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EVALUATION OF PHENOLIC CONTENTS AND ANTIOXIDANT ACTIVITY OF ETHANOL FRUIT EXTRACT OF *VIGNA SINENSIS*

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ABSTRACT

Free radicals can contribute to innumerable precarious diseases. The ethanolic fruit extract of *Vigna sinensis* was screened for polyphenolic compounds with HPLC, and antioxidant activity using reducing power assay, total phenolic content, flavonoid content and total antioxidant capacity determination methods. Major polyphenolic compounds like catechin hydrate, vanillic acid, *p*-coumaric acid, rutin hydrate and quercetin contents were measured in the extract by reverse-phase HPLC (60.82 ± 1.59, 5.06 ± 0.25, 3.58 ± 0.12 15.30 ± 0.72 and 21.76 ± 0.932 mg/100g of dry extract, respectively). The polyphenolic compounds in the fruit extract of *Vigna sinensis* might be responsible for the antioxidant activity exhibited by the sample. The extract was observed to demonstrate average reducing power ability (0.0791 ± 0.007 at 250 µg/ml), which increased with increasing concentration of the sample. The total phenolic and flavanoid contents were 56.24 ± 1.19 mg/g of gallic acid and 137.4 ± 12.5 mg/g of quercetin equivalent, respectively, while the total antioxidant capacity of *Vigna sinensis* was 147.8 ± 2.11 mg of ascorbic acid/g, which was considerably higher in the extract. The data from the present study reveal that *Vigna sinensis* fruit acts as an antioxidant agent due to the presence of its polyphenolic compounds.

KEYWORDS: *Vigna sinensis*, HPLC, rutin hydrate, vanillic acid, quercetin, total antioxidant capacity.

INTRODUCTION

Vigna sinensis (*V. sinensis*) belongs to the genus Vigna, and the family Fabaceae. The plant is also known as bora or the cowpea, which are one of the most important food legume crops grown in the semiarid tropics covering Asia, Africa, southern Europe and Central and South America. Some of the more well known common names for cultivated cowpeas include black-eye pea, southern pea, yardlong bean, catjang and crowder Pea. [1] Cowpeas are generally considered as warm-weather crops and are better suited to dry regions. They are able to grow in poor soil with low levels of phosphorous and organic matter making them a particularly important crop in the desert regions. [2]

The plants are rich in proteins, minerals, vitamin A and C, thiamine, riboflavin, folate, iron, phosphorous, potassium, magnesium and manganese, and are low in anti-nutritional factors. [3] Other studies have revealed the presence of hydroxybenzoic acids such as gallic, vanillic, p-hydroxybenzoic and protocatechuic, along with flavonols such as amyricetin glucoside, mono- and di-glycosides of quercetin and a quercetin diglycoside acylated with ferulic acid. [4] The antioxidant and anti-inflammatory activity has also been evaluated. Ethyl acetate and *n*-butanol fractions of *V. sinensis* seeds were found to strongly inhibit nitric oxide production. These compounds inhibiting NO production were isolated and identified. The active compounds included oleanolic acid, linolenic acid, linoleic acid, soyasaponin etc.[5] Furthermore, fermentation followed by heating has been shown to be a very effective process to increase the functionality of this variety of *V sinensis*. For this reason, this cowpea variety could be used as an ingredient to obtain high value-added flours.

Since very little research work has been done on the ethanol fruit extracts of *Vigna sinensis* grown in Bangladesh, an attempt has been made to develop a more intensive understanding of the major bioactive polyphenolic compounds and the antioxidant activity of the plant.

MATERIALS AND METHODS

Sample collection

The raw *V. sinensis* fruits were obtained from the local market of Khulna, Bangladesh during June 2013 (Accession no: DACB 36747). The fruits were carefully selected in order to obtain a uniform batch in relation to size and degree of maturity.

Extraction

The fruits were cleaned and cut into small pieces before being dried in an oven at 50°C. After

drying, the samples were ground to a fine powder in a mechanical blender and kept at room temperature prior to extraction. The powdered sample was extracted with 90% ethanol in an orbital shaker for 1 week at ambient temperature and pressure. The extracts were first filtered in a clean cotton plug to remove any plant debris, and then through Whatman filter paper no. 1. Afterwards, the extracts were concentrated in a rotary vacuum evaporator to remove excess solvent.

Chemicals

Standards of Gallic acid (GA), (+)-catechin hydrate (CH), vanillic acid (VA), caffeic acid (CA), (-)-epicatechin (EC), *p*-coumaric acid (PCA), rutin hydrate (RH), ellagic acid (EA), quercetin (QU), ascorbic acid, ABTS, folin-ciocalteu's phenol reagent were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC), methanol (HPLC), acetic acid (HPLC), ethanol, trichloroacetic acid (TCA), phosphate buffer (pH 6.6), potassium ferricyanide [$K_3Fe(CN)_6$], ferric chloride ($FeCl_3$), sodium phosphate, EDTA, ammonium molybdate and sodium carbonate were of analytical grade and acquired from Merck (Darmstadt, Germany).

Preparation of standard solutions

Standard stock solutions of (+) catechin, (-) epicatechin, quercetin, *p*-coumaric acid, caffeic acid, ellagic acid, rutin hydrate, vanillic acid were prepared in ethanol at a concentration of 100 µg/ml and stored in the refrigerator at 5°C until use. All stock solutions were further diluted with ethanol to appropriate concentrations to make standard solutions of 20 µg/ml for all the polyphenols excluding caffeic acid, which was made up to 8 µg/ml, and quercetin that was made up to 6 µg/ml.

Preparation of sample solutions

The extracted fruit sample of *V. sinensis* was prepared with ethanol to a concentration of 5 mg/ml by mixing for 30 min. All the solutions were kept in the dark at low temperature (5°C), and spiked with phenolic standards to identify the individual polyphenols. Sample solutions were filtered through 0.20 µm nylon syringe filter (Sartorius, Germany), degassed in an ultrasonic bath (Hwashin, Korea) for 15 min, and then separated by RP-HPLC to obtain chromatograms for the polyphenolic compounds.[6, 7]

Optimization of Chromatographic Conditions

Suitable separation conditions for the determination of polyphenolic compounds were established. HPLC analysis was performed with Thermo Scientific Dionex UltiMate 3000 Rapid Separation LC (RSLC) systems (Thermo Fisher Scientific Inc., MA, USA), equipped with a quaternary rapid separation pump system (LPG-3400RS), Ultimate 3000RS autosamplier (WPS-3000) and rapid separation diode array detector (DAD-3000RS).

The phenolic compounds were detected in Acclaim® C18 (4.6 x 250 mm; 5µm) column (Dionix, USA) with a flow rate of 1 ml/min. The column was operated at a temperature of 30°C using a temperature controlled column compartment (TCC-3000). Data collection such as, acquisition, peak integration, and calibrations were done with Dionix Chromeleon software (Version 6.80 RS 10). Separations were carried out in a mobile phase consisting of acetonitrile (solvent A), acetic acid solution pH 3.0 (solvent B), and methanol (solvent C). The solvent gradient elution program was as follows: 0 min, 5%A/95%B; 10 min, 10%A/80%B/10%C; 20 min, 20%A/60%B/20%C and 30min, 100%A. The injection volume for all samples was 20 µl. The detection wavelength was: λ 280 nm held for 18.0 min, changed to λ 320 nm and held for 6 min, and finally changed to λ 380 nm and held for the rest of the analysis and the diode array detector was set at an acquisition range from 200 nm to 700 nm. The detection and quantification of GA, CH, VA, CA, and EC was done at 280 nm, of PCA, RH, and EA at 320 nm, and of QU at 380 nm, respectively. The phenolic compounds were analysed by matching the retention time and their spectral characteristics against those of standards. Analyses were performed in triplicate.

Antioxidant activities

Reducing power assay

The reducing power of *V. sinensis* was studied using the method of Hemayet et al. and Dehpour et al.[8, 9] The extract at different concentrations was mixed with 1 ml ethanol, 2.5 ml phosphate buffer (0.2 M, pH 6.6), and 2.5 ml potassium ferricyanide [$K_3Fe(CN)_6$] (1%). The sample solutions were next incubated at 50°C for 20 min and a 10% solution of trichloroacetic acid (2.5 ml) was added to them. They were then centrifuged at 3000 rpm for 10 min. The top layer of the mixture (2.5 ml) was mixed with 2.5 ml distilled water and 0.5 ml of 0.1% $FeCl_3$. The absorbance was measured at 700 nm with a spectrophotometer. All determinations were carried out in triplicate.

Total antioxidant capacity

The total antioxidant capacity was measured by the method of Prieto et al. [10] The ethanol extract was prepared in its respective solvent and mixed with 1 ml of the reagent solution (0.6M H_2SO_4, 28 mM sodium phosphate, 4 mM ammonium molybdate mixture). The tubes were incubated for 90 min at 95ºC. The mixture was cooled to room temperature and the absorbance was read at 695 nm against a blank sample. Ascorbic acid equivalents were calculated using the standard graph for ascorbic acid. The experiment was conducted in triplicates and values were expressed as equivalents of ascorbic acid in mg per gram of extract.

Total phenolic content

Total phenolic content of the extract was determined using the modified Folin-Ciocaltu method.[11, 12] 0.5 ml of extract (1 mg/ml), 5 ml Folin-Ciocaltu reagent (1:10 v/v distilled water) and 4 ml (75 g/l) of sodium carbonate were mixed with the sample solutions and incubated at 40°C for the next 30 min for color development. The absorbance was measured at 765 nm. The total content of phenolic compounds in the extract was expressed as gallic acid equivalents (GAE) mg/g of the dry extract.

Total flavonoid content

The total flavonoid content was determined by reactions of the aluminium chloride colorimetric method with some modifications.[13, 14] 0.25 ml of each fraction dissolved in their respective solvents was mixed with 1.25 ml of distilled water, followed by addition of 75 µl of a 5% (w/v) sodium nitrite solution. After 6 min, 150 µl of 10% aluminium chloride solution were added, and the mixture was allowed to stand for another 5 min before 0.5 ml of 1M NaOH was added. The mixture was made upto 2.5 ml with distilled water and mixed well. The absorbance of the reaction mixture was measured at 430 nm with a double beam Analykjena UV/Visible spectrophotometer (Model 205, Jena, Germany).

Statistical analysis

Data were statistically elaborated and presented as mean ± standard deviation (S.D).

RESULTS AND DISCUSSION

HPLC Fingerprint Profiling

Five analysed major phenolic compounds (catechin hydrate, vanillic acid, *p*-coumaric acid, rutin hydrate and quercetin) were resolved under the chromatographic conditions (Figure 1,

Table 1). Chromatographic analysis of the extracted samples of *V. sinensis* fruit revealed that catechin hydrate was found in appreciable concentration (60.82 ± 1.59 mg/100 g of dry extract). Other phenolic acids found in moderate concentrations include: rutin hydrate and quercetin (15.30 ± 0.72 and 21.76 ± 0.93 mg/100 g dry extract, respectively). This was followed by vanillic acid and *p*-coumaric acid, which were detected at a relatively low amount (5.06 ± 0.25, 3.58 ± 0.12 mg/100 g dry extract).

Phenlic compounds and flavnonoids are thought to have potent antioxidant activities and positive effects on human health.[15] These act as effective scavengers of oxidizing molecules and various free radicals[16] that are helpful in the treatment of diseases.

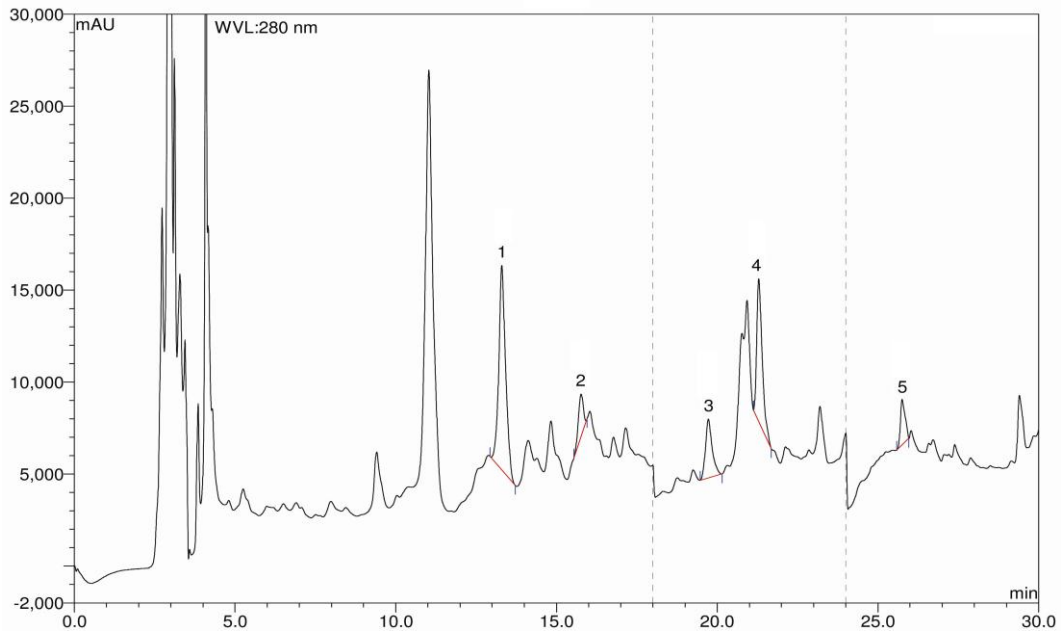

Figure 1: HPLC chromatogram of *V. sinensis* ethanol extract, Peaks: 1, (+)-catechin; 2, vanillic acid; 3, *p*-coumaric acid; 4, rutin hydrate; 5, quercetin.

Table 1: Contents of polyphenolic compounds in the ethanol fruit extract of *V. sinensis* (n = 5).

Polyphenolic Compound	*V. sinensis* ethanol fruit extract	
	Content (mg/100 g of dry extract)	% RSD
CA	60.82	1.59
VA	5.06	0.25
PCA	3.58	0.12
RH	15.30	0.72
QU	21.76	0.93

Reducing power assay

Based on the relative maximum absorbance of the ethanol fruit extract of *V. sinensis*, reducing power activity was evaluated (Figure 2). Reducing power assay was determined by observing the reduction of Fe^{3+} to Fe^{2+}, which was visualized by forming the blue color complex.[17] The antioxidants in the sample donate electrons to free radicals neutralizing the radicals. The higher the absorbance value, the stronger the reducing power of the samples. At 250 µg/ml, the maximum absorbance for the fruit ethanol extract of *V. sinensis* was found to be 0.0791 ± 0.007, while the standard ascorbic acid showed an absorbance of 1.1115 ± 0.009. The extract showed concentration dependent reducing power. The presence of phenolic compounds in the ethanol fruit extract of *V. sinensis* justifies its reducing power ability. Antioxidant compounds are able to donate electrons to reactive radicals, thus forming more stable compounds.

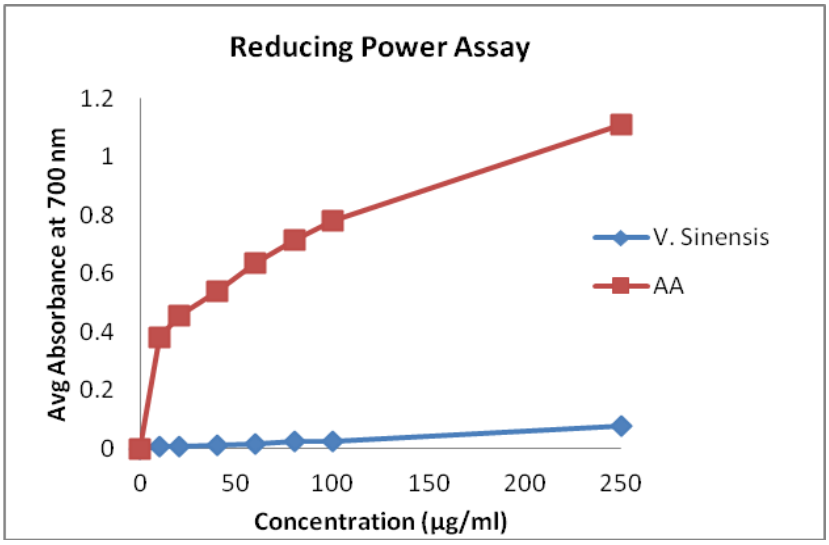

Figure 2. Reducing power assay of ethanol fruit extract of *V. sinensis*.

Total antioxidant capacity

The ethanol fruit extract of *V. sinensis* showed significant total antioxidant capacity in comparison to the standard ascorbic acid (147.1 ± 2.11 per g of extract). The total antioxidant activity is expressed as the number of equivalents of ascorbic acid (Table 2). The total antioxidant capacity was measured spectrophotometrically based on the phosphomolybdenum method.[18] It depends on the reduction of Mo (VI) to Mo (V) by the test sample and the subsequent formation of green phosphate/Mo (V) complex at an acidic pH. Recent studies show that flavonoids and related polyphenols contribute greatly to the scavenging activity of medicinal plants. [19, 20]

Table 2: Total antioxidant capacity of ethanolic fruit extract of *V. sinensis*.

Extract	Total antioxidant capacity mg of ascorbic acid equivalent (AAE) per g of dry extract
V. sinensis fruit	147.1 ± 2.11

The values are expressed as mean ± standard deviation (n=3).

Total phenolic and flavonoid content

Total phenolic content was estimated using Folin-Ciocalteu reagent. Total phenolic content of the ethanol fruit extract of *V. sinensis* was expressed as milligrams of gallic acid (GAE). Figure 3 summarizes the total phenolic content of the test sample (33.29 ± 1.13 mg/g of gallic acid equivalent). As is observed from the results, the plant sample exhibited considerable total phenolic content. Moderate quantity of total flavonoid content was also obtained from the plant fruit extract (22.16 ± 1.03 mg/g of quercetin, respectively) (Figure 3). Plant materials rich in phenolics are able to retard oxidative degradation of lipids with the help of their hydroxyl groups, thus improving food quality. These are of great interest to the industries. [21]

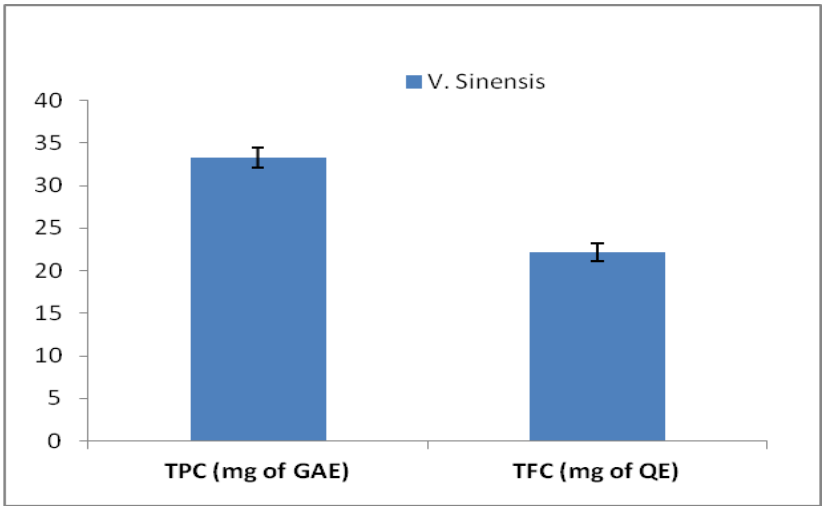

Figure 3: Total antioxidant capacity, total phenolic content and total flavonoid content determination of ethanol fruit extract of *V. sinensis*.

CONCLUSION

Research on phenolic compounds is of current interest since they have important biological and pharmacological properties. HPLC, with various detection possibilities has been a preferred technique for routine analysis of phenolics. It is apparent from the results of the present study that the presence of phenolics in *V. sinensis* fruits might be responsible for the reducing power assay. This also indicated that the ethanol fruit extract of this plant contained

potential antioxidant bioactive compounds. A more extensive study of the plant, *V. sinensis* could lead to many more chemically interesting and biologically active drug constituents.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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