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Screening of Antioxidant and Radical Scavenging Activity of *Vigna ungiculata*, *Bidens pilosa* and *Cleome gynandra*

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Abstract: This study was conducted to investigate the total phenolics content and the antioxidant activities from methanolic extracts of *Vigna ungiculata*, *Bidens pilosa* and *Cleome gynandra*. Antioxidant and free radical scavenging activity were studied using DPPH and reducing power assays, a β-carotene linoleic acid model system and the inhibition of lipid peroxidation in rat brain. Phenolic compounds were also quantified using HPLC. Total phenolic compounds for *Vigna ungiculata*, *Bidens pilosa* and *Cleome gynandra* were 1136.603±3.869 g g^{-1}, 1102.797±2.239 mg g^{-1} and 1327.333±1.658 mg g^{-1} dry mass, respectively. All three vegetable extracts contained vanillin, caffeic acid, p-coumaric acid and ferulic acid. There was an increase in the reducing power effects and inhibition of lipid peroxidation in all samples. All samples showed a time dependent decrease in radical scavenging of DPPH and β-carotene.

Key words: Antioxidant, free radical, phenolic acids, wild fruits

INTRODUCTION

Indigenous vegetables could play a crucial role in overcoming malnutrition in Zimbabwe (Tredgold, 1986). *Bidens pilosa* (blackjack, *mhuwu*) is a weak annual herb usually a metre or less in height which has many applications including the treatment of coughs, conjunctivitis, dysentery and respiratory infections (Shava, 2000).

Vigna ungiculata (cowpea, *munyemba*) is a trailing vigorous-growing annual herb with large seeds (Shava, 2000). *Hausa* and *Edo* tribes use cowpeas medicinally to treat stubborn boils (Tredgold, 1986).

Cleome gynandra (spider plant, *nyevhe*) is an annual herb which can grow up to a size of 60 cm. The leaves and flower buds are washed and boiled in water with a little salt. A relatively long cooking time (2 h) is normally used to remove the bitter flavour (Tredgold, 1986).

There is now increasing interest in antioxidant activity of phytochemicals present in the diet (Ou *et al.*, 2002). An antioxidant can be defined as any substance that when present at low concentrations compared to that of an oxidisable substrate, significantly delays or inhibits the oxidation of that substrate (Atoui *et al*., 2005). Antioxidants prevent damage to cellular components arising as a consequence of chemical reactions involving free radicals (Vertuani *et al*., 2002). Most antioxidants isolated from higher plants are polyphenols, which show biological activity as antibacterial, anti-carcinogenic, anti-inflammatory, antiviral and immune-stimulating effects.

The antioxidant effect of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Shon *et al.*, 2003). The objective of this research was to estimate the phenolic content, evaluate the antioxidant activity and determine the phenolic profile of the methanolic extracts of *Bidens pilosa*, *Vigna ungiculata* and *Cleome gynandra*.

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MATERIALS AND METHODS

Sample Collection and Preparation

Bidens pilosa, *Vigna ungiculata* and *Cleome gynandra* were obtained form Mbare green market, sundried and ground into a fine powder and stored at room temperature until further analysis.

Chemicals

The chemical standards used were all of analytical grade. Catechin, vanillin, caffeic acid, p-coumaric acid, protocatechuic acid, ferulic acid, p-hydroxy-benzoic acid, p-hydroxybenzaldehyde, 1, 1-dipheny l-2 picrylhydrazyl radical (DPPH), ascorbic acid, trichloroacetic acid (TCA) and potassium ferricyanide were obtained from Sigma-Aldrich Chemie (Steinheim, Germany).Thiobarbituric acid (TBA) and β-carotene were obtained from Boehringer, Manheim, Germany. Sodium carbonate, methanol (HPLC grade), ascorbic acid, acetonitrile (super purity solvent) and acetic acid were obtained locally.

Folin-Ciocalteu reagent (1 N), 20% sodium carbonate, standard gallic acid (0.5 mg mL^{-1}), 50% methanol in distilled water (1:1 v/v) and ferric chloride were used for analysis.

Extraction and Determination of the Total Phenolics in the Vegetables

Total phenolic compounds were extracted from the vegetables as described by Makkar (1999). The sample of vegetable (2 g) was extracted twice with cold 50% aqueous methanol (10 mL). The two extracts were combined, made up to 20 mL with 50% aqueous methanol, centrifuged at 3000 rpm for 10 min sand transferred into small sample bottles for analysis. Total phenolic compounds were determined following the method by Makkar (1999). To a sample (50 µL), distilled water (950 µL) was added to make up to 1 mL followed by 1N Folin C. reagent (500 µL) and sodium carbonate (2.5 mL). After 40 min at room temperature absorbance was read at 725 nm on a Spectronic 20® Genesys™ spectrophotometer against a blank that contained methanol instead of sample. Total phenolics were in terms of gallic acid equivalent.

Evaluation of Antioxidant Activity of the Extracts

Antiradical Activity

The radical scavenging activity was determined following the method by Kuda *et al*. (2005). Methanolic solutions of DPPH (1990 µL, 1 mM) containing 10 µL sample were monitored at 517 nm for 30 min on a Spectronic 20® Genesys™ spectrophotometer. Ascorbic acid was used as a positive control. The scavenging activity was calculated as:

$$\text{Scavenging activity (\%)} = [1\text{-}(\text{Abs sample}/\text{Abs control})]$$

Where A_0 is the initial absorbance at increasing time, t. The antiradical activity was expressed as k.

Reducing Power Assay

Reducing power effects were determined following the method by Kuda *et al*. (2005). Up to 80 µL sample or ascorbic acid control solution was mixed with phosphate buffer (0.2 mL, 0.2 M pH 7.2) and 1% potassium ferricyanide (0.2 mL). The mixture was incubated at 50°C for 20 min after which TCA (0.2 mL, 10%) was added. After transferring an aliquot of the mixture (0.125 mL) into microtitre plate, distilled water (0.125 mL) and $FeCl_3$ (0.02 mL, 0.1%) was added. The absorbance at 655 nm was measured on a Spectra MAX 340 (USA, Sunnyvale, California) microtitre plate spectrophotometer.

Inhibition of Phospholipid Peroxidation

Homogenization of rat brain (2 g) was done with a chloroform:methanol mixture (2:1, v/v) followed by centrifugation at 3000xg for 5 min. The supernatant obtained was used as the phospholipids source.

The blank contained the phospholipid solution (50 µL) mixed with distilled water (0.5 mL) and 50% methanol (0.2 mL). The test run contained the phospholipids solution (50 µL), the vegetable extract (0.5 mL), 50% methanol (0.2 mL), $FeSO_4$ (0.5 mL), TBA (0.5 mL) and TCA (4 mL). Ascorbic acid (5%) was used as the positive control. Incubation at 37°C was followed by the addition of TBA and TCA and the solution was then heated in a boiling water bath for 15 min. After cooling on ice, absorbance was read at 532 nm.

The β-carotene-linoleic Acid Model System

The inhibition of lipid oxidation was assayed using the method of Amin and Tan (2002). β-Carotene 0.2 mg mL^{-1} (1 mL) solution in chloroform was placed into 100 mL round bottomed flask. The mixture was evaporated for 10 min using a rotary vapor to remove the chloroform. Linoleic acid (40 µL) and Tween 80 (400 µL) and the mixture was diluted immediately with distilled water to 100 mL and agitated vigorously to form an emulsion. Emulsion (4.8 mL) was placed in test tubes and sample extracts (20 µL) were added to each test tube. The test mixture was shaken and incubated at 50°C for 2 h. The absorbance was read at 470 nm at 15 min interval. A control was also set with 50% methanol being added instead of sample. The antioxidant activity was calculated as the degree of inhibition of lipid oxidation using the formula:

$$\text{Antioxidant activity} = 100 \times \{1-[(A_0-A_t) / A^\circ_0-A^\circ_t)]\}$$

where A_0 and A°_0 are the absorbance values measured at the initial incubation time for the samples and control, respectively while A_t and A°_t are the absorbance values measured in the samples or standards and control at t = 120 min.

HPLC Analysis for Phenolic Acids

A Shimadzu HPLC system with a SCL-6B Shimadzu system controller, C-R AX Shimadzu Chromatopac, Shimadzu SPD-10 AV UV-V is detector equipped with a Dynamax 60 A C18 column was used for analysis of phenolic compounds. Five microlitres of sample were injected and the flow rate set at 1 mL min^{-1}. All samples in duplicate were filtered through a 0.22 µm filter unit (Millex®-GV, Molsheim, France) before injection and the solvents were filtered through a 0.45 µm filter (Whatman, Maidstone, England). Two mobile phases, A, which contained water: acetic acid (98:2 v/v) and B, which contained water-acetonitrile-acetic acid (78: 20: 2 v/v/v), were used. The gradient profile used had 0% Solvent B at the start, rising to 80% within 55 min, remaining at 80% up to 70 min and falling back to 0% at time 80 min. Detection was carried out by measuring absorbance at 280 nm according to Pena-Neira *et al*. (2000). After each run, the system was reconditioned for 15 min before analysis of the next sample.

Statistical Analysis

Samples were analysed in duplicate and results are given as averages ±standard deviations. One-way ANOVA and the Student's t-test, both packaged in the Statistical Package for Social Sciences (SPSS) for Windows Standard Version 8.0.0 were used for the statistical evaluation with $p<0.05$ considered statistical significant.

RESULTS AND DISCUSSION

Among all the vegetable extracts, *Cleome gynandra* had the highest phenolic content (1327.333±1.658 mg g^{-1}) followed by *Vigna ungiculata* (1136.603±3.869 mg g^{-1}) and *Bidens pilosa* (1102.797±2.239 mg g^{-1}) (Fig. 1).

The vegetable extracts showed a time dependent scavenging of DPPH, which may be attributed to its hydrogen-donating ability (Fig. 2). When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced (Kuda *et al*., 2005). The bleaching (changes in colour from deep-violet to light yellow) that occur at 515 nm consequently result in the decrease in scavenging effect of all vegetable extracts.

The ferric reducing power assay which measures antioxidant capacity by the reduction of the ferric tripyridyltriazine complex to the blue ferrous complex was done (Lee *et al*., 2005). For all samples, there was a general increase in absorbance with the increase in volume of the vegetable extracts as shown in Fig. 3. Increased absorbance at 655 nm of the reaction mixture indicates an increased reducing power (Ou *et al*., 2002).

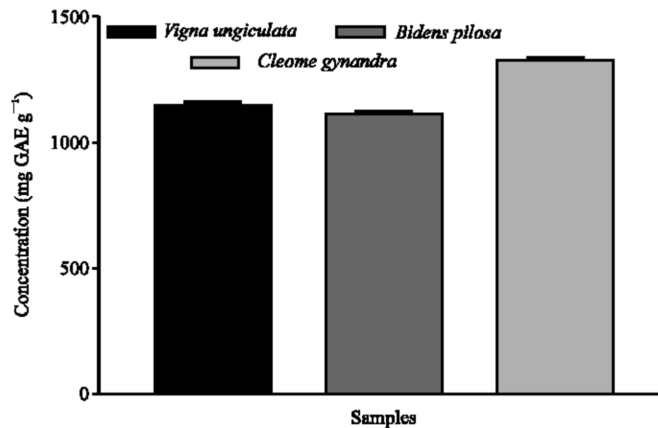

Fig. 1: Total phenolic contents

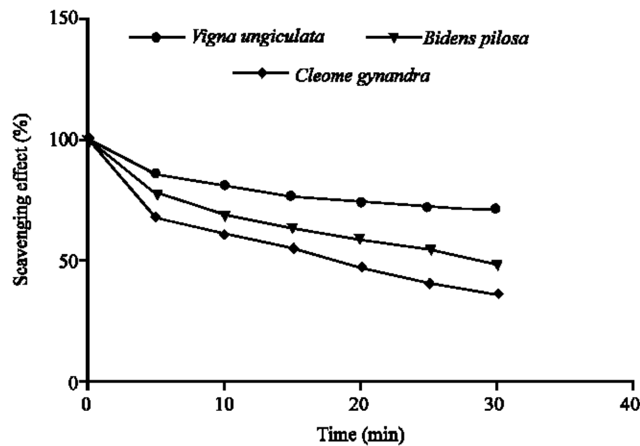

Fig. 2: DPPH radical scavenging assay

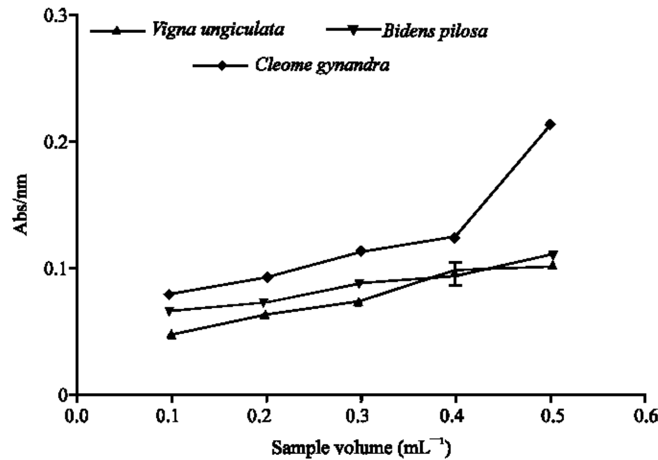

Fig. 3: Reducing power effect

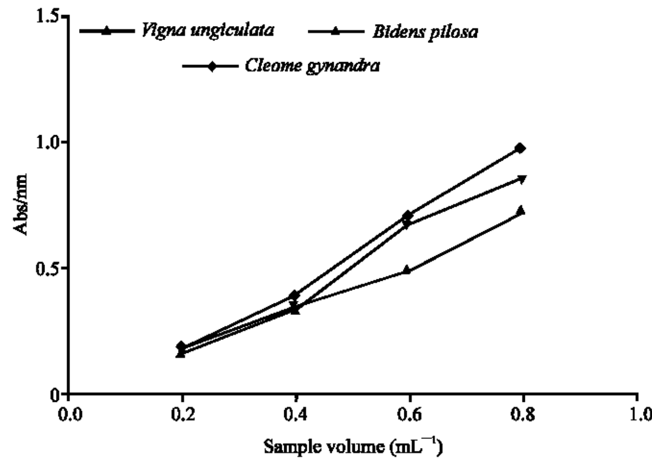

Fig. 4: Inhibition of lipid peroxidation in rat brain

The capacity to inhibit lipid peroxidation in rat brain by the vegetable extracts was performed. *Cleome gynandra* exhibited the highest inhibitory effect on rat brain peroxidation whilst *Vigna ungiculata* exhibited the lowest inhibitory effect on peroxidation as shown in Fig. 4. Biological specimens such as rat brain contain a mixture of thiobarbituric acid reactive substances (TBARS), including lipid hydroperoxides and aldehydes, which increase as a result of oxidative stress (Capecka *et al*., 2005). TBARS return to normal levels over time, depending upon the presence of antioxidants (Wangensteen *et al*., 2004).

The total antioxidant activity, which reflected the ability of the vegetable extracts to inhibit the bleaching of the β-carotene, was measured and compared with that of the control. All vegetable extracts showed similar trends with a significant decrease ($p<0.05$) of the absorbance values. This indicates that the samples acted as effective antioxidants in the β-carotene linoleate system, which inhibited the oxidation activity of β-carotene (Fig. 5). There was a decrease in absorbance values of β-carotene in the absence of vegetable extracts due to the oxidation of β-carotene and linoleic acid. The high absorbance values indicated that vegetable extracts possessed antioxidant activity. Antioxidant activity

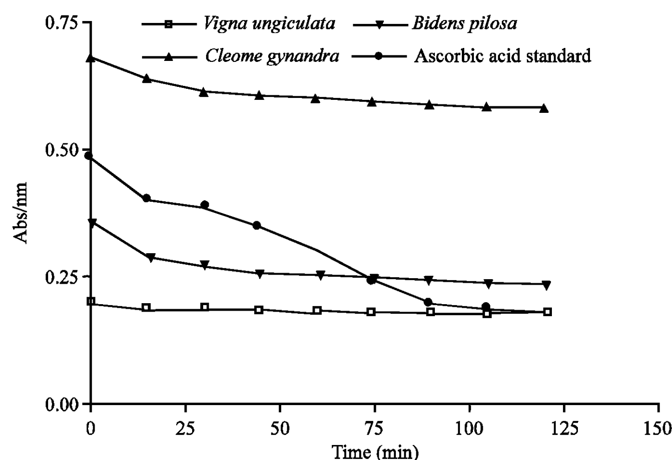

Fig. 5: Beta-carotene linoleic acid model system

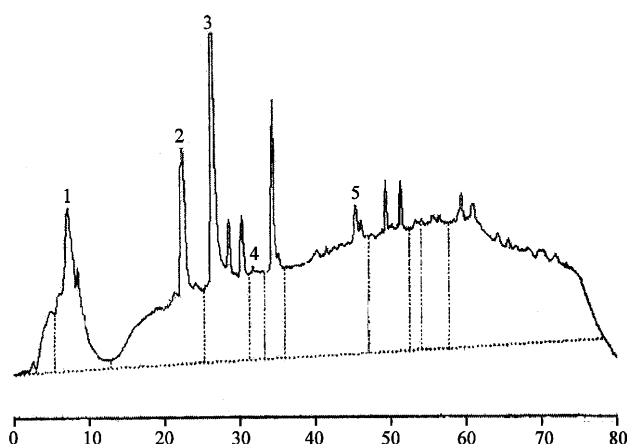

Fig. 6: HPLC separation of dried, *Vigna ungiculata*. (1) Gallic acid, (2) p-Hydroxybenzoic acid, (3) Vanillin, (4) Caffeic acid and (5) Ferulic acid

of vegetable extracts depend on the type and polarity of the extracting solvent, the isolation procedures and purity of active compounds as well as the assay techniques and substrates used (Chun *et al.*, 2005).

Qualitative analysis of the vegetable extracts was done using HPLC and the chromatograms obtained are shown in Fig. 6-8. All three vegetable extracts contained vanillin, caffeic acid, p-coumaric acid and ferullic acid. However, protochatechuic acid and catechin were detected only in *Cleome gynandra*. There is a wide degree of variation between different phenolic compounds in their effectiveness as antioxidant (Ismail *et al.*, 2004). Ferulic acid and other hydroxycinnamic acids (caffeic and p-coumaric acid derivatives) have been found to have good antioxidant activities. The presence of the CH = CH-COOH group in the hydroxycinnamic acids is considered to be key for the significantly higher antioxidative efficiency than the COOH in the hydroxybenzoicacids (Kim *et al.*, 2006). The presence of flavonoids in *Cleome gynandra* could explain the high antioxidant activity observed in all the assays performed. Flavonoids generally have more hydroxyl groups if compared to ferulic acids.

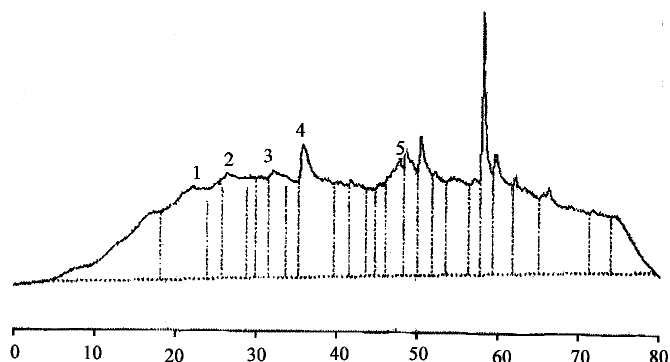

Fig. 7: HPLC separation of *Bidens pilosa*. (1) p-Hydroxybenzaldehyde, (2) Vanillin, (3) Caffeic acid, (4) p-Coumaric acid and (5) Ferulic acid

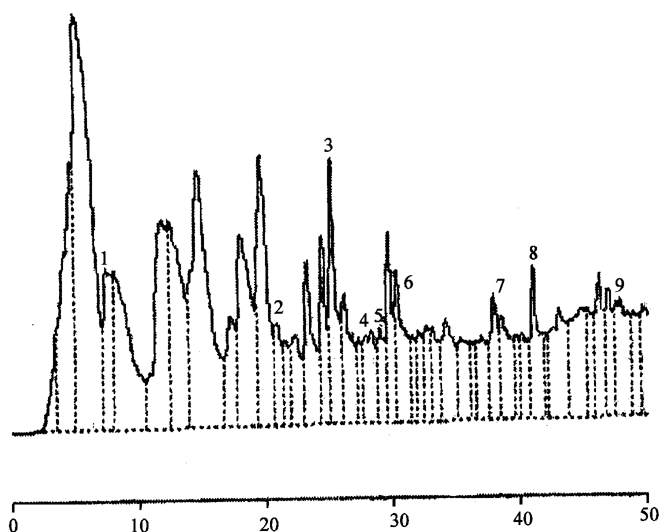

Fig. 8: HPLC separation of *Cleome gynandra*. (1) Gallic acid, (2) Protocatechuic acid, (3) p-Hydroxybenzoic acid, (4) p-Hydroxybenzaldehyde, (5) Catechin, (6) Vanillin, (7) Caffeic acid, (8) p-Coumaric acid and (9) Ferulic acid

Besides, orthosubstitution with electron-donating alkyl or methoxy groups of flavonoids increases the stability of the free radical and hence its antioxidant potential (Ismail *et al*., 2004).

Several studies have reported on the relationships between phenolic content and antioxidant activity. Some authors have found a correlation between the phenolic content and the antioxidant activity, while others found no such relationship. Velioglu *et al*. (1998) reported a strong relationship between total phenolic content and the antioxidant activity in selected fruits, vegetables and grain products. No correlation between antioxidant activity and phenolic content was found in the study by Kahkonen *et al*. (1999) on some plants extracts containing phenolic compounds (Ismail *et al*., 2004).

In this study, the findings do not show a significant relationship between antioxidant activity and total phenolic contents. For example, *Cleome gynandra* had the lowest antioxidant activity (61%) at 10 min in the DPPH radical scavenging assay and yet its total phenolic content (TPH) was higher than that of *Bidens pilosa* and *Vigna ungiculata*. The correlation between total phenolic contents and the antioxidant activity such as the DPPH radical scavenging activity of the vegetable extracts where

$R^2 = 0.58$ for TPH/DPPH was not highly significant according to the Pearson's test. It is known that the antioxidant properties of single compounds within a group vary remarkably so that the same levels of phenolics do not necessarily correspond to the same antioxidant responses. Moreover, the response of phenolics in the Folin-C assay also depends on their chemical structure and thus the radical scavenging capacity of an extract cannot be predicted on the basis of its total phenolic content (Atoui *et al.*, 2005).

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