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TOTAL PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY OF METHANOLIC EXTRACT OF SELECTED WILD LEAFY VEGETABLES GROWN IN BANGLADESH: A CHEAPEST SOURCE OF ANTIOXIDANTS

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ABSTRACT

Nowadays, more attention has been paid on wild plants as new source of natural antioxidants. Therefore, methanolic extracts of 10 traditionally consumed wild leafy vegetables of Bangladesh were analyzed for their total phenolic content **(**TPC**)** and free radical scavenging activity. Folin–Ciocalteu method followed by spectrophotometric measurement was used to quantify the TPC of the selected wild leafy vegetables. Free radical scavenging activity was examined utilizing 1,1-diphenyl-2-picryl-hydrazyl **(**DPPH**)** assay. Different concentrations of the plant extract were applied to ascertain the dose response relationship in inhibiting DPPH free radical**.** The results revealed that the TPC ranged from 102.20 to 710.42 mg GAE.100g^{-1} dry weight **(**DW**)**. The highest TPC was observed in *Bauhinia acuminata* **(**Shetokanchan**)** while *Leucas aspera* **(**Shetodhron**)** exhibited the lowest TPC among the undertaken vegetables. The studied samples proportionately inhibited DPPH with increasing concentrations. At high concentration **(**500 µg.mL^{-1}**)**, the percentage inhibition of DPPH radical by plant extract ranged from 68.1 ±2.65% to 93.1 ±1.23%. The highest DPPH radical inhibition was observed in *Bauhinia acuminata* **(**Shetokanchan**) (**93.10 ±1.23%**)**, followed by *Commelina benghalensis* **(**Bat baittashak**) (**91.97 ±1.31%**)**, *Hydrocotyle sibthorpiodes L.* **(**Sakumubakla**) (**91.83 ±2.13%**)**. The lowest DPPH radical inhibition among the studied samples was observed in *Leucas aspera* **(**Shetodhron**) (**68.1 ±2.65%**)**. IC_{50} values measured by DPPH assay in this study ranged from 11.64 to 313.79 µg.mL^{-1}. The study findings indicated that the samples under study possesses strong activity against DPPH, and thus could be used as natural antioxidants in the food and/or pharmaceutical industry.

Keywords: antioxidants; Bangladesh; DPPH; wild vegetables; total phenolic content

INTRODUCTION

Antioxidant is termed as a compound whose major function is to inhibit the oxidation of biological molecules **(**lipids, proteins or other molecules**)** and hence provides a defensive effect against ROS **(**Reactive oxygen species**)** such as hydrogen peroxide **(**H_2O_2**)**, superoxide **(**$O_2^{\bullet-}$**)**, hydroxyl radical **(**$OH^{\bullet}$**)**, peroxyl radical **(**$ROO^{\bullet}$**)**, and singlet oxygen **(**$_1O^2$**) (Moyo et al., 2013; Brindza et al., 2019)**. These ROS are produced in the body either as a by-product of normal cellular aerobic respiration or exposure to environmental factors such as pollution, radiation, cigarette smoke and herbicides **(Thomas et al., 2010)**.

The production of ROS in a healthy individuals is governed by the antioxidant defense mechanism **(Anahita, Asmah and Fauziah, 2015)**. Overproduction of ROS disrupts the antioxidant defense mechanism in the body and generates oxidative stress as a result of damaging effects on nucleic acids, proteins, enzymes and other biological molecules containing a lipid component of polyunsaturated fatty acids through oxidation **(Alam, Rana and Akhtaruzzaman, 2017a; Alam, Rana and Akhtaruzzaman 2017b; Škrovánková et al., 2018)**. Dietary antioxidant nutrients, which include phenolic acids, polyphenols, flavonoids vitamin E, vitamin C, and carotenoids are believed to scavenge free radicals and thus inhibit the oxidative mechanisms which are responsible for many disorders and diseases in humans such as infections, diabetes, arthritis, cardiovascular diseases, cancer, Alzheimer's diseases, AIDS etc. **(Mendoza-Wilson et al., 2016; Bystrická et al., 2017; Lenková et al., 2017)**.

Antioxidants are generally classified into two categories, synthetic and natural **(Gülçin, 2012)**. However, toxic effects of synthetic antioxidants **(**fx. butylated hydroxyanisole **(**BHA**)**, butylated hydroxytoluene **(**BHT**))** has led to search for an alternative natural antioxidants which have no toxic effects **(Gülçin, 2012; Baba and Malik, 2015; Mendoza-Wilson et al., 2016)**. Wild/traditional plants as a source of natural antioxidants

Figure 1 Photograph of selected wild leafy vegetables of Bangladesh.

are recently received much attention from the researchers **(Gülçin, 2012; Baba and Malik, 2015; Mendoza-Wilson et al., 2016)**. These plants contain significant amounts of antioxidants, such as polyphenols, flavonoids, terpenoids, vitamin C, vitamin E, selenium, β-carotene, other carotenoids, which play an important role neutralizing free radicals **(Gülçin, 2012; Mendoza-Wilson et al., 2016)**. Several studies have indicated the inverse relationship between intake of traditional plants and chronic diseases **(Wang et al., 2011; Baba and Malik, 2015)**. Thus, increasing intake of these antioxidant rich plants can lower and/or prevent the generation of free radicals associated health problems **(Wang et al., 2011; Gülçin, 2012; Baba and Malik, 2015; Mendoza-Wilson et al., 2016)**.

Bangladesh is blessed with a rich biodiversity of plant foods. Indigenous people residing in different areas of the country rely on local plant and plant products to meet their daily requirements for micro and macro nutrients **(Shajib et al., 2013)**. Despite widespread use of wild plants as staple and medicines in Bangladesh, little is known about the antioxidant potential of these plants. Therefore, in this study an effort has been made to study and report the total phenolic content **(**TPC**)** and antioxidant activity of some wild leafy vegetables of Bangladesh consumed by the indigenous community. The preliminary data from these vegetables could be incorporated into food composition database to enrich it and used for increasing awareness to preserve these and maintain biodiversity.

Scientific hypothesis

The content of total polyphenols and antioxidant activity were evaluated in different types of leafy vegetables consumed by specific community of Bangladesh. We presumed that there exist a significant difference with respect to total polyphenol content and antioxidant activity, measured by DPPH method, in different indigenous leafy vegetable species.

MATERIAL AND METHODOLOGY

Reagents

Folin-Ciocaltu reagent and gallic acid were obtained from Sigma–Aldrich Co. **(**St. Louis, MO, USA**)**. The analytical grade acetone, petroleum ether, and methanol were purchased from Merck **(**Darmstadt, Germany**)**.

Sample plan

Muli-region sampling plan was employed for the vegetable sampling. In order to conform the representative sample principle – "what the mass people consume' and from where they collect it"? **(Greenfield and Southgate, 2003)**, the vegetables were collected from the retail markets located at wholesale markets where the vegetables

are arrived from different geographical regions of the country and also from cultivation fields. It was, thus, ensured the representative sample.

Identification of vegetable sample

A taxonomist who also accompanied the collection team, confirmed the sample identity. The identified samples undertaken in this study were *Cajanus cajan, Stereospermum suaveolens, Calamus tenuis, Commelina benghalensis, Enhydra fluctuans, Albizia procera, Tamarindus indica, Hydrocotyle sibthorpiodes, Leucas aspera,* and *Bauhinia acuminate*. Photographs of the studied vegetables are also of given in the Figure 1.

Sample processing

The samples were collected fresh, packed into autoseal poly bags with little water spray and brought to the laboratory for processing and analysis. Two to three samples **(**250 – 500 g**)** were collected for each of the items from every market and growing fields, which were then mixed to make three analytes or composite test samples. Samples were first gently washed with tap water to remove sand and other extraneous material before being washed with distilled water. Surface water was removed with tissue paper, air dried, cut into small pieces. The sample was freeze-dried at -48 °C **(**il Shin lab.Co. Ltd., Korea**)** and stored in refrigerator for phenolics analysis.

Extraction of phenolics

Approximately two grams of freeze-dried powdered sample was taken into a 250 mL conical flask and 42.5 mL methanol and 7.5 mL 1N hydrochloric acid were added. It was soaked for 24 hours at room temperature with intermittent shaking. Extracts were filtered through No. 1 Whitman filter paper and the filtrate was concentrated using a rotary evaporator at low temperature under reduced pressure. Methanol was added to make a final concentration of 1 $mg.mL^{-1}$ to be used as stock solution.

Analysis of phenolic content

The total phenolics content was estimated by Folin-Ciocalteu colorimetric **(Alam, Rana and Islam, 2016)**. In brief,

150 µL diluted sample extract was added to 225 µL of x2 diluted Folin-Ciocalteu reagent and was kept for 5 minutes at room temperature. Then 1.125 mL of 2% Na_2CO_3 solution was added, mixed well and kept for 15 minutes at room temperature and finally, the absorbance was measured at 750 nm by UV-VIS Spectrophotometer **(**UV-1800, Shimadzu, Japan**)**. Total phenol content was calculated using a standard curve made by standard gallic acid. Results were expressed as mg gallic acid equivalent **(**GAE**)** per 100 g dry weight **(**DW**)**.

DPPH free radical scavenging assay

The antioxidant activity of the plant extracts was evaluated by utilizing 1,1-diphenyl-2-pycrylhydrazyl **(**DPPH**)** free radical according to **Piang-Siong et al. (2017)** with slight modification. Briefly, DPPH stock solution was prepared by dissolving 6.5 mg of DPPH in 5 mL of 100% methanol and protected from light. 100 µL of varying concentrations **(**100 – 500 $\mu g.mL^{-1}$**)** of the plant sample extracts were taken and the volume was made up to 200 µL using methanol. Each of the samples was then further diluted with methanol up to 4 mL and to each 200 µL of DPPH stock solution was added. The mixture was then shaken vigorously and allowed to stand at room temperature for 30 min in the dark. After 30 min, the mixture was measured spectrophotometrically at 520 nm **(**UV-1800, Shimadzu, Kyoto, Japan**)**. Absorbance of the control **(**200 µL DPPH in 4 mL methanol**)** and blank **(**methanol without DPPH**)** were also measured. The DPPH free radical inhibition capacity was calculated according to the following equation:

$$\% \text{ DPPH inhibition} = \left(\left(1 - \left(\left(Abs_{sample} - Abs_{blank}\right)/\left(Abs_{control} - Abs_{blank}\right)\right)\right)\right) \times 100$$

where, Abs_{blank} is the absorbance of the blank **(**containing only methanol**)**, $Abs_{control}$ is the absorbance of the control reaction **(**containing all reagents minus plant extracts**)**, and Abs_{sample} is the absorbance of the plant extracts. The plant extracts concentration required for 50% inhibition of DPPH free radical **(**IC_{50}**)** was estimated from the dose-response graph plotted with percentage inhibition and concentrations of plant extract.

Statistic analysis

Descriptive statistics were performed and values were expressed as mean ± standard deviation. One-way analysis of variance **(**ANOVA**)** was employed to evaluate the differences among varieties for total polyphenol content and antioxidant activity and was declared significant when $p < 0.05$ at 5% level of significance. SPSS **(**version 20.0 SPSS Inc, IL, USA**)** was used to analyze the data.

RESULTS AND DISCUSSION

Total phenolic contents of selected wild vegetables

The TPC of selected wild vegetables was analyzed using the Folin–Ciocalteu method, and also presented in Table 1. Generally, these vegetables had high TPC. *Bauhinia acuminata* had the highest phenolic content **(**710.42 ±4.32 mg GAE.$100g^{-1}$ DW**)**, followed by *Commelina benghalensis* L. **(**701.33 ±5.01 mg GAE.$100g^{-1}$ DW**)**, *Hydrocotyle sibthorpiodes* L. **(**633.45 ±3.73 mg GAE.$100g^{-1}$ DW**)**, *Albizia procera* **(**550.89 ±1.58 mg GAE.$100g^{-1}$ DW**)**, *Cajanus cajan* Millsp. **(**491.51 ±2.71 mg GAE.$100g^{-1}$ DW**)**. The lowest TPC was found in *Leucas aspera* **(**102.20 ±1.10 mg GAE.$100g^{-1}$ DW**)**. The TPC in the wild leafy vegetables of the present study ranged from 102.20 to 710.42 mg GAE.$100g^{-1}$ DW. Previous study reported that the TPC of some common Indian leafy vegetables was in the range of 5 – 69.5 mg of tannic acid.g^{-1} of extract **(Shyamala et al., 2005)**. Compared to other studies, the wild vegetables under investigation had higher TPC than some frequently consumed leafy and non-leafy vegetables **(Uusiku et al., 2010; Kaur and Kapoor, 2002; Gupta et al., 2005; Salvatore et al., 2005; Gupta and Prakash, 2009; Mohankumar, Uthira and Maheswari, 2018)**.

Table 1 Total phenolic content of the selected wild leafy vegetables.

Local Name	English Name	Scientific Name	Total phenolic content (mg GAE.100g^{-1} DW)
Orohorpata	Pigeon pea	*Cajanus cajan* Millsp.	491.51 ±2.71^{e}
Parul	Rose flower fragrant	*Stereospermum suaveolens*	405.64 ±2.97^{g}
Bet gach	Korok bet	*Calamus tenuis* Roxb.	340.53 ±2.85^{i}
Bat baittashak	Blue commelina	*Commelina benghalensis* L.	701.33 ±5.01^{b}
Helencha	Buffalo spinach	*Enhydra fluctuans*	435.12 ±3.48^{f}
Shada koroi	Labbec tree	*Albizia procera*	550.89 ±1.58^{d}
Tetul pata	Tamarind leaf	*Tamarindus indica*	380.25 ±2.05^{h}
Sakumubakla	Lawn marsh	*Hydrocotyle sibthorpiodes* L.	633.45 ±3.73^{c}
Shetodhron	Unavailable	*Leucas aspera*	102.20 ±1.10^{j}
Shetokanchan	white orchid-tree	*Bauhinia acuminata*	710.42 ±4.32^{a}

Note: different superscript letters in each column indicates the significant differences in the mean at p <0.05.

Table 1 DPPH free radical scavenging activity of selected wild leafy vegetables.

Local Name	English Name	Scientific Name	% of DPPH$^{\cdot}$ Inhibition at different concentration				
			100	200	300	400	500
Orohorpata	Pigeon pea	*Cajanus cajan* Millsp.	48.24 ±1.05^{b,c}	62.03 ±0.18^{b,c}	75.1 ±2.45^{b,c}	84.66 ±4.37^{a,b}	90.79 ±5.81^{a,b}
Parul	Rose flower fragrant	*Stereospermum suaveolens*	45.55 ±6.08^{b,c}	56.03 ±0.06^{c,d}	70.72 ±0.18^{d}	82.41 ±0.88^{a,b}	89.31 ±1.11^{a,b}
Bat baittashak	Blue commelina	*Commelina benghalensis* L.	53.28 ±1.02^{a,b,c}	66.72 ±1.28^{a,b}	79.34 ±1.05^{a}	85.2 ±1.23^{a,b}	91.97 ±1.31^{a}
Helencha	Buffalo spinach	*Enhydra fluctuans*	49.66 ±2.28^{a,b,c}	63.45 ±1.10^{b,c}	73.48 ±0.88^{c,d}	81.07 ±1.93^{a,b}	90.10 ±0.89^{a,b}
Shada Koroi	Labbec tree	*Albizia procera*	48.10 ±3.50^{b,c}	60.34 ±0.71^{b,c,d}	71.55 ±0.92^{c,d}	87.76 ±3.50^{a}	91.21 ±4.90^{a}
Tetul pata	Tamarind leaf	*Tamarindus indica*	48.10 ±4.40^{b,c}	58.1 ±2.28^{c,d}	63.1 ±1.59^{e}	73.79 ±2.12^{b}	84.62 ±2.81^{a,b}
Sakumubakla	Lawn marsh	*Hydrocotyle sibthorpiodes* L.	56.03 ±0.67^{a,b}	67.38 ±1.05^{a,b}	77.59 ±1.23^{a,b}	84.31 ±1.28^{a,b}	91.83 ±2.13^{a}
Shetodhron	Unavailable	*Leucas aspera*	21.03 ±8.81^{d}	41.55 ±7.11^{e}	50.03 ±2.08^{f}	61.38 ±10.49^{c}	68.1 ±2.65^{c}
Shetokanchan	White orchid-tree	*Bauhinia acuminata*	60.24 ±2.27^{a}	72.66 ±1.58^{a}	80.21 ±0.35^{a}	88.76 ±0.95^{a}	93.1 ±1.23^{a}

Note: different superscript letters in each column indicates the significant differences in the mean at p <0.05.

Secondary plant metabolites, such as aromatic phenolic compounds, are widely distributed throughout the plant kingdom and related with colour, sensory qualities and nutritional and antioxidant attributes of food. The antioxidant activity of phenolic compounds is mainly due to redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers, heavy metal chelators and hydroxy radical quenchers **(Kaur and Kapoor, 2002)**. Phenolic compounds such as flavonoids, phenolic acid and tannins exerts varied biological activities such as anti-inflammatory, anti-carcinogenic and anti-atherosclerotic activities and these effects could be attributed to their antioxidant activity **(Podsędek, 2007; Mertz et al., 2009)**.

However, it has been reported that the TPC of vegetables varies widely depending on the variety of vegetable, climatic conditions, stage of maturation, soil features, extraction methods, especially the extraction solvent, time and pH **(Kamffer, Bindon and Oberholster, 2010)** and thus makes it difficult for effective comparison, as different standard compounds have been used for their analysis.

Several studies have reported an inverse relation between flavonoid intake and risk of lung cancer, cardiovascular diseases **(Wang et al., 2011; Bystrická et al., 2017; Lenková et al., 2017; Brindza et al., 2019)** and biomarkers of inflammation **(Shaik et al., 2006)**. Hence, the consumption of such wild vegetables, rich in phenolic compound, could ameliorate or prevent the generation of chronic diseases.

DPPH free radical scavenging activity of selected wild vegetables

The DPPH free radical assay is an easy, reliable and quick way to evaluate antioxidant potential of various extracts **(Piang-Siong et al., 2017)**. This method is simple and do not call for a special reaction and device. In this method, the antioxidant activity is assessed on the basis of the ability of the antioxidant to donate hydrogen or electron to DPPH$^{\cdot}$ to produce a stable DPPH-H (diphenylhydrazine) molecule. Changes in the color occur as DPPH radicals in the environment decreases and this **(Piang-Siong et al., 2017)**. Hence the more potent antioxidant, more decrease in absorbance is seen and accordingly the IC_{50} value will be minimum.

$$DPPH^{\cdot} + A\text{-}H = DPPH\text{-}H + A^{\cdot}$$

In this study, the extracts of the undertaken vegetable samples were assessed for the antioxidant potential by utilizing the above principle of DPPH radical scavenging method. Table 2 represents the DPPH$^{\cdot}$ radical scavenging abilities of the wild vegetables used in this study. At 500 μg.mL^{-1}, *Bauhinia acuminata* (Shetokanchan) showed the highest DPPH inhibition (93.10 ±1.23%), followed by *Commelina benghalensis* (Bat baittashak) (91.97 ±1.31%), *Hydrocotyle sibthorpiodes L.* (Sakumubakla) (91.83 ±2.13%), *Albizia procera* (Shada Koroi) (91.21 ±4.90%), *Cajanus cajan* (Orohorpata) (90.79 ±5.81%). The lowest DPPH radical inhibition among the studied samples was observed in *Leucas aspera* (Shetodhron) (68.1 ±2.65%) compared to other samples studied. In this study, all extracts prepared from the vegetable leaves contain varying degrees of DPPH scavenging activity. At a higher concentration, these extracts exhibit more significant DPPH free radical scavenging activity. From Table 2, we can also observe that there is a dose response relationship in inhibiting DPPH$^{\cdot}$ free radical.

Fabaceae species are one of the several indigenous vegetables that have been reported to be rich in antioxidant compounds **(Gođevac et al., 2008)**. As stated, this was the first study to evaluate the antioxidant capacities of the undertaken wild vegetables, no reference data was available to compare. Thus, only few studies could be found examining the other members of the same species. In a study by **Gođevac et al., (2008)** using nine different Fabaceae species, they found that the Fabaceae species possess good antiradical activity. Similar results was also reported by **Srinivasan et al. (2016)**. Similar to our study, few other studies have also reported that the *Tamarindus indica*, *Commelina nudiflora* L., *Bauhinia variegata*, *Leucas aspera* exhibited potent DPPH free radical scavengers **(Rajani and Ashok, 2009; Das et al., 2011; Chew et al., 2012; Kuppusamy et al., 2015; Mbaye et al., 2017)**. It is speculated that the antioxidant activity of the extract is attributed to the presence of phenolic components **(Rajani and Ashok, 2009; Das et al., 2011; Chew, Jessica and Sasidharan, 2012; Kuppusamy et al., 2015; Mbaye et al., 2017)**.

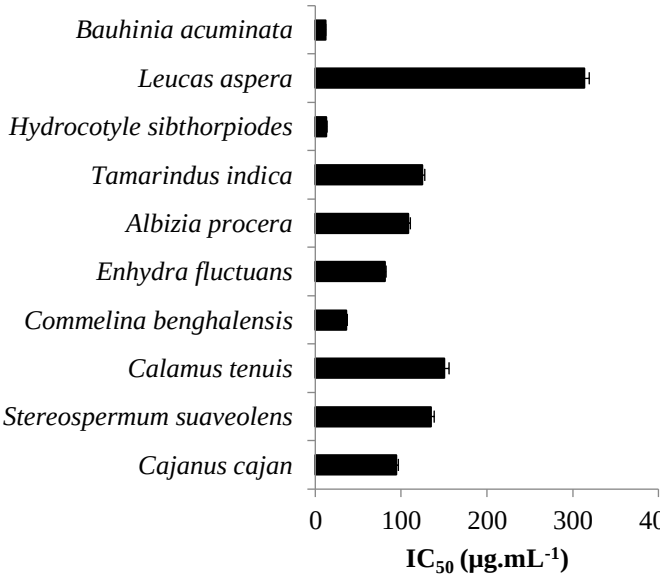

Figure 2 IC_{50} values of selected wild leafy vegetables of Bangladesh.

The DPPH radical scavenging activity was further expressed as the effective concentration (IC_{50}) at which antioxidant activity was 50% (Figure 2). The lowest IC_{50} values for the vegetables were recorded of *Bauhinia acuminata* (11.64 μg.mL^{-1}), *Hydrocotyle sibthorpiodes* L. (12.78 μg.mL^{-1}), *Enhydra fluctuans* (81.19 μg.mL^{-1}), *Cajanus cajan* (94.28 μg.mL^{-1}), whereas *Leucas aspera* (313.79 μg.mL^{-1}) exhibited the highest IC_{50} value in all the studied samples.

The IC_{50} values observed in this study were varying from other reports **(Gođevac et al., 2008; Rajani and Ashok, 2009; Das et al., 2011; Chew, Jessica and Sasidharan, 2012; Kuppusamy et al., 2015; Srinivasan et al., 2016; Mbaye et al., 2017)**. Ethanolic extract of *L. aspera* root exhibited a significant DPPH radical scavenging activity having an IC_{50} value of 7.5 μg.mL^{-1} **(Rahman, Sadhu and Hasan, 2007)** whereas we found 313.79 μg.mL^{-1} in methanolic extract of the leaves. Moreover, the ethanolic extract of whole *L. aspera* plant showed an IC_{50} value of 176.46 mg.mL^{-1} **(Das et al., 2011)**. This difference in scavenging activity could be due to the different solvent extract system, growing conditions, fractions of the plant and some other variables **(Rahman Sadhu and Hasan, 2007; Chew, Jessica and Sasidharan, 2012)**.

CONCLUSION

The selected wild plant extracts investigated in this study showed potent antioxidant activity and varying degree of phenolic content. Among the studied samples, *Bauhinia acuminata* (Shetokanchan) exhibited the highest phenolic content (710.42 mg GAE.100g^{-1}) and free radical scavenging activity (up to 93.1 ±1.23% inhibition). As the plant extracts are quite safe and the use of synthetic

antioxidant has been limited because of their toxicity, therefore, these wild vegetables could be employed as antioxidant additives or as nutritional supplements. However, further studies are required to isolate and characterize the individual components from these plants which are actually responsible for their antioxidant activities and develop their applications for food and pharmaceutical industries.

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