International Journal of Food Science and Nutrition
ISSN: 2455-4898; Impact Factor: RJIF 5.14
Received: 09-07-2020; Accepted: 28-07-2020; Published: 09-08-2020
www.foodsciencejournal.com
Volume 5; Issue 4; 2020; Page No. 93-101

Horse Gram: an incredible food grain as a potential source of functional and nutritional food ingredient

HM Theja Herath[1*], MDW Samaranayake[2], S Liyanage[3], WKSM Abeysekera[4]

[1-3] Food Technology Section (FTS), Modern Research & Development Complex (MRDC), Industrial Technology Institute (ITI), 503A, Halbarawa Gardens, Malabe, Sri Lanka

[4] Department of Agricultural Technology, Faculty of Technology, College House, 94, Cumarathunga Munidasa Mawatha, University of Colombo, Colombo, Sri Lanka

Abstract

Horse gram (Spieces; *Macrotyloma uniflorum)* is an incredible food legume which has been underutilized due to the poor cooking qualities and unflavours compounds. The present study provides unexploited nutritional and functional properties of two horse gram varieties of Sri Lanka showing high content of protein (22.0 -24.2%), crude fiber (6.7- 6.9%) and minerals (3.6%) specially containing iron, zinc and Ca. Besides nutritional importance horse gram contained high content of Dietary Fiber (DF); 21.2% and Resistant Starch (RS); 10.5% leading to low Glycaemic Index (GI); 39.8. Anti-nutritional factors present in seeds of ANK brown and ANK black were phytic acid; 4.55± 0.55 mg/g, 2.60±0.26 mg/g, saponins;10.06±0.73 mg/g, 11.52±0.78 mg/g and tripsin inhibitors 0.65± 0.02mg/g, 0.69± 0.01mg/g respectively and those contents significantly ($p < 0.05$) decreased with cooking. Total Polyphenolic Content (TPC) and Total Flavonoid Content (TFC) of seeds were 3.09±0.11, 3.88±0.21 mg Gallic Acid Equivalent (GAE) /g and 1.40±0.04, 0.88±0.03mg Quercetin Equivalent (QCE) /g while in cooked form were 1.30±0.03, 1.68±0.12 mg (GAE) /g and 0.87±0.07, 1.35±0.13 (QCE) /g respectively. Antioxidant activity exhibited by DPPH, ABTS and FRAP significantly ($p<0.05$) decreased with respect to varieties and cooking process.

Keywords: horse gram; functional foods; antioxidant properties; anti nutritional factors; autoclaving

1. Introduction

Horse gram is an unexploited food legume of the family *Fabaceae* and grouped under the species; *Macrotyloma uniflorum* [1]. It is subdivided into two varieties namely ANK Black and ANK Brown which can be distinguished from one another by different physical characteristics, specially the seed coat colour. This plant is a herbaceous legume showing indeterminate growth pattern. It has a considerable adaptation to warm climates with adequate rainfall and wide range of temperature regimes [2]. Horse gram is cultivated across Southeast Asia, Africa, Australia, Burma and West India [3]. It is considered as a good source of protein, fiber, minerals, carbohydrates and energy [4].

According to the recent estimates, more than one-third of all child deaths across the worldwide are affected from malnutrition while in developing countries 54% of child deaths were associated with child malnutrition [5, 6]. National Nutrition Surveillance System of Sri Lanka 2010 was reported, nearly 50% children are malnourished (22 % underweight, 16% wasted and 18 % stunting) and 30% of the children are iron deficient [7].

Malnutrition and micro-nutrient deficiency are mainly a result of the consumption of highly refined cereal-based meal, which is bulky, high energy and less nutritious specially lack of protein and minerals [5, 6]. Horse gram is an excellent source of protein (17.9 - 25.3%), carbohydrates (51.9 - 60.9%), essential amino acids, low content of lipid (0.58-2.06%), source of iron, molybdenum [4], other minerals [8] and vitamins. The total protein content of legume is an approximately two to four-fold greater than cereals and tuber crops. Legumes along with cereals can be considered as main plant sources of energy and good quality proteins. Cereal proteins are deficient in certain essential amino acids, particularly in lysine whereas they are rich in cysteine. However, legume proteins are rich in lysine [9,10]. Therefore, the combined consumption of beans and cereals can ensure a balanced protein diet due to the nutritional complementation of essential amino acids. Further horse gram could be promoted as a high-quality protein constituent of the daily diet among economically depressed communities in developing countries and potential application in reducing the high prevalence of protein and energy malnutrition.

Food legumes are considered as an incredible source of health-promoting functional components due to their soluble and insoluble dietary fiber, resistant starch, minerals, vitamins (e.g., vitamins B, D and E), phenolic compounds, phytoestrogens (lignans) and other phytochemicals [11, 12, 13]. These compounds may influence biological functions individually or synergistically. Further anti-nutrient bio active compounds exert wide range of biological actions in the human body. Those anti-nutrient components cause a number of health beneficial effects by reacting as a potential antioxidant (e.g. phytic acids, saponins, phenolic compounds [14].

Phytic acid (*myo*-inositol hexakisphosphate) is one of the major Anti-Nutritional Factors (ANFs) present in legumes. Phytic acid chelates divalent cations such as Ca^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, $Fe^{2+/3+}$ and thereby reducing their bioavailability. Many phytate-mineral complexes are insoluble and therefore they may become unavailable for absorption under normal physiological condition [15]. On the other hand it has potential activities of antioxidant and anti-carcinogenic [16]. Certain evidences showed that saponins provide neuro protective effects on attenuation of central nervous system disorders, such as Parkinson's disease, stroke, Huntington's disease and Alzheimer's disease, along some *in-vivo* studies showing saponins have tumor-inhibitory effects and antifungal activity [17].

The excessive generation of free radicals in human body leads to a condition known as oxidative stress[18,19] and it is linked to the development and progression of several degenerative diseases such as intestinal diseases (colon cancer, constipation, gallstones, diverticulosis etc.), diabetes, heart diseases, neurological diseases, inflammatory diseases, blood pressure and aging [20]. Antioxidants are the compounds which have a capability to reduce the oxidative stress and thereby will decreases risk of getting such diseases [18, 19]. Therefore, diets rich in antioxidants play a vital role in the prevention and management of such diseases while performing as radical scavengers, reducing agents and chelator of metal ions [21, 22].

Further, epidemiological evidence indicates that the consumption of horse gram exerts protective effects against the development of several chronic diseases, such as gastrointestinal disorders, cardiovascular diseases, hypercholesterolemia, obesity, diabetes and several types of cancer [3]. In addition, the literature also reports on functional ingredients in horse gram that aid in weight loss, improve digestion and strengthen blood circulation. The low glycaemic index of horse gram is attributed by the action of RS and DF [3].

2. Materials and Methods

2.1 Preparation of sample

Two horse gram (*Macrotyloma uniflorum*) varieties of locally grown ANK-Black and ANK-Brown were used in this study. They were obtained from the Grain Legumes and Oil Crops Research and Development Centre (GLOCRDC), Angunakolapellessa. Cleaned and dried horse gram seeds were ground to pass through a 0.5mm (500µm) sieve in a Hammer Mill (RETSCH S/S CROSS BEATER Sk1) prior to chemical analysis of raw sample.

For the preparation of cooked sample autoclaving of seeds was performed. The seeds (25g) were soaked in distilled water (1:10; seeds: water; w/v) at overnight in room temperature (25 ± 2°C). After decanting water, the soaked seeds with water (1:5; seeds: water; w/v) were subject to autoclaving at 120°C for 10 min. Soon after decanting the liquid, the cooled autoclaved seeds were freeze-dried. The freeze-dried seed samples were ground to a fine powder to pass through a 0.5mm sieve and stored (4°C) untill analysis.

2.2 Chemicals

Standard stock solutions (calcium, potassium, iron and zinc 1000 mg/L) for mineral analysis, Spectrosol standards were purchased from BDH, England to plot calibration curve of each analyzed minerals.

Anion exchange Resin: AGI-X4, chloride form, 100-200 mesh was purchased from Bio-Rad Company, USA and used for phytate analysis.

Dialysis tubings of Spectro/Por MMCO 12,000 - 14,000 Da were obtained from Thomas Scientific, USA for dialysis.

Benzoyl-DL-Arginine-p-Nitroanalide Hydrochloride (BAPA) purchased from Sigma-Aldrich, USA for tripsin inhibitor activity.

Chemicals for antioxidant activity assay

2,2ˈ-Azino-bis (3-ethylbenzothiazoline-6- sulfonic acid) diammonium salt (ABTS), 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), 2,2-diphenyl-2- picrylhydrazine (DPPH), 6-hydroxy2,5,7,8-tetramethylchroman-2carboxylic acid (Trolox), gallic acid, Quercetin, 2,4,6-tripyridyls-triazine (TPTZ), potassium persulfate, ferric chloride and Folin Ciocalteu reagent were purchased from Sigma-Aldrich, USA.

All the other chemicals used for the preparation of buffers and solvents were of analytical grade (Sigma, Aldrich).

Enzymes

Tripsin from bovine pancreas, Denmark, Alpha amylase from *Bacillus sp.* Novozymes A/S, Amyloglucosidase from *aspergillus niger*, Novozymes A/S were purchased from Denmark, Sigma Aldrich.

Assay kits

K-RSTAR, Megazyme international Ireland, Bray Business Park, Bray, Co. Wicklow, Ireland was used to determine Resistant Starch and Non-Resistant Starch.

2.3 Compositional analysis of horse gram

Horse gram samples were tested for proximate composition, major mineral content, dietary fibre content, starch fractions; soluble and resistant starch and Predicted Glycemic Index (pGI) with acceptable standard protocols.

2.3.1 Proximate composition

Proximate composition of horse gram seeds were carried out according to the methods described in AOAC, 2012 [23]. Each determination of composition value was performed in triplicates. Moisture content of the horse gram seed flour was determined according to the oven drying method as described in AOAC, 2012; 925.09B [23]. Crude protein content of the seed flour was determined by Kjeldahl method as specified in AOAC, 2012; 920.87 [23]. Crude fat content was determined by soxhlet extraction method according to AOAC, 2012; 920.39C [23]. Crude fibre content was determined according to the method described in AOAC, 2012; 962.09E [23]. Ash content was determined as specified in AOAC, 2012; 923.03 by dry ashing [23]. Total carbohydrate content was determined according to the by difference method.

2.3.2 Mineral analysis

Varian SpectrAA 220 Fast Sequential Atomic Absorption Spectrophotometer was used for the analysis of calcium, potassium, iron and zinc by following the method of 975.03 as specified in AOAC, 2012 [23]. Phosphorous content was determined using sodium molybdate, according to the method 995.11 as specified in AOAC, 2000 [24].

2.3.3 Total DF content

Total DF contents of horse gram seed flour were determined by the enzymatic gravimetric method as described by Asp *et al.* 1983 [25]. In this method, starch and protein are digested using enzymes into small fragments. Ethanol is added to the filtrate to precipitate the soluble fiber and total fiber (both soluble and insoluble) is recovered by filtration.

2.3.4 Resistant Starch (RS) content

The RS content of the samples were determined using an enzymatic assay using megazyme assay kit. This method allows the measurement of RS, solubilized starch and total starch content of samples. The procedure has been subjected to inter laboratory evaluation under the auspices of AOAC International and AACC international and accepted by both associations of AOAC Official Method, 2002.02; AACC, 2000 Method 32-40.01[26, 27].

2.3.5 Total sugar content
Sugar content of samples was determined by spectrophotometrically using Phenol-Sulphuric method as described in Dubois *et al.* 1956 [28].

2.3.6 Predicted Glycaemic Index value
In-vitro starch digestibility was determined using the method as described by Jenkins *et al.* 1987 [29].
Available carbohydrate portions of each of the cooked horse gram samples (around 2 g) were digested using human saliva and digested samples were dialyzed at 37°C using a membrane. Sugar content of the dialysate was determined by DNS [30] reagent and hydrolysis index values were calculated from the starch digestibility curve. The predicted GI was calculated by using an equation [31].

2.4 Anti-nutritional compounds
2.4.1Phytate content
Phytate content in horse gram varieties was determined on eluted acid extracted fraction from anion exchange chromatographic technique followed by wet digestion and quantification by spectrophotometrically, using ammonium molybdate (as a colour reagent) using AOAC, 2000; 986.11[24].

2.4.2 Saponin content
Saponin content of horse gram varieties was determined using the double solvent extraction technique following gravimetric method as described by Cheok *et al.* 2014 [17].
2.00 g of sample was extracted twice using 20 ml of 20% ethanol aqueous and combined extraction which was reduced to 5 ml over a water bath at about 90^0C, was shaken with 10 ml of diethyl ether. Aqueous layer was recovered while ether layer was discarded. Extracts were twice washed with 6ml of n-butanol containing 1 ml of 5 % aqueous sodium chloride. The remaining solution was heated in water bath. After evaporation of water sample was dried in an oven at 105^0C to a constant weight and Saponin content was calculated as a percentage.

2.4.3 Tripsin inhibitors
Trypsin inhibitor content of horse gram was determined according to the method described by Hamerstrand *et al.* 2008[32] in accordance with AACC International method [33].
Accurately weighed (around 1.000 g) the ground seed powder was extracted with 50 ml of 0.01M sodium hydroxide at room temperature for 3 hrs. pH of the extract was adjusted between 8.4 -10.0 and diluted to 100 ml with distilled water. 0.25 ml of the suspension was mixed with 0.5 ml of trypsin solution and incubated for 10 minutes at room temperature. Then 1.25 ml of 0.04% Benzoyl-DL-Arginine-p-Nitroanalide Hydrochloride (BAPA) was added and further incubated for 10 minutes at room temperature. Reaction was terminated by adding 0.25 ml of 30% acetic acid. Absorbance was measured at 410 nm using 96 well micro plate reader (SpectrMax Plus[384], Molecular Devices, USA) and Tripsin Inhibitors Activity (TIA) was calculated (mg/g) using given equation.

2.5 Total Polyphenolic Content (TPC) and Total Flavonoid Content (TFC)
2.5.1 Extraction of sample
The ground grain powder (2.5g) was shaken overnight at room temperature (25±2°C) with 20 times the sample weight of 70% Ethanol. Extracts were then centrifuged at 3000g for 15 min, evaporated under vacuum in a rotary evaporator and resulted solution was freeze dried as described in Abesekara *et al.* 2017[22].

2.5.2 Total Polyphenol Content (TPC)
Total phenolic content of legume extract was determined using the Folin-Ciocalteu reagent (Sigma-Aldrich, USA) as described by Singleton, *et al.* 1999[34]. Each extract was diluted in distilled water (2 mg/mL) and 20µL of sample was mixed with 110µL of 10 times diluted Folin-Ciocalteu reagent and 70µL of 10% Sodium Carbonate (Na_2CO_3) solution. After incubating 30 min at room temperature (25±2°C), absorbance of the mixture was measured at 765nm using a 96-well micro plate reader (SpectrMax Plus [384], Molecular Devices, USA) using Gallic acid as the standard and TPC was expressed as mg Gallic acid equivalents (GAE)/g of the horse gram flour in dry weight basis.

2.5.3 Total Flavonoid Content (TFC)
Total Flavonoid content of legume extract was determined using the method as described by Pourmorad *et al.* 2006 [35].
Total Flavonoid Content of horse gram extract was determined using a reaction volume of 200µL, containing 100µL of 2% Aluminium Chloride solution and 100µL of (2mg/mL) sample. After incubating 10 min at room temperature (25±2°C), absorbance of the mixture was measured at 415nm using Quercetin as the standard and results was expressed as mg Quercetin equivalent (QCE)/g of the whole grain legume flour in dry weight basis.

2.6 Antioxidant Activity
2.6.1 DPPH Radical Scavenging Activity
DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of horse gram grain extract was determined as method described by Blois, 1958[36]. A reaction volume of 200µL, containing 125 µL of DPPH radical and 50 µL (2 mg/mL) of sample was incubated at 25±2°C for 10 min. The absorbance values were recorded at 517 nm using Trolox as the standard. DPPH radical scavenging activity of each horse gram flour extract was calculated and results were expressed as mg Trolox equivalent (TE)/ g of the horse gram flour on dry weight basis.

2.6.2 Ferric Reducing Antioxidant Power (FRAP) Assay
Ferric reducing antioxidant power (FRAP) of grain extracts was performed according to the method described by Benzie & Szeto, 1999[37]. The working FRAP reagent was prepared by mixing 300 mM of acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triaine (TPTZ) solution in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ in a ratio of 10:1:1. The mixture was incubated at 37°C for 10 min. A reaction volume of 200 µL was prepared using 150µL of working FRAP reagent, 30 µL acetate buffer and 10 µL (2 mg/mL) of sample. The mixture was incubated at room temperature (25±2°C) for 8 min.

Absorbance was recorded at 600 nm using Trolox as the standard and results was expressed as mg Trolox equivalent (TE)/ g of the horse gram flour on dry weight basis.

2.6.3 ABTS+ radical scavenging activity

The ABTS+ radical scavenging activity of grain extracts were determined according to the method described by Re *et al.* 1999[38]. A stable stock solution of ABTS+ radical was produced by reacting 7.8 mM of ABTS in potassium persulphate at 37ºC for 16 h in dark. A reaction volume of 200 µL, containing 40 µL of seven times diluted ABTS stock solution, 150 µL phosphate buffer and 5 µL (2 mg/mL) of sample was incubated at 25±2ºC for 10 min. Absorbance values were recorded at 734 nm using Trolox as the standard and results was expressed as mg Trolox equivalent (TE)/100 g of the horse gram flour on dry weight basis.

2.7 Statistical analysis

Statistical analysis of data was done by one-way ANOVA to identify the significant differences among the samples at 95% confidence interval using SPSS statistical software (IBM SPSS statistical version 20). Paired -T test was used to identify whether there is a significant difference among raw and cooked forms of each variety in antioxidant properties.

3. Results & Discussion

Physical and some nutritional properties of locally grown nine legume varieties of Sri Lanka were studied and this work has been reported by Herath *et al.* 2018[39]. Since the horse gram had identified as the best variety among other legume varieties, steps were taken to extensively investigate their functional properties and the overall data was compiled in present paper to access for the scientists, nutritionists and breeders to study properties of horse gram in one sight.

3.1 Physical and morphological characteristics of horse gram varieties

Among morphological characteristics, 100 seed mass, seed size, seed shape, seed coat color and seed coat texture have been observed in horse gram varieties were given in the Table 1. The distinctive fruit of horse gram principally grows into a pod that contains the seeds of the plant. Legume seeds have a characteristic structure consisting of three major parts, seed coat (hull), cotyledons and hypocotyl.

Table 1: Morphological characteristics of horse gram varieties

Variety	Seed weight (g)	Seed size	Seed shape	Seed coat color	Seed coat texture
ANK Brown	3.1	Small	Rhomboid	Light brown	Smooth
ANK Black	3.3	Small	Rhomboid	Jet black	Smooth

Seed weight refers to the weight (g) of 100 seeds.
Seed size is defined as varieties with seed weight < 15 g as Small size; 15.1-20 g as Medium size; >20.1-25 g as Large.

Horse gram had small size seed among varieties where ANK Black and ANK Brown had 3.3g/ in 100 seeds and 3.1g/ in 100 seeds respectively. Seed weight of legume variety could be a useful criterion for determining suitability for a particular end-use application. Seed coat texture predominantly showed the smooth nature in horse gram varieties. Seeds with smooth seed coat texture tend to absorb less water than seeds with wrinkled seed coat [40]. Moreover, seed coat texture could be an important selection criterion when processing seeds into flour. Ease of soaking and dehulling characteristics in smooth seed coat are important to give light color to flour. Color of the horse gram varieties are varies and it influences consumer acceptance.

3.2 Proximate composition of horse gram seeds

Proximate composition of moisture, fat, protein, ash and fiber content of whole horse gram seed flour were determined by standard procedures [23] and carbohydrate content was calculated following 'by difference' method on dry weight basis. Proximate composition of selected horse gram varieties is given in Table 2.

Table 2: Nutritional and functional properties of horse gram varieties

Nutritional and functional parameters (on dry weight basis)	Variety	
	ANK brown	ANK Black
Caloric value (K cal)	363.2[a]	361.6[b]
Protein (%)	24.2±0.25[a]	22.0±0.37[b]
Carbohydrates (%)	64.6[b]	66.8[a]
Dietary fibre (%)	21.2±1.30[a]	21.2±0.27[a]
Nonresistant starch (%)	32.7±1.0 [b]	36.3±1.13[a]
Total starch (%)	43.1±1.66[b]	46.9±1.29[a]
Resistant Starch (%)	10.5±0.62[a]	10.5±0.28[a]
Total sugars (%)	6.05±0.64	5.91±0.48
Fat (%)	0.8±0.02[a]	0.8±0.12[a]
Total mineral (%)	3.6±0.09[a]	3.6±0.02[a]
Fe (mg/kg)	115.5±0.61[a]	104.0±1.75[b]
Ca (mg/kg)	1571.6±2.00[b]	1286.8±3.00[a]
Zn (mg/kg)	30.6±0.95[a]	30.6±0.81[a]
K (%)	1.0±0.21[a]	1.0±0.12[a]
P(mg/100g) PGI	369.2[a]±1.73[a] 39.8 ±0.64[a]	361.0±3.00[b] 39.8±0.25[a]

Data presented as Mean ± SE (n=3). Mean values in a row superscripted by different letters are significantly different at $p < 0.05$.
pGI= Predicted Glycaemic Index

The cotyledons part of horse gram seeds provide majority of the nutritional components, with the exception of fibre and Ca of which significant portion of those components are found in the seed coat [41]. However, legume seeds are varying greatly in their composition, depending on the type and variety of seed, soil conditions and environmental factors [42].

The crude protein content of the whole ground horse gram (undehulled) ranged between 22.0 ±0.37% (ANK Black) to 24.2±0.25% (ANK Brown) on dry weight basis. In this context, significant difference (p <0.05) was observed between the protein content within two varieties of horse gram. The findings of Adam *et al.* 1989 were in conformity with these values which showed that crude protein content of the selected legumes ranged from 15- 45% [43]. In horse gram out of total protein content, albumin-globulin protein fractions contribute from 75.27% to 78.76% while glutenin and residual protein varies from 9.93%-17.52% to 6.56% - 11.0% respectively [44]. It was reported that horse gram had high lysine content (0.052g g^{-1}) as an essential amino acid [45].

The variations in protein content have been observed owing to analytical methods, genotype, different environments and agricultural practices. Protein concentration is reported to be highest in the embryo, followed by cotyledons and least in the seed coat (22.6, 18.6% and 9.1% on dry weight basis) respectively [3]. The low-fat content in horse gram is an advantage during processing it into flour, since there is no need for defatting step in seed flour production [3]. The significant difference (p >0.05) was not shown between the fat contents values among each other. Legumes contained high fibre content than any major food group comprising soluble and insoluble. In most legumes consumed by humans, the crude fibre content ranges from 8% to nearly 28% [46]. The mean values for total ash contents between two varieties were similar values and there is no significant difference observed (p >0.05). Carbohydrate content of horse gram varieties ranged from 64.8 (ANK Brown) to 66.7% (ANK Black) and values were significantly different (p <0.05).

3.3 Carbohydrate; Dietary Fibre, Starch and Sugars

In the most of legumes, the largest part of the carbohydrate fraction is starch, accounting around 35% – 45% of the seed weight depending on the legume species [47]. Starch which is not digestible at small intestine reaches large intestine where it is fermented by colonic micro flora is known as RS [20]. RS contents two horse gram varieties are the same 10.5% and it accounts around 15-16% of total carbohydrate. Fermented RS produces the butyrate which has a protective effect on colorectal cancer [48]). Further it was reported that resistant starch rich foods are beneficial for management of diabetic [49].

Legumes are good source of DF of both soluble and insoluble fibre [50]. According to previous research studies, DF contents were high in legumes rather than cereals and rich in metabolically active soluble DF [51]. It was reported that the total amount of DF contained in different legumes varies between 3g and 6g/ 75g serve of cooked legume [52]. The DF content of horse gram varieties is shown in Table 2. DF contents of whole grains of both varieties had similar amounts (i.e. 21.1%). In this context, no significant difference (P> 0.05) showed between horse gram varieties. The mean values for DF fibre content of selected locally consume legume varieties were reported and ranged from the lowest of 13.07 ± 0.51% (cowpea-Bombay) to the highest of 21.38 ± 0.22% (horse gram-ANK Black) [39]. The main physiological functions of DF were reported to be beneficial in the reduction of constipation, blood glucose level, blood pressure, obesity, cholesterol level and increase in prebiotic effects and prevention of certain cancer [3]. The total soluble sugar content of horse gram varieties ranged from 5.91±0.48% to 6.05±0.64%.

3.4 Mineral contents

Results of the mineral analysis are presented in Table 2. Iqbal *et al*. 2006 indicated that potassium is the most abundant mineral among legume seeds [53]. It has been observed from the present study and similar values (i.e. 1.0 %) were contained in both varieties. Phosphorous, copper, iron, calcium and magnesium are some of other important minerals found in legumes in significant amount [42]. Mineral contents will vary in response to both genetic and environmental factors.

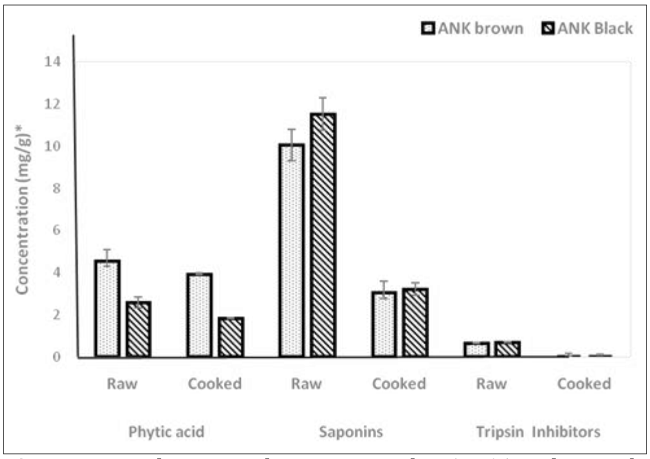
3.5 Anti nutritional Compounds

The contents of phytic acid, saponin and tripsin inhibitors are tabulated in Table 3 (Figure 1).

Table 3: Anti nutritional Compounds in raw and cooked seeds of horse gram

Variety	Phytic acid (mg/g)		Saponins (mg/g)		Tripsin Inhibitors (mg/g)	
	Raw	Cooked	Raw	Cooked	Raw	Cooked
ANK brown	4.55± 0.55^a	3.91± 0.08^a	10.06±0.73^b	3.05±0.52^b	0.65± 0.02^a	0.04±0.13^b
ANK Black	2.60±0.26^b	1.82±0.02^b	11.52±0.78^a	3.22±0.30^a	0.69± 0.01^a	0.11± 0.01^a

Data presented as Mean ± SE (n=3).
Mean values in a column superscripted by different letters are significantly different at p < 0.05.
Anti-nutritional factors are expressed as (mg/g) in dry weight basis



*Anti-nutritional compounds are expressed as (mg/g) in dry weight basis

Fig 1: Anti nutritional compounds in raw and cooked seeds of horse gram

The phytic acid contents in raw ANK brown and ANK Black were 4.55±0.55 mg/g and 2.60±0.26 mg/g where as in cooked form 3.91±0.08 mg/g and 1.82±0.02 mg/g respectively. In both raw and cooked form in two varieties phytic acid contents were significantly differed. Phytic acid contents in raw seeds were in accordance with the range stated by Deshpande *et al.* 1982 whereas the phytic acid content of legumes varies from 4.0 - 20.0 mg/g [54]. Phytic acid has been implicated in influencing the cooking quality of legumes. Phytic acid chelates divalent cations (Ca, Mg) and prevents their cross linking with pectin, thereby facilitating cell wall dissolution during the cooking process [55]. The saponin contents in raw ANK brown and ANK Black were 10.65±0.73 mg/g and 11.52±0.76 mg/g where as in cooked form 3.05±0.16 mg/g and 3.22±0.19 mg/g respectively. In both raw and cooked form in two varieties, saponin content were significantly (p<0.05) differed. The result of saponin reduction as a percentage after autoclaving is ~70%. The tripsin inhibitory compounds in raw ANK brown and ANK Black were 0.65±0.02 mg/g and 0.69±0.01 mg/g where as in cooked form 0.04±0.13 mg/g and 0.11±0.01 mg/g respectively. In the cooked form of two varieties, tripsin inhibitor contents were significantly (p<0.05) differed while in raw form a significant different was not seen. It was observed that in the process of autoclaving the most of the tripsin inhibitory compounds were destroyed.

3.6 Anti-oxidant properties

Natural phenolic compounds exert their health beneficial effects mainly through their antioxidant activity by protecting against oxidative damage [56].

The widely accepted significant amount of antioxidant activity is present in the phenolic compounds. Those compounds are capable of scavenging initial free radicals, binding metal ions, decomposing primary products of oxidation to non-radical species and breaking chains to prevent continuous hydrogen abstraction from substances [57]. Different solvent extraction methods have been used to extract plant materials e.g. Water, acetone, methanol (absolute, 70%), ethanol (absolute, 70%), acetone (50%, 80%) etc. However, completeness of extraction and subsequent effects of extraction on antioxidant activity are not fully understood. Generally, the chemical extractions methodologies depend on the type of solvent, pH, extraction time, temperature and physical characteristics of sample [58].

Antioxidant properties were determined as total reducing capacity by Ferric Reducing Antioxidant Power (FRAP), Oxygen Radical Absorbance Capacity (ORAC), DPPH Radical and ABTS Radical Scavenging capacity [59,60,61,62]. In the present study anti-oxidant properties of raw and cooked form of horse gram seeds were presented in Table 4 and Table 5 (Figure 2).

Table 4: Anti-oxidant properties of raw seeds of horse gram varieties

Variety	Antioxidant Properties				
	TPC	TFC	DPPH	ABTS	FRAP
ANK brown (mg/g)	3.09±0.11^{b}	1.40±0.04^{a}	4.71±0.42 ^{a}	8.22±0.49^{b}	0.27±0.08^{a}
ANK black (mg/g)	3.88±0.21^{a}	0.88±0.03^{b}	4.11±0.42 ^{b}	8.87±0.10^{a}	0.24±0.08^{b}

Data presented as Mean ± SE (n=3). Mean values in a column superscripted by different letters are significantly different at $p < 0.05$.

TPC: Total Polyphenolic Content TFC: Total Flavonoids Content

TPC expressed as: mg Gallic acid equivalents / g, TFC expressed as mg Quercertin equivalents/ g of whole seed in flour in dry weight basis.

Activity of DPPH, ABTS, FRAP are expressed as mg Trolox equivalents/ g whole seed flour in dry weight basis.

Table 5: Anti-oxidant properties of cooked seeds of horse gram varieties

Variety	Antioxidant Properties				
	TPC	TFC	DPPH	ABTS	FRAP
ANK brown (mg/g)	1.30±0.03^{b}	0.87±0.07	2.18^{b} ±0.45	2.76±0.08^{b}	0.11±0.00^{b}
ANK black(mg/g)	1.68±0.12^{a}	1.35±0.13	3.17±0.60 ^{b}	3.38±0.45^{a}	0.17±0.01^{b}

Data presented as Mean ± SE (n=3). Mean values in a column superscripted by different letters are significantly different at $p < 0.05$.

TPC: Total Polyphenolic Content TFC: Total Flavonoids Content

TPC expressed as mg Gallic acid equivalents/ g TFC expressed as mg Quercertin equivalents/ g of whole grain flour in dry weight basis.

Activity of DPPH, ABTS, FRAP are expressed as mg Trolox equivalents/ g whole grain flour in dry weight basis.

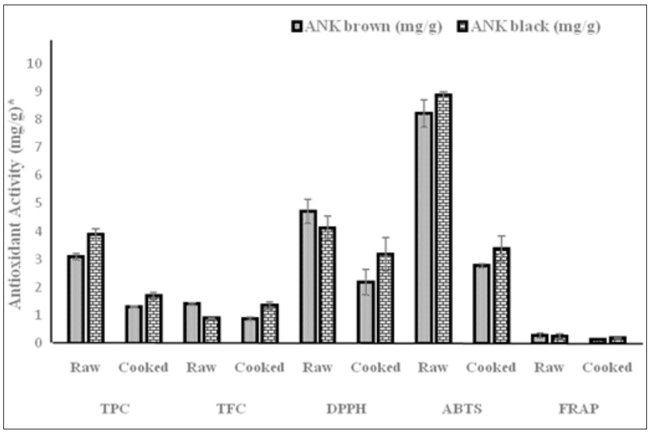


Fig 2: Antioxidant properties in raw and cooked varieties of horse gram

TPC: Total Polyphenolic Content TFC: Total Flavonoids Content

*TPC: mg Gallic acid equivalents/ g and TFC: mg Quercertin equivalents/ g of whole grain flour in dry weight basis.

*Activity of DPPH, ABTS, FRAP are expressed as mg Trolox equivalents/ g whole grain flour in dry weight basis.

The TPC of 70% ethanolic extracts of ANK-Brown and ANK-Black are 3.09 ±0.11 mg/g and 3.88 ± 0.21mg/g respectively and they are significantly ($p < 0.05$) varied. The previous study reported that the phenolic content of legumes varied in the range of 0.325 -6.378 mg GAE/g [63]. Further author had reported that horse gram is under the high phenolic acid group and it contained 3.58±0.072 mg GAE/g. Sreerama *et al.* 2010, reported that the polyphenolic content of Indian pulses and legumes ranged from 0.62-4.18 mg/g[64]. Those results are already in agreement with the present results. The cooked horse gram seeds were shown around 2-fold reduction of total polyphenolic content (Table 4). The order of potency of ethanolic extracts of horse gram for TPC were raw > cooked form. The principal phenolic compound of horse gram seeds is flavonoids (quercetin) and contents were given in Table 4. Flavonoids are widely spread secondary metabolites including flavones, flavanols etc. and which have shown the protective mechanism against oxidative stress [58]. Further they have analyzed the TFC in legume varieties including green pea, black bean, chickpea, lentil, soya beans and kidney beans which ranged from 0.08 - 3.21mg/g. Those results are already in agreement of the present study. It is seen that cooking reduced the TFC in ANK brown while in ANK black reduction has not been shown. It may be due to the solubility of the compounds during the cooking process.

3.7 Anti-oxidant activity
The free radicals produced in the body are associated with the chronic diseases specially a certain type of cancers. The dietary antioxidants are capable of capturing free radicals and preventing risk of diseases. Therefore, it is important to study the radical scavenging activity in food. DPPH is a stable free radical which produces violet colour in ethanol. Reduction of DPPH radicals by antioxidants reflects by loss of absorbance. The radical scavenging activity of added substance correlates to the degree of discoloration and thereby measurements were interpreted by DPPH radical scavenging activity of raw and cooked (70% ethanolic extracts) form of horse gram in Table 4 and Table 5. The significant differences ($p < 0.05$) were observed between horse gram varieties as well as form of raw and cooked. Ethanolic extract of raw seeds exhibited significantly high ($p < 0.05$) DPPH radical scavenging activity compared to ethanolic extracts of cooked form. The order of potency of ethanolic extracts of horse gram for DPPH radical scavenging activity is raw > cooked form. DPPH activity of commonly consumed legumes varieties were reported by Sreerama *et al.* 2010 and results were varied from 0.26 - 1.07 mg/g [64]. But horse gram was not included in their study.

Ferric Reducing Antioxidant Power (FRAP) is basically depends on the reduction of ferric tripyridyltriazine (Fe (111)-TPTZ) complex to ferrous tripyridyltriazine (Fe (11)-TPTZ) complex by a reduction of antioxidant at low pH. In comparison to other test of antioxidant power this assay produces simply, speedy, inexpensive and highly reproducible results [37]. FRAP of 70% ethanolic extracts of horse gram is given in Table 4 and Table 5. Results demonstrated the significant differences ($p < 0.05$) between horse gram varieties as well as form of raw and cooked. Raw form of seeds exhibited significantly high ($p < 0.05$) for FRAP. The order of potency of ethanolic extracts of horse gram for FRAP was raw> cooked.

ABTS radical scavenging activity of 70% ethanolic extracts of raw and cooked horse gram varieties are presented in Table 4 and Table 5. ABTS radical scavenging activity showed a significant difference ($p < 0.05$) between varieties as well as forms of raw and cooked. Raw seeds showed the highest activities for ABTS. The order of potency of ethanolic extracts of horse gram for FRAP was raw> cooked.

4. Conclusion
According to the results obtained horse gram contained high content of protein (22.0 -24.2%), crude fiber (6.7- 6.9%) and minerals (3.6%) specially iron, zinc and Ca. Besides nutritional importance horse gram had high content of Dietary Fiber (DF); 21.2% and Resistant Starch (RS); 10.5% leading to low Glycaemic Index (GI); 39.8. The antioxidant properties in seeds exhibited by TPC, TFC, DPPH, ABTS and FRAP were significantly ($p<0.05$) differed with respect to varieties and cooking.

5. Acknowledgement
Authors are acknowledged the financial support given by the Treasury Grant (TG18/147) which was given to ITI by Ministry of Science and Technology.

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