

Antioxidant, Anti-inflammatory, and Anticoagulation Properties of *Aegiceras corniculatum* and *Acanthus ilicifolius*

Rana Biswas¹, S. M. Mahbubur Rahman¹, Kazi Mohammed Didarul Islam¹, Md. Morsaline Billah^{1,2}, Afiya Aunjum¹, Tauhidur Rahman Nurunnabi¹, Sujan Kumar Kundu¹, Md. Emdadul Islam^{1*}

¹Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna-9208, Bangladesh

²Interdisciplinary Chair in Biobusiness, University of Lincoln, UK

*Corresponding author: Md. Emdadul Islam, Associate Professor of Biotechnology and Genetic Engineering, Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna-9208, Bangladesh. P.O. Box: 9208, Tel: +88-(0)1712773266, Fax: +88-(0)41751038, Email: emdad950711@yahoo.com

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Abstract

Free radical production from different biological and environmental sources is due to an imbalance of natural antioxidants, which further leads to inflammation. Antioxidant metabolites are often characterized by anti-inflammatory and anticoagulation activity. Mangrove plants synthesize different classes of metabolites, including antioxidants, to minimize the devastating effect of oxidation resulting from the elevated salinity, UV, and other unique geochemical components. Accordingly, this study aimed at investigating the antioxidant, anti-inflammatory, and anticoagulation properties, as well as polyphenol content of the two selected mangrove plant species: *Aegiceras corniculatum* and *Acanthus ilicifolius*. We used the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, reducing power, ferric reducing antioxidant power (FRAP) assay, β -carotene-linoleic acid bleaching assay (BCB), total phenolic content (TPC), total flavonoid content (TFC) and total tannin content (TTC) to determine antioxidant activity of the ethanol extract of *A. corniculatum* bark and leaves and *A. ilicifolius* leaves. Furthermore, human red blood cell (HRBC) membrane stabilization assay, lipoxygenase (LOX) inhibition assay, and prothrombin time (PT) test were performed for determining anti-inflammatory activity of the samples. *A. corniculatum* bark is a potent antioxidant (IC_{50} 20.49 \pm 2.14 μ g/mL in DPPH assay) with anti-inflammatory (IC_{50} 23.58 \pm 1.75 μ g/mL in LOX inhibition assay) and anticoagulation activity (18.19 \pm 0.13 min in prothrombin time assay) compared to other extracts. All extracts were found with significant ($P < 0.001$) antioxidant, anti-inflammatory, and anticoagulation properties. Further studies on liquid chromatography-mass spectrometry (LC-MS) analysis, anti-inflammatory, and anticoagulation are recommended.

Keywords: Antioxidant, Anti-inflammatory, Anticoagulation, Mangrove, Sundarbans

Introduction

Free radicals like reactive oxygen species (ROS) and reactive nitrogen species (RNS) are the result of several cellular signaling pathways as a defense mechanism (1). An excessive amount of free radicals cause oxidative stress that damages cells, tissues, and organs, which can result in several diseases, like cancer, cardiovascular diseases, Alzheimer disease (AD), and even accelerate ageing process (2). Antioxidants can slow down or prevent the resulting damages by free radicals through delaying, intercepting, and inhibiting their activities or via breaking the chain reaction of oxidation (3). Regular tidal inundation and elevated salinity help mangrove species to

combat the oxidative stress that leads to the excessive production of ROS, such as superoxide anion ($O_2\bullet$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH\bullet$), particularly in the chloroplast and mitochondria. Plants possess several antioxidant enzymes, including glutathione reductase, ascorbate peroxidase, superoxide dismutase, etc, which are protective against damaging effects of ROS (4). In addition to morphological adaptation, specific biochemical changes occur in halophytes. Plants have an efficient system for scavenging ROS, which can protect them against the destructive oxidative reaction. Antioxidant enzymes play a vital role in defense mechanisms in salt stress. Successful salt

management is achieved either by tolerance or plants' mechanisms to deal with the imposed stress (5). This kind of oxidation is neutralized by antioxidants that relieve oxidative stress. Antioxidants are enzymatic or non-enzymatic. Non-enzymatic antioxidants include polyphenolic compounds, such as phenols, tannin, and flavonoids. Recently, it has been proposed that flavonoids and polyphenolic compounds act as reducing agents either as enzyme cofactors or electron donors (6). Accumulation of these agents can be observed in tissues under stress (7).

Aegiceras corniculatum is a species of shrub or tree mangrove belonging to the Myrsinaceae family, commonly known as Black Mangrove, River Mangrove, or Khalisha. It is found in coastal and estuarine regions from India, South East Asia, Southern China, to New Guinea and Australia. It has been used in the treatment of asthma, diabetes, rheumatism and fish poisoning (8). *Acanthus ilicifolius* Linn. belongs to the Acanthaceae family is another mangrove plant locally known as "Hargoja" and has been used to treat rheumatism, paralysis, asthma and snake bite. Its root is expectorant and used to treat cough and asthma (9).

Inflammation is a defensive mechanism against the pathogens in the host body (10). Free radicals as the crucial signaling components lead to membrane dysfunction as well as tissue damage at the inflammation site. During inflammation, oxidative stress causes the opening of interendothelial junctions to allow the migration of inflammatory cells, which are involved in tissue injury (11). The enzyme lipoxygenase (LOX) plays a crucial role in the inflammation process and allergic reactions. LOX inhibitors can be a potent source of novel compounds for the development of therapeutic agents to treat cancer and inflammatory diseases, like rheumatic disorders and osteoarthritis (12).

Inflammation reduces the natural anticoagulation activity and weakens the fibrinolytic system. Inflammatory cytokines are considered as the key mediators for activating coagulation. The natural anticoagulants are responsible for dampening the elevated level of cytokine. Besides, the factors associated with natural anticoagulant cascades (e.g., thrombomodulin) make endothelial cells less responsive to inflammatory mediators, which in turn can facilitate the neutralization of some inflammatory mediators. Therefore, promotion of thrombosis and amplification of the inflammatory process both happen through downregulation of anticoagulant pathways. Catastrophic events, such as severe sepsis or inflammatory bowel disease happen when the inflammation-coagulation interactions overwhelm the natural defense systems (13).

Overall, ROS production enhances, which in turn can initiate clotting. Considering the association between anticoagulant and anti-inflammatory properties with antioxidant properties, our study aimed at investigating the antioxidant activity of the two selected mangrove species. We chose *A. corniculatum* and *A. ilicifolius* because of their traditional uses and reported properties. We employed the following assays to determine the

antioxidant activity of the studied samples: 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH), reducing power, ferric-ion reducing antioxidant power (FRAP) and β -carotene bleaching (BCB) assays. To quantify the antioxidant molecules, we measured their polyphenol contents (phenol, flavonoid and tannin contents). In the next step, human red blood cell (HRBC) membrane stabilization assay and LOX inhibition assay were performed for evaluating anti-inflammatory activity and also prothrombin time (PT) assay for measuring anticoagulation activity of the samples.

Materials and methods

Chemicals: We bought ascorbic acid, quercetin, butylated hydroxytoluene (BHT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), potassium ferricyanide, ferric chloride, 2,4,6-tripyridyltriazine (TPTZ), β -carotene (type I \geq 93%), linoleic acid, Folin-Ciocalteu reagent, gallic acid, sodium carbonate, sodium nitrite, aluminum chloride, sodium hydroxide, hydrogen peroxide (H_2O_2), calcium chloride and soybean LOX from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were obtained from local commercial suppliers.

Collection of plant material: Fresh bark and leaves of *A. corniculatum* and also the leaves of *A. ilicifolius* were collected from Chadpai range, East zone of the Sundarbans, Khulna, Bangladesh on the 10th of January 2018. The collected samples were identified, and voucher specimens (RB/AA-KU-2018009 and RB-KU-2018010 for *A. corniculatum* and *A. ilicifolius*, respectively) were deposited at the herbarium of the Forestry and Wood Technology Discipline, Khulna University, Khulna, Bangladesh.

Extraction: Before the extraction, all the plant parts were separated and appropriately washed using distilled water and dried for four weeks at room temperature in a well-ventilated room. The air-dried plant materials were crushed using a grinder, and the powdered materials were preserved in a dry, cool, and dark place in airtight container until analysis started. About 100 g of the grinded leaves and bark of *A. corniculatum* as well as leaves of *A. ilicifolius* were extracted separately using 100 mL of 70% (v/v) aqueous ethanol. These mixtures were soaked for 72 h at 25 °C with occasional stirring and shaking. The resulting compounds were then filtered using Whatman grade No. 41 filter paper followed by drying under reduced pressure in a rotary evaporator at 40 °C to generate aqueous ethanol crude extract. The crude extract was weighed and kept at -20°C for further analysis. The herbarium codes of *A. corniculatum* and *A. ilicifolius* were RB/AA-KU-2018009 and RB-KU-2018010, respectively.

Determination of antioxidant activity

2, 2-diphenyl-1-picrylhydrazyl free radical scavenging assay: The DPPH assay was performed as described by Afrin et al. (14) with slight modifications. The DPPH solution was freshly prepared by dissolving DPPH (0.2

mM) in absolute ethanol. Two milliliters of the plant extracts and several standard different concentrations (6.25, 12.5, 25, 50, and 100 µg/mL) were added to 2 mL of DPPH solution followed by mixing properly and incubating at dark for 30 min. The inhibition percentage was calculated by recording absorbance at 517 nm against ethanol blank in a UV-visible spectrophotometer (UV-VIS Spectrophotometer - 2375, Labmatrix Manufacturing LLP). The following formula was employed to calculate scavenging activity:

$$\text{Scavenging ability for DPPH radicals (\%)} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

where $Abs_{control}$ refers to the absorbance of DPPH radical solution without samples, and Abs_{sample} denotes the absorbance of DPPH radical solution with samples. Ascorbic acid was used as the standard antioxidant. The 50% inhibitory concentration (IC_{50}) was determined by a non-linear regression curve of percent inhibition against the sample concentration.

Reducing power assay: The reducing power was determined according to the method developed by Afrin et al. (14) with some modifications. Various concentrations of extracts (25, 50, 100, 200, and 400 µg/mL) were mixed with 1.25 mL of phosphate buffer and 1.25 mL of potassium ferricyanide. After incubating at 50 °C for 20 min in water baths, 1.25 mL of trichloroacetic acid (10%) was added and centrifuged at 3000 rpm for 10 min. Then, 1.25 mL of the solution from the upper layer was mixed with an equal volume of distilled water and 250 µL freshly prepared ferric chloride solution (0.1%). The absorbance was taken at 700 nm. Ascorbic acid was used as the standard.

Ferric-ion reducing antioxidant power assay: FRAP assay is another method developed to determine the reducing power. We performed its protocol as described by Shokrzadeh et al. (15) with slight modifications. A standard curve was plotted by adding the FRAP reagent to Fe^{2+} solutions of known concentrations ranging from 0 to 100 µM. FRAP reagent for the calibration curve was prepared by mixing 25 mL of acetate buffer (300 mM; pH 3.6), 2.5 mL of TPTZ (2, 4, 6-tripyridyl-s-triazine) solution and 2.5 mL of H_2O followed by heating at 37 °C before the experiment. Likewise, FRAP reagent for the sample and standard ascorbic acid was prepared using 2.5 mL of ferric chloride [$FeCl_3 \cdot 6H_2O$ (20 mM)] instead of H_2O . A total of 100 µL of plant extract/ascorbic acid, 900 µL of distilled H_2O , and 2 mL of FRAP reagent were added to prepare reaction mixture followed by incubation in water bath (dark condition) at 37 °C for 30 min. After cooling, the absorbance was recorded at 593 nm.

β-Carotene bleaching assay: The β-carotene-linoleic acid bleaching assay was carried out based on the method by Ksiksi et al. (2012) (16). In brief, 1 mL of β-carotene solution (100 µg/mL), 200 µL of linoleic acid, and 200 µL of Tween-40 were adequately mixed. Then, the

chloroform was removed using rotary evaporator at 40 °C. In the next step, 100 mL of oxygenated distilled water was added, and emulsion solution was prepared by shaking vigorously. About 3 mL of β-carotene emulsion and 200 µL of various concentrations of extracts (6.25, 12.5, 25, 50, and 100 µg/mL) were added into the test tubes. BHT was used as the standard. After preparing the reaction mixtures, an initial absorbance was immediately taken at 460 nm against a blank, which was made by an emulsion containing all except β-carotene. After the incubation of reaction mixtures at 50 °C in water bath, the final absorbance was remeasured at 470 nm. Percent inhibition of the samples was calculated by following equation:

$$\text{Inhibition of bleaching (\%)} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

where $Abs_{control}$ refers to the absorbance of β-carotene linoleic acid solution without samples. BHT was used as the standard antioxidant. The IC_{50} value calculation for β-carotene bleaching inhibition was similar to that of the DPPH radical scavenging activity assay.

Screening of Phytochemical constituent: Phytochemical components of the bark and leaf extract of *A. corniculatum* and also leaf extract of *A. ilicifolius* were screened according to the standard protocols (17) to identify the presence of phenolic compounds, flavonoids, tannins, saponins, carbohydrate, alkaloids, and fats.

Polyphenolic compound analysis of plant extracts

Total phenolic content : The total phenolic content (TPC) of plant extracts was quantified using the Folin-Ciocalteu method (18) with some modifications. In brief, 1 mL of the extract solution (100 µg/mL) was diluted to 9 mL with water. Then, 1 mL of Folin-Ciocalteu (FC) reagent and 10 mL of 7% sodium carbonate (Na_2CO_3 , w/v) were added. That mixture was then incubated at room temperature for 60 min followed by the adjustment of volume to 25 mL with distilled water and mixing. The absorbance was measured at 750 nm against the blank on a spectrophotometer. The TPC was calculated using the gallic acid standard curve and expressed as mg of gallic acid equivalent (GAE) per mg of dry extract (mg GAE/g dw).

Total flavonoid content: The total flavonoid content (TFC) was estimated using the protocol as previously described (18) with minor modifications. In brief, the plant extract (100 µg/mL, 1 mL) was added to 5 mL of distilled water plus 0.3 mL of $NaNO_2$ solution (5%, w/v) and the mixture was shaken and left to stand for 5 min. Then, 0.6 mL of $AlCl_3$ (10%) solution was added, and after 5 min, we added 2 mL of NaOH solution (1 M). After mixing properly, the solution was allowed to stand at room temperature for 5 min. The absorbance of reaction mixture was recorded at 510 nm against the blank. The TFC was calculated using the calibration curve of

quercetin. The values were expressed as mg of quercetin equivalents (QEs) per mg of dry extract (mg QE/g dw).

Total tannin content: Total tannin content (TTC) was determined based on the method as described earlier (19) with minor modifications. The plants' extracts (100 µg/mL, 1 mL), 0.5 mL of FC reagent, and also 7.5 mL of distilled water were mixed and left for 5 min. In the next step, 1 mL of 35% (w/v) Na₂CO₃ solution was added. After adjusting the volume to 10 mL, the mixture was incubated at room temperature for 30 min. The absorbance of the reaction mixtures was measured at 725 nm using a blank. Gallic acid was used for the preparation of standard curve, and the TTC levels were determined in mg of gallic acid equivalents (GAEs) per mg of dry extract (mg GAE/g dw).

Determination of anti-inflammatory activity

Human RBC membrane stabilization activity: It was studied by the hemolysis of human RBC using either hypotonic solution or hydrogen peroxide (H₂O₂). Five milliliters of blood was collected from the human volunteers and kept at an EDTA vacuum test tube to prevent blood coagulation.

- **H₂O₂ induced HRBCMS activity:** This assay was performed based on the method as previously described (20). The collected blood sample was centrifuged at 3000 rpm for 15 min. After removing the supernatant, the remaining cells were washed thrice using PBS (10x, pH 7.4), and then 4% (v/v) RBC suspension was prepared by diluting in PBS. To prepare the reaction mixture, we mixed 1 mL of the RBC suspension and 0.5 mL of various concentrations of plants' extracts (50-800 µg/mL) and incubated it for 5 min at room temperature. Then, 0.5 mL H₂O₂ (100 µM) was added and shaken well. Ascorbic acid was used as the standard drug. The control was prepared by omitting the sample (plant extract or standard). The percentage inhibition of hemolysis was calculated by the following formula:

$$\% \text{ inhibition} = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100$$

- **Hypotonic solution induced HRBCMS activity:** We followed the previously used method to perform this assay (21) with minor modifications. An equal volume of blood and sterilized Alsever's solution (2% dextrose, 0.8% sodium citrate, 0.42% NaCl, and 0.05% citric acid in distilled water) were centrifuged at 3000 rpm for 10 min. The supernatant was carefully separated using Pasteur pipette, and isosaline (0.85%, pH 7.2) was used to wash the cells three times and prepare 10% (v/v) RBC suspension. The assay mixtures consisted of 2 mL of hyposaline (0.25% w/v), 1 mL of phosphate buffer saline (0.15 M, pH 7.4), 0.5 mL RBC suspension and various concentrations of the extracts and standard (50-800 µg/mL). Diclofenac sodium was used as the reference standard. The control was prepared with

saline by omitting the extracts. After 30 min of incubation at 37 °C in a water bath, the reaction mixture was cooled and centrifuged for 10 min at 5000 rpm. The absorbance of the released hemoglobin was measured at 540 nm. The percentage inhibition of hemolysis was determined through the following equation:

$$\% \text{ inhibition} = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100$$

Lipoxygenase inhibition assay: The inhibition of LOX activity was determined according to the previous study (22) with slight modifications. LOX (final concentration of 167 U/mL) was prepared in borate buffer solution (0.2 M, pH 9.00), and linoleic acid (134 µM) was used as a substrate. Different concentrations of the samples' extracts (6.25 - 100 µg/mL) were prepared with 1.6% DMSO solutions. The enzyme solution was stored on ice throughout the assay. In the next step, 25 µL of the extracts and also 975 µL of the enzyme solution were added, and the mixture was kept at room temperature for 5 min. Then, 1 mL of linoleic acid was added to the solution, and its absorbance was measured at 234 nm against a blank (25 µL DMSO, 975 µL borate buffer, and 1 mL linoleic acid) for 10 min with 1-min interval. Quercetin was used as a positive control. The LOX inhibition percent was calculated using the following equation:

$$\% \text{ inhibition} = \frac{A - B}{A} \times 100$$

where A refers to the absorbance at 234 nm without a test sample, and B represents absorbance at 234 nm with a test sample. The IC₅₀ values were estimated by interpolating from the equation of inhibition activity against concentration.

Determination of anticoagulation activity

Prothrombin time (PT) determination assay: The PT was determined as the previous study (23). Five milliliters of blood was drawn into EDTA vacuum tube from the median cubital vein of 15 healthy volunteers. The blood samples were then centrifuged for 15 min at 3000 rpm. The obtained plasma samples of each individual were pooled together in a plane container using automatic pipette and stored at 4 °C until the use. About 200 µL of plasma was mixed with 100 µL of different concentrations of plants' samples (50-800 µg/mL) followed by shaking well to mix them properly. Then, 300 µL of CaCl₂ (25 mM) was added to the reaction tubes and incubated at 37 °C in a water bath. To allow the reaction, the tubes were shaken gently and tilted back and forth in 5-s intervals. The coagulation time was determined in seconds using a stopwatch.

Statistical analysis: Three replications were considered for all the experiments, and the results were expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) followed by Bonferroni test were performed in Graphpad Prism Version 6.01. The P values of less than 0.05 were considered significant.

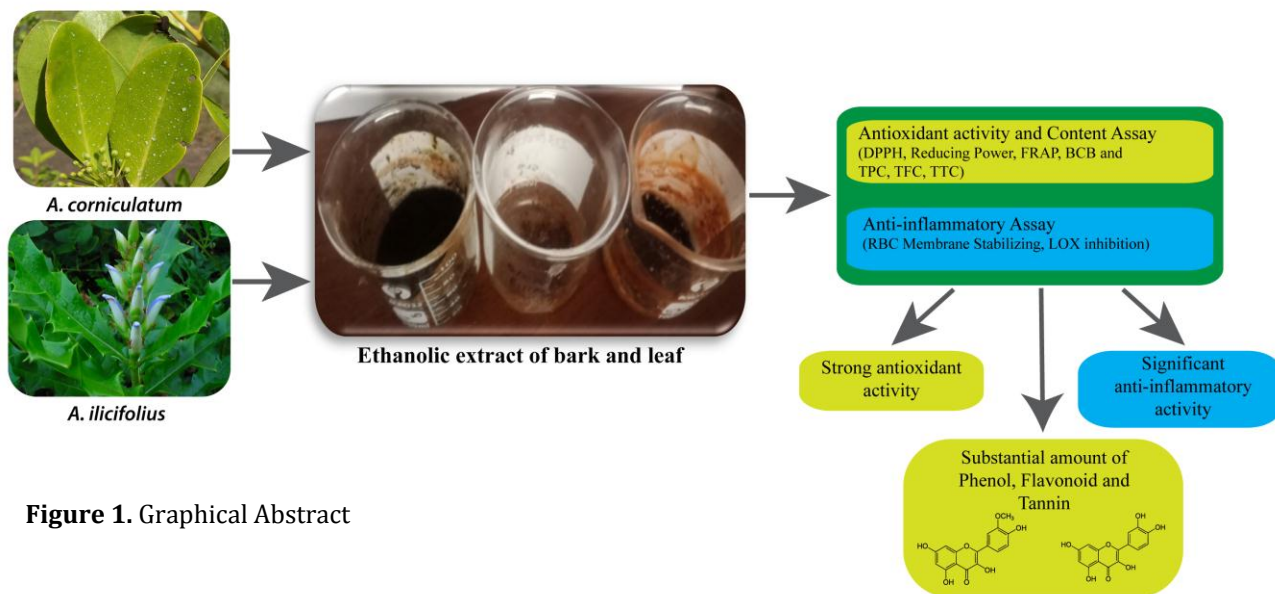


Figure 1. Graphical Abstract

Results

Each compound produced in plant parts has its antioxidant activity, which is very difficult to estimate by a single antioxidant assay (24). Various methods, including DPPH, BCB and FRAP methods were used to determine the antioxidant properties of the plants' extracts. The inhibition activity of DPPH free radical scavenging increased linearly in a concentration-dependent pattern (Fig. 2a).

The bark and leaf extract of *A. corniculatum* showed significant ($P < 0.001$) antioxidant activity with IC_{50} values of $20.49 \pm 2.14 \mu\text{g/mL}$ and $33.51 \pm 1.60 \mu\text{g/mL}$, respectively compared to the standard ascorbic acid ($IC_{50} = 10.08 \pm 1.79 \mu\text{g/mL}$) (Table 1). *A. ilicifolius* leaf extract exhibited less potent antioxidant activity with higher IC_{50} value ($78.90 \pm 1.87 \mu\text{g/mL}$).

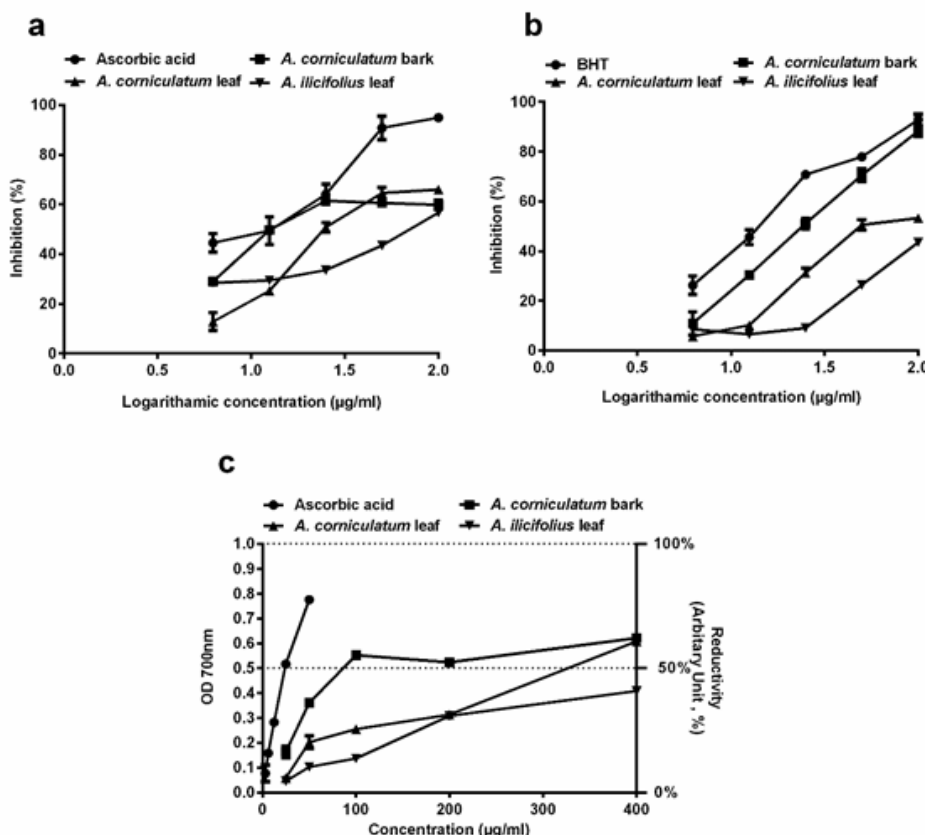


Figure 2. Antioxidant properties of plant extracts and standards (a: DPPH free radical scavenging activity, b: BCB inhibition activity, c: Reducing power activity). Statistical analysis of all samples antioxidant activities were significantly different from the control groups ($P < 0.001$).

Table 1. Antioxidant, anti-inflammatory, and anticoagulation properties of plants' extracts and standards. Values are expressed as mean \pm SD. Statistical analysis of all activities showed significant differences ($P < 0.001$) compared with the control group.

Sample	Antioxidant activity				Quantitative analysis of antioxidant molecule			Anti-inflammatory activity			Anticoagulation activity
	DPPH IC ₅₀ (μ g/mL)	Reducing power EC ₅₀ (μ g/mL)	FRAP Fe ²⁺ eq. (μ M/100 μ g dw)	BCB IC ₅₀ (μ g/mL)	TPC (mg GAE/g dw)	TFC (mg QE/g dw)	TTC (mg GAE/g dw)	RBC Membrane Stabilization			PT at 800 μ g/mL (min)
								H ₂ O ₂ mediated IC ₅₀ (μ g/mL)	Hypotonic solution mediated		
									LOX inhibition IC ₅₀ (μ g/mL)	IC ₅₀ (μ g/mL)	
Standard	10.08 \pm 1.79 ^a	28.49 \pm 0.39 ^a	84.11 \pm 0.93 ^a	14.67 \pm 0.03 ^b	-	-	-	32.15 \pm 0.03 ^a	53.57 \pm 0.019 ^a	23.58 \pm 1.75 ^c	35.24 \pm 0.05 ^d
<i>A. corniculatum</i> Bark	20.49 \pm 2.14	215.21 \pm 4.71	72.80 \pm 1.20	24.77 \pm 0.02	499.04 \pm 0.01	568.33 \pm 0.01	545.47 \pm 9.47	63.93 \pm 0.02	360.51 \pm 0.002	26.79 \pm 1.31	18.19 \pm 0.13
<i>A. corniculatum</i> Leaf	33.51 \pm 1.59	319.41 \pm 3.71	29.36 \pm 0.43	68.94 \pm 0.02	277.11 \pm 0.01	210.00 \pm 0.01	205.54 \pm 6.15	120.78 \pm 0.01	525.10 \pm 0.001	59.51 \pm 2.45	10.33 \pm 0.14
<i>A. ilicifolius</i> Leaf	78.90 \pm 1.87	465.81 \pm 5.65	13.25 \pm 0.21	278.67 \pm 0.01	128.86 \pm 0.01	18.33 \pm 0.01	31.12 \pm 1.16	352.93 \pm 0.001	886.98 \pm 0.001	108.61 \pm 2.72	8.87 \pm 0.41

^a = Ascorbic acid, ^b = BHT, ^c = Quercetin, ^d = Warfarin

The reducing power of all studied extracts and also the standards were found with the increased reducing capability, which increased in a concentration-dependent manner (Fig. 2c). An arbitrary unit was assumed, where 1.0 absorbance was considered as 100% reducing capacity, according to Ferreira et al. (25). Therefore, 50% effective concentration (EC₅₀) was defined as the concentration that the reducing power showed 0.5 of absorbance. As shown in Table 1, *A. corniculatum* bark was found with relatively lower EC₅₀ value (215.21 \pm 4.71 μ g/mL) compared to the standard ascorbic acid (EC₅₀ = 28.49 \pm 0.39 μ g/mL). In contrast, the reducing power of *A. corniculatum* and *A. ilicifolius* leaves (EC₅₀ = 319.409 \pm 3.71 and 465.81 \pm 5.65 μ g/mL, respectively) was less than that of *A. corniculatum* bark.

The *A. corniculatum* bark extract showed effective reduction ability among the extracts, with the FRAP value of 72.80 \pm 1.20 μ M Fe (II)/100 μ g dry weight, whereas ascorbic acid was observed with FRAP value of 84.11 \pm 0.93 μ M Fe (II)/100 μ g dry weight (Table 1).

Like DPPH assay, in BCB assay, all extracts showed the increased concentration dependency (Fig. 2b). The antioxidant activity is shown in Table 1. The respective IC₅₀ value of the bark and leaf extract of *A. corniculatum* and leaf extract of *A. ilicifolius* in BCB assay was 24.77 \pm 0.02 μ g/mL,

68.94 \pm 0.02 μ g/mL and 278.67 \pm 0.01 μ g/mL, respectively.

The TPC and TTC of extracts were expressed as mg GAE/g dw, whereas TFC was expressed as mg QE/g dw. It is noticeable from the Table 1 that the bark extract of *A. corniculatum* possessed higher amount of phenol (499.04 \pm 0.01 mg GAE/g dw), flavonoid (568.33 \pm 0.01 mg QE/g dw) and tannin (545.47 \pm 9.47 mg GAE/g dw), whereas, *A. corniculatum* leaf extract was found with phenolic content of 277.11 \pm 0.01 mg GAE/g dw, flavonoid content of 210.00 \pm 0.01 mg QE/g dw and tannin content of 205.54 \pm 6.15 mg GAE/g dw. In contrast, *A. ilicifolius* leaf extract showed lower amount of these polyphenol contents.

In HRBC membrane stabilization activity assay, ascorbic acid was chosen as the standard. All samples exhibited inhibition activity in a concentration-dependent pattern (Fig. 3a, b). In particular, *A. corniculatum* bark was found with IC₅₀ value of 32.15 \pm 0.03 μ g/mL and 53.57 \pm 0.019 μ g/mL, respectively (Table 1). The LOX inhibitory activity of the extracts was determined and compared with that of quercetin (Fig. 3c). All extracts showed an inhibitory effect in a concentration-dependent manner. We found that *A. corniculatum* bark had lower IC₅₀ value of 26.79 \pm 1.31 μ g/mL, which was very close to the standard quercetin (IC₅₀ = 23.58 \pm 1.75 μ g/mL).

Also, *A. corniculatum* and *A. ilicifolius* leaves showed considerable amounts of LOX inhibition activity ($IC_{50} = 59.51 \pm 2.45$ and $108.61 \pm 2.72 \mu\text{g/mL}$, respectively). In PT assay, the clotting time of all extracts showed a concentration-dependent manner (Table 2). Standard warfarin took the highest time to clot (35.24 ± 0.05 min at $800 \mu\text{g/mL}$). *A. corniculatum* bark needed 18.19 ± 0.13 min and *A. corniculatum* leaf took 10.33 ± 0.14 min for complete clotting at highest concentration ($800 \mu\text{g/mL}$). *A. corniculatum* leaf prevented the coagulation within 8.87 ± 0.41 min.

Discussion

Oxidative stress-mediated inflammation is a common risk factor in the pathogenesis of numerous diseases. Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used against inflammatory conditions. However, various complications associated with NSAIDs have resulted in considering alternative pharmacotherapies. In this regard, plant-derived antioxidant metabolites with anti-inflammatory properties can be an attractive proponent. In the present study, two mangrove plants, namely *A. corniculatum* and *A. ilicifolius* were explored as potential

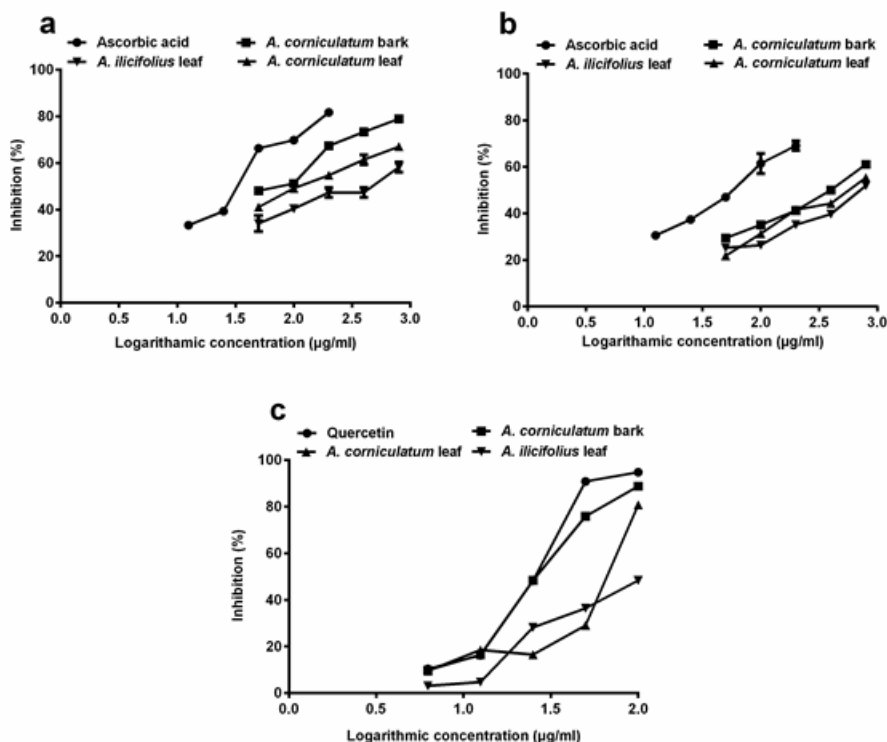


Figure 3. Anti-inflammatory properties of the extracts and standards (a: H_2O_2 induced HRBCMS activity, b: Hypotonic solution-induced HR BCMS activity, c: LOX inhibition activity). Statistical analysis of anti-inflammatory activities revealed significant differences between the samples and control groups ($P < 0.001$).

Table 2. Prothrombin time (PT) of the extracts and standard. Values are expressed as mean \pm SD. All values are statistically significant ($P < 0.001$) compared with the control group.

Concentration ($\mu\text{g/mL}$)	PT Time (min)			
	Warfarin (standard)	<i>A. corniculatum</i> bark	<i>A. corniculatum</i> leaf	<i>A. ilicifolius</i> leaf
800	35.24 ± 0.05	18.19 ± 0.13	10.33 ± 0.14	8.87 ± 0.41
400	32.00 ± 0.39	16.45 ± 0.13	9.30 ± 0.10	8.25 ± 0.16
200	30.17 ± 0.12	14.38 ± 0.19	8.43 ± 0.11	7.4 ± 0.06
100	27.23 ± 0.12	13.07 ± 0.06	7.39 ± 0.10	6.95 ± 0.36
50	25.41 ± 0.12	10.35 ± 0.07	6.91 ± 0.33	5.96 ± 0.34

In the present study, two mangrove plants, namely *A. corniculatum* and *A. ilicifolius* were explored as potential sources of natural antioxidants with anti-inflammatory properties.

In DPPH inhibition assay, *A. corniculatum* was found as a more significant potential source of antioxidant than *A. ilicifolius*. Compounds with reducing power have electron donation capacity and can reduce oxidized intermediate of the lipid peroxidation process. Accordingly, they are considered as primary and secondary antioxidants (14). Therefore, to obtain accurate results regarding antioxidant activity, we evaluated reducing power and FRAP value of the studied extracts. We found *A. corniculatum* bark more potential reducing capabilities than other extracts. In the FRAP assay, we also quantified the reduction power equivalent to the Fe (II) ion. By extrapolating the absorbance in the equation of Fe (II) ion standard curve, we estimated the reducing power of plants' extracts. *A. corniculatum*, and *A. ilicifolius* leaves samples were observed with lower reducing ability than *A. corniculatum* bark sample. Contrary to the findings of Bidve et al. (8), we found higher amount of reducing capacity, which could be due to the difference in the used solvents. Ngo et al. (26) showed that aqueous ethanol extract had higher antioxidant capacity than absolute methanol. Similarly, it is also reported that the combination of organic solvents with water can extract highest antioxidant capacities (27). In general, antioxidant molecules can prolong the length of rapid color degradation process of β -carotene (coupled with linoleic acid) by neutralizing the peroxide products of linoleic acid. Accordingly, BCB method can estimate the ability of an antioxidant to inhibit lipid peroxidation (16). The findings of BCB assay (Table 1) indicated that the bark extract of *A. corniculatum* possesses considerable activity, which is close to the positive control BHT ($IC_{50} = 14.67 \pm 0.03 \mu\text{g/mL}$).

Phytochemical screening of all ethanolic extracts of both plants demonstrated the presence of phenolics, flavonoids, tannins, and alkaloids, whereas proteins and oil were not present in those extracts. Although saponins were found in *A. corniculatum* bark, they were absent in *A. corniculatum*, and *A. ilicifolius* leaves extracts. Generally, phenolics flavonoids and tannins are mostly associated with the antioxidant activity of plants.

The higher antioxidant activity of these extracts made us take the leap of quantitative analysis of polyphenol content. Therefore, the total polyphenolic contents were determined as the quantification of antioxidant compounds correlated with the antioxidant properties of the studied samples. The findings of TPC are consistent with the results reported by Wei et al. (28). Flavonoids, like Isorhamnetin (29), Quercetin (30), and several tannins were isolated from the aerial parts of *A. corniculatum* (28). We postulate the presence of such compounds might be the reason for the higher amount of flavonoid and tannin contents in our study. Moreover, the obtained polyphenol content was correlated with antioxidant properties.

Previous studies showed that neutrophils, defensive blood cells of the host, mainly can generate ROS (31). Apart from having antioxidant properties, the phenolic compounds and flavonoids are also anti-inflammatory factors (32). In our study, three different methods were performed to determine the anti-inflammatory properties of the extracts. In HRBC membrane stabilization activity assay, we used various concentrations of the extracts to protect the bursting of RBC, which was mediated by H_2O_2 and hypotonicity. *A. corniculatum* bark was found with the highest potential to protect the RBC lysis both by H_2O_2 and hyposaline at relatively lower IC_{50} value. In contrast, *A. corniculatum* and *A. ilicifolius* leaves had the lowest activity (Table 1). The enzymatic antioxidants are directly involved in the neutralization of ROS. Compared to standard quercetin, *A. corniculatum* bark showed potential LOX inhibition activity. Consistent with antioxidant properties, anti-inflammatory activity of the extracts also showed similar results. These results confirmed their antioxidant properties as they showed significant anti-inflammatory properties compared with the standards, suggesting that all specimens possess antioxidant properties.

PT was determined by clotting times at different concentrations of the extracts. High anticoagulation effect of *A. corniculatum* extracts was reported in previous studies (33). Substances with high antioxidant activity also exhibit anticoagulant activity; therefore, flavonoid groups also have anticoagulant effects (34). Isorhamnetin (as a flavonoid) was isolated from the bark of *A. corniculatum* (29). It is reported that isorhamnetin possesses anticoagulation (antithrombotic) properties (35). This isorhamnetin along with other flavonoids could modulate the anticoagulation activity. In our study, *A. corniculatum* bark and leaf demonstrated significant ($P < 0.001$) PT. According to the results, all extracts delayed coagulation time, which indicated their potential anticoagulation properties.

Based on the current study, *A. corniculatum* bark possesses significant antioxidant potential with a higher amount of polyphenol content (phenol, flavonoid, and Tannin). Besides, it also showed significant anti-inflammatory and anticoagulation properties.

Conclusion

Two mangrove species, namely *A. corniculatum* and *A. ilicifolius* were investigated in this study for their antioxidant and anti-inflammatory properties. *A. corniculatum* ethanolic bark extract demonstrated potent antioxidant, anti-inflammatory, and anticoagulation properties compared with all evaluated extracts. We infer that polyphenols, particularly flavonoid compounds, might be responsible for the observed biological properties as we could demonstrate a significant positive correlation between metabolic content and biological activity. An LC-MS analysis needs to be carried out to reveal the metabolic profile of *A. corniculatum* and *A. ilicifolius*, particularly for the ethanolic bark extract of *A. corniculatum*, which demonstrated potent antioxidant, anti-inflammatory, and anticoagulation effects.

Ethical statement

This study was approved by Khulna University, Bangladesh (Ethical code: KUAEC-2017/08/15). All ethical considerations were observed throughout the research. To draw human blood (RBC membrane-stabilizing assay), the procedure and rules of Bangladesh Medical Research Council were strictly followed.

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Authors' contributions

Rana Biswas and Afiya Anjum; Concept, literature search, experimental studies, data acquisition, data analysis, statistical analysis, and manuscript preparation.

Tauhidur Rahman Nurunnabi and Sujan Kumar Kundu; Sample collection, herbarium preparation, data acquisition, data analysis, statistical analysis, and manuscript preparation.

S. M. Mahbubur Rahman and Kazi Mohammed Didarul Islam; Definition of intellectual content, data analysis, manuscript preparation, manuscript editing, and manuscript review.

Md. Morsaline Billah and Md. Emdadul Islam; Concept, experimental design, data analysis, statistical analysis, manuscript editing, and manuscript review.

Conflicts of interests

The authors declare no conflicts of interest.

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