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Research Article

Nutritional and Antioxidant Properties of the Seeds of *Vigna unguiculata* subsp. *sesquipedalis* (L.) Verdc. – An Underutilized Legume of West Bengal

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Abstract

The exploration of underutilized crops for nutritional benefits is one of the major strategies to feed the ever-increasing population. There are many nutritionally rich leguminous crops (*Vigna unguiculata*, *Cassia hirsuta*, *Canavalia ensiformis*, *Dolichos biflorus*, etc.) that have remained unexplored for a long time, though could provide a cheap and alternative food source. The present study was conducted to assess the nutritional and antioxidant properties of the seeds of an underutilized legume *Vigna unguiculata* subsp. *sesquipedalis* (L.) Verdc. The methanolic seed extract showed concentration-dependent radical scavenging activities against DPPH, ABTS, NO, OH and FRAP. The seed extract also showed the presence of nutritional and antioxidative phytochemicals *viz.* alkaloids, saponins, tannins, phenolics, flavonoids, ascorbic acid etc. The presence of a considerably high amount of protein and a low amount of total sugar can also be regarded as beneficial for regular consumption. Moreover, HPLC-MS analysis also revealed the presence of several phenolic derivatives (gallic acid, pyrogallol, chlorogenic acid, catechol etc.) that might also contribute to the antioxidative property of the seeds. Further research on the isolation, purification and characterization of the antioxidative phytocompounds would help decipher their mechanism of action as well as increase the sustainable utilization of the nutritionally rich legume crop.

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Introduction

Legumes are one of the most important crops that have been cultivated worldwide for ages (Messina, 1999). The nutritional value of legumes has long been appreciated and occupied a major part of traditional diets in many countries (Setchell and Radd, 2000). Legumes belong to the family Leguminosae (Fabaceae) which are consumed directly by human beings commonly as mature dry seeds but occasionally as immature seeds enclosed in pods (Adel et al., 1980). The leguminous grains also known as pulses are valued for their high-quality protein with a good amino acid profile (Amarteifio and Mololo, 1998). They also contain complex carbohydrates (oligosaccharides, dietary fibres and resistant starch), vitamins (vitamin-B, folates, ascorbic acid and tocopherols), minerals as well as antioxidants. (Tiwari et al., 2011; Venter and Eyssen, 2001). Legumes are also very good sources of water-soluble vitamins, especially thiamine (vitamin B_1), riboflavin (vitamin B_2), niacin (vitamin B_3), pyridoxine (vitamin B_6) and folate. They are an excellent source of several minerals, including calcium, copper, iron, magnesium, phosphorus, potassium and zinc (Venter and Eyssen, 2001; Mirabelli and Shehab, 2008). In addition to their food value, legumes are important in agricultural systems because of their ability to fix atmospheric

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nitrogen thereby increasing the overall fertility of the soil (Tiwari et al., 2011).

Recently, population explosion and limitations in crop production pose a big challenge for sustainable utilization of food crops all over the world. Due to scarcity, high cost and unreliable supply of healthy food in developing and underdeveloped countries, the cheap and alternative sources of healthy and nutritious foods need to be explored. The use of underutilized legume plants in this regard can help fight malnutrition associated problems and therefore enhance the overall health status of the rural and economically backward population (Salvi and Katewa, 2016). The use of indigenous and locally cultivated plants has long been an intimate part of many communities worldwide. Also, the use of underutilized species plays a key role in keeping alive the cultural diversity associated with food habits, health practices, religious rituals and social exchanges. Focusing attention on neglected and underutilized species is therefore an effective way to combat micronutrient deficiency, the so-called 'hidden hunger' and other dietary deficiencies, particularly among the economically vulnerable social groups in developing countries (Salvi and Katewa, 2006).

Underutilized plants contribute immensely to food security and serve as means of survival during unfavourable situations of drought, famine, flood etc. (Assefa and Abebe, 2011). They can also supplement nutritional requirements owing to their better nutritional value (Van Andel, 2006). With an alarming increase in human population and depletion of natural resources, it has been felt necessary to explore the possibility of using under-explored plant resources having potential for food, fodder, energy and industrial usage. Many neglected and underutilized species are relatively nutritionally rich and are also adapted to low input agriculture. The erosion of these species can have immediate consequences on the nutritional status and food security of the poor (Vodouh et al., 2012). As the lower income group of the population is particularly vulnerable, it is suggested that attention must be given to easily available, accessible, cheap but nutritious plant protein sources to improve the nutritional status of the low-income group of the population (Iqbal et al., 2006; Khattab et al., 2009). Different types of underutilized legumes have been reported to be used by people including *Vigna unguiculata* ssp. *cylindrica*, *Mallotus subulatus*, *Cassia hirsutta*, *Canavalia ensiformis*, *Cajanus cajan*, *Sphenostylis sterocarpa*, *Vigna subterranean*, *Vigna racemosa, Clitoria fairchildiana*, *Cucumis dipsaceus*, *Lupinus angustifolius*, *Afzelia africana*, *Brachystegi acurycoma*, *Detarium microcarpum*, *Mucuna flagellipes*, *Mucuna pruriens*, *Mucuna deeringiana*, *Mucuna monosperma*, *Macrotyloma uniflorum*, *Dolichos biflorus*, *Phaseolus mungo*, *Rhynchosia filipes*, *Vigna trilobata*, *Vigna mungo*, *Atylosis scarabacoides*, *Dolichos trilobus*, *Teramnus labialis*, *Cicer arietinum* (Fernandez and Berry, 1988; Rajyalakshmi and Geervani,1994; Arinathan et al., 2003).

In the present study, an assessment of some nutritional and antioxidant properties of *Vigna unguiculata* seeds – an underutilized legume of West Bengal has been undertaken. The legume though consumed in the villages as a vegetable and rarely as a pulse is not so popular among the people even in smaller towns and cities and is not even considered an important crop from the point of food consumption. Therefore, the analysis of its nutritional and antioxidant properties would reveal some important findings to consider for wider usage as a food crop.

Materials and Methods

Collection of plant material

The seeds of Vigna unguiculata subsp. sesquipedalis (L.) Verdc. (Local name: Borboti) was collected from the market of the Mokdumpur area of Malda District (**Fig. 1a**).

Extraction of seed components for antioxidant analysis

The seeds were washed 2-3 times with distilled water, shade dried for 5-7 days, ground to a fine powder and stored in an airtight container at room temperature in the dark for further use. 10 g of shade-dried powder was extracted with 50 mL methanol in an orbital shaking incubator at room temperature for 48 h (Patil and Gaikward, 2010). The solvent extract was then filtered using Whatman no. 1 filter paper and concentrated at 60 ºC in a hot air oven. After drying, the extract was finally dissolved in dimethyl sulfoxide (DMSO) and stored in a refrigerator at 4 ºC for further use (**Fig. 1b,c**).

Solvent extractive value

The methanolic extractive value of the seed extract was calculated as the percentage yield of the final residue according to the following formula:

Solvent extractive value (%) = W2 – W1 / W2 X 100

Where W1 is the weight of the final residue after extraction (g) and W2 is the weight of powder taken for extraction (g).

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Fig. 1 (a) Seeds of *Vigna unguiculata* subsp. *sesquipedalis*, (b) Powdered seeds, (c) Methanolic extract of seeds

Phytochemical screening of the seed extract

Terpenes
To 2 mL of methanolic extract 2 mL of 5% $FeCl_3$ was added. The formation of yellow-brown precipitate indicated the presence of tannins in the sample (Parekh and Chanda, 2007).

Alkaloids
2 mL of extract was added to 1.5 mL of 1(N) HCl and the mixture was incubated in a water bath. After that, 6 drops of Wagner's reagent were added. The formation of orange precipitate indicated the presence of alkaloids (Ogunyemi, 1979).

Saponins
Aqueous extract of 2 g powder was made and subjected to the frothing test. Frothing persistence indicated the presence of saponins. Later the froth was mixed with a few drops of olive oil. The formation of emulsion further confirmed the presence of saponins (Sofowora, 1993).

Cardiac glycosides
To 2 mL of extract, 1 mL of glacial acetic acid and 1-2 drops of $FeCl_3$ were added followed by 1 mL of concentrated H_2SO_4. The appearance of a brown ring at the interface of two liquids indicated the presence of cardiac glycosides (Trease and Evans, 1989).

Flavonoids
To 2 mL of methanolic extract, a few drops of concentrated HCl was added followed by the addition of 0.5 g of zinc powder. After 3 min, the appearance of magenta-red colour indicated the presence of flavonoids (Parekh and Chanda, 2007).

Phenolics
1 mL of 1% ferric chloride solution was added to 2 mL of extract. The blue colour developed indicated the presence of phenolics (Martinez and Valencia, 2003).

Free amino acids
To 1 mL extract, 0.25% w/v ninhydrin reagent was added and boiled for a few mins. The formation of blue colour indicated the presence of amino acids.

Tannins
2 mL of extract was mixed with 5mL of $CHCl_3$, and 1 mL of conc. H_2SO_4. Reddish-brown colouration at the junction of two layers was observed which indicated the presence of terpenes.

Anthraquinones
5 mL of the methanolic extract was boiled with 5 mL of H_2SO_4 and filtered hot. The filtrate was shaken with 10 mL of $CHCl_3$. The $CHCl_3$ layer was pipetted into another test tube and 1mL of 10% ammonia was added. The resulting solution was observed for the appearance of a rose pink colour which indicated the presence of anthraquinones.

Phlobatannins
To 1mL of extract, 1% aqueous HCl was added and boiled with the help of a hot plate stirrer. The formation of red coloured precipitate confirmed a positive result.

Quantitative evaluation of some nutritional and antioxidative components

Estimation of total and reducing sugar
Total and reducing sugar was extracted following the method of Harborne (1973). 1g of seed powder was extracted in 10 mL of 95% ethanol and the alcoholic fraction was evaporated in a boiling water bath. The residue was re-extracted with ethanol and the process was repeated three times. Then the residue was dissolved in distilled water and the final volume was made up to 5 mL which was then centrifuged at

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5000 rpm for 10 mins. The supernatant was collected and used for estimation.

Estimation of total sugar was done by Anthrone reagent following the method of Plummer (1978). To 1 mL of test solution, 4 mL of Anthrone reagent (0.2% Antrone in conc. H_2SO_4) was added. Then the reaction mixture was cooled under running tap water and absorbance was measured in a colorimeter at 620 nm and the total sugar content was quantified using a standard curve of D-glucose.

Reducing sugar was estimated by the Nelson-Somogyi method as described by Plummer (1978). 1 mL of the test solution was mixed with 1 mL of alkaline copper tartarate solution (2 g $CuSO_4$, 12 g Na_2CO_3 anhydrous, 8 g Na-K tartrate, 90 g Na_2SO_4 anhydrous in 500 mL of distilled water) and heated over a boiling water bath for 20 mins. The reaction mixture was then cooled under running tap water. After that, 1 mL of Nelson Arseno-molybdate reagent was added along with 2 mL of distilled water and mixed vigorously. A blue colour was developed, the absorbance of which was measured in a colorimeter at 515nm. Quantification of reducing sugar content was done using a standard curve of D-glucose.

Estimation of protein

Soluble protein from the seeds was extracted by the previously described standard protocol (Chakraborty et al., 1995). 1g of tissue was homogenized in a pre-chilled mortar and pestle using 5mL of 50 mM sodium phosphate buffer (pH 7.2) and PVP (polyvinyl pyrrolidone) under ice-cold conditions and centrifuged at 10,000 rpm at 4 °C for 15 mins. The supernatant was used for the estimation of protein.

Quantification of soluble protein was done following the method of Lowry (1951). To 1 mL of supernatant, 5 mL of alkaline reagent (A: 2% sodium carbonate in 0.1(N) NaOH and B: 2% sodium potassium tartrate and 1% $CuSO_4$, then mix A and B in 50:1 ratio) was added, mixed well and incubated at room temperature for 15 mins. After incubation, 0.5 mL of Folin-ciocalteau's reagent was added to the mixture and incubated for 10-15 min. The absorbance was measured at 660 nm.

Estimation of total and ortho-dihydroxy phenol

The phenols were extracted by the method given by Mahadevan and Sridhar (1982). 1 g of seed powder was soaked in 5mL of boiling absolute alcohol in dark for 10 mins. After cooling, the sample was crushed with 80% alcohol and then filtered in a dark chamber. The residue was re-extracted with 80% alcohol and then the final volume was made up to 10 mL with 80% alcohol.

Total phenols were estimated following the previously described protocol of Bray and Thorpe (1954). To 1 mL of extract, 1mL of diluted Folin ciocalteu's reagent (1:1 v/v) and 2 ml of 20% Na_2CO_3 solution was added. The reaction mixture was boiled in a water bath for 1 min and then cooled under running tap water and the final volume was made up to 25 mL by adding distilled water. The absorbance was measured at 650 nm in a colorimeter and quantified using a standard curve of catechol.

Estimation of ortho-dihydroxy phenols was done by the method of Arnow (1937). 1 mL of extract was added with 2 mL of 0.5N HCl, 1 mL of Arnow's reagent (10g $NaNO_2$ and Na_2MoO_4 in 100 ml of dH_2O) and 2 mL of 1 N NaOH. A pink colour was developed and the volume of the reaction mixture was made up to 10 mL with distilled water. After vigorous shaking, the absorbance was measured at 515 nm in a colorimeter and quantified using a standard curve of caffeic acid.

Estimation of ascorbate

1 g of seed powder was crushed with 6% trichloroacetic acid and then filtered using Whatman no. 1 filter paper following the protocol of Mukherjee and Choudhuri (1983). The final volume of the filtrate was made up to 10 mL by adding distilled water. 4 mL of extract was added with 2 mL DNPH (2% dinitrophenyl hydrazine). The reaction mixture was kept in a boiling water bath for 10 mins and cooled at room temperature. Then 5 mL of 80% v/v H_2SO_4 was added and absorbance was read at 530 nm. The amount of ascorbate was calculated using a standard curve of ascorbic acid.

Estimation of saponins

Total saponin content was determined based on vanillin-sulphuric acid colorimetric reaction with some modifications (Makkar et al., 2007). About 0.5 mL of plant extract was added with 0.5 mL of distilled water. About 0.5 mL of vanillin reagent (800 mg of vanillin in 10 ml of 100% ethanol) and 2.5 mL of 72% sulphuric acid were added to the mixture and mixed well. This solution was kept in a water bath at 60 ºC for 10 min. After 10 min, it was cooled under ice-cold water and the absorbance was read at 544 nm. The amount of saponins was expressed as diosgenin equivalents (mg DE/ g extract) derived from a standard curve of diosgenin.

Estimation of alkaloids

1g of seed powder was extracted with 40 mL of 10% acetic acid in a 250 mL beaker and covered to stand

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for 4 h. After that, the mixture was filtered and the volume was reduced to one quarter using a water bath. To this sample, concentrated NH_4OH solution was added drop-wise until the precipitation was completed. The solution was allowed to settle and the precipitate was collected by filtration and weighed. The amount of total alkaloid was calculated as the residual weight obtained after extraction (Senguttuvan et al., 2014).

Estimation of tannins

The total tannin content was estimated using the procedure described by Broadhurst et al. (1978). To 0.5 mL of seed extract, 3 mL of vanillin reagent (4% in methanol) was added. Then 1.5 mL of concentrated HCl was added and the mixture was incubated for 15 min. The absorbance was measured at 500 nm (Senguttuvan et al., 2014).

Estimation of total flavonoids

The total flavonoid content was estimated following a previously standardized protocol (Senguttuvan et al. 2014). 1 mL of seed extract was diluted with 0.2 mL of distilled water followed by the addition of 0.15 mL of sodium nitrite (5%) solution. This mixture was incubated for 5 min and then 0.15 mL of aluminium chloride (10%) solution was added and allowed to stand for 6 min. Then 2 mL of sodium hydroxide (4%) solution was added and the final volume was made up to 5 mL with distilled water. The mixture was shaken well and allowed to stand for 15 min at room temperature. The absorbance was measured at 510 nm. The appearance of the pink colour showed the presence of flavonoid content. The total flavonoid content was expressed as rutin equivalent mg RE / g extract on a dry weight basis using the standard curve of rutin.

In vitro antioxidant activity of seed extract

DPPH free radical scavenging activity

Different concentrations of plant extracts (0.1mL) were put in the test tube and 2.9 mL of a methanol solution of DPPH (0.1 mM) was added (Blois et al., 1958). The mixture was kept in the dark at room temperature for 30 min and absorbance was measured at 517 nm against a blank. Ascorbic acid was used as standard. The following equation was used to determine the percentage of the radical scavenging activity of each extract.

DPPH scavenging (%) = 100 X $(A_0 - A_s) / A_0$

Where A_0 is the absorbance of blank and A_s is the absorbance of the sample.

ABTS free radical scavenging activity

The ABTS (2,2-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging activity was done following the method of Re et al. (1999). The ABTS radical cation ($ABTS^+$) was produced by the reaction of a 7 mM ABTS solution with potassium persulphate (2.45mM). The $ABTS^+$ solution was diluted with ethanol to an absorbance of 0.70 ± 0.05 at 734 nm. The mixture was stored in the dark at room temperature for 12 h. before use. After the addition of 0.25 mL of extract to 2 mL of diluted $ABTS^+$ solution, absorbance was measured at 734 nm after exactly 6 min. The decrease in absorption was used for calculating scavenging effect values. Ascorbic acid was used as standard. The following equation was used to determine the percentage of the radical scavenging activity of each extract.

ABTS scavening (%) = 100 X $(A_0 - A_s) / A_0$

Where A_0 is the absorbance of blank and A_s is the absorbance of the sample.

FRAP assay

The FRAP (Ferric reducing antioxidant potential) was determined using a previously described method with slight modifications (Benzie and Strain, 1996). The fresh FRAP reagent was prepared by mixing 500 mL of acetate buffer (300 mM; pH 3.6), 50 mL of 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) (10 mM), and 50 mL of $FeCl_3 \cdot 6H_2O$ (50 mM). The spectrophotometric measurement was performed at 593 nm and the measurement was monitored with 75 µL of each extract and 2 mL of FRAP reagent.

Hydroxyl radical scavenging activity

The scavenging activity of the extracts on hydroxyl radical was measured according to a previously described method (Yu et al., 2004). To 2 mL of extract, 400 µL of $FeCl_2$ (1 mM), 800 µL of 1,10-phenanthroline (1 mM) and 1 mL of H_2O_2 (0.6 %) were added. The mixture was then homogenized using a vortex and incubated at room temperature for 5 min. The absorbance was read at 560 nm against the blank. The percentage of the radical scavenging activity of each extract was calculated from the equation below:

OH radical scavenging (%) = 100 X $(A_0 - A_s) / A_0$

Where A_0 is the absorbance of blank and A_s is the absorbance of the sample.

NO scavenging activity

NO (Nitric oxide) scavenging activity was determined according to the Griess-Illosvoy reaction (Garratt, 1964). The reaction mixture contained 2

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mL of sodium nitroprusside (10 mM) in 0.5 mL phosphate buffer (0.5 M; pH 7.4). Various concentrations of the extract (0.5 ml) were added in a final volume of 3 mL. After incubation for 60 min at 37 ºC, Griess reagent [N-(1-Naphthyl) ethylenediamine (0.1%) and sulphanilic acid (1%) in H_3PO4 (5%)] were added. The pink chromophore generated during diazotization of nitrite ions with sulphanilamide and subsequent coupling with N-(1-Naphthyl) ethylenediamine was measured spectrophotometrically at 540 nm. Ascorbic acid was used as a positive control. The scavenging ability (%) of the nitric oxide was calculated using the formula:

NO scavenging effect (%) = 100 X (A_0 - A_s) / A_0

Where A_0 is the absorbance of blank and A_s is the absorbance of the sample.

HPLC of phenolics

Extraction of total phenolics from the dried samples for HPLC analysis was done following the standard protocol with slight modifications (Pari and Latha, 2005). 2 g of powdered sample was soaked in 10 mL of methanol and kept overnight in dark. After 12 h of soaking, the suspension was filtered and the filtrate was completely evaporated using a rotary evaporator at 40°C and lyophilized. The lyophilized extract was re-dissolved in 1 mL of HPLC grade methanol and filtered through a millipore membrane filter (0.45µm). HPLC analysis of total phenolics present in the sample was done following the method described by Pari et al. (2007). The analysis was done using High-Performance Liquid Chromatography (Shimadzu) equipped with HPLC pumps (model LC 10ATVP), UV-Vis detector (SPD-10AVP) and C18 column. The flow rate of 1mL/min, injection volume of 20 µL and binary gradient elution of HPLC grade acetonitrile-water–acetic acid (5:93:2, v/v/v) [solvent A] and acetonitrile-water–acetic acid (40:58:2, v/v/v) [solvent B], starting with solvent B from 0 to 100% over 20 min were applied. The separation of compounds was monitored at 280 nm. The identification of the phenolic compounds was done using the correlations with the standards such as gallic acid, pyrogallol, resorcinol, catechol, catechin, chlorogenic acid, caffeic acid and vanillic acid. The retention time of the standard phenolics are given below:

Table 1 Retention time of standard phenolic compounds

Sl. No.	Phenolic standards	Retention time (in mins)
1	Gallic acid	5.33 - 5.34
2	Pyrogallol	5.75 - 5.77
3	Resorcinol	9.06 - 9.07
4	Catechol	10.15 - 10.25
5	Catechin	14.91 - 14.95
6	Chlorogenic acid	15.65 - 15.69
7	Caffeic acid	16.64 - 16.68
8	Vanillic acid	17.23 - 17.54

Results

Qualitative screening of the seed extract

The phytochemical screening of the seed extract revealed the presence of several phytoconstituents *viz.* tannins, alkaloids, saponins, cardiac glycosides, flavonoids, phenolics, phlobatannins, amino acids, and protein was observed. Among the phytoconstituents, a stronger presence of phenols, flavonoids, alkaloids, tannins, terpenes, anthraquinones and proteins as enlisted in Table 2.

Antioxidant and nutritional status of seed extract

Methanolic extractive value of Vigna seed powder was calculated to be 9% which means that approximately 9 g of phytoconstituents could be extracted using methanol from 100 g of the crude powder. Among the secondary metabolites, a considerable amount of phenols, flavonoids and alkaloids was observed (Table 3). The amount of phenols (1.7 ± 0.11 g/100g) was the highest among

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the secondary metabolites followed by that of the alkaloids (1.14 ± 0.06 g/100g). Other antioxidants like ascorbic acid (0.43 ± 0.09 g/100g) were also present in a significant amount. Quantitative estimation showed that the amount of proteins (8.15 ± 0.5 g/100g) in the seed extract was highest in comparison to the other nutritional components (Table 3). Other nutritional components like sugars, fats and free fatty acids were also quantified and presented in Table 3.

Table 2 Phytochemical screening of the seed extracts

Phytoconstituents	Qualitative tests	Observation	Inference
Tannin	Ferric chloride test	Yellow-brown precipitate	++
Alkaloids	Wagners test	Orange precipitate	++
Saponins	Frothing test	Formation of frothing bubbles	+
Cardiac glycosides	Keller-Kiliani test	Violet ring bellow the brown ring	+
Flavonoids	Shinoda's test	Pink colour	++
Phenolics	Folin-ciocalteau test	Blue colour	+++
Phlobatannins	HCl test	Red precipitate	+
Amino acids	Ninhydrin test	Blue colour	+
Terpenes	H_2SO_4 test	Reddish-brown precipitate	+
Anthraquinolins	Ammonia	Rose pink	+
Protein	Biuret test	Violet colour	++

+++ Strong presence; ++ Moderate presence; + Low presence

In vitro antioxidative activity of the seed extract

DPPH radical scavenging activity

The DPPH scavenging activity of the seed extract showed a concentration-dependent activity as shown in **Fig. 2a**. The standard antioxidant ascorbic acid showed a relatively higher DPPH radical scavenging activity. The DPPH scavenging of *Vigna* seed extract ranged from 0-40% for the tested concentrations; whereas the scavenging activity of ascorbic acid ranged from 90-100%. Here IC_{50} value could not be calculated. The inhibition percentage against the concentration of each extract required to reduce 25% of the DPPH radical was therefore determined. The IC_{25} value for *Vigna* seed extract was calculated to be 3.9 mg/mL.

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ABTS cation scavenging activity

The ABTS scavenging activity of the seed extract was comparatively better in comparison to that of the DPPH scavenging activity. The ABTS scavenging activity of ascorbic acid was relatively higher than the seed extract (**Fig. 2b**). The ABTS scavenging of *Vigna* seed extract ranged from 10-80% for the tested concentrations, whereas the scavenging activity of ascorbic acid ranged from 80-100%. The IC_{50} value for the *Vigna* extract was calculated to be 0.48 mg/mL.

Table 3 Amount of some important antioxidant and nutritional components present in seed extract

Parameters quantified	Amount (g/100g)
Antioxidants components	
Total phenol	1.7 ± 0.11
Ortho-dihydroxy phenol	0.31 ± 0.04
Flavonoids	0.15 ± 0.05
Saponins	0.011 ± 0.006
Tannins	0.015 ± 0.007
Ascorbic acid	0.43 ± 0.09
Alkaloids	1.14 ± 0.06
Nutritional components	
Protein	8.15 ± 0.5
Total sugar	1.2 ± 0.07
Reducing sugar	0.081 ± 0.05
Fats	0.5 ± 0.03
Free fatty acids	0.08 ± 0.007

NO radical scavenging activity

The NO radical scavenging activity of the seed extract compared to ascorbic acid is shown in **Fig. 2c**. The NO radical scavenging activity of *Vigna* seed extract ranged from 5-30% for the tested concentrations, whereas the scavenging activity of ascorbic acid ranged from 78-92%. Here IC_{50} value could not be calculated. The IC_{25} value for the extract was determined to be 0.53 mg/mL.

OH radical scavenging activity

The OH radical scavenging activity of the extract compared to ascorbic acid is shown in **Fig. 2d**. The OH radical scavenging activity of *Vigna* seed extract ranged from 42-80% for the tested concentration, whereas the scavenging activity of ascorbic acid ranged from 4-62%. Therefore, the OH radical scavenging activity was somewhat comparable to ascorbic acid. The IC_{50} value for the seed extract was calculated to be about 0.48 mg/mL.

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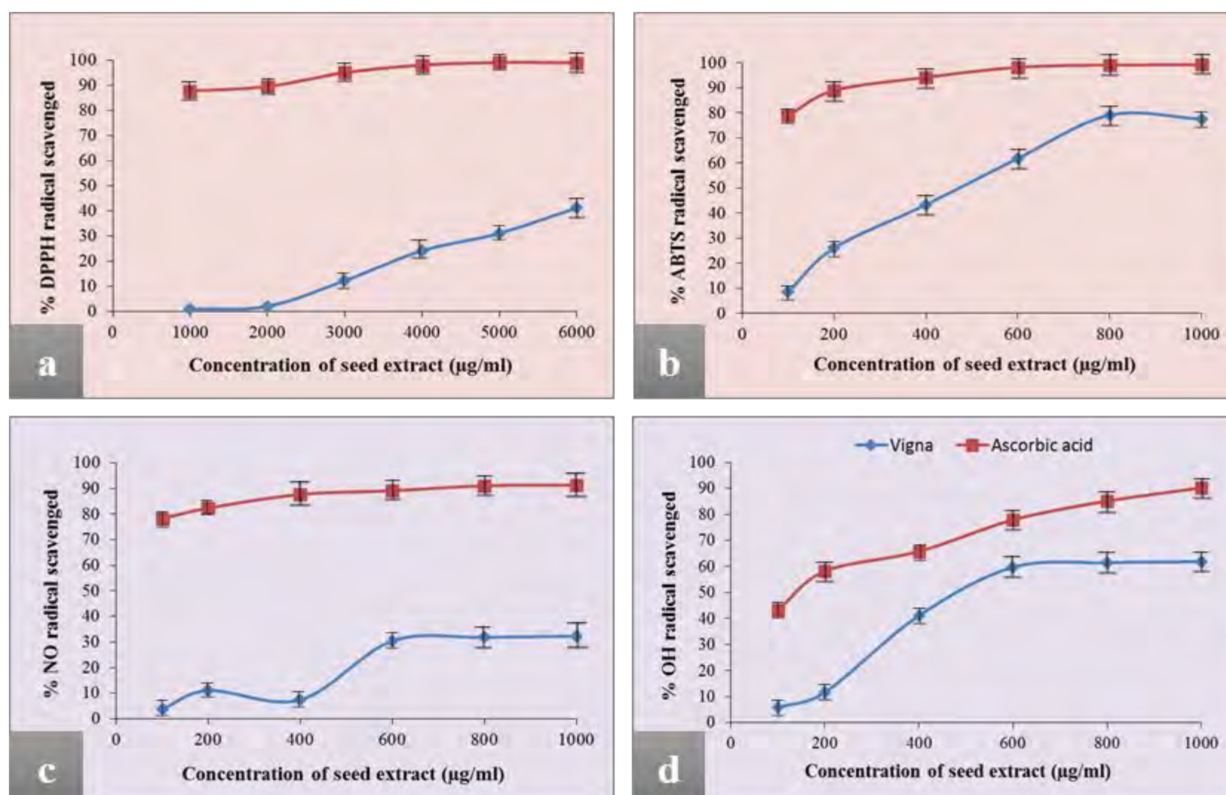

Fig. 2 (a) DPPH scavenging activity, (b) ABTS scavenging activity, (c) NO radical scavenging activity (d) OH radical scavenging activity of the *Vigna* seed extract.

FRAP activity

The scavenging effect of the FRAP radical assay also showed a concentration-dependent activity which was calculated using a standard curve of $FeSO_4$. The FRAP activities of ascorbic acid and *Vigna* seed extract were determined at a concentration of 1mg/mL. Ascorbic acid showed relatively higher FRAP activity (Table 4). The reducing power of seed extract was however found to be 0.053 Fe^{+2} equiv./mg extract.

Table 4 FRAP activities as vcalculated using standard curve of $FeSO_4$

Samples	Conc. of sample (mg/mL)	O.D. (1mg/mL)	Conc. from SC (mM Fe^{2+} equiv.)	FRAP (mM Fe^{2+} equiv./μg extract)
Vigna	1	1.364	1.074015748	0.053700787
Ascorbic acid	1	1.924	1.51496063	0.075748031

HPLC analysis of phenolics

The HPLC chromatogram of the *Vigna* seed extract has shown the presence of some phenolic compounds (**Fig. 3**). Among these some of these compunds has been identified as gallic acid, pyrogallol, catechol, chlorogenic acid, and caffeic acid (peak nos. 5,6,18 31, 34) based on the retention time of standard compounds as shown in Table 5.

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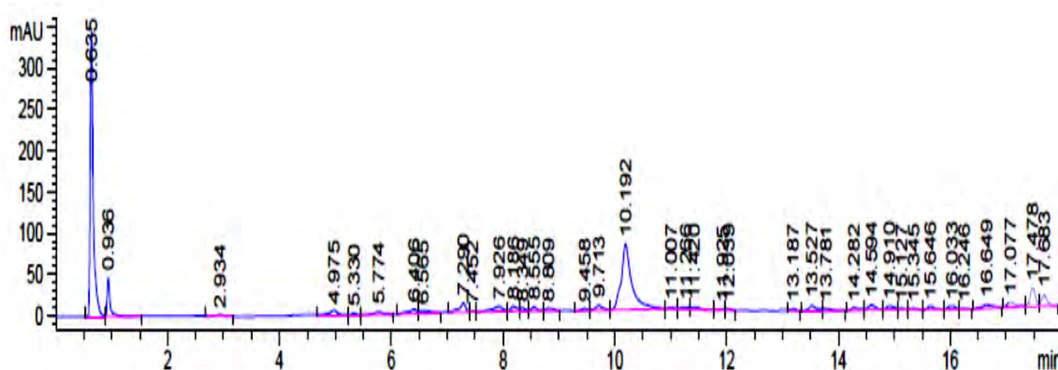

Fig. 3 HPLC profile of phenolics extracted from *Vigna* seed extract.

Table 5 Details of major peaks detected with retention time and peak area

Peak #	RetTime [min]	Width [min]	Area [mAU*s]	Area %
1	0.635	0.0566	1316.45032	33.5963
2	0.936	0.0563	176.71410	4.5098
3	2.934	0.1738	17.21987	0.4395
4	4.975	0.1257	47.35107	1.2084
5	5.330	0.0829	6.31764	0.1612
6	5.774	0.1459	38.93552	0.9936
7	6.406	0.1500	61.49410	1.5694
8	6.565	0.1786	40.61675	1.0366
9	7.290	0.1218	95.85567	2.4463
10	7.452	0.0635	5.38934	0.1375
11	7.926	0.1664	82.05089	2.0940
12	8.186	0.1173	44.81142	1.1436
13	8.349	0.0805	21.68813	0.5535
14	8.555	0.0937	26.04545	0.6647
15	8.809	0.1185	26.35175	0.6725
16	9.458	0.0997	20.73409	0.5291
17	9.713	0.1332	57.09259	1.4570
18	10.192	0.1804	1039.02869	26.5164

Discussion

Legumes are known to be a good source of plant proteins. According to Singh (2017) pulses are an excellent source of protein, sugars, dietary fibre, vitamins and minerals. In the present study, the crude protein content of *Vigna unguiculata* subsp. *sesquipedalis* – an unconventional legume was found to be the highest among all the nutritional and antioxidant components, which is a characteristic feature of the legume seeds. This has been realized that the protein content of the underutilized legume is close to that of other legumes as reported by Bravo et al. (1999) for other lesser-known pulses in India. Therefore, the seemingly higher content of proteins in *Vigna* seeds suggests the potential of these lesser-known legumes being used as alternative sources of protein for the seemingly underprivileged population. Also, the total sugar and reducing sugar content of the *Vigna* seeds were found to be considerably lower enhancing the overall nutritional aspect of the legume.

According to Zia-Ul-Haq et al. (2013), legumes like cowpea are rich in polyphenolic compounds more than other leguminous seeds. In the present study, the presence of a significant number of polyphenolic compounds like phenols, flavonoids and tannins in the *Vigna* seed extract was found. It has also been known that polyphenolic compounds also have beneficial effects due to their antioxidant activity and the ability of phenolic substances including phenolic acids to act as antioxidants has been extensively investigated (Rice-Evans et al., 1997). Tannin is also known to exert beneficial nutritive effects by its ability to form a complex with

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dietary proteins and inhibit endogenous proteins, such as digestive enzymes (Liener, 1994). Researchers have even demonstrated that intake of food rich in flavonoids protects humans against diseases linked with oxidative stress (Shahidi et al., 1995).

Phenolic compounds also consist of phenolic acids produced in plants, and the abundance of these chemicals depends on species and stage of growth (López-Amorós et al., 2006). The presence of phenolics also confers the major proportion of the antioxidant potential of the plant extracts. Sebei et al. (2013) determined eight phenolic compounds in peanut kernels including caffeic acid, dihydroxyphenylacetic acid, syringic acid, p-coumaric acid, rutin trihydrate, naphtoresorcinol, trans-2-hydroxycinnamic acid and dihydrate quercetin. In addition, p-hydroxybenzoic acid and chlorogenic acid were also detected in both peanut kernels and skin (Win et al., 2011). In the present study, the HPLC analysis of phenolics has confirmed the presence of several phenolic compounds in the *Vigna* seed extract.

Apart from being a source of plant proteins, legumes have been known to be rich in polyphenolics making them a very good candidate for free radical scavenging. The DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical is a stable free radical, which has been widely accepted as a tool for estimating the free radical-scavenging activities of antioxidants (Park et al., 2005). Similarly, the ABTS assay is very useful for the assessment of total antioxidant capacity. It is based on the inhibition by antioxidants of the absorbance of the radical cation 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonate), which has an absorption at 734 nm (Igbinosa et al., 2011). Ferric reducing/antioxidant power (FRAP) reflects total antioxidant power involving the single electron transfer reaction (Igbinosa et al., 2011). Nitric oxide (NO) is a key signaling molecule that plays a crucial role in the pathogenesis of various diseases associated with inflammation (Gates et al., 2008). The OH radical generated by the Fenton reaction is a highly reactive free radical. This free radical can be formed from hydrogen peroxide and the superoxide anion and may be generated in the human body under certain physiological conditions (Lee et al, 2004). In the present study, the determination of free radical scavenging activity of the seed extract pointed towards a considerable degree of antioxidant properties present in the seeds. Among the free radicals, the seed extract showed a comparatively better result in the scavenging of ABTS and NO radicals. However, the results were not comparable to the standard antioxidant - ascorbic acid, this may be due to the use of the standard antioxidant in its purest form whereas the antioxidant components in the seed extracts were present along with the other components. Therefore, the purification and identification of antioxidant components from the seed extract will be important to understanding the free radical scavenging attributes of the seed extract.

Conclusion

From the above experimental results, it is evident that the seed extract of *Vigna unguiculata* subsp. *sesquipedalis* showed considerable antioxidant activities *in vitro*. The seed extract showed concentration-dependent scavenging of free radicals viz. DPPH, ABTS, NO, OH and FRAP. The presence of active phytochemicals like alkaloids, phenolics, flavonoids, saponins, tannins etc. further confirm the nutritional benefits of the seeds. HPLC-MS analysis of phenolics also showed the abundance of several phenolic derivatives *viz.* catechol, pyrogallol, gallic acid, chlorogenic acid etc. which might be responsible for the antioxidative properties. The seeds were also found to be considerably rich in protein, whereas the amount of total sugar and reducing sugar was found to be comparatively lower. Therefore, the results suggest that the underutilized legume *Vigna unguiculata* subsp. *sesquipedalis* can be used as a nutritionally rich alternative food in our

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daily diet. Further research on the isolation, purification and characterization of the antioxidative active compounds would help upgrade the nutritional profile of this underutilized legume.

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