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Antioxidant Activity and HPLC Fingerprinting Profile of Ethanolic Extract of *Euphorbia cotinifolia* Bark from Bangladesh

Tanzir Ahmed Khan[1], Md. Mahfuzur Rahman[1], Md. Alamgir Kabir[1], Shaikh Emdadur Rahman[2], Proity Nayeeb Akbar[3], Hemayet Hossain[3*], and Ismet Ara Jahan[3]

[1]Institute of Food Science and Technology (IFST), Bangladesh Council of Scientific and Industrial Research, Dr. Qudrat-E-Khuda Road, Dhaka-1205, Bangladesh.
[2]Pharmacy Discipline, Life Science School, Khulna University, Khulna-9208, Bangladesh.
[3]BCSIR Laboratories, Bangladesh Council of Scientific and Industrial Research, Dr. Qudrat-E-Khuda Road, Dhaka-1205, Bangladesh.

Authors' contributions

This work was carried out in collaboration between all authors. Authors TAK, MMR, HH, PNA and IAJ designed the study, wrote the protocol and wrote the first draft of the manuscript. Author MAK managed the literature searches, analyses of the study performed the spectroscopy analysis and author MMR managed the experimental process and author HH identified the species of plant. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The present study was designed to investigate the antioxidant activity and High Performance Liquid Chromatography (HPLC) fingerprinting profiles of the ethanolic stem bark extract of *Euphorbia cotinifolia* growing in Bangladesh.
Methodology: *In-vitro* antioxidant activity of the ethanol extract was carried out using ABTS (2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid) radical scavenging activity, reducing power assay, total antioxidant activity, total phenolic and flavonoid content determination. The

**Corresponding author: Email: hemayethossain02@yahoo.com;*

polyphenolics content in the ethanol extract was identified and quantified through HPLC with Diode-Array Detection method.
Results: The ABTS radical scavenging activity demonstrated an IC_{50} (Inhibitory concentration 50) of 18.50 ml, while the maximum absorbance of reducing power was found to be 0.1148 at 250 ml, respectively. The total antioxidant capacity, total phenolic and flavonoid contents were found at significant level (347.8 mg of ascorbic acid/g, 56.24 mg/g of gallic acid, and 137.4 mg/g of quercetin equivalent), respectively. Catechin, caffeic acid, epicatechin, *p*-coumeric acid, ellagic acid and quercetin were quantified in the ethanol extract by reverse-phase HPLC (175.50, 4.61, 67.73, 4.01, 558.31 and 4.35 mg/100g of dry extract, respectively).
Conclusion: These results may be due to the higher polyphenolics content of the ethanol extract of *Euphorbia cotinifolia* bark, which also accounts for the significant antioxidant activity observed. Hence, it can be suggested that bioactive polyphenolics compound in *Euphorbia cotinifolia* might be responsible for the antioxidant activities.

Keywords: Euphorbia cotinifolia; HPLC; rutin hydrate; ellagic acid; quercetin; ABTS.

1. INTRODUCTION

Euphorbia belongs to the family Euphorbiaceae, which consists of about 300 genera and 5000 species distributed in tropical countries [1]. *Euphorbia cotinifolia* (*E. cotinifolia*) is one of the species of *Euphorbia*. *Euphorbia cotinifolia* is known to have biological and molluscidal activity [2] in addition to antioxidant, antibacterial, anti-cancer and anti-tumor properties [3]. Previous studies have reported that the dichloromethane extract of this plant showed mediocre antiviral activity with cytotoxic properties [4]. The leaves of the plant contain Ingenol esters [5], which induce apoptosis (anticancer, and anti-HIV properties) as reported by Blanco-Molina et al 2001 [6]. Spectroscopic analysis of leaves and small branches of the plant detected seventeen polyphenols including two new ellagitannins, and a trigalloylglucopyranose [7].

Therefore, this experiment was performed to investigate the antioxidant activities of the ethanol extract of *E. cotinifolia* bark growing in Bangladesh, and quantify the major bioactive polyphenolics compound present in the ethanol extract through HPLC.

2. MATERIALS AND METHODS

2.1 Plant Material

Barks of *E. cotinifolia* were collected from Khulna, Bangladesh during January 2013. All materials were properly washed with clean water, air-dried under the sun, powdered with a grinder, and stored in an airtight container until further analysis. The samples were identified by Sarder Nasir Uddin, Senior Scientific Officer, Bangladesh National Herbarium, Mirpur, Dhaka, Bangladesh. A voucher specimen has been deposited for further reference (Accession no: DACB 36713).

2.2 Extraction

The powdered sample was extracted with 90% ethanol in an orbital shaker for 1 week at ambient temperature (25°C). Afterwards, the extracts were filtered in a clean cotton plug to remove any plant debris, and then through Whatman filter paper no. 1. The extract was concentrated in a rotary vacuum evaporator (R-205, Buchi, Switzerland) at a reduced pressure.

2.3 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).

2.4 Antioxidant Activities

2.4.1 ABTS radical scavenging activity test

The method of decolourisation of free radical $ABTS^+$ was performed according to Fan et al.

2009 with some modifications [8]. ABTS radical cation was prepared by mixing 7 mM ABTS solution with 2.45 mM potassium persulfate. The mixture was allowed to stand for 12-16 h at room temperature in the dark until reaching a stable oxidative state. The ABTS solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 with pH 7.4 phosphate buffered saline (PBS) solution at 734 nm, before use. 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:

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

Where,

A_o = Absorbance of control and A_s = Absorbance of sample

2.4.2 Reducing power assay

The reducing power of *E. cotinifolia* was studied using the method of Ismet et al. 2014. and Dehpour et al. 2009. [9,10]. The extract at different concentrations was 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 sample solutions were next incubated at 50°C for 20 min and a 10% solution of trichloroacetic acid (2.5 ml) was added to them. They were then centrifuged at 3000 rpm for 10 min. The top 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. All determinations were carried out in triplicate.

2.4.3 Total antioxidant capacity

The total antioxidant capacity was measured by the method of Prieto et al. 1999 [11]. The ethanol extract was prepared in its respective solvent and mixed with 1 ml of the reagent solution (0.6 M H_2SO_4, 28 mM sodium phosphate, 4 mM ammonium molybdate mixture). The tubes were incubated for 90 min at 95°C. The mixture was cooled to room temperature and the absorbance was read at 695 nm 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.

2.4.4 Total phenolic content

Total phenolic content of the extract was determined using the modified Folin-Ciocaltu method [12,13]. After reacting 0.5 ml of extract (1 ml), 5 ml Folin-Ciocaltu reagent (1:10 v/v distilled water) and 4 ml (75 g/l) of sodium carbonate, the sample solutions were mixed and left to stand at 40°C for the next 30 min for colour development. The absorbance was read at 765 nm. The total phenolic content was calculated and expressed as mg of gallic acid equivalent per gram using the equation obtained from the standard gallic acid calibration curve, $y = 6.993x + 0.0379$, $R^2 = 0.9995$.

2.4.5 Total flavonoid content

The total flavonoid content was determined by reactions of the aluminium chloride colorimetric method with some modifications [14,15]. The absorbance of the reaction mixture was measured at 430 nm with a double beam Analykjena UV/Visible spectrophotometer (Model 205, Jena, Germany). Quercetin was used for calibration of a standard curve ($y = 6.2548x + 0.0925$; $R^2 = 0.998$) and the results were expressed as mg of quercetin equivalent per g of dry extract.

2.5 Quantification of Major Polyohenols of *E. cotinifolia* Bark by HPLC

2.5.1 HPLC system

HPLC analysis was carried out with Thermo Scientific Dionex UltiMate 3000 Rapid Separation LC (RSLC) systems (Thermo Fisher Scientific Inc., MA, USA), equipped with a quaternary rapid separation pump system (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 collection such as, acquisition, peak integration, and calibrations were done with Dionix Chromeleon software (Version 6.80 RS 10).

2.5.2 Chromatographic conditions

The mobile phase consisted of acetonitrile (solvent A), acetic acid solution pH 3.0 (solvent B), and methanol (solvent C). The gradient program was set as follows: 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. The flow rate was kept constant throughout the analysis at 1 ml/min. The injection volume was 20 µl, and the detection wavelength was: 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. Analyses were performed in triplicate.

2.5.3 Standard and sample preparation

Ethanol stock solutions (100 µg/ml) containing phenolic compounds were prepared and diluted to appropriate concentrations to make standard solutions of 20 µg/ml for all the polyphenols excluding caffeic acid, which was made up to 8 µg/ml, and quercetin that was made up to 6 µg/ml. All the standard solutions were stored in the dark at 5°C.

A solution of *E. cotinifolia* bark extract at a concentration of 5 mg/ml was prepared in ethanol by mixing in a vortex machine for 30 min. The solution was then degassed in an ultrasonic bath (Hwashin, Korea) for the next 15 min. Sample solutions were filtered through 0.20 µm nylon syringe filter (Sartorius, Germany), and analysed by RP-HPLC to obtain chromatograms for the polyphenolics compound [16,17]. The calibration curves were constructed by plotting the peak under the curve area versus the concentration of the analytes.

2.6 Statistical Analysis

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

3. RESULTS AND DISCUSSION

3.1 Antioxidant Activities

The ethanol extract of the bark sample of *E. cotinifolia* was evaluated for its possible antioxidant activities as follows:

3.1.1 ABTS radical scavenging activity

The scavenging ability of ABTS was quantified in terms of percentage inhibition of the radical cation by antioxidants in the sample (Table 1). The sample showed the capacity to neutralise the radical cation $ABTS^+$. At the concentration of 250 µg/ml the highest activity obtained by the bark extract of *E. cotinifolia* was 26.84 ± 0.82 µg/ml with a percentage inhibition value even exceeding that of the standard ascorbic acid (13.99 ± 0.11). The IC_{50} value of the bark extract was found to be 18.50 ± 0.16 µg/ml, which was similar to that of the ascorbic acid (12.01 ± 0.12 µg/ml). ABTS assay is often used in evaluating the total antioxidant power of single compounds and complex mixtures of various plants [18]. In the previous study, the leaf extract of *E. cotinifolia* demonstrated an IC_{50} value of 95.60 ± 0.19 µg/ml, which is lower in comparison to that of the presently investigated bark extract [19].

3.1.2 Reducing power assay

Reducing power activity evaluated the antioxidant properties of samples based on their relative maximum absorbance (Table 2). At 250 µg/ml, the maximum absorbance for the bark ethanolic extract of *E. cotinifolia* was found to be 0.1148 ± 0.015, while the standard ascorbic acid showed an absorbance of 1.1115 ± 0.009. The absorbance of the extract increased with an increasing concentration. As Fe^{3+} oxidizes to Fe^{2+} and donates a hydrogen atom, the free radical chain is interrupted causing the reductones to exert an antioxidant response [20]. Due to the higher phenolic compound content in the ethanol bark extract of *E. cotinifolia*, it causes a faster reduction of Fe^{3+}/ ferricyanide complex to its ferrous form (Fe^{2+}), exhibiting stronger reducing power ability. In comparison to the reducing power assay of the leaf extract of *E. cotinifolia*, at 250 µg/ml the ethanol bark extract was found to show a more significant maximum absorbance of 0.1148 ± 0.015 [19].

3.1.3 Total antioxidant capacity

The total antioxidant capacity depends on the reduction of Mo (VI) to Mo (V) by the extract and the subsequent formation of green phosphate/Mo (V) complex at an acidic pH. The ethanol extract of *E. cotinifolia* showed very potent total antioxidant capacity (Table 3). The total antioxidant activity is expressed as the number of equivalents of ascorbic acid [21,22,23]. In details, the bark extract of *E. cotinifolia* possessed a high antioxidant capacity with a value of 347.80 ± 3.14 mg of ascorbic acid/g of extract. The total antioxidant capacity of the ethanolic extracts was also found in relatively significant quantity when compared to the standard ascorbic acid per g of

extract. While the ethanol leaf extract of *E. cotinifolia* established a total antioxidant capacity of 328.7 ± 3.01, the bark extract was found to have a close, yet higher value of 347.8 ± 3.14 mg of ascorbic acid equivalent (AAE) per g of dry extract, respectively [19].

3.1.4 Total phenolic and flavonoid content

The bark extract of *E. cotinifolia* contained a high average amount of total phenolic content (56.24 ± 1.19 mg/g of gallic acid equivalent). Significant total flavonoid content was also observed in the ethanol bark extract of *E. cotinifolia* (137.4 ± 12.5 mg/g of quercetin, respectively) (Table 4). Phenolic and flavonoid compounds are of great interest in nutrition and medicine because of their strong antioxidant activity and innumerable other health benefits [24,25]. The high inhibition value in the ethanol extract is most likely because of the presence of high concentration of these compounds. Phenols have hydroxyl groups that account for their scavenging ability [26]. Certain flavonoids are also reported as potent free-radical scavengers [27,28].

Table 1. ABTS radical scavenging activity of *E. cotinifolia* bark extract with standard ascorbic acid

Concentration (μg/ml)	ABTS radical scavenging activity of *E. cotinifolia* bark and standard	
	***E. cotinifolia* bark extract**	**Ascorbic acid**
10	37.88 ± 0.34	48.60 ± 0.17
20	59.98 ± 0.41	85.79 ± 0.25
40	71.44 ± 0.07	99.19 ± 0.21
60	83.54 ± 0.05	99.25 ± 0.29
80	89.71 ± 0.04	99.53 ± 0.24
100	92.12 ± 0.14	95.58 ± 0.18
250	97.43 ± 0.17	99.85 ± 0.27
IC_{50}	18.50 ± 0.16	12.01 ± 0.12

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

Table 2. Reducing power assay of *E. cotinifolia* bark extract with standard ascorbic acid

Concentration (μg/ml)	Reducing power assay of *E. cotinifolia* bark extract and standard	
	***E. cotinifolia* bark extract**	**Ascorbic acid**
20	0.0159 ± 0.007	0.4577 ± 0.017
40	0.0191 ± 0.021	0.5398 ± 0.023
60	0.0283 ± 0.007	0.6345 ± 0.037
80	0.0513 ± 0.012	0.7125 ± 0.013
100	0.0872 ± 0.017	0.7811 ± 0.029
250	0.1148 ± 0.015	1.1115 ± 0.009

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

Table 3. Total antioxidant capacity of ethanolic bark extract of *E. Cotinifolia*

Extract	Total antioxidant capacity
	mg of ascorbic acid equivalent (AAE) per g of dry extract
E. cotinifolia bark extract	347.8 ± 3.14

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

Table 4. Total phenolic and flavonoid content of ethanolic bark extract of *E. cotinifolia*

Extract	Total phenolic content	Total flavonoid content
	mg of gallic acid equivalent (GAE) per g of dry extract	**mg of quercetin equivalent (QE) per g of dry extract**
E. cotinifolia bark extract	56.24 ± 1.19	137.4 ± 12.5

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

Therefore, it can be reported that the antioxidant activity of *E. cotinifolia* closely associates with the presence of these phenolics, flavonoids, etc. In looking at the results of the total phenolic and flavonoid content of *E. cotinifolia* leaf extract (64.64 ± 2.14 mg of GAE per g of dry extract and 81.72 ± 6.05 mg of QE per g of dry extract), it can be stated that the bark extract demonstrates a lower total phenolic content (56.24 ± 1.19 mg of GAE per g of dry extract), but a higher total flavonoid content (137.4 ± 12.5 mg of QE per g of dry extract) [19].

3.2 Phenolic Compounds of *E. cotinifolia* Based on HPLC Assay

The contents of the phenolic compoounds in the bark extracts of *E. cotinifolia* were analyzed by RP-HPLC. Based on the comparison of the retention times with those of the standard peaks, six phenolic compounds: (+) catechin, caffeic acid, epicatechin, p-coumeric acid, ellagic acid and quercetin were identified, respectively (Fig. 1). The most abundant phenolic compounds obtained from the ethanol bark extract of *E. cotinifolia* were catechin and ellagic acid (175.50 ± 1.09 and 558.31 ± 5.96 mg/100 g dry extract, respectively) followed by epicatechin (67.73 ± 0.87 mg/100 g of dry extract) (Table 5). The remaining polyphenolics compound were present, but at a lower concentration, with *p*-coumeric acid being the lowest in amount (4.01 ± 0.02 mg/100 g dry extract).

Phenolic compounds such as catechin, ellagic acid, rutin hydrate, etc are all well-known human health antioxidants [26]. The present results showed that *E. cotinifolia* was rich in these active components, and could be used as a potential source of antioxidants for the food and drug industries.

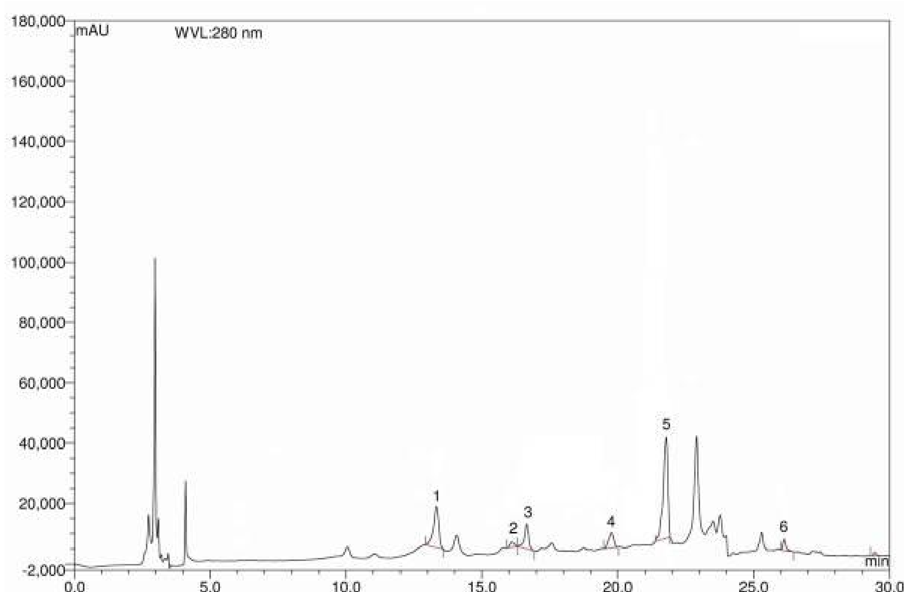

Fig. 1. HPLC chromatogram of ethanol extract of *E. cotinifolia* bark. Peaks: 1, (+)-catechin; 2, caffeic acid; 3, (–)-epicatechin; 4, *p*-coumaric acid; 5, ellagic acid; 6, quercetin

Table 5. Contents of polyphenolics compound in the ethanolic bark extract of *E. cotinifolia* (n=3)

Polyphenolic compound	Ethanol extract of *E. cotinifolia* bark	
	Content (mg/100 g of dry extract)	% RSD
CH	175.50	1.09
CA	4.61	0.05
EC	67.73	0.87
PCA	4.01	0.02
EA	558.31	5.96
QU	4.35	0.05

4. CONCLUSION

The results from the present study indicated that the ethanolic bark extract of *E. cotinifolia* showed potent antioxidant activity. It can be inferred that the *E. cotinifolia* sample exhibit antioxidant properties most probably due to the presence of high polyphenolics compound. The present study provides meaningful information for the collection and application of *E. cotinifolia* in both healthcare and the food industry. Nevertheless, a large, systematic study of *E. cotinifolia* from different sources would be helpful.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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