1) to 3.71 ± 0.12 mg (GAE) /g (Dhawala) respectively. Statistically significant differences ($p < 0.05$) were observed for TPC among the grain legume varieties in both conditions of raw and cooked separately. ANKCP2 and ANK-Black showed the significantly highest TPC among raw whole grain legumes whereas Dhawala had shown the highest TPC content in cooked form. ANKCP2 had bicoloured white-brown seed coat colour whereas ANK-Black had jet black seed coat colour. Significantly high value for TPC in cooked form is obtained for the variety having cream coloured seed coat Dhawala and it might be due to the thermal processing by which increase in extractability of bounded compounds during thermal degradation of cellular constituents. All the analyzed varieties

Table 2. Total Phenolics Content (TPC) and Total Flavonoid Content (TFC) of raw and cooked form of local legume varieties

Variety	Total Phenolic Content		Total Flavonoid Content	
	Raw	Cooked	Raw	Cooked
ANK-Brown	3.09 ± 0.11^{bc}	1.30 ± 0.03^{c}	1.40 ± 0.04^{cd}	0.86 ± 0.07^{fgh}
ANK-Black	3.87 ± 0.21^{ab}	1.68 ± 0.12^{b}	0.88 ± 0.03^{e}	1.34 ± 0.13^{d}
MISB1	2.12 ± 0.08^{de}	0.84 ± 0.04^{e}	1.09 ± 0.02^{de}	2.26 ± 0.11^{b}
Pb 1	1.06 ± 0.00^{gh}	0.73 ± 0.02^{e}	0.95 ± 0.06^{de}	1.39 ± 0.18^{d}
MI 6	2.23 ± 0.07^{cde}	0.84 ± 0.12^{e}	1.82 ± 0.05^{b}	1.76 ± 0.04^{c}
MI 5	1.80 ± 0.07^{defg}	1.87 ± 0.11^{b}	2.01 ± 0.08^{ab}	1.33 ± 0.08^{d}
ANKCP2	4.34 ± 0.15^{a}	0.83 ± 0.05^{e}	1.45 ± 0.11^{c}	1.14 ± 0.06^{def}
ANKCP1	1.43 ± 0.07^{efgh}	0.85 ± 0.04^{e}	1.23 ± 0.02^{cd}	0.82 ± 0.03^{gh}
MICP1	0.84 ± 0.04^{h}	0.30 ± 0.04^{f}	1.43 ± 0.13^{c}	1.24 ± 0.03^{de}
Waruni	3.09 ± 0.11^{bc}	0.94 ± 0.04^{de}	2.19 ± 0.04^{a}	0.62 ± 0.04^{h}
Dhawala	1.07 ± 0.01^{fgh}	3.71 ± 0.12^{a}	1.83 ± 0.34^{b}	8.33 ± 0.16^{a}
Bombay	2.02 ±0.98^{def}	1.15 ± 0.07^{cd}	1.47± 0.03^{c}	0.98 ± 0.13^{efg}

Data represented as mean ± standard deviation (n=3) on dry weight basis (db).
Mean values in a column superscripted by different letters are significantly different at p < 0.05.
TPC: mg gallic acid equivalents (GAE) /g whole grain legume, TFC: mg quercetin equivalents (QE) /g whole grain legume

except Dhawala and MI 5 had shown the similar reducing pattern of TPC contents with effect of cooking. The TPC of MI 5 did not show any change with cooking. Varieties ANK-brown and Waruni had similar contents of TPC in raw form while varieties of MISB1, MI 6, ANKCP2 and ANKCP1 also showed similar results in cooked form. TPC of the most of the analyzed varieties (58.3%) were in the range of 1.0-3.0 mg (TE)/ g in the raw form while the most of the varieties (58.3%) were less than 1 mg (TE)/ g in cooked form. Horse gram varieties had significantly high TPC in both form of raw and cooked.

It was reported that phenolic contents of legumes were in the range of 0.325–6.378 mg (GAE) /g [25]. According to the data reported by Sreeramulu *et al.*, 2009 the TPC of legumes in India ranged from 0.62 to 4.18 mg (GAE)/g and the values are in agreement of the present results [26]. Zhao *et al.*, 2014 reported that TPC of legumes extracts ranged 9.5 - 47.6 mg (GAE) /g [10]. Further it was reported that the TPC of legumes were significantly higher than that corn, millets, wheat and rice [19] [26]. The data reported by Xu *et al.*, 2007, analyzing twenty one varieties of legumes, the TPC ranged from 0.57 - 9.60 mg (GAE)/g were in the same range of our varieties [27]. The differences in total phenolic contents in different studies were resulted from genetic reasons, variation between cultivars, extraction procedures and solvents, climatic and environmental factors [28].

Two-way ANOVA has shown the interaction between the TPC of legume variety in its both form of raw and cooked (Variety * Raw Cook, $p<0.05$). It was observed that TPC of most of the varieties had reduced contents with cooking except Dhawala. In comparison of TPC of raw and cooked form of each variety using paired t-test, it was observed that the significantly differences ($p<0.05$) in all analyzed varieties except MI 5.

3.1.2 Total Flavonoids Content (TFC)

Flavonoids are widely spread plant secondary metabolites. However less reported studies were on the identification and quantification of flavanoids [27] [29]. Flavonoids contents of ethanolic extracts of selected raw and cooked form of whole grain legumes are given in Table 2. The TFC of raw and cooked form of different legumes varieties ranged from 0.88 ± 0.03 (ANK-Black) to 2.19 ± 0.04 mg (QE) /g (Waruni) and 0.62 ± 0.04 (Waruni) to 8.33 ± 0.16 mg (QE) /g (Dhawala) respectively. It is seen that statistical significant differences ($p < 0.05$) for TFC among the grain legume varieties in both conditions of raw and cooked separately. Waruni seed coat colour with reddish brown and MI 5 seed coat colour with green showed significantly highest TFC among raw form of whole grain legumes whereas Dhawala seed coat colour with cream colour had shown the highest TFC content in cooked form. The TFC contents of varieties of ANK-Brown, ANKCP2, MICP1 and Bombay had similar values in raw form whereas in the cooked form the most of varieties were in the range of 1 .00 – 2.00 mg (QE) /g. Dhawala had shown the

highest value for TFC in cooked form may be due to the degradation and converting chemical structures of cell wall materials during cooking process. It is seen that results obtained for flavonoids content was higher than phenolics content in MI 5 and MICP1, may be due to the changes in the constituents the extracting process.

According to the data reported by Xu *et al.*, 2007, the TFC (as catechin equivalent) of legumes were in the range of 0.05 to 4.54 mg/g and those values could not compare with present study due to the our results were presented as quercetin equivalent for TFC [27]. According to the Malilla & Mishra, 2017 the TFC (as quercetin equivalents) of legumes ranged from 0.14 - 0.46 mg/g and those values are comparable with our range [29].

Two-way ANOVA has shown a significant interaction between the TFC of legume variety in its both form of raw and cooked (Variety * Raw Cook, $p<0.05$). In comparison of TFC of raw and cooked form of each variety using paired t-test, it was observed that the significantly differences ($p<0.05$) in all varieties except varieties of MI6, ANKCP2 and MICP1.

3.2 Antioxidant Activity of Raw and Cooked Form of Grain Legume Seeds

In the present study antioxidant activities of raw and cooked form of legumes were studied using DPPH, FRAP and ABTS assays.

3.2.1 DPPH assay

The DPPH activity of methanolic extracts of selected raw and cooked form of whole grain legumes are given in Table 3. The DPPH activity of the varieties of legumes was shown only in the varieties of ANK-Black, ANK- Brown and Waruni while other analyzed varieties were not shown the activity at the assay concentration of 0.5 mg/ml (i.e. at well concentration of sample). Higher concentrations of samples may give positive results for DPPH activity for other varieties. The DPPH activity of raw and cooked form of different legume varieties ranged from 3.38± 0.18 (Waruni) to 4.95± 0.42 mg (TE)/ g (ANK Brown) and 1.51± 0.13 (Waruni) to 3.71 ± 0.12 mg (TE)/ g (ANK Black) respectively. It was predominantly seen that varieties with dark coloured seed coat of brown, black and reddish brown in raw form of ANK Brown, ANK Black and Waruni respectively relation to the high activity for DPPH. Results were shown that reduction of DPPH activity in legume varieties with cooking process may be due to the destruction of activity related chemical structures. It was observed that statistical significant differences ($p < 0.05$) for DPPH activity among the legume varieties in both conditions of raw and cooked separately.

ANK-brown had the significantly ($p < 0.05$) highest DPPH activity followed by ANK-Black in the raw form where both varieties were belong to the Horse gram. Several studies were reported the activity of DPPH in legume varieties by Xu *et al.*, 2007 [27] in China, but it was difficult to make comparison due to the external factors of differences in agricultural practices, soil conditions extraction solvent, concentrations etc. According to the data reported by Sreeramalu *et al.*, 2009 the DPPH activity of legumes including dhal, gram, green gram, lentils, soya bean ranged from 0.26-1.07 mg (TE)/g but horse gram and cowpea varieties were not included in their study [26]. A previous study reported that the highest DPPH scavenging activity among legumes extracts was from raw and dry heated horse gram seeds [30]. The differences in DPPH activity may be affecting from the plant origin, environmental factors and solvent used for extraction [28].

Two-way ANOVA has shown a significant interaction between the DPPH activity of legume variety in its both form of raw and cooked (Variety * Raw Cook, $p < 0.05$).

In comparison of DPPH activity in raw and cooked form of each variety using paired t-test, it was observed that the significantly differences ($p<0.05$) within all varieties of except variety of ANK- Black.

Extracts with high antioxidant activity in raw and cooked form of varieties i.e. ANK-Black, ANK-Brown and Waruni were subjected to the dose response studies to obtain the IC_{50} values as shown in Fig. 1 and Fig. 2.

The order of radical scavenging for DPPH in raw and cooked forms of varieties was ANK-Brown > ANK-Black > Waruni > and ANK-Black > Waruni > ANK-Brown respectively. However cooked forms of ANK- Black and Waruni had shown marked DPPH activity.

Pair-wise comparison among each variety for IC_{50} of DPPH assay in raw and cooked form showed a significant difference ($p < 0.05$).

Table 3. Antioxidant activity of raw and cooked form of legume varieties

Variety	DPPH		ABTS		FRAP	
	Raw	Cooked	Raw	Cooked	Raw	Cooked
ANK-Brown	4.95± 0.42^{a}	2.18± 0.45^{b}	8.22 ± 0.48^{a}	2.76 ± 0.07^{c}	0.27 ± 0.01^{a}	0.11± 0.00^{cd}
ANK-Black	4.11± 0.41^{b}	3.17± 0.60^{a}	8.87 ± 0.01^{a}	3.37± 0.45^{b}	0.24 ± 0.01^{a}	0.17 ± 0.01^{b}
MISB1	NA	NA	6.09 ± 0.23^{ab}	1.86 ± 0.06^{def}	0.06 ± 0.00^{de}	0.04 ± 0.00^{gh}
Pb1	NA	NA	4.31 ± 0.63^{bc}	1.91 ± 0.06^{de}	0.07 ± 0.00^{de}	0.04 ± 0.00^{gh}
MI 6	NA	NA	2.08 ± 0.54^{c}	2.23 ± 0.74^{d}	0.05 ± 0.01^{de}	0.06± 0.00^{fg}
MI 5	NA	NA	1.64 ± 0.02^{c}	1.62 ± 0.02^{ef}	0.05 ± 0.00^{de}	0.13± 0.00^{c}
ANKCP2	NA	NA	1.74 ± 0.06^{c}	1.39 ± 0.05^{f}	0.09± 0.01^{cde}	0.06 ± 0.01^{ef}
ANKCP1	NA	NA	1.87 ± 0.08^{c}	1.65 ± 0.08^{ef}	0.12 ± 0.00^{bcd}	0.08± 0.00^{ef}
MICP1	NA	NA	1.96 ± 0.29^{c}	0.61 ± 0.05^{g}	0.03 ± 0.01^{e}	0.02 ± 0.00^{h}
Waruni	3.38± 0.18^{c}	1.51± 0.13^{c}	7.62 ± 0.73^{b}	1.66 ± 0.10^{ef}	0.21 ±0.01^{ab}	0.09 ± 0.00^{de}
Dhawala	NA	NA	2.58 ± 0.07^{c}	11.74 ± 0.26^{a}	0.04 ± 0.00^{de}	0.32 ± 0.02^{a}
Bombay	NA	NA	4.27 ± 0.33^{bc}	1.86 ± 0.10^{def}	0.16 ±0.06^{abc}	0.10 ±0.00^{d}

Data represented as mean ± standard deviation (n=3) on dry weight basis. Mean values in a column superscripted by different letters are significantly different at $p < 0.05$.
DPPH, ABTS and FRAP expressed as mg Trolox Equivalents (TE) / g whole grain legume
NA; Not Activity at assay concentration 0.5 mg/mL (i.e. at well concentration of sample)

3.2.2 FRAP assay

The FRAP activity of methanolic extracts of raw and cooked form of whole grain legumes are given in Table 3.

The results of FRAP activity in varieties of legumes was shown in all analyzed varieties. The FRAP of raw and cooked form of different legume varieties ranged from 0.03±0.01 (MICP1) to 0.27±0.01 mg (TE)/g (ANK-Brown) and 0.02 ± 0.00 (MICP1) to 0.32 ± 0.02 mg (TE)/g (Dhawala) respectively. It was observed a statistical significant difference ($p < 0.05$) for FRAP among the grain legume varieties in both conditions of raw and cooked separately.

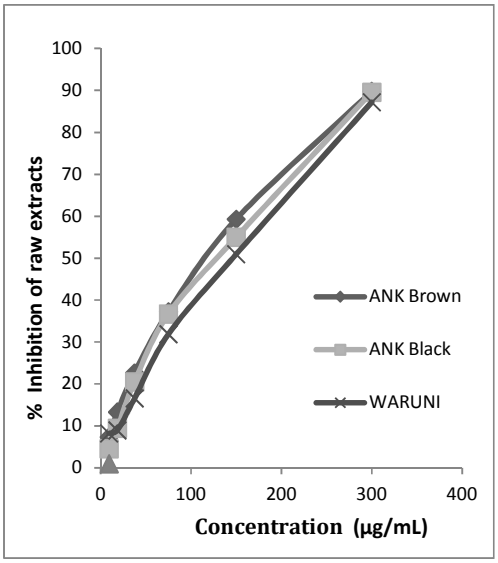

Fig. 1. Dose response relationship of selected raw legume varieties for DPPH radical scavenging activity

IC_{50} values: ANK-Brown: 100.92±0.89^{a}; ANK-Black: 138.35±1.18^{b} and Waruni: 149.47±7.8^{c} µg/mL.
IC_{50} values superscripted by different letters are significantly different at $p < 0.05$.
Assay concentration: 1mg/mL

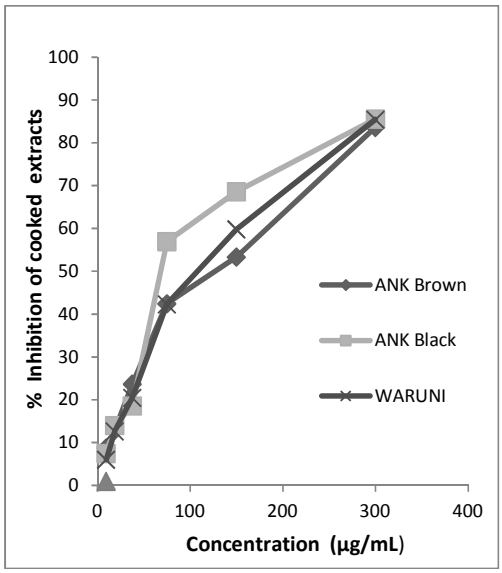

Fig. 2. Dose response relationship of selected cooked legume varieties for DPPH radical scavenging activity

IC_{50} values: ANK Brown: 123.32±9.02^{c}; ANK Black: 66.20±1.40^{a} and Waruni: 97.51±2.12^{b} µg/mL.
IC_{50} values superscripted by different letters are significantly different at $p < 0.05$.
Assay concentration: 1mg/mL

In the raw form of ANK-brown had the significantly highest FRAP activity followed by ANK- Black in which both varieties were belong to the Horse gram. It was predominantly seen that varieties with dark coloured seed coat of brown, black and reddish brown in raw form of ANK Brown, ANK Black and Waruni respectively relation to the high activity for FRAP. The results of FRAP assay of the most of analyzed varieties were in the range of 0.00-0.10 (TE)/ g of whole grain legume. Several studies were reported the results of FRAP assay of legume varieties by Xu *et al.*, 2007 but the difficult to make comparison due to the differences in agricultural practices, soil conditions, extraction solvents, concentrations etc [27]. According to the data reported by Sreeramalu *et al.*, 2009, the FRAP activity of legumes including dhal, gram, green gram, lentils, soya bean ranged from 52.85 to 372.76 µmol (TE)/g but horse gram and cowpea varieties were not included in their study [26]. The differences in FRAP results may be affected from source of plant extract, environmental factors and extraction solvent [28].

Two-way ANOVA has shown a significant interaction between the FRAP activity of legume variety in its both form of raw and cooked (Variety * Raw Cook, $p < 0.05$).

In comparison of FRAP in raw and cooked form of each variety using paired t-test, it was observed that the significantly differences ($p<0.05$) within all varieties of except variety of MI 6.

3.2.3 ABTS assay

ABTS is soluble in both aqueous and organic solvents. Therefore it can be used to detect both hydrophilic and lipophilic antioxidant capacities of plant extracts [31].

The ABTS activity of varieties of legumes was shown in all analyzed varieties. The ABTS activity of raw and cooked form of different legumes varieties ranged from 1.64 ± 0.02 (MI 5) to 8.87± 0.01 mg (TE)/g (ANK Black) and 0.61 ± 0.05 (MICP1) to 11.74 ± 0.26 mg (TE)/g (Dhawala) respectively. Statistically significant differences ($p < 0.05$) were observed for ABTS activity among the legumes varieties in both conditions of raw and cooked separately.

It was predominantly seen that varieties with dark coloured seed coat of brown, black, reddish brown and grey brown in raw form ANK Brown, ANK Black, Waruni and Bombay respectively relation to the high activity for ABTS. Most of the varieties were shown the reduction of ABTS activity with cooking process. A significant ($p < 0.05$) increment of activity of ABTS with relation to the cooking process was shown in variety Dhawala and MI 6. It may be due the changes occurred during thermal processing by which increase in extractability of bounded compounds. It is seen ABTS activity of variety MI 5 had similar values in raw and cooked forms. Two-way ANOVA has shown a significant interaction between the ABTS activity of legume variety in its both form of raw and cooked (Variety * Raw Cook, $p<0.05$). In comparison of ABTS of raw and cooked form of each variety using paired t-test, it was observed the significant differences ($p<0.05$) within each variety.

Extracts with high antioxidant activity in raw and cooked form of varieties i.e. ANK-Black, ANK-Brown and Waruni and cooked form of Dhawala were subjected to the dose response studies obtain the IC_{50} values as shown in Fig. 3 and Fig. 4. The highest scavenging activity of ABTS radicals was shown for the legume extract of raw form of ANK- Black and ANK-Brown where the IC_{50} values were 60.28±0.64 µg/mL and 60.97±3.59 µg/mL respectively.

The order of scavenging for ABTS in raw and cooked forms of varieties was ANK-Black = ANK-Brown > Waruni > and ANK-Black > ANK-Brown > Waruni > Dhawala respectively.

Similarly pair-wise comparison among each variety for IC_{50} of ABTS assay in raw and cooked form showed a significant difference ($p < 0.05$).

3.3 Overall Antioxidant Potential of Legume Varieties

In generally, DPPH radical scavenging activity showed lower activity when compared to the ABTS radical scavenging activity. In the DPPH scavenging assay, the small molecules have a better potential to approach to the radical site than the large molecules due to the presence of steric hindrance of radical molecule structure [31]. This would be resulted for the differences observed in scavenging potencies of legumes extracts. Since the DPPH assay is base on the alcoholic media, the molecules which are soluble in alcohol only react with DPPH. In ABTS assay the molecules which are soluble in water are involved. Therefore, the molecules with potential activity which are not available in methanolic

solution will not be responded for DPPH scavenging activity [25]. Further, present study had shown a predominant relationship between the varieties with dark coloured seed coat of brown, black and reddish brown in raw form of ANK Brown, ANK Black and Waruni respectively with antioxidant activity. Further most of those activities were not observed in cooked form due to the loss of activity related compounds in cooking process.

Present study showed that the varieties of *Macrotyloma uniflorum* (common name horse gram), ANK- Black and ANK-brown in both forms; raw and cooked had overall high antioxidant potential determined using for TPC, DPPH, ABTS and FRAP assays (in methanolic extract). Horse gram has been reported worldwide for its antioxidant potential and also reported to be the antioxidant activity is varietal dependent [26] [28]. It was reported that phenolic contents of legumes were in the range of 0.325–6.378 mg (GAE)/g and the high TPC was found in horse gram varieties (i.e. 3.570 mg/GAE g) [25]. It was observed that our horse gram varieties had also similar values for TPC. Since whole grain horse gram is rich source of dietary fiber, vitamins, minerals, polyphenolic compounds and high antioxidant potential, it is good for prevention and management of non communicable diseases such as diabetes, cancers, cardiovascular diseases, neurological diseases and inflammatory diseases [32] [33] [34]. Many studies reported that antioxidant activity in terms of radical scavenging activity of legumes are involved in the prevention of non communicable diseases including diabetes, cardiovascular diseases, cancers, neuro degenerative diseases and inflammatory diseases [35] [36]. Further, it was revealed an inverse relationship between the intake of diets rich in antioxidants and the human degenerative diseases [35].

A significantly the highest results of antioxidant content of TPC was found in the variety ANKCP 2 in raw form but the antioxidant activities of DPPH, ABTS or FRAP were not seen in any form of raw and cooked. Variety Dhawala in cooked form had a significantly high antioxidant potential determined using TPC, TFC, ABTS and FRAP assays whereas the same observation is not seen in raw form. The high antioxidant potential in cooked form is a good advantage of gaining beneficial health effects to consumer. The thermal processing leads to the increase in extractability and solubilisation of phenolic and antioxidant compounds due to the degradation of cellular materials. It may cause the by rupturing plant cell walls and disruption of the structure of complex compounds [37].

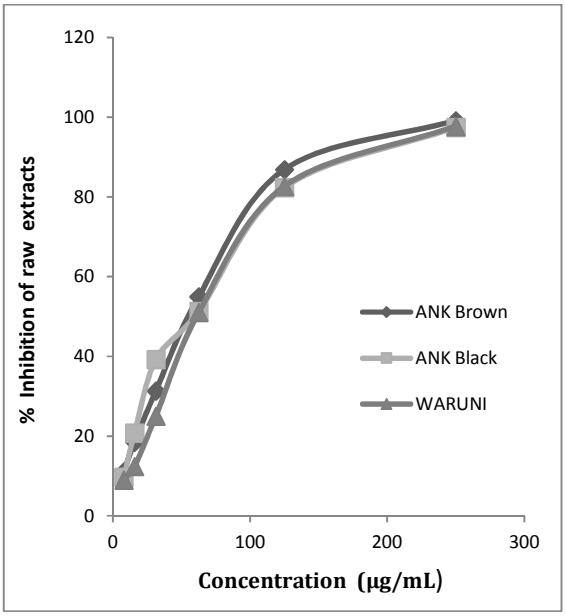

Fig. 3. Dose response relationship of selected raw legume varieties for ABTS radical scavenging activity

IC_{50} values: ANK Brown: 60.97±3.59[a]; ANK Black: 60.28±0.64[a] and Waruni: 66.63±6.17[b] µg/mL.
IC_{50} values superscripted by different letters are significantly different at $p < 0.05$.
Assay concentration: 1mg/mL

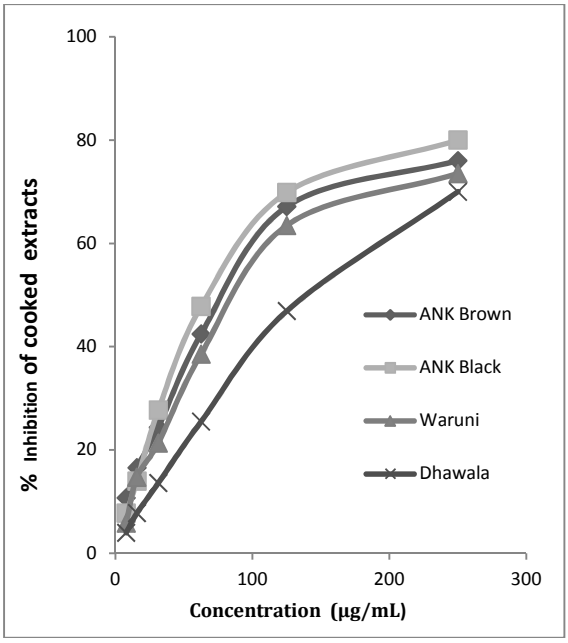

Fig. 4. Dose response relationship of selected cooked legume varieties for ABTS radical scavenging activity

IC_{50} values: ANK Brown: 85.68±2.37^{b}; ANK Black: 64.05±2.59^{a}; Waruni: 92.32±6.04^{c}; Dhawala : 169.00±4.81^{d} µg/mL. IC_{50} values superscripted by different letters are significantly different at $p < 0.05$.
Assay concentration: 1mg/mL

4. CONCLUSION

Present study on local legumes had gained a wide recognition for developing health-promoting and functional foods. Among the studied twelve legumes varieties having dark coloured seed coat of ANK black, ANK-brown and Waruni in form of raw had shown the high antioxidant potential in terms of TPC, DPPH and ABTS. Among those varieties in cooked form showed high activity for DPPH and ABTS only in ANK Black and ANK Brown. In cooked form of variety Dhawala had shown the potent antioxidant activity. Since this is the first systematic study of antioxidant potential of locally grown legume varieties, study will provide data for varietal identification. Further this study indicates the potential utilization of legume varieties in Sri Lanka for consumption and developing functional foods.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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