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Phytochemical screening and evaluation of antioxidant and thrombolytic activities of methanolic extract of *Antidesma bunius* L. (Family Euphorbiaceae) leaves

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Abstract
The purpose of this study was to evaluate total antioxidant capacity, free radical scavenging activity, thrombolytic activity and screening of phytochemical constituents. Total antioxidant capacity and free radical scavenging activity were determined by standard methods. In thrombolytic test clot lysis method was used. Phytochemical screening was done by characteristic color changes or color precipitate using standard phytochemical reaction methods. Results of the preliminary phytochemical screening of the methanolic extract of *A. bunius* L. leaves revealed the presence of various bioactive components which include saponins, steroids, alkaloids, flavonoids and proteins in the extract. The total antioxidant capacity of crude methanolic extract was also found very good compared to standard catechin. In DPPH assay the scavenging activity of the methanolic extract (IC_{50} 3.10µg/ml) showed very good activity compared to standard BHT (IC_{50} 2.30 µg/ml).The methanolic extract of leaves of *A. bunius* exhibited thrombolytic activity 21.1 %, thrombolytic activity respectively compared to standard streptokinase (61.31%) lysis of clot. It can be concluded that the methanolic extract of *A. bunius* leaves could be used in drug formulation because of its effective pharmacological properties. Hence, the plant may further be explored for its various pharmacological activities.

Keywords: Phytochemical, Antioxidant, *Antidesma bunius* leaves, Methanolic extract, Thrombolytic activity

1. Introduction
The therapeutic efficacy of many native plants for various diseases has been stated by traditional herbal medicinal practitioners [1]. Medicinal plants as the major medicament in traditional system of medicine have been used in medical purposes. Major people towards natural products, natural medicine is attracting more than allopathic medicine. Furthermore this system of medicine is least toxic without side effects [2]. *A. bunius* L. (Family-Euphorbiaceae) is traditionally used as sudorific and in the treatment of snakebite; decoction is used to promote perspiration in febrile condition; juice of the plant is useful in the treatment of insomnia. Fresh juice of the fruits is used in the manufacture of wine as an antioxidant. Roots and leaves are antihelminthic and also used in indigestion cough and stomachache. The seeds are used against round worms and threadworms, coughs, flatulence, intestinal colic and also used as pesticide [3]. Antioxidants protect human body against free radicals by scavenging them [4]. Free radicals are liable for devastation of protein, as well as that lipid and DNA by oxidative process during aerobic metabolism in the biological system [5], lead to many degenerative disease and long-term or chronic diseases together with atherosclerosis, carcinogenesis, diabetes mellitus, ischemic cardiac disease, immunocompromised and neurodegenerative diseases [6]. Thromboembolic disorders are one of the main causes of morbidity and mortality in Bangladesh [7]. Nowadays, cardiovascular disease caused by blood clot formation is one of the most dangerous diseases which are increasing day by day [8]. Therefore, thrombosis is the formation of a blood clot (thrombus) inside a blood vessel, stopping the flow of blood through the circulatory system. Using thrombolysis medications are used to dissolve clots formed in blood vessels and in the management of thrombosis in patients [9-10]. Thrombolytic drugs such as alteplase, anistreplase, streptokinase, urokinase and tissue plasminogen activator (TPA) are commonly used all over the world for the treatment of thrombosis [11-12], but their use is associated with hyper risk of haemorrhage [13], anaphylactic reaction and lacks specificity [14]. So,

There is a try to develop improvement of natural constituents from various animal and plants sources which have thrombolytic activity [15-16], antiplatelet [17-18], antithrombotic [20] and anticoagulant [20-21].

1.1 Plant description: The tree may be shrubby, 10 to 26 ft (3-8 m) high, or may reach up to 50 or even 100 ft (15-30 m). Leaves oblong, 4 to 9 in (10-22.5 cm) long, 2 to 3 in (5-7.5 cm). The tiny, odorous, reddish, axillary or terminal racemes 3 to 8 in (7.5-20 cm) long. The round or ovoid fruits, up to 1/3 in (8 mm) across, are borne in grapelike, pendent clusters (often paired), fruits are very acid, slightly sweet, Some tasters detect a bitter or "unpleasant aftertaste", straw-colored stone, an irregular, flattened oval, ridged or fluted, very hard, 3/8 in (1 cm) long, 1/4 in (6 mm) wide.

2. Methods and materials

2.1 Collection of sample and preparation of extract: For this investigation *A. bunius* fresh leaves were collected during the month of April, 2017, from Chittagong in fresh condition and identified by an expert taxonomist. A voucher specimen was submitted to the national herbarium, Mirpur, Dhaka, Bangladesh. Accession number: DACB 43490. About 400g of dried and powdered plant material was soaked in 1.7 liter of methanol in an amber glass container for about 14 days at room temperature with occasional shaking. After 14 days, the solution was filtered using cotton filter and Whitman's filter paper number 1. The filtrate was concentrated to solid mass by using a rotary evaporator [22-23].

2.2 Phytochemical analysis of methanolic crude extract: Phytochemical screening of the leaves extract were tested for the presences of active principles such as alkaloids, flavonoids, tannins, proteins etc. Determined using the standard procedures were used.

2.2.1 Test for Tannins (Braymer's Test): 2ml of the extract was stirred with 2ml of distilled water and 2-3 drops of $FeCl_3$ (5%) solution were added. The formation of green color precipitate indicates the presence of tannins [24]. Observation: Green color precipitate indicates the presence of tannins.

2.2.2 Test for Flavonoids: 1ml of the extract, 1ml of 10% lead acetate (Pb (OAc) solution is added [25]. Observation: Yellow color precipitate was taken as a positive result for flavonoids.

2.2.3 Test for Terpenoids: 2ml of the extract was dissolved in 2ml of chloroform $(CH_3CO)_2O$ and evaporated to dryness. 2ml of concentrated sulphuric acid (H_2SO_4) was added and heated for about 2 min [26]. Observation: Deep red color indicates the presence of terpenoids.

2.2.4 Test for Saponins (Foam Test): 5ml of the extract was shaken vigorously with an equal volume of distilled water in a test tube and the mixture was warmed [27]. Observation: The formation of emulsion forms or stable foam was taken as an indication of presence of saponins.

2.2.5 Test for Steroids (Salkowski Test): 2ml of the extract was mixed with few drops of acetic anhydride, boiled and colored. 2ml concentrated sulphuric acid was then added from the sides of the test tube [27]. Observation: The formation of reddish brown ring at the junction of two layers and green color of the upper layer and the formation of deep red color in the lower layer would indicate a positive test for steroids.

2.2.6 Test for Phlobatannins (Precipitate Test): 2ml of the extract was added to 2ml of HCl (1%) and the extract was boiled [27]. Observation: Deposition of a red precipitate was taken as an indication of presence of phlobatannins.

2.2.7 Test for Carbohydrates (Molisch's Test): 2ml of the extract was treated with 2 drops of ethanolic α-naphthol (20%) solution in a test tube [27]. Observation: Formation of the reddish violet ring at the junction indicates the presence of carbohydrate.

2.2.8 Test for Coumarins: 2ml of the extract was added to 3ml of NaOH (10%) [28]. Observation: Formation of the yellow color indicates the presence of coumarins.

2.2.9 Test for Alkaloids (Hager's Test): 3ml of the extract solution was treated with few drops of Hager's reagent (saturated picric acid solution) [28]. Observation: Presence of alkaloids confirmed by the formation of yellow coloured precipitate.

2.2.10 Test for Proteins (Xanthoproteic Test): The extracts were treated with few drops of conc. nitric acid [27]. Observation: Formation of yellow color indicates the presence of proteins.

2.2.11 Test for Anthraquinones (Borntrager's Test): 3ml of the extract was treated with 3ml of benzene and then 5ml aqueous NH_3 (10%) was added in a test tube [27]. Observation: After shaking, change in color of aqueous layer was observed. Observation: Pink, violet or red color in aqueous layer indicated the presence of anthraquinones.

2.2.12 Test for Anthocyanins: 2ml of the extract was treated with 2ml of HCl (2N) and then added NH_3 in a test tube [27]. Observation: formation of pinkish red to bluish violet color indicates the presence of anthocyanins.

2.3 Antioxidant evaluation

2.3.1 Determination of total antioxidant capacity: Total antioxidant capacity of sample was determined by the method of Prieto *et al.* [29], with some modifications. The phosphomolybdenum method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and subsequent formation of a green phosphate/ Mo (V) complex at acidic pH. 0.5 mL of plant extract /standard at different concentrations (7.825–250 µg/mL) was mixed with 3 mL of reaction mixture containing 0.6M sulphuric acid, 28 mM sodium phosphate and 1% ammonium molybdate was added into the test tubes. The test tubes was incubated at 95 °C for 10 min to complete the reaction. Then, the absorbance of the solution was measured at 695nm using a spectrophotometer against a blank solution to room temperature. Catechin was used as standard, a reference sample. A typical blank solution contained 3 mL of reaction mixture and the appropriate volume (500 µl) of the same solvent used for the samples/standard, and it was incubated under the same conditions as the rest of the samples solution. Increased absorbance of the reaction mixture

introduces increased total antioxidant capacity. We used standard/samples at six different concentrations ranges from (7.825 to 250 µg/mL) for each antioxidant assay. The experiment was repeated three times at each concentration.

2.3.2 DPPH radical scavenging assay: DPPH was used to evaluate the free radical scavenging activity of various compounds and medicinal plants according to Desmarcheliar et al. [30], with some modifications. The 1, 1-diphenyl-2-picrylhydrazyl (DPPH) has been widely used to evaluate the free radical scavenging capacity of antioxidants. DPPH free radical is reduced to the corresponding hydrazine when it reacts with hydrogen donors. DPPH can make stable free radicals in aqueous or methanol solution. Resulting, DPPH produces purple/violet color in methanol solution and fades to shades of yellow color in the presence of antioxidants. 2 ml of methanol solution of plant extract or standard sample at different concentration (3.906–150µg/mL) was mixed with 3 ml of methanol solution of DPPH was added into the test tube. The test tube was incubated at room temperature for 30 minutes in dark place to complete the reaction. Then the absorbance of the solution was measured at 517nm using a spectrophotometer against a blank solution. A typical blank solution contained all reagents except plant extract or standard solution. The percentage (%) inhibition activity was calculated by the following equation:

% DPPH radical scavenging activity = $\{(A_0 - A_1)/A_0\} \times 100$

Where, A_0 is the absorbance of the control, and A_1 is the absorbance of the extract/standard. Then % of inhibition was plotted against concentration, and from the graph IC_{50} was calculated. The experiment was repeated three times at each concentration.

2.4 Thrombolytic activity: In vitro clot lysis activity of the leaves of *A. bunius* was carried out according to the method of Prasad *et al.* [31], with minor modification. Aliquots (5ml) of venous blood were drawn from healthy volunteers who were distributed in ten different pre weighed sterile vials (1ml/vial) and incubated at 37°C for 45 minutes. After clot formation, the serum was completely removed without disturbing the clot and each vial having clot was again weighed to determine the clot weight (clot weight = weight of clot containing vial – weight of vial alone). To each vial containing pre-weighed clot, 100µl aqueous solution of the crude extract was added separately. As a positive control, 100µl of streptokinase (SK) and as a negative non thrombolytic control, 100µl of distilled water were separately added to the control vials. All the vials were then incubated at 37 °C for 90 minutes and observed for clot lysis. Therefore, after incubation, the released of fluid was removed and vials were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis as shown below:

% clot lysis = (Weight of the lysis clot / Weight of clot before lysis) × 100

3. Results and discussion

The preliminary phytochemical screening of the extract was done to ascertain the presence or absence of bioactive components. Saponins, Tannins, Flavonoids, Steroids, Phlobatannins, Carbohydrates, Coumarins, Alkaloids, Proteins, Emodins, and alkaloids were presence (Table1) whereas, Anthraquinones, Anthocyanins, Carbohydrates, Terpenoids were absent.

Table 1: Qualitative analysis of the phytochemical screening of the *A. bunius* L.

Phytochemical tests	Crude methanol extract
Saponins	+
Tannins	+
Flavonoids	+
Terpenoids	-
Steroids	+
Phlobatannins	+
Carbohydrates	-
Coumarins	+
Alkaloids	+
Proteins	+
Emodins	+
Anthraquinones	-
Anthocyanins	-

Here, + = Present, = Not present.

3.1 Total antioxidant capacity: The antioxidant potential of the methanolic extracts of *A. bunius* was estimated from their ability to reduce the reduction of Mo (VI) to Mo (V) by the antioxidant compound and subsequent formation of a green phosphate/Mo (V) complex with a maximal 695nm. Total antioxidant activity of plant extracts and (+) catechin (standard) were depicted in (Table 2) in (Figure.1, 2). As shown in Fig. 1, 2 crude methanolic extract showed considerable antioxidant activity compared to (+)-catechin (Standard). The activity was less than that of catechin. At the concentration 62.50µg/ml, the absorbance of crude methanol extract and (+)-catechin were 0.665 and 0.775 respectively. The extract was found to increase the total antioxidant activity with the increasing concentration of the extract. At 250 µg/ml, the absorbance of crude methanol extract and (+)-catechin were 1.537 and 3.457 respectivel

Table 2: Total antioxidant activity of the crude methanol extract of *A. bunius* and (+)-catechin (standard) at different concentrations.

Name of sample	Conc.(µg/ml)	Absorbance			Absorbance Mean ±STD
		a	b	c	
(+)-Catechin	7.825	0.072	0.073	0.075	0.073 ± 0.0012
	15.625	0.145	0.148	0.141	0.145 ± 0.0029
	31.25	0.723	0.720	0.718	0.720 ± 0.0020
	62.50	0.876	0.874	0.877	0.875 ± 0.0012
	125	1.461	1.463	1.469	1.469 ± 0.0033
	250	2.457	2.459	2.456	2.457 ± 0.0012
Crude methanol extract	7.825	0.061	0.065	0.068	0.064± 0.0028
	15.625	0.133	0.136	0.131	0.133± 0.0020
	31.25	0.334	0.336	0.339	0.336± 0.0020

	62.5	0.663	0.665	0.668	0.665± 0.0020
	125	0.812	0.815	0.818	0.815 ± 0.0024
	250	1.506	1.504	1.601	1.537 ± 0.0452

Each value is the average of three analyses ± standard deviation.

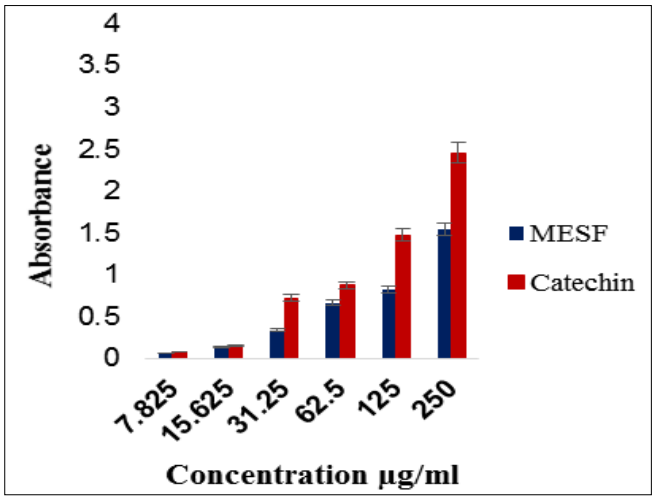

Fig 1

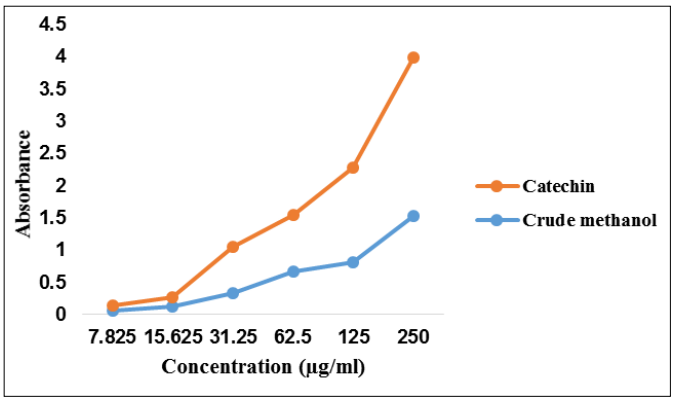

Fig 2

3.2 DPPH radical scavenging activity: The model of Scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples including plant extracts. DPPH antioxidants Assay is based on the ability of 1, 1 diphenyl-2-picryl-hydrazyl (DPPH), and a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 517 nm and also for a visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which can be quantitatively measured from the change in absorbance and % of scavenging activity is calculated. The activity was increased by increasing the concentration of the sample extract. The antioxidant activity of the extractives of *A. bunius* was evaluated by DPPH radical scavenging assays. The results of DPPH radical scavenging assays of plant extracts and BHT (butylatedhydroxy toluene) are given in (Table 3) and in figure (3, 4). The scavenging activity of the crude methanol extract was very close to that of BHT (standard). IC_{50} of standard and crude methanol extract were 2.30µg/ml and 3.75µg/ml respectively.

Table 3: DPPH radical scavenging activity of the crude methanol extract of *A. bunius* and BHT (Standard) at different concentrations.

Name of sample	Conc. (µg/ml)	% of scavenging			% of scavenging Mean ± STD	IC_{50} (µg/ml)
		a	b	c		
BHT	3.906	84.7	84.9	85.12	84.91 ±0.171	2.30±0.002
	7.8125	88.74	89.32	88.72	88.93 ±0.278	
	15.626	93.02	91.89	92.78	92.56 ±0.486	
	31.25	94.37	93.67	94.10	94.05± 0.288	
	62.5	94.60	94.68	94.62	94.63 ±0.033	
	125	96.13	96.23	95.78	96.05 ±0.192	
Crude methanol extract	3.909	51.65	51.25	53.37	52.09±0.919	3.75±0.079
	7.8125	54.06	54.98	55.01	54.68±0.440	
	15.625	60.6	62.12	61.55	61.42±0.626	
	31.25	64.67	66.42	65.33	65.47±0.883	
	62.5	80.96	81.77	83.66	82.13±1.131	
	125	92.82	91.88	91.68	92.12±0.497	

Each value is the average of three analyses ± standard deviation.

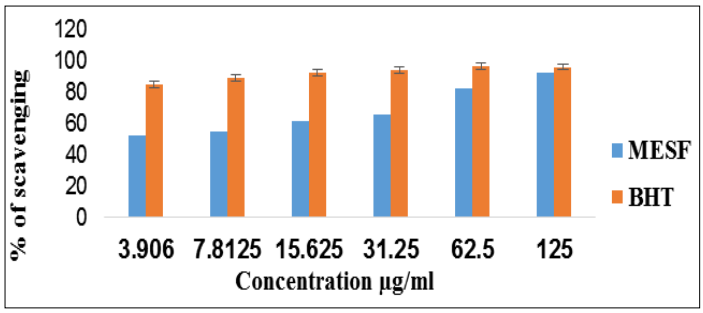

Fig 3

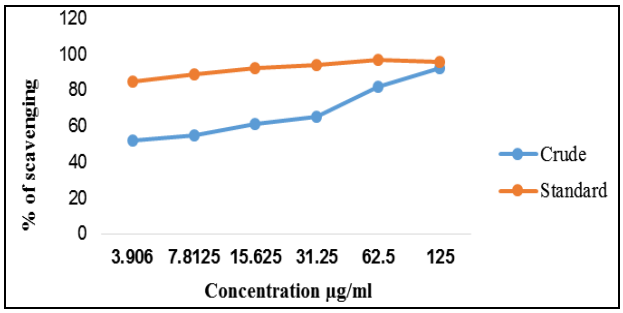

Fig 4

3.3 Thrombolytic activity: In present study, as a part of discovery cardio protective agents from natural sources, the extractives of *A. bunius* leaves were assessed for Thrombolytic activity and the results are presented in the following table 4.

Table 4: Thrombolytic activity (in terms of % of clot lysis) of the extractives of *A. bunius* leaves.

Fractions	W_1mg	W_2 (mg)	W_3 (mg)	W_4 (mg)	W_5 (mg)	(W_5/W_4)X 100%
Sample	813	1160	1087	347	73	21.1%
Blank	815	1151	1130	336	21	6.26%
SK	835	1520	1100	685	420	61.31%

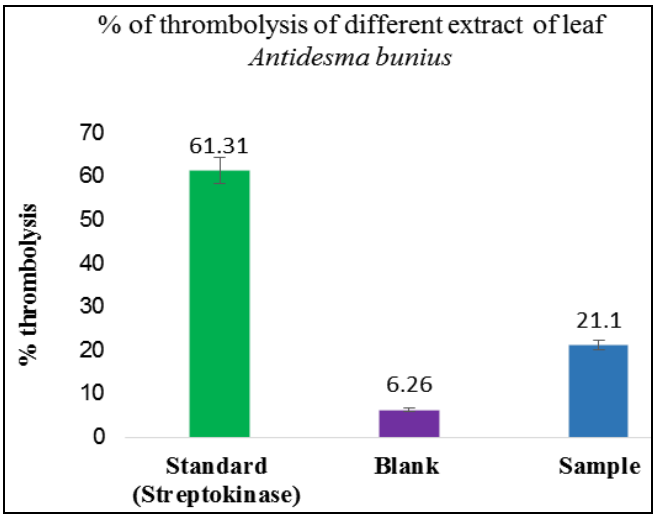

Fig 5: Thrombolytic activity of the extractives of the *A. bunius* leaves.

4. Discussion

The preliminary phytochemical screening tests may be beneficial in the detection of the bioactive components and may lead to the drug formulation and great importance in the field of drug research or drug discovery field. These tests facilitate their qualitative separation and quantitative estimation of pharmacologically active chemical compounds [32]. The results of the phytochemical screening of methanolic extract of *A. bunius* leaves has revealed the presence of tannins, flavonoids, saponins, phenols, alkaloids which could be responsible for the versatile medicinal properties or pharmacological actions of this plant part, like anti-diabetic, antioxidant, antiradical, cytotoxicity activity and pesticide agent [33-34]. The antioxidant capacity of the methanolic extract was assessed by phosphomolybdenum method. The phosphomolybdenum method was based on the reduction of Mo (V1) to Mo (V) by the antioxidant compound and the formation of green phosphate color molybdenum (v) compounds indicates the antioxidant capacity of the plant extract [35]. Many studies that total antioxidant capacity depends on the presence of flavonoid, carotenoids, vitamin E and C and polyphenols within the plant [36-37]. This study confirmed that the methanolic extract showed a good total antioxidant activity that increasing concentration (Table 2). The antioxidant activity of the plant extract based on free radical scavenging is measured by DPPH test. This test measured the hydrogen donating ability of the plant extract to reduce DPPH to DPPH-H. In the DPPH assay, the conversion of purple color of DPPH solution to yellow to different grade depending on the existence and amount of antioxidant compounds within the extract. Our present study inspected that the methanolic extract of *A. bunius* had a similar free radical scavenging activity when compared with standard BHT (Table 3). In this study, the methanolic extract of leaves of *A. bunius* exhibited thrombolytic activity 21.1% compared to standard streptokinase 61.31%.

5. Conclusion

From this study, it was concluded that the phytochemical screening of the methanolic extract of the leaves of *A. bunius* contain some bioactive components and these pharmacological properties are attributed to their phytochemical constituents. So, further studies are in progress to isolate the active constituents responsible for the observed effect.

6. Acknowledgements

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7. Ethical approval

The conducted research is not related to either human or animals use.

8. Competing interests

Authors have declared that no competing interests exist.

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