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Antioxidant Properties and HPLC Assay of Bioactive Polyphenols of the Ethanol Extract of *Excoecaria agallocha* Stem Bark Growing in Bangladesh

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Authors' contributions

This work was carried out in collaboration between all authors. Authors IAJ, HH, SER and PNA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors SER, MMR and TAK managed the analyses of the study. Author MASS managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The ethanolic extract of *Excoecaria agallocha* stem bark was evaluated for antioxidant properties and quantification of bioactive polyphenols by HPLC-DAD. **Methodology:** ABTS assay, total antioxidant capacity, reducing power, total phenolic and flavonoid contents were used for determining antioxidant activities.

Results: In ABTS assay, the extract showed 74.11% inhibition at 10 μg/ml and IC_{50} of 4.80 μg/ml, which was significant compared to ascorbic acid (12.20 μg/ml). The antioxidant capacity was calculated as 686 mg of ascorbic acid/g of extract. The maximum absorbance for reducing power assay was 0.73 at 250 μg/ml. The total phenolic and flavonoid contents were found to be significant (375.46 mg/g of gallic acid, 22.44 mg/g of quercetin equivalent, respectively). In HPLC assay, catechin hydrate had the highest content (713.91 mg/100 g of dry extract).

Conclusion: The obtained results suggest the antioxidant activities with high concentration of catechin hydrate in the extract of *Excoecaria agallocha* stem bark.

Keywords: Excoecaria agallocha; total antioxidant capacity; catechin hydrate; vanillic acid.

1. INTRODUCTION

Excoecaria agallocha belonging to the family of Euphorbiaceae is a small mangrove tree, which is widely grown in swamps, tidal forests of Sundarbans and other littoral areas of Bangladesh [1,2]. The plant is widely available in the temperate and tropical countries of Asia, Southwestern Pacific and Australia especially at higher elevations where salinity is low [2]. The plant has traditionally been used for the treatment of sores, ulcers, leprosy and as an emetic and a purgative [1]. Previous reports on the bark oil of *E. agallocha* reveal its effectiveness against leprosy, rheumatism and paralysis. Clinical trials have presented anticancer, anti-HIV, antiviral and antibacterial agent properties from this plant [3]. Phytochemical studies on *E. agallocha* have shown the presence of diterpenoids [4-10], triterpenoids [11], and flavonoids [12]. In addition, a novel pharbol ester, an anti-HIV principle has also been isolated from the leaves and stem of this plant [13]. Hence, in this experiment, we attempted to investigate the antioxidant activities of ethanol extract of *E. agallocha* stem barks as well as quantify the major polyphenolics compounds of the bark extract through HPLC-DAD.

2. MATERIALS AND METHODS

Collection and identification of plant materials: The plant, *E. agallocha* was collected from the Sunderbans of Mongla range, Bangladesh in July 2012 and identified by Bangladesh National Herbarium, Mirpur, Dhaka (Accession no: DACB 36612).

2.1 Preparation of Ethanol Extract

The stem barks of *E. agallocha* were separated from foreign substances. The plant materials were chopped and air-dried first in the shade and then in an electric oven at 40ºC. The dried plant materials were powdered in a mechanical grinder. Approximately 200 g of the powdered plant material was taken in a clean, flat-bottomed glass container and soaked in 500 ml of 95% ethanol. The container with its contents was sealed and left to stand for a period of 4 days accompanied by occasional shaking and stirring. After the ethanol extract

was filtered using a Buchner funnel, the filtrate was concentrated in rotary evaporator at bath temperature (not exceeding 40ºC) to get a gummy concentrate.

2.2 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, DPPH, ABTS, and folin-ciocalteu's phenol reagent were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC grade), methanol (HPLC grade), acetic acid (HPLC grade), 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).

2.3 Antioxidant Activities

2.3.1 ABTS radical scavenging activity test

The antioxidant activity was determined by ABTS radical cation described by Fan et al. [14] with some modifications. ABTS radical cation was produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate and the mixture was allowed to stand in the dark at room temperature for 16 h. Right before use, the ABTS solution was diluted with ethanol to an absorbance of 0.70±0.02 at 734 nm. 1 ml of each sample of different concentrations (10 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 effect was calculated as follows:

$$\text{ABTS scavenging effect} = \left(\frac{A_o - A_s}{A_o}\right) \times 100$$

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

2.3.2 Total antioxidant capacity

The total antioxidant capacity was measured by spectrophotometeric method of Prieto et al. [15]. At different concentration ranges, aqueous extracts were prepared in their respective solvents and mixed with 1ml of the reagent solution (0.6 M H_2SO_4, 28 mM sodium phosphate, 4 mM ammonium molybdate mixture). The solutions 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 standard graph of ascorbic acid. The experiment was conducted in triplicates and the values are expressed as equivalents of ascorbic acid in mg per g of extract.

2.3.3 Reducing power assay

The method of Dehpour et al. [16] was followed to determine the reducing power of the ethanol extract *E. agallocha* barks. Different concentrations of the extract (10-250 µg/ml) in 1 ml of methanol were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was then incubated at 50ºC 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 distilled water (2.5 ml) and 0.5 ml, 0.1% $FeCl_3$ and the absorbance of the mixture was measured at 700 nm with the same spectrophotometer. Increased absorbance of the reaction mixture indicated increased reducing power. All the determinations were carried out thrice and average of the results was taken. Ascorbic acid and butylated hydroxy anisole (BHA) were used as the standard reference compounds in this study.

2.3.4 Total phenolic content determination

The Folin-Ciocaltu method followed to determine the total phenolic content of the extract [17]. 1.0 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 then vortexed for 15 second for color development and allowed to stand for 30 min at 40ºC.The absorbance was read at 765 nm with the same spectrophotometer. Total phenolic content was calculated as mg of gallic acid equivalent per gram using the equation obtained from a standard gallic acid calibration curve y=6.993x + 0.0379, R^2=0.9995.

2.3.5 Total flavonoid content determination

Aluminium chloride colorimetric method was used for the determination of total flavonoids concentration of the ethanol extract [18]. The ethanol extracts (5 ml, 1 mg ml^{-1}) were mixed with 2.5 ml of aluminum chloride reagent (133 mg aluminium chloride and 400 mg sodium acetate in 100 ml of de-ionized water). It was allowed to stand for 30 min at room temperature and the absorbance of the reaction mixture was measured at 430 nm with a double beam Analykjena UV/Visible spectrophotometer (Model 205, Jena, Germany). Total flavonoids content was determined as mg of Quercetin equivalent per gram using the equation obtained from quercetin calibration curve y=6.2548x + 0.0925; R^2 = 0.998.

2.4 Quantification of Major Polyphenols of *E. agallocha* by HPLC

2.4.1 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 autosampler (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 30ºC using a temperature controlled column compartment (TCC-3000). Data acquisition, peak integration, and calibrations were performed with Dionix Chromeleon software (Version 6.80 RS 10).

2.4.2 Chromatographic conditions

The phenolic composition of *E. agallocha* was determined by HPLC, as described by Khirul et al. [19] and Sarunya & Sukon [20]. The mobile phase consisted of acetonitrile (solvent A), acetic acid solution pH 3.0 (solvent B), and methanol (solvent C). The system was run with 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. There was a 5 min post run at 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 where it was held for the rest of the analysis; the diode array detector was set at an acquisition range of 200-700 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.

2.4.3 Standard and sample preparation

A stock standard solution (100 µg/ml) of each phenolic compound was prepared in methanol by weighing out approximately 0.0050 g of the analyte into 50 ml volumetric flask. The mixed standard solution was prepared by dilution the mixed stock standard solutions were prepared in methanol to give a concentration of 20 µg/ml for each of the polyphenols except for caffeic acid (8 µg/ml) and quercetin (6 µg/ml). All standard solutions were stored in the dark at 5ºC and were stable for at least three months.

The calibration curves of the standards were made by serial dilution of the stock standards (five set of standard dilutions) with methanol 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 produced from the chromatograms as peak area vs. concentration of standard. A solution of *E. agallocha* at a concentration of 5 mg/ml was prepared in ethanol by vortex mixing (Branson, USA) for 30 min. The samples were stored in the dark at low temperature (5℃). The sample solution was spiked with phenolic standards for further identification of 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. The compounds were identified by comparing with standards of each identified compound using the retention time, the absorbance spectrum profile and also by running the samples after the addition of pure standards.

2.5 Statistical Analysis

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

3. RESULTS AND DISCUSSION

3.1 Antioxidant Activities

Ethanolic extract of *E. agallocha* was evaluated for its possible antioxidant activities. Five complementary test systems, namely ABTS radical scavenging activity, total antioxidant capacity, reducing power, total phenolic and flavonoid content determinations were followed for this analysis.

3.1.1 ABTS radicals scavenging activity

ABTS assay is used in evaluating total antioxidant power of single compounds and complex mixtures of various plants [21]. Specific absorbance at 734 nm can be used in both organic and aqueous solvents as an index reflecting the antioxidant activity [22]. Their scavenging powers correlated well with increasing concentrations (Table 1). At 10 µg/ml, the ethanol extract showed greater percentage inhibition (74.11%) than that of the standard, ascorbic acid (45.43%). The highest percentage scavenging activity was shown at 250 µg/ml by each

of the extracts and the standard (98.60 and 99.94%, respectively). The IC_{50} value for the ABTS assay of the extract (4.80 µg/ml) was also found to be more significant in comparison to that of the standard (12.20 µg/ml).

Table 1. ABTS radical scavenging activity of ethanol extract of *E. agallocha* barks

Concentration (µg/ml)	% ABTS radical inhibition of the extract and standard	
	Ethanol extract of *E. agallocha*	**Ascorbic acid (standard)**
10	74.11±0.10	45.43±0.12
20	85.73±015	99.16±0.15
40	94.99±0.16	99.25±0.17
60	97.19±0.20	99.42±0.14
80	97.28±0.17	99.58±0.12
100	98.08±0.12	99.84±0.15
250	98.60±0.13	99.94±0.13
IC_{50}	4.80±0.02	12.20±0.05

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

3.1.2 Total antioxidant capacity

The total antioxidant capacity by phosphomolybdenum method assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of green phosphate/Mo (V) complex at acidic pH. The phosphomolybdenum method is quantitative since the total antioxidant activity is expressed as the number of equivalents of ascorbic acid [15]. Extracts of *E. agallocha* showed very potent total antioxidant capacity. Ascorbic acid equivalents are presented in (Table 2). In ethanolic extracts, total antioxidant capacity was found to be 656.00 mg of ascorbic acid/g of extract.

Table 2. Total antioxidant capacity of ethanol extract of *E. agallocha* bark

Extract	Avg. absorbance at 695 nm	Total antioxidant capacity
		mg of ascorbic acid equivalent (AAE) per g of dry extract
Ethanol extract of *E. agallocha* bark	0.34±0.01	656.00±0.28

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

3.1.3 Reducing power assay

The reducing power assay of the ethanol extract of *E. agallocha* was determined and presented in (Table 3). The maximum absorbance for the ethanol extract was found to be 0.73±0.014 at 250 µg/ml, while the standard ascorbic acid was found to be 1.11±0.09) at the same concentration. With an increase in concentration, the absorbance of the extract and the standard was found to increase.

3.1.4 Total phenolic content

The total phenolic content was found to be quite high in the ethanolic crude extract of *E. agallocha* (375.46±0.0809 mg/g of gallic acid equivalent) (Table 4).

Table 3. Reducing Power (RP) assay of the ethanolic extract of *E. agallocha* barks

Concentration (µg/ml)	% RP inhibition of the extract and standard at different concentration	
	Ethanol extract of *E. agallocha*	Ascorbic acid (standard)
10	0.03±0.007	0.38±0.012
20	0.07±0.007	0.46±0.017
40	0.14±0.014	0.54±0.023
60	0.19±0.014	0.63±0.037
80	0.26±0.021	0.71±0.013
100	0.34±0.014	0.78±0.029
250	0.73±0.014	1.11±0.009

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

Table 4. Total phenolic content of the ethanol extract of *E. agallocha* bark

Extract	Avg. absorbance at 765 nm	Total phenolic content
		mg of gallic acid equivalent (GAE) per g of dry extract
Ethanol extract of *E. agallocha* bark	1.35±0.15	375.46±10.809

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

Phytochemical compounds, especially phenolics (such as flavonoids, phyenyl propanoids, phenolic acids, tannins etc.) are very important components for the free radical scavenging and antioxidant activities of plants. Polyphenols are generally of the chemical patterns; phenolic groups react as hydrogen donors and neutralize the free radicals [23,24,25]. In the present study, the total amount of phenolic compounds present in the ethanol extract was calculated and found to be quite high in the *E. agallocha* stem bark. The results show that the presence of phenolic components in the extract is possibly the cause of the inhibition value of the extract. Hence, we know that phenols are important components of plants.

3.1.5 Total flavonoid content

The total flavonoid content was calculated as significant in ethanolic extract of *E. agallocha* (22.44 mg/g of quercetin equivalent per g of dry extract) (Table 5).

Table 5. Total flavonoid content of ethanol extract of *E. agallocha* bark

Extract	Avg. absorbance at 415 nm	Total flavonoid content
		Mg of quercetin equivalent (QE) per g of dry extract
Ethanol extract of *E. agallocha* bark	0.61±0.013	22.44±0.91

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

Flavonoids are considered as effective scavengers of oxidizing molecules, including singlet oxygen, and different free radicals [26]. Hence, in comparison to other literature findings [27], our results suggested that phenolics and flavonoids could be the major contributors for the antioxidant activity.

3.2 Phenolic Contents of *E. agallocha* from HPLC-DAD

Identification and quantification of individual phenolic compounds in the *E. agallocha* was analysed by HPLC. The chromatographic separations of phenolic compounds present in ethanol extract are shown in (Fig. 1). The contents of the phenolic compounds in ethanol extract were calculated from the corresponding calibration curve as stated in Khirul et al. [19] and presented in (Table 6).

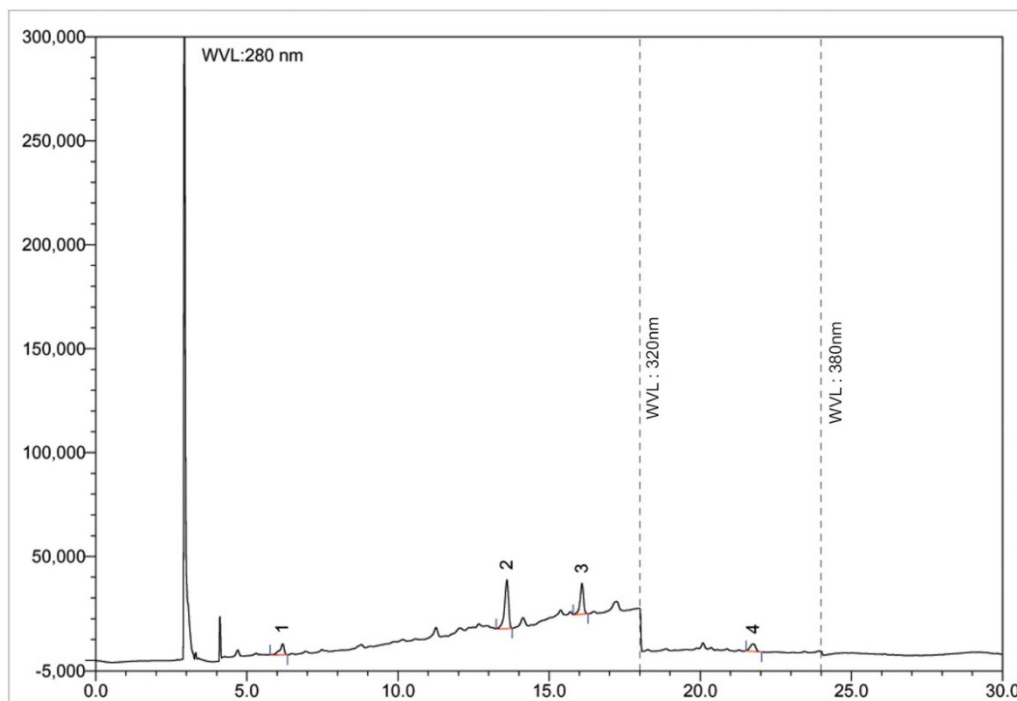

Fig. 1. HPLC chromatogram of *E. agallocha*. Peaks: 1, gallic acid; 2, catechin; 3, vanillic acid; 4, ellagic acid

Table 6. Contents of polyphenolic compounds in the ethanol extract of *E. agallocha* bark (n=5)

Polyphenolic compound	*E. agallocha* stem bark	
	Content (mg/100g of dry extract)	% RSD (relative standard deviation)
GA	42.61	0.59
CH	713.91	2.55
VA	96.88	1.29
EA	52.08	0.91

The experimental results indicated that *E. agallocha* contained an especially high concentration of (+)catechin (713.91 mg/100 g of dry extract). It was also seen that vanillic acid and ellagic acid were detected but at moderate concentration in the ethanol extract (96.88 and 52.08 mg/100 g of dry extract, respectively). Gallic acid was also detected, but in

a lower concentration (42.61 mg/100 g of dry extract) than the rest. No other polyphenolic compounds were detected in the *E. agallocha*.

4. CONCLUSION

The potential of the bark extract of *E. agallocha* as antioxidant agents may be due to the presence of phytoconstituents like polyphenolics compounds (gallic acid, catechin hydrate, vanillic acid, ellagic acid) and might be responsible for its activity. The above-mentioned polyphenolic compounds have been determined and reported for the first time ever. However, a more extensive study is necessary to determine the exact mechanism(s) of action of the extract.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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