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HPLC PROFILING OF POLYPHENOLIC COMPOUNDS AND FREE RADICAL ASSAY OF THE ETHANOLIC EXTRACT OF *ANNONA RETICULATA* GROWING IN BANGLADESH

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ABSTRACT

The present study investigates the HPLC profiling of phenolic compounds, and antioxidant activities of *Annona reticulata*. Through HPLC assay, high levels of rutin hydrate (603.88 mg/100 g extract) and moderate amount of catechin, epicatechin, and *p*-coumeric acid were determined (99.00, 78.19, 85.38 mg/100 g extract, respectively). Some amount of vanillic acid, ellagic acid, and quercetin were also detected (19.80, 31.56, 8.83 mg/100 g extract, respectively). Antioxidant activity tests such as, ABTS radical scavenging activity, reducing power, total antioxidant capacity, total phenolic and flavanoid content determination, were also performed. At 250 µg/ml, the percentage inhibition value of *Annona reticulata* for ABTS scavenging was 99.93 µg/ml, and the maximum absorbance for reducing power assay was 0.195. The total antioxidant capacity, and phenolic and flavonoid contents were calculated to be 419.20 mg of ascorbic acid/g, 120.07 mg/g of gallic acid, and 90.05 mg/g of quercetin equivalent, respectively. These results suggested that polyphenolic compounds in *Annona reticulata* might be responsible for their antioxidant activities.

KEYWORDS: *Annona reticulata*, HPLC, rutin hydrate, ellagic acid, quercetin, ABTS.

INTRODUCTION

Annona reticulata (*A. reticulata*) belongs to the family of Annonaceae. It is a small deciduous tree, well known particularly for its fruits, also called the custard apple. Plants of these kinds have leaves that are straight and pointed at the apex and flowers that are yellow-green and generally, in clusters of three or four. The fruits are of variable shapes and lengths. The flavor is sweet and pleasant, similar to the taste of traditional custard, but it also varies considerably across cultivars and individuals. The plant has a lower ratio of seed and skin to pulp, and some types produce seedless fruits. [1, 2] *A. reticulata* are native to the Caribbean and Central America. However, it is cultivated and occasionally naturalized in many tropical countries, including Southeast Asia, Taiwan, India, Bangladesh, Pakistan, Australia, and Africa. [3, 4] Fruits and leaves of *A. reticulata* are used as insecticides, anthelmintic, styptic and antiproliferative. [5] The barks are very astringent and used against diarrhea, dysentery, toothaches, fevers etc. [6] The plant shows various pharmacological activities such as antioxidant, anticancer, anti-inflammatory, analgesic, CNS depressant, antimalerial, anthelmintic, in syphills, etc. The bioactive phytochemicals vary widely within the plant and play an important role in health and medicinal activity. These include mainly phenolic and flavanoid compounds, which are responsible for

antioxidant activity. The main bioactive phytochemicals found in *A. reticulata* reported to date are acetogenins, aliphatic ketones, alkaloids, benzenoids, cyclic peptides, essential oils, lignans, long chain fatty acids, organic acids, terpenoids, purines, steroids, tryptamine and volatiles. [7, 8, 9, 10] Thus, in this study, we attempted to investigate the antioxidant activities and determine the phenolic compounds by HPLC of the ethanolic extract of *A. reticulata*.

MATERIALS AND METHODS

Plant material: The leaves of *A. reticulata* were collected from Khulna, Bangladesh during June 2012. The plant parts were properly washed, shed dried, and grinded by a mechanical grinder into coarse powder. The plant powder was stored in an airtight container until the extraction was done.

Extraction: The powdered plant materials were dissolved in ethanol and extracted with 90% ethanol in an orbital shaker for a week at room temperature to obtain the ethanolic extract. The extract was first filtered in a clean cotton plug to remove any plant debris, and then through Whatman filter paper no. 1. The solvent was removed under reduced pressure in a rotary vacuum evaporator. The plant yielded 5.02% extract of the dried plant material and was stored for further analysis.

Chemicals: 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 purchased from Merck (Darmstadt, Germany).

Quantification of major polyphenols of A. reticulata by HPLC

HPLC system: Chromatographic analyses were carried out on a Thermo Scientific Dionex UltiMate 3000 Rapid Separation LC (RSLC) systems (Thermo Fisher Scientific Inc., MA, USA), coupled to a quaternary rapid separation pump (LPG-3400RS), Ultimate 3000RS autosamplier (WPS-3000) and rapid separation diode array detector (DAD-3000RS). Phenolic compounds were separated in Acclaim® C18 (4.6 x 250 mm; 5µm) column (Dionix, USA) which was controlled at 30°C using a temperature controlled column compartment (TCC-3000). Data acquisition, peak integration, and calibrations were done with Dionix Chromeleon software (Version 6.80 RS 10).

Chromatographic conditions: The phenolic composition of *A. reticulata* was determined by HPLC, as previously described method. [11, 12] The mobile phase consisted of acetonitrile (solvent A), acetic acid solution pH 3.0 (solvent B), and methanol (solvent C). The system was run at the following gradient elution program: 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. A 5 min post run preceded the initial conditions for equilibration of the column. The flow rate was kept constant throughout the analysis at 1 ml/min and the injection volume was 20 µl. For DAD detection, the wavelength program was optimized to monitor phenolic compounds at their respective maximum absorbance wavelengths as follows: 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.

Standard and sample preparation: Standard stock solutions (100 µg/ml) of each phenolic compound were prepared in ethanol by weighing out approximately 0.0050 g of the analyte into 50 ml volumetric flask. The standards were prepared by diluting the standard stock solutions in ethanol to make a solution of concentration of 20 µg/ml for each of the polyphenols except caffeic acid, which was made up to 8 µg/ml, and quercetin (6 µg/ml). All the standard solutions were stored in the dark at 5°C and were stable for at least three months.

The calibration curves of the standards were prepared by serial dilution of the standard stock solutions (five set) with ethanol to yield 1.25 - 20 µg/ml for GA, CH, VA, EC, PCA, RH, EA; 0.5 - 8.0 µg/ml for CA, and 0.375 - 6.0 µg/ml for QU. The calibration curves were drawn from the chromatograms as peak area vs. concentration of standard.

A solution of *A. reticulata* at a concentration of 5 mg/ml was prepared in ethanol by mixing (Branson, USA) for 30 min. The samples were stored in the dark at low temperature (5°C). Spiking of the solution samples with phenolic standards was done to

identify the individual polyphenols. Prior to HPLC analysis, all solutions (mixed standards, sample, and spiked solutions) were filtered through 0.20 µm nylon syringe filter (Sartorius, Germany) and degassed in an ultrasonic bath (Hwashin, Korea) for 15 min.

Antioxidant activities

ABTS radical scavenging activity test: The method of Fan et al. [13] with some modifications was used to determine ABTS radical scavenging. ABTS radical cation was produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate. The mixture was allowed to stand in the dark at room temperature for 16 h. In the moment of use, the ABTS solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. One ml of each of the sample with various concentrations (5 to 250 µg/ml) were added to 1 ml of ABTS solution and mixed vigorously. The reaction mixture was allowed to stand at room temperature for 6 min and the absorbance at 734 nm was immediately recorded. The ABTS scavenging activity was calculated as follows:

ABTS scavenging effect = I (%) = $(A_o - A_s / A_o) \times 100$

Where, A_o = Absorbance of control and A_s = Absorbance of sample

Reducing power assay: The reducing power of *A. reticulata* was studied using the method of Hemayet et al. and Dehpour et al. [14, 15] Different concentrations of the extract (5-250 µg/ml) were 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 mixture was then incubated at 50°C for 20 min and a 10% solution of trichloroacetic acid (2.5 ml) was added to it. It was then centrifuged at 3000 rpm for 10 min. The upper 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. An increase in the absorbance of the reaction mixture indicated an increase in the reducing power. All the determinations were carried out thrice and an average was taken. Ascorbic acid were used as the standard reference compounds.

***Total antioxidant capacity*:** The total antioxidant capacity was measured by the spectrophotometeric method of Prieto et al. [16] At different concentration ranges, ethanol extract was prepared in their respective solvents and mixed with 1ml of the reagent solution (0.6M H_2SO_4, 28mM sodium phosphate, 4mM ammonium molybdate mixture). The tubes were incubated for 90min at 95ºC. The mixture was cooled to room temperature and the absorbance was read at 695nm 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: The modified Folin-Ciocaltu method [17, 18] was followed to determine the total phenolic content of the extract. A 0.5 ml of each extract (1 mg/ml) 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 seconds and allowed to stand for the next 30 min at 40°C for color development. Afterwards, the absorbance was read at 765 nm with the same spectrophotometer. The total phenolic content was calculated as mg of Gallic acid equivalent per gram using the equation obtained from the standard gallic acid calibration curve $y=6.993x + 0.0379$, $R^2=0.9995$.

Total flavonoid content: Aluminium chloride colorimetric method was used for the determination of total flavonoid content of the ethanol extract. [19, 20] The extract (5 ml, 1 mg ml^{-1}) in ethanol was mixed with 2.5 ml of aluminium chloride reagent (133 mg aluminium chloride and 400 mg sodium acetate in 100 ml of DI water), and allowed to stand for 30 min at room temperature. The absorbance of the reaction mixture was measured at 430 nm with a double beam Analykjena UV/Visible spectrophotometer (Model 205, Jena, Germany). The total flavonoid content was calculated as mg of Quercetin equivalent per gram using the equation obtained from Quercetin calibration curve $y=6.2548x + 0.0925$; $R^2 = 0.998$.

Statistical analysis

Data were presented as mean ± Standard deviation (S.D).

RESULTS AND DISCUSSION

HPLC assay of A. reticulata: Individual phenolic compounds of *A. reticulata* were identified and quantified by HPLC. The chromatographic separations of phenolic compounds in the ethanol extract are shown in Figure 1. The experimental results indicated that *A. reticulata* contained an especially high concentration of rutin hydrate (603.88 mg/100 g of dry extract, respectively). (+) Catechin, epicatechin, p-coumeric acid were also detected in moderate quantity (99.00, 78.19 and 85.38 mg/100 g of dry extract, respectively). In addition, the presence

of vanillic acid, ellagic acid and quercetin was also observed, but at a relatively low concentration (19.80, 31.56, and 8.83 mg/100 g of dry extract) (Table 1).

Antioxidant activities: *A. reticulata* was evaluated for its possible antioxidant activities. Five complementary test systems, namely ABTS radical scavenging activity, reducing power assay, total antioxidant capacity, total phenolic content and total flavonoid content were followed for the analysis.

ABTS radical scavenging activity: The scavenging powers of ABTS increase with an increasing concentration (Table 2). At minimum concentration (5 µg/ml), the extract showed greater inhibition (26.39%) than that of the standard ascorbic acid (13.99%). The IC_{50} value of the extract was found to be slightly greater (16.82 µg/ml) in comparison to that of the ascorbic acid (12.61 µg/ml). In evaluating the total antioxidant power of single compounds and complex mixtures of various plants, ABTS assay is often used. [21] The relative absorbance at 734 nm is used as an index to portray the antioxidant activity in both organic and aqueous solvents. [22]

Reducing power assay: Ascorbic acid was used as a positive control to determine the reducing power activity of *A. reticulata* (Table 3). At 250 µg/ml, the maximum absorbance for *A. reticulata* was found to be 0.195, while the standard ascorbic acid showed an absorbance of 1.111. The absorbance of the extract increased as the concentration was incremented. The reducing power ability depends on the conversion of Fe^{3+} into Fe^{2+}. The mechanism for the reducing power assay begins with the compound donating a hydrogen atom. This causes the free radical chain to break and results in the reductones (antioxidants) to exert the antioxidant response. [23] The phenolic compounds present in *A. reticulata* cause Fe^{3+}/ ferricyanide complex to reduce to its ferrous form (Fe^{2+}), and thus exhibiting reducing power ability.

Total antioxidant capacity*:* Phosphomolybdenum method is used to describe the total antioxidant capacity. It is based on the reduction of Mo (VI) to Mo (V) by the sample and the subsequent formation of green phosphate/Mo (V) complex at an acidic pH. *A. reticulata* showed very potent total antioxidant capacity. Ascorbic acid equivalents are presented in (Table 4). The total antioxidant capacity in the ethanolic extract was found to be 419.20 mg of ascorbic acid/g of extract. The total antioxidant activity is expressed as the number of equivalents of ascorbic acid. [24, 25, 26] The total antioxidant capacity of the ethanolic extract was found in relatively significant quantity when compared to the standard ascorbic acid per g of extract.

Total phenolic and flavonoid content: The total phenolic content calculated in *A. reticulata* was quite high (120.07 mg/g of gallic acid equivalent). The total flavonoid content was found in significant amount in *A. reticulata* (90.05 mg/g of quercetin equivalent per gm of dry extract) (Table 5). The high inhibition value in the ethanol extract could most likely be due to the presence of high concentration of phenolic compounds. Phenols have hydroxyl groups that account for their scavenging ability. [27] Polyphenolic compounds can inhibit mutagenesis and carcinogenesis in humans when ingested up to 1 g from a diet rich in fruits and vegetables on a daily basis. [28] Phenolic compounds are also good natural antioxidants. Phytochemical components, especially polyphenols, such as flavonoids, phyenyl propanoids, phenolic acids, etc are responsible for the free radical scavenging and antioxidant activities of plants. Certain flavonoids are also reported as potent free-radical scavengers, and their activity against the DPPH radical is closely associated with their chemical structure. [29, 30, 31] Therefore, the antioxidant activity of the *A. reticulata* closely associates with the presence of these phenolics, flavonoids, etc.

CONCLUSION

The potential of the *A. reticulata* as an antioxidant agent may be due to the presence of phytoconstituents like polyphenolics compounds (rutin hydrate, catechin, epicatechin, p-coumeric acid, vanillic acid, ellagic acid, and quercetin) and hence, are responsible for its activity. It can be inferred that *A. reticulata* exhibits antioxidant properties because of high content of polyphenolics compounds. However, a more extensive study would be required to determine the exact mechanism(s) of action of the extract.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

Table 1: Contents of polyphenolic compounds in the ethanol extract of *A. reticulata* (n=5).

Polyphenolic compound	*A. reticulata*	
	Content (mg/100 g of dry extract)	% RSD
CH	99.00	1.29
VA	19.80	0.51
EC	78.19	1.15
PCA	85.38	0.88
RH	603.88	2.98
EA	31.56	0.69
QU	8.83	0.02

Table 2: ABTS radical scavenging activity of *A. reticulata* and standard.

Concentration (µg/ml)	% Inhibition at different concentration	
	A. reticulata	Ascorbic acid
5	26.39 ± 0.16	13.99 ± 0.11
10	35.42 ± 0.21	48.60 ± 0.17
20	58.40 ± 0.19	85.79 ± 0.25
40	99.37 ± 0.15	99.19 ± 0.21
60	99.64 ± 0.18	99.25 ± 0.29
80	99.74 ± 0.22	99.53 ± 0.24
100	99.82 ± 0.24	95.58 ± 0.18
250	99.93 ± 0.27	99.85 ± 0.27
IC_{50}	18.52 ± 0.15	12.01 ± 0.12

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

Table 3: Reducing power assay of the *A. reticulata* and standard.

Concentration (µg/ml)	Average absorbance at 700nm	
	A. reticulata	Ascorbic acid
10	0.010 ± 0.001	0.380 ± 0.012
20	0.021 ± 0.001	0.457 ± 0.017
40	0.037 ± 0.002	0.539 ± 0.023
60	0.043 ± 0.003	0.634 ± 0.037
80	0.062 ± 0.005	0.712 ± 0.013
100	0.081 ± 0.006	0.781 ± 0.029
250	0.195 ± 0.011	1.111 ± 0.019

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

Table 4: Total antioxidant capacity of *A. reticulata*.

Extract	Avg. absorbance at 695 nm	Total antioxidant capacity
		mg of ascorbic acid equivalent (AAE) per gm of dry extract
A. reticulata	0.22 ± 0.08	419.20 ± 7.14

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

Table 5: Total phenolic and flavonoid content of the *A. reticulata*.

Extract	Total phenolic content	Total flavonoid content
	mg of gallic acid equivalent (GAE) per g of dry extract	mg of quercetin equivalent (QE) per gm of dry extract
A.reticulata	120.07 ± 5.06	90.05 ± 1.21

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

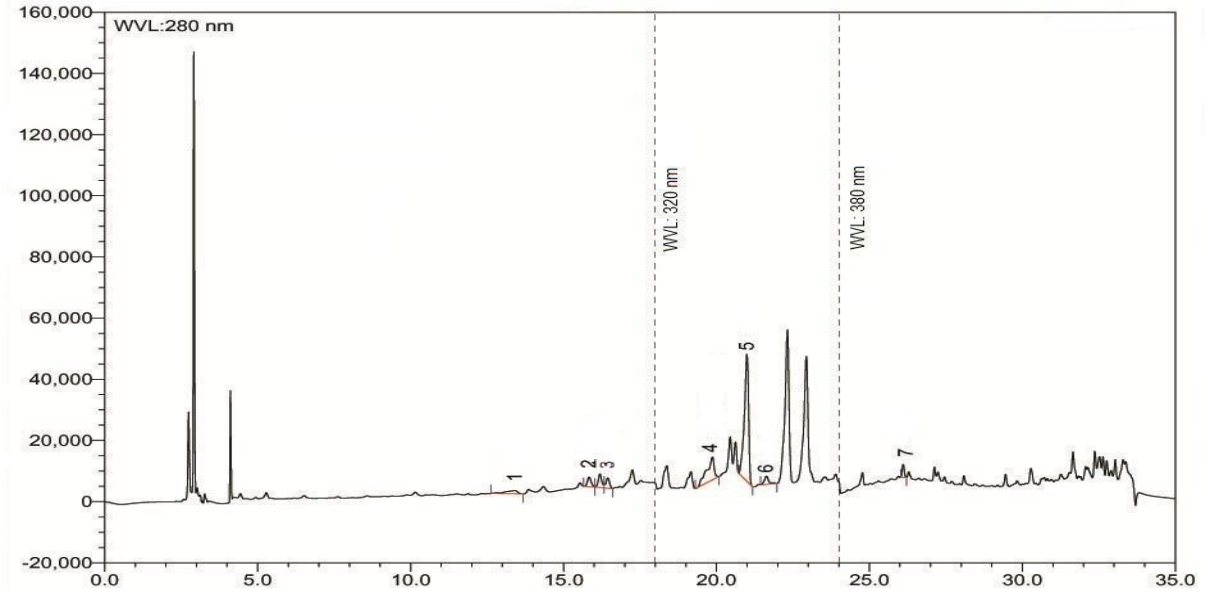

Figure 1. HPLC chromatogram of *A. reticulata* Peaks: 1, catechin hydrate; 2, vanillic acid; 3, epi-catechin; 4, *p*-coumeric acid; 5, rutin hydrate; 6, ellagic acid; 7, quercetin.

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