A Comparative Study on Antioxidant Properties of Two *Phyllanthus* Species Growing in Bangladesh

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ABSTRACT: A comparative study of antioxidant properties on two varieties of *Phyllanthus* (*Phyllanthus niruiri* L. & *Phyllanthus urinaria* L. leaves) growing in Bangladesh was done in the present study. Five complimentary test 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 species of *Phyllanthus*. In the present investigation for the evaluation of antioxidant activities the concentration is expressed by the ratio of crude sample per solvent volume instead of extract weight per solvent volume. Based on these concentrations the investigated *P. niruri* L. and *P. urinaria* L. leaves extracts exhibited significant results. At 2 mg/ml concentration DPPH radical scavenging capacity of the methanol extract of both the plants was found to show significant (>90%) activity which is comparable to Ascorbic acid and BHA. In case of reducing power tests for both the extract, the activity of *P. urinaria* is comparable to that of BHA and AA and that of *P. niruiri* is comparable to BHA. *P. niruiri* showed better antioxidant potential than that of *P. urinaria* based on the observed results of their corresponding methanol extract.

Key words: Antioxidant, DPPH free radical scavenging, Reducing power assay, Total antioxidant capacity, Total phenolic, Total flavonoid.

INTRODUCTION

Phylanthus niruri, is a small plant which grows mainly in tropical and subtropical regions in Central and South American countries, India and East Asia. Locally it is known as "Vui amla". 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] An aqueous infusion of the whole plant, which is a typical preparation, is employed as a stomachic, aperitive, antispasmodic, laxative, diuretic, carminative, against constipation, fever including malaria, dysentery, gonorrhea, syphilis, tuberculosis, cough, diarrhea and vaginitis.[2,3] It has antispasmodic, pain- relieving, .anti-inflammatory[4] and antiviral activity.[5-7] It also has action on Kidney Stones & Uric Acid.[8] Chemical investigation of this plant has been carried out and several constituents were isolated such as lignans, alkaloids, flavonoids, tannins, phthalic acid, gallic acid and terpenoids.[9,10]

Phyllanthus urinaria L. (Euphorbiaceae) is widely used as a traditional folk medicine for inflammatory relief.[11]. It was also reported to have anti-diabetic, anti-cancer and anti-inflammation properties and was shown to have anti-mutagenic and anti-carcinogenic effects in India.[12]

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

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metabolites is the genus *Phyllanthus*. *P. niruri* and *P. urinaria*.[12] Research on *Phyllanthus* sp. has been widely conducted in India[13] reported that antioxidant activity and hepato protective potential found in *P.niruri*.

Although there has been some reports on the health benefits of *Phyllanthus* species elsewhere but information regarding the comparative study on antioxidant activities of these two species growing in Bangladesh is not found. This study was therefore undertaken to compare the two *Phyllanthus* 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 materials. For *in vitro* screening of antioxidant potential of *Phyllanthus niruri* L. and *Phyllanthus urinaria* L. (local name, Bhui Amla) were collected from BCSIR campus, Dhaka, Bangladesh, during June, 2010.

Preparation of sample. The foreign materials were separated from the collected samples of *P. niruri & P. urinaria* leaves and washed separately with clean water. The leaves were then cut into small pieces and dried under shade. The dried samples were taken in an airtight container and stored in a cool place. The samples were grinded into powder before analysis.

Preparation of sample extracts. *The dry powdered* samples of *P. niruri & P. urinaria* leaves (100-1000 mg) were weighed and, 50 ml of methanol and water were then added separately into each of the samples and allowed to stand for 24 hours with occasional stirring. The ratios of sample weight to solvent volume were 2, 4, 8, 12, 16 and 20 mg/ml. The extracts were then vacuum filtered and used as stock solutions for the following tests.

Antioxidant screening. *In vitro* antioxidant screening of the above dried plant materials were carried out using the five established methods, DPPH (1, l-diphenyl-2-picrylhydrazyl) scavenging activity, reducing power assay, total antioxidant capacity, total phenolic content and total flavonoid content determination.

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).

DPPH free radical scavenging activity. DPPH radical scavenging activity of *Phyllanthus* samples were studied by the method of Oyaizu as followed (Oyaizu, 1986). During this experiment the test samples methanol and water extracts of two varities of *Phyllanthus* (5mL) accompanied with standard compounds Ascorbic acid and Butylated hydroxy anisole (BHA) methanolic solutions (5 ml) were separately mixed with freshly prepared DPPH methanolic solution (1 mM, 1 ml), vortex and allowed to stand at the dark place at 25°C for 30 min for the reaction to occur. After 30 min of incubation period, the absorbance was read against a blank at 517 nm with a double beam Analykjena UV/Visible spectrophotometer (Model 205, Jena, Germany). The radical scavenging activity was expressed as the inhibition percentage (1%) and calculated as per the equation: [(A blank - A sample)/A blank x 100] Where A blank is the absorbance of the control reaction (containing all reagents except the test compound), and A sample is the absorbance of the test compound with all reagents. An average of three experimental results was recorded and is presented in Table 1. Here, synthetic antioxidants butylated hydroxyl anisole (BHA) and L-ascorbic acid were used as positive control standard.

Test for reducing power activity. The reducing powers of *Phyllanthus* samples were determined by the following methods developed by Oyaizu.[14] Methanol and water extracts of *Phyllanthus* (10 ml)

accompanied with Ascorbic acid and BHA methanolic solutions (10 ml) were spiked separately with phosphate buffer (2.5 ml, 0.2M, pH 6.6) and 1% potassium ferricyanide (2.5 ml). The mixture was then incubated at 50°C for 20 minutes. The resulting solution was then cooled rapidly, spiked with trichloroacetic acid (2.5 ml, 10%), and centrifuged at 3000 rpm for 10 minutes. The supernatant (5 ml) was then mixed with distilled water (5 ml) and ferric chloride (1 ml, 0.1%). The mixture was then allowed to stand for 10 minutes to complete the reaction and the absorbance was recorded at 700 nm. The higher the absorbance represents the stronger the reducing power. The experiment was repeated thrice and the mean of the results is presented in Table 2.

Determination of total antioxidant capacity. The antioxidant activity of the extract was evaluated by the phosphomolybdenum method according to the procedure describe by Prieto.[15] The assay is based on the reduction of Mo (VI)–Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH. A 0.3 ml extract was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95ºC for 90 min. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer against blank after cooling to room temperature. Methanol (0.3 ml) in the place of extract is used as the blank. The antioxidant activity is expressed as the number of gram equivalents of ascorbic acid from the calibration curve $y=1.203x -0.0843$, $R^2=0.9901$.

Total phenolic content determination. The total phenolic content of the extract was determined by the modified Folin-Ciocaltu method.[16] 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 mg of gallic acid equivalent per gram using the equation obtained from a standard gallic acid calibration curve $y=6.2548x -0.0925$, $R^2=0.9962$.

Assay for total flavonoids conctent. Aluminium chloride colorimetric method[17] 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 mg 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 $P<0.05$.

RESULTS AND DISCUSSION

A comparative study of antioxidant activity on two variations of *Phyllanthus* 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.[18] Tannins and Flavonoids, commonly found in plants have been reported to have significant antioxidant activity.[19]

The DPPH radical scavenging activity of different solvent extracts (methanol and water) and the standard antioxidants ascorbic acid and butylated hydroxyanisole (BHA) in methanol solutions at different concentrations are shown in Table 1. The result of the study expressed that ascorbic acid and BHA possessed 98.2213±0.826 and 96.0101±0.226%

radical inhibitory power at a concentration of 2mg/ml. The methanol extracts of both the *P. niruri* and *P. urinaria* leaves found to possess highest radical scavenging activity 90.6813±0.362 and 90.0917±0.23 % respectively at 2 mg/ml concentration. The inhibitory activity of both the standards were found to be almost consistent from 2 mg/ml to 20 mg/ml concentrations, whereas the inhibition percentage of both the methanol extracts were found to be decreased gradually with the increase of concentration. At 20 mg/ml concentration *P. niruri* methanol extract exhibited 72.2213±0.196% inhibition whereas at the same concentration *P. urinaria* methanol extract gave 61.8977±0.211% inhibition of DPPH radical. In case of water extract maximum inhibition was also observed at 2mg/ml for both the plants. *P. niruri* water extract showed 55.3731±0.295% inhibition and the *P. urinaria* exhibited 52.9100±0.335% inhibition at this concentration. In this case also the activity was decreased gradually with the increase of concentration of extracts for both the plants. At 20 mg/ml concentration, the inhibitions were decreased to 2.6187±0.360 and 1.2492±0.236% respectively for *P. niruri* and *P. urinaria* leaves water extracts. The results showed the scavenging activity of *P niruiri*> *P. urinaria.*

The reducing properties of plant extracts are generally associated with the presence of reductones[20] which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom.[21] Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. Table 2 is showing the results of reducing power activity of methanol and water extracts of *P. niruri* and *P. urinaria* leaves. In this study, it is observed that with the increase of concentration of extract the reducing power increased. At 2 mg/ml concentration Ascorbic acid and BHA exhibited relatively high reducing power than all extracts of *Phyllanthus* except *P. urinaria* water extract. The reducing power of *P. urinaria* water extract at this concentration is comparable to that of BHA at the same concentration (absorbance 1.4473±0.0369 and 1.5222±0.023 respectively). The reducing power was found to be increased with the increase of concentration for both the extracts of the two plants. The activity of water extract of *P. niruri* at higher concentration (20 mg/ml) is moderate whereas that of *P. urinaria* at the same concentration is significant and comparable to both ascorbic acid and BHA. At the highest concentration (20 mg/ml) *P. urinaria* methanol extract exhibited higher activity (2.8274±0.0.1939) than the *P. niruri* methanol extract at the same concentration (2.0874±0.0.036). Both of these activities are comparable to BHA at the same concentration. In comparison to the activity of ascorbic acid at 20 mg/ml concentration the activity of *P. urinaria* water extract at the same concentration is significant.

Total antioxidant capacity of methanol and water extracts of *P. niruri* and *P. urinaria* leaves, expressed as the number of gram equivalents of ascorbic acid, is shown in Table 3. By comparing activity of total antioxidant activity to ascorbic acid extracts from *in vitro* culture was best among other extract. *Phyllunthus* sp. contain alkaloids hydrolysable tannins, phenolics, polyphenols and flavonoids.[22,23] The antioxidative effect is mainly due to phenolic components, such as phenolic acids, and phenolic diterpenes.[24] At 2 mg/ml concentration the total antioxidant capacity of both the methanol and water extract of *P. niruri* was found to be better than the extract of *P. urinaria* at the same concentration. The activity was found to be increased gradually for all the extracts with the increase of concentrations. From 8 mg/ml to 20 mg/ml concentrations the activity of water extract of *P. urinaria* (1.83±0.017-1.49±0.037) was found to be higher than the activity of *P. urinaria* water extract (0.78±0.011-1.33±0.041). But in case of methanol extract the activity was found to be higher for *P. niruri* than for *P. urinaria* at all concentrations (0.39±0.017-1.92±0.032) and (0.25±0.12-1.08±0.37) respectively.

Table 1. DPPH radical scavenging activity of methanol and water extracts of *Phylanthus niruiri & Phylanthus urinaria* and standards (ascorbic acid and BHA).

Concentration	*P. niruiri*		*P. urinaria*		Standards	
	Methanol extract	Water extract	Methanol extract	Water extract	Ascorbic Acid	BHA
2mg/ml	90.68±0.463	55.37±0.295	90.09±0.236	52.91±0.335	98.22±0.362	96.01±0.226
4mg/ml	87.94±0.386	46.03±0.562	84.28±0.123	45.82±0.235	97.99±0.548	96.01±0.658
8mg/ml	85.38±0.269	26.15±0.365	80.30±0.365	23.73±0.222	97.88±0.136	95.60±0.462
12mg/ml	81.25±0.131	17.44±0.489	73.48±0.369	16.77±0.236	97.98±0.351	95.14±0.251
16mg/ml	75.61±0.499	10.93±0. 39	68.20±0.323	8.47±0.287	97.01±0.302	95.88±0.533
20mg/ml	72.22±0.196	2.61±0.366	61.89±0.211	1.24±0.236	98.00±0.368	96.08±0.377

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

Table 2. Reducing power activity of methanol and water extracts of *Phylanthus niruiri & Phylanthus urinaria* and standards (ascorbic acid and BHA).

Concentration	*P. niruiri*		*P. urinaria*		Standards	
	Methanol extract	Water extract	Methanol extract	Water extract	Ascorbic Acid	BHA
2mg/ml	0.135±0.011	0.573±0.02	0.164±0.045	1.447±0.036	3.441±0.01	1.523±0.023
4mg/ml	0.185±0.016	0.702±0.01	0.592±0.057	1.630±0.023	3.492±0.05	1.589±0.019
8mg/ml	0.625±0.029	1.014±0.04	0.668±0.023	1.723±0.039	3.681±0.03	1.696±0.027
12mg/ml	1.528±0.018	1.054±0.06	1.714±0.04	1.845±0.030	3.711±0.01	1.737±0.028
16mg/ml	1.609±0.018	1.077±0.05	1.918±0.043	2.082±0.019	3.751±0.02	1.817±0.036
20mg/ml	2.087±0.036	1.086±0.04	2.827±0.193	2.494±0.178	3.787±0.02	1.858±.0361

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

Table 3. Total Antioxidant Capacity of methanol and water extracts of *Phylanthus niruiri & Phylanthus urinaria*.

Plant names	Extracts	Different concentration of extracts Values are expressed as no. of gram equivalents of ascorbic acid					
		2mg/ml	4mg/ml	8mg/ml	12mg/ml	16mg/ml	20mg/ml
Phylanthus niruiri	Methanol	0.39±0.017	0.67±0.015	0.98±0.023	1.24±0.047	1.50±0.046	1.92±0.032
	Water	0.31±0.015	0.47±0.012	0.78±0.011	0.88±0.013	1.26±0.047	1.33±0.041
Phylanthus urinaria	Methanol	0.25±0.012	0.35±0.019	0.49±0.016	0.64±0.011	0.84±0.014	1.08±0.037
	Water	0.39±0.018	0.50±0.020	0.83±0.017	1.22±0.046	1.27±0.048	1.49±0.037

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

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.[25] 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.

Table 4 and 5 revealed the results of total phenolic content and total flavonoid contents in the methanol and water extracts of *P. niruri* and *P. urinaria*. The water extract of *P. niruri* was found to contain 86.197 mg of gallic acid equivalent per gram of dry powder. Whereas, the same extract of *P. urinaria* was found to possess 65.850 mg of gallic acid equivalence per gram of dry powder. On the other hand the methanol extract of *P. niruri* and *P. urinaria* were found to contain 60.920 and 32.510 mg of gallic acid equivalence per gram of dry powder.

Table 4. Total phenolic content of *Phyllanthus niruri & Phylanthus urinaria*
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Name of plants	Extracts	Total phenolic content (mg of gallic acid equivalent per g of dry extract)
Phyllanthus niruri	Methanol	72.56± 0.52
	Water	43.68± 0.68
Phyllanthus urinaria	Methanol	67.21± 0.39
	Water	53.29± 0.75

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

Table 5. Total flavonoid content of *Phyllanthus niruri & Phylanthus urinaria*

Name of plants	Extracts	Total flavonoid content (mg of Quercetin equivalent per g of dry extract)
Phyllanthus niruri	Methanol	139.69 ± 1.26
	Water	83.52 ± 0.58
Phyllanthus urinaria	Methanol	121.39 ± 1.01
	Water	90.28 ± 0.39

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

From Table 5 it is observed that *P. niruri* and *P. urinaria* methanol extracts contained 130.6±1.62 and 121.33±1.29 mg of quercetin equivalent per gram of dry powder and the water extract contained 30.52±0.621 and 60.55±0.792 mg of quercetin equivalent respectively per gram of dry extract.

CONCLUSION

For DPPH scavenging test of both the methanol and water extracts for *P. niruiri* showed higher activity than that of *P. urinaria* (*P. niruri* > *P. urinaria*). Reducing power activity of *P. urinaria* is comparable to that of BHA and AA and that of *P. niruiri* is comparable to BHA. The results of total antioxidant capacity showed that methanol extract of *P niruiri* > *P urinaria*, where as for water extract *P. urinaria* >*P. niruiri*. The results for the total phenolic content analysis indicate that all *Phyllanthus* species contain significant amounts of phenolic compounds as revealed by the concentrations when compared to the positive control. When comparing the two *Phyllanthus* species, quantity of phenolics in the methanol extract was *P. niruri* > *P.urinaria*. However, this order was reversed for water extract, which is *P. niruri* < *P. urinaria*.

Overall it can be observed from Table 1 that both the total phenolic content and total antioxidant activity was consistently higher in the methanol extract when compared with the water extract. The probable reason for this is due to the difference in the polarity of solvents used. The polarity index for water and methanol are 9.0 and 6.6. Methanol is more efficient in extracting wider range of phenolic compounds (from polar to semipolar) found in *Phyllanthus*. This research finding is supported by investigation carried out by (Masturah et al., 2006) where the major components of *Phyllanthus* sp are active hydrolysable tannins that can be extracted using ethanol-water mixture which are semipolar compounds such as ellagitannins and gallotannins.[26] A significant difference of antioxidant potential between the two *Phyllanthus* species was observed for both water and methanol crude extract. Between the two tested species, crude methanol and water extract of *P. niruri* scored the highest antioxidant potential activity followed by *P. urinaria.* The finding was supported by Harish and co-workers that leaf and fruit extracts from *P. niruri* exhibited antioxidant activity. From the above results and discussion it can be concluded that the methanol extract of *Phyllunthus niruri* possesses the potent antioxidant substances and which may be responsible for its anti-tumor, anti-carcinogenic and remedy for hepatitis B viral infection mechanism as well as justify the basis of using this plant's extract as folkloric remedies.

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