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(Research Article)

Comparative Studies on Antioxidant Potential of *Andrographis paniculata* Grown in Different Places of Bangladesh

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ABSTRACT

The antioxidant activity of methanol cold extract and n-hexane hot extract of leaf and stem part of Andrographis paniculata (Burm.f) growing in four different areas of Bangladesh was done in the present study. Five complimentary in-vitro methods namely DPPH free radical scavenging activity, reducing power assay, total antioxidant capacity, total phenolic and flavonoid contents determination were used for the evaluation of antioxidant properties of two extracts of Andrographis paniculata. In DPPH free radical scavenging test, IC_{50} value for methanolic cold extract in different places was found significant i.e.187.92, 179.70, 53.42, 11.77 µg/ml respectively while compared to the IC_{50} value of the reference standards ascorbic acid and Butylated Hydroxy Anisole (BHA) (6.12 and 7.82 µg/ml) respectively. The result revealed that the methanol cold extract demonstrated the present of highest amount of phenolic and flavonoid compounds (83.1838±0.172 µg GAE/g of sample and 552.5377±0.381µg GAE/g of sample) and also showed significant antioxidant activity (IC_{50} value 11.7729±0.23 µg/ml). A positive correlation was observed between the total phenolic content and total antioxidant capacity of Andrographis paniculata having correlation coefficient (R^2) of 0.98. Measurement of reducing power through ferric and cupric reducing antioxidant capacity was evaluated on cold and hot extract; methanol cold extract was showed significant reducing capacity (0.9521±0.033 Avg. absorbance at 700 nm).

Key Words: *A. paniculata*, DPPH, Free radical scavenging activity, Total phenolic content, Total flavonoids content, Total antioxidant capacity.

INTRODUCTION

Andrographis paniculata (Burm.f) is a herbaceous plant in the family Acanthaceae which grows mainly in tropical and subtropical regions in South and Southeast Asian countries, India, Sri Lanka and China. Locally it is known as "King of bitters". *Andrographis paniculata* (*A. paniculata*) is an annual herb having 30-110 cm height. It is one of the most important medicinal plants used by people in these countries for treatment of jaundice, asthma, hepatitis, urolithic disease, fever, malaria, stomachache and tuberculosis[1]. Traditionally, this plant is used in antimicrobial activity[2], hepatoprotective capacity[3], anti-malarial activity[4], anti-diarrheal potential[5]. The plant's juice and the leaves (dried or fresh) are used in traditional Asian medicines and various other herbal combinations. The therapeutic activities of this plant are attributed to the diterpene andrographolide and the related neoandrographolide. Andrographolide is the major chemical of the plant, and *in vitro* studies on cancer cells showed it to have cytostatic and cytotoxic activity in neoplastic cells[6,7], anti-inflammatory[8] and hepatoprotective activity against galactosamine and paracetamol intoxication[9]. In recent studies andrographolide has been found to be inhibitor against the HIV virus *in vitro*[10]. Neoandrographolide has better activity against malaria[11] and is hepatoprotective against carbon tetrachloride[12]. Toxicity data in animals suggest the safe use of *A. paniculata* extracts and its chemicals for various clinical conditions[13,10].

Free radicals generated in the human body may increase the risk of chronic diseases such as cancer and cardiovascular diseases. These free radicals are usually produced through aerobic respiration. Although the human body produces antioxidant enzymes to neutralize free radicals[14], a diet rich in edible antioxidants is recommended to assist the human body to protect itself from food born free radicals.

Bangladeshi's rainforest being part of the world's tropical rain forest is also considered as one of the most evolved and diverse rainforest in the world. This biodiversity supports numerous species of medicinal plants. One of such genus

that have been used for in folk medicine for decades and known to possess great diversity of secondary metabolites is the genus *Andrographis*[15]. Research on *A. paniculata* has been widely conducted in India[16] reported that antioxidant activity and hepato protective potential found in *A. paniculata.*

Although there has been some reports on the health benefits of *A. paniculata* species elsewhere but information regarding the comparative study on antioxidant activities of these four species growing in Bangladesh is not found. This study was therefore undertaken to compare the four *A. paniculata* species in terms of its potential antioxidant activity growing in Bangladesh. Additionally, two types of extraction method were used in this study and their efficiencies in extracting the beneficial phytochemical were compared through antioxidant assays.

MATERIALS AND METHODS

Collection of Plant Material

Whole plants of *A. paniculata* (Burm.f) were collected in middle of 2011 from Gaibandha, Natore and Kushtia and a voucher specimen representing this collection has been deposited in Bangladesh National Herbarium (Accession number DACB-35939), Mirpur, Dhaka.

Chemicals

1,1-Diphenyl-2-picryl hydrazyl (DPPH), L-Ascorbic acid, Butylated hydroxy anisole (BHA), Folin-Ciocaltu phenol reagent, Gallic acid, Quercetin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Ferric chloride, potassium ferricyanide, trichloroacetic acid (TCA), phosphate buffer (pH 6.6), sodium phosphate, methanol, ammonium molybdate, sulfuric acid, sodium carbonate, aluminum chloride and potassium acetate were of analytical grade and purchased from Merck (Darmstat, Germany).

Preparation of Extracts

The collected and identified plant parts were dried (after cutting and slicing where necessary) in the sun and finally in a mechanical dryer at 60 – 70^0 C. The dried samples were ground to coarse powder with a Grinding Mill and powdered samples were kept in clean closed glass containers pending extraction. During grinding of sample, the grinder was thoroughly cleaned to avoid contamination with any remnant of previously ground material or other foreign matter deposited on the grinder. Methanol cold extract and n-hexane hot extract (50 mg) were weighed; 50 ml of methanol and CH_2Cl_2 were then added separately and mixed with stirring. The concentrations of these solutions were 1 mg/ml. Then were prepared in respective solvent systems from which serial dilutions were carried out to obtain the concentrations of 1, 5, 10, 50, 100, 200 µg/ml and used as stock solutions for the antioxidant screening and reducing power assay.

DPPH Free Radical Scavenging Assay

The antioxidant activity of the methanol cold and n-hexane hot extract obtained from it was measured by evaluating the scavenging activity of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical[17]. In this assay, 2 ml of 0.2 mµ methanolic DPPH solution was added to 2 ml of extract solution at different concentrations and the contents were stirred vigorously for 15 seconds. Then the solutions were allowed to stand at dark place at room temperature for 30 min for reaction to occur. After 30 min, absorbance was measured against a blank at 517 nm with a double beam Analykjena UV/Visible spectrophotometer (Model 205, Germany). The percentage of DPPH radical-scavenging activity of each plant extract was calculated as follows:

DPPH radical-scavenging activity,

$$I\% = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100$$

Where, A_{blank} is the absorbance of the control solution (containing all reagents except plant extracts); A_{sample} is the absorbance of the DPPH solution containing plant extract. The DPPH radical-scavenging activity (%) was plotted against the plant extract concentration to determine the concentration of extract necessary to decrease DPPH radical-scavenging by 50% (called IC_{50}). The IC_{50} value of each extract was estimated by sigmoid non-linear regression, using Sigma Plot 2000 Demo (SPSS Inc., Chicago, IL, USA). These values were changed to antiradical activity, defined as $1/EC_{50}$, since this parameter increases with antioxidant activity. All determinations were performed in triplicate. An average of six experimental results was recorded and is presented in Table-1 and 2, Fig-1and 2. Here, synthetic antioxidants butylated hydroxyl anisole (BHA) and L-ascorbic acid were used as positive control standard.

Reducing Power Assay

This assay was determined according to the method reported by Oyaizu with slight modifications. Briefly, 10 ml of extract solution of different concentrations was mixed with 2.5ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide [$K_3Fe(CN)_6$] (1%, w/v). The mixture was incubated at 50°C for 20 min. The reaction was terminated by adding 2.5 ml of Trichloroacetic acid (10%, w/v), then the mixture was centrifuged at 3000 rpm for 10 min. The supernatant solution (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml, 0.1%, w/v) solution. Then the optical density (OD) was measured at 700 nm against a blank. Increased OD value of the reaction mixture indicates increased reducing power. Three replicates were made for each test sample.

Total Antioxidant Capacity (TAC)

The total antioxidant activity of the extract was evaluated by the phosphomolybdenum assay method[18] which is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate-Mo (V) complex in acidic condition. The extract (2.0 µg/ml, 0.3 ml) was allowed to mix up with 3.0 ml of reagent solution (0.6 M H_2SO_4, 28 mM Na_3PO_4, 4 mM ammonium molybdate) and the reaction mixture was incubated at 95^0 C for 90 minutes. After cooling at room temperature, the absorbance of the solution was measured at 695 nm using a UV-Visible spectrophotometer against an appropriate blank. The antioxidant activity was expressed as the number of gram equivalents of ascorbic acid.

Total Phenolic Content

The total phenolic content of the extract was determined by the modified Folin-Ciocaltu method[19]. Briefly, 0.5 mL of

extract was mixed with 5 ml Folin-Ciocaltu reagent (1:10 v/v distilled water) and 4 ml (75 g/L) of sodium carbonate. The mixture was vortexed for 15 s and allowed to stand for 30 min at 40°C for color development .The absorbance was read at 765 nm
with a double beam Analykjena UV/Visible spectrophotometer (Model 205, Jena, Germany). Total phenolic content was determined as µg of gallic acid equivalent per gram using the equation obtained from a standard gallic acid calibration curve y=6.91x −0.093, R^2=0.9972.

Total Flavonoid Content

Aluminium chloride colorimetric method[20] was used for determination of total flavonoids concentration in the samples of *phylanthus sps.* leaf. Each extract and fraction (0.5 ml, 1:10 gm/L) in methanol were separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. It was allowed to stand for 30 min at room temperature and the absorbance of the reaction mixture was measured at 415 nm. Total flavonoids content was determined as µg of Quercetin equivalent per gram using the equation obtained from a standard Quercetin calibration curve y=4.7385x + 0.0355; R^2 = 0.9933.

Statistical Analysis

Data were presented as mean ± S.D. Statistical differences between control and treated groups were tested by Student's t-test. The differences were considered significant at P<0.05.

A comparative study of antioxidant activity on four variations of *A. paniculata* growing in Bangladesh was carried out in this study. Antioxidant properties were determined following five established methods like DPPH free radical scavenging activity, reducing power assay, total antioxidant capacity, total phenolic and flavonoid content determination.

A method based on the scavenging of the stable radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH) has been used extensively to predict the antioxidant activities of extracts of plants[21]. Tannins and Flavonoids, commonly found in plants have been reported to have significant antioxidant activity[22]. The DPPH radical scavenging activity of different solvent extracts (methanol and n-hexane) and the standard antioxidants ascorbic acid and butylated hydroxyanisole (BHA) in methanol solutions at different concentrations are shown in table-1 and table-2. The IC_{50} values (The half maximal inhibitory concentration) of Ascorbic acid and BHA were found as 6.12±0.13 µg/ml and 7.82±0.15 µg/ml respectively and the IC_{50} values of methanol extract of APLK-M, APLG-M, APLN-M, and $APLN_s$-M were found as 187.92±0.49, 179.70±0.41, 53.42±0.36, and 11.77±0.23 µg/ml respectively. Both the APLN-M and $APLN_s$-M (collected from Natore) samples were exhibited highest DPPH free radical scavenging activity than the other samples. And best IC_{50} values exhibited in Natore samples. In case of methanol cold extracts, both the APLN and $APLN_s$ (collected from Natore) samples showed best activity. So, the order of DPPH free radical scavenging activity of methanol extracts of the test samples is $APLN_s$-M >APLN-M >APLK-M >APLG-M.

RESULT AND DISCUSSION

Table-1: Comparison of DPPH radical scavenging activity of methanol cold extracts among four *A. paniculata* samples and Standards.

Concentration (µg/ml)	% Inhibition of methanol cold extracts and Standards at different concentration					
	APLK-M	APLG-M	APLN-M	$APLN_s$-M	AA (Standard)	BHA (Standard)
1	2.92±0.11	2.71±0.12	4.11±0.12	5.92±0.21	35.01±0.21	31.81±0.24
5	9.14±0.14	0.23±0.15	10.05±0.23	15.23±0.24	47.91±0.23	41.42±0.19
10	11.85±0.12	2.19±0.24	25.23±0.31	42.02±0.35	69.01±0.19	62.32±0.16
50	15.52±0.21	11.94±0.32	46.79±0.24	80.78±0.31	81.49±0.27	78.84±0.17
100	25.80±0.23	26.20±0.23	81.78±0.16	91.08±0.34	95.05±0.31	91.46±0.32
200	53.21±0.25	55.64±0.19	86.28±0.33	93.38±0.35	96.69±0.18	93.69±0.41
IC_{50} (µg/ml)	187.92±0.4	179.70±0.5	53.42±0.33	11.77±0.23	6.12±0.13	7.89±0.15

The average values of the extract are presented as mean ± S.D. AA=Ascorbic acid; BHA=Butylated Hydroxy Anisole; APLK-M=A. paniculata leaf with stem from Kushtia methanol cold extract; APLG-M= A. paniculata leaf with stem from Gaibandha methanol cold extract; APLN-M=A. paniculata leaf with stem from Natore methanol cold extract; APLNs-M=A. paniculata leaf with stem from Natore a standard sample methanol cold extract; S.D=Standard Deviation.

Table-2: Comparison of DPPH radical scavenging activity of n-hexane hot extracts among four *A. paniculata* samples and Standards.

Concentration (µg/ml)	% Inhibition of n-hexane hot extracts and Standards at different concentration					
	APLK-H	APLG-H	APLN-H	$APLN_s$-H	AA (Standard)	BHA (Standard)
1	12.49±0.13	12.84±0.23	29.33±0.12	25.41±0.23	35.01±0.21	31.82±0.24
5	14.85±0.21	15.99±0.31	30.03±0.19	27.08±0.21	47.91±0.23	41.51±0.19
10	15.39±0.25	16.33±0.15	32.52±0.21	28.54±0.15	69.01±0.19	62.32±0.16
50	17.52±0.22	26.28±0.22	34.42±0.31	30.108±0.32	81.44±0.27	78.87±0.17
100	20.75±0.12	26.76±0.32	38.72±0.23	40.33±0.42	95.25±0.31	91.96±0.32
200	25.35±0.35	30.48±0.33	56.03±0.18	54.52±0.53	96.29±0.18	93.69±0.41
IC_{50} (µg/ml)	-	-	178.3±0.29	174.32±0.1	6.10±0.13	7.89±0.15

The average values of the extract are presented as mean ± S.D. AA=Ascorbic acid; BHA=Butylated Hydroxy Anisole; APLK-H=A. paniculata leaf with stem from Kushtia n-hexane hot extract; APLG-H= A. paniculata leaf with stem from Gaibandha n-hexane hot extract; APLN-H=A. paniculata leaf with stem from Natore n-hexane hot extract; APLNs-H=A. paniculata leaf with stem from Natore a standard sample n-hexane hot extract; S.D=Standard Deviation.

The reducing properties of plant extracts are generally associated with the presence of reductones[23] which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom[24]. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. Reducing power activity of methanol and n-hexane extracts of both the APLN and $APLN_s$ (collected from Natore) samples can be considered best (0.84±0.027 and 0.95±0.033 respectively) in compare to the activity of standard samples (2.85±0.036 for Ascorbic acid and 1.91±0.041 for BHA) that are presented at table-3 and table-4. Where, reducing power activity of APLK-M and APLG-M were found moderate (0.67±0.029 and 0.55±0.036). So, in overall consideration it is clear that the reducing power activity of both the APLN and $APLN_s$ (collected from Natore) samples is better than the activity of both APLK (Kushtia sample) and APLG (Gaibandha sample) samples and the order of reducing power activity of methanol cold extracts of the test samples is $APLN_s$-M >APLN-M >APLK-M >APLG-M.

Table-3: Comparison of reducing power assay of Methanol cold Extract among the four *A. paniculata* samples and Standard following the extraction method.

Concentration (µg/ml)		Average Absorbance at 700 nm methanol cold extracts and Standards at different concentration				
	APLK-M	APLG-M	APLN-M	$APLN_s$-M	AA (Standard)	BHA (Standard)
1	0.10±0.012	0.09±0.011	0.20±0.018	0.20±0.016	0.17±0.022	0.15±0.031
5	0.20±0.018	0.19±0.018	0.30±0.011	0.36±0.029	0.43±0.023	0.40±0.015
10	0.32±0.025	0.31±0.013	0.45±0.026	0.45±0.015	0.58±0.029	0.51±0.015
50	0.43±0.017	0.38±0.023	0.60±0.013	0.60±0.017	0.90±0.018	0.81±0.036
100	0.56±0.034	0.49±0.024	0.74±0.017	0.88±0.028	1.74±0.031	1.02±0.029
200	0.67±0.029	0.55±0.036	0.84±0.027	0.95±0.033	2.85±0.036	1.91±0.041

The average values of the extract are presented as mean ± S.D. AA=Ascorbic acid; BHA=Butylated Hydroxy Anisole.

Table-4: Comparison of reducing power assay of n-Hexane hot extract among the four *A. paniculata* samples and Standard following the extraction method.

Concentration (µg/ml)		Average Absorbance at 700 nm of n-Hexane hot extracts and Standards at different concentration				
	APLK-H	APLG-H	APLN-H	APLNs-H	AA (Standard)	BHA (Standard)
1	0.05±0.007	0.09±0.009	0.08±0.007	0.05±0.008	0.17±0.022	0.15±0.03
5	0.10±0.002	0.12±0.005	0.10±0.002	0.09±0.001	0.43±0.023	0.40±0.01
10	0.10±0.004	0.15±0.0012	0.15±0.0013	0.11±0.001	0.58±0.029	0.51±0.01
50	0.13±0.001	0.15±0.0014	0.18±0.0011	0.15±0.001	0.90±0.018	0.81±0.03
100	0.18±0.009	0.18±0.007	0.26±0.005	0.20±0.009	1.74±0.031	1.02±0.02
200	0.25±0.007	0.19±0.0013	0.36±0.0014	0.30±0.001	2.85±0.036	1.91±0.04

The average values of the extract are presented as mean ± S.D. AA=Ascorbic acid; BHA=Butylated Hydroxy Anisole.

Total antioxidant capacity of methanol extracts of *A. paniculata* expressed as the number of µg equivalents of ascorbic acid, is shown in Table-5. By comparing activity of total antioxidant activity to ascorbic acid extracts from *in vitro* culture was best among other extract. *A. paniculata* contain alkaloids[25] hydrolysable tannins, phenolics, polyphenols and flavonoids[26] The antioxidative effect is mainly due to phenolic components, such as phenolic acids, and phenolic diterpenes[27]. Total antioxidant capacity of methanol extract of both the APLN and $APLN_s$ (collected from Natore) samples can be considered best (0.33±0.02 and 0.50±0.01 respectively) in compare to Kushtia and Gaibandha sample. A positive correlation was seen between total phenolic content and total antioxidant capacity of *A. paniculata* having correlation coefficient (R^2) values of 0.98 which are presented at figure-1.

Table-5: Total antioxidant capacity, total phenolic content, total flavonoid content and free radical scavenging activity of different samples of *A. paniculata.*

samples	Total phenolic content (µg of GAE/gm of dried extract)	Free radical scavenging activity (IC_{50} µg/ml)	Total antioxidant capacity (µg of ascorbic acid/100 g of extract)	Total flavonoids content (µg of QE gm of dried extract)
AA	-	6.12±0.13	-	-
BHA	-	7.82±0.15	-	-
APLK-M	18.21±0.134	187.92±5.49	0.05±0.03	282.00±10.206
APLG-M	39.78±0.108	179.70±4.41	0.15±0.02	313.55±9.117
APLN-M	65.60±0.171	53.42±0.36	0.33±0.02	552.53±7.381
$APLN_s$-M	83.18±0.172	11.77±0.23	0.50±0.01	440.43±5.278

The average values of the extract are presented as mean ± S.D. AA=Ascorbic acid; BHA=Butylated Hydroxy Anisole.

The Total phenolic content (TPC) assay is a common assay widely used to estimate relative amounts of phenolic compounds present in an extract. The TPC results were expressed as mg gallic acid equivalent as this compound represents the most simple form of a phenolic compound. Phenolic compounds present in the extract undergo a complex redox reaction with phosphotungstic and phosphomolybdic acids present in the TPC reagent[28]. Depending on the number of phenolic groups present, different response can be observed in terms of the color change due to oxidation of the TPC reagent. The total phenolic content of different Kushtia, Gaibandha Natore and Natore Standard ($APLN_s$) samples in CH_3-OH cold extract was found to be demonstrated 18.2691±0.134, 39.7829±0.108, 65.6005±0.171 and 83.1838±0.172 µg GAE/gm of sample, respectively. The result indicated the presence of highest amount of phenolic compounds in the Natore standard sample ($APLN_s$) which are stated at table-6.

Table-6 and table-7 revealed that the results of total phenolic content and total flavonoids contents in the methanol extract of *A. paniculata* respectively. The methanol cold extract of *A. paniculata* was found to contain 83.1838±0.172 µg of gallic acid equivalent per gram of dry powder.

From table-7 it is observed that methanol cold extract of *A. paniculata* was found to contain 552.5377±0.381 µg of quercetin equivalent per gram of dry powder and the n-hexane hot extract contained 501.1079±0.17 µg of quercetin equivalent respectively per gram of dry extract.

$$y=141.72x+15.21 \ (R^2=0.98)$$

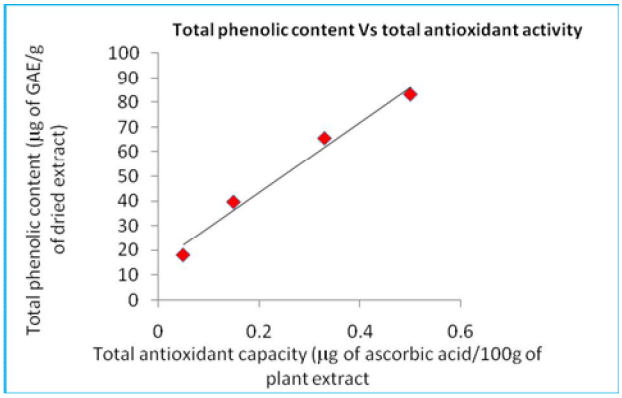

Fig-1: Correlation between the total phenolic content and total antioxidant capacity.

Table-6: Total phenolic content of methanol cold extract of *A. paniculata* sample

Extract Name	Avg. absorbance at 765 nm	Total phenolic content (µg GAE/ g of dry ext.)
Methanol Extract (APLK-M)	0.033 ±0.019	18.2691±1.134
Methanol Extract (APLG-M)	0.1819 ±0.018	39.7829±2.108
Methanol Extract (APLN-M)	0.3603 ±0.011	65.6005±2.171
Methanol Extract ($APLN_s$-M)	0.4818±0.014	83.1838±2.172

Values are expressed as mean ± SD (n = 3) GAE: Gallic acid equivalent

Table-7: Total flavonoids content of methanol cold extracts of *A. paniculata* samples.

Extract Name	Avg. absorbance at 430 nm	Total flavonoid content (µg QE/ g of dry ext.)
Methanol Extract (APLK-M)	1.3718±0.021	282.0091±5.206
Methanol Extract (APLG-M)	1.5213±0.025	313.5591±8.117
Methanol Extract (APLN-M)	2.6537±0.014	552.5377±10.381
Methanol Extract ($APLN_s$-M)	2.1225±0.027	440.4347±9.278

Values are expressed as mean ± SD (n = 3) QE: Quercetin equivalent.

So, from the above discussions it has been observed that the antioxidant activity of both the APLN and $APLN_s$ (collected from Natore) *A. paniculata* samples is better than the activity of APLK (collected from Kushtia) and APLG (collected from Gaibandha) *A. paniculata* samples. So, in conclusion, it can be said that the medicinal properties of *A. paniculata* samples of Natore are better than that of *A. paniculata* samples of Kushtia and Gaibandha. It is also observed that through these methods, which we used during this research (for determination of antioxidant activity), the quality of four different *A. paniculata* (Kalmegh) samples could be clearly differentiated. So, we can say that, these method, specially, determination of total phenolics contents, determination of total flavonoids contents and DPPH scavenging activity methods can be used as tools for standardization of *A. paniculata* (Kalmegh) samples.

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