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IN-VITRO DETERMINATION OF TOTAL PHENOLIC AND FLAVONOID CONTENT, AND THROMBOLYTIC, ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES OF ABROMA AUGUSTA LNN. EXTRACT

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Abstract

The aim of the present study was to explore the existence of phytochemical constituents and presence of some potent pharmacological activity of *Abroma augusta* using standard method. Phytochemical analysis revealed the presence of phenol and flavonoid. Aqueous soluble fraction (AQSF) exhibited highest percentage (46.58%) of clot lysis activity compared to standard drug, streptokinase (69.52%). Dichloromethane soluble fraction (DCMSF) inhibited the highest diameter of clear zone against both gram positive (19.60 ± 0.12 mm) and gram negative (20.00 ± 0.20 mm) bacteria compared to standard antibiotic, Kanamycin (50.00 ± 0.19). The methyl soluble fraction (MSF) showed the lowest level of IC_{50} value (36.70 ± 0.32 µg/ml) in comparison to ascorbic acid (12.48 ± 0.09 µg/ml), while MSF disclosed the maximum level (62.19 ± 0.26 mg of GAE/gm of extract) of total phenolic content (52.09 ± 0.16 mg of QE/gm) in the extracts of *Abroma augusta (A. augusta)*.

Keywords: DPPH, total phenolic, flavonoid content, thrombolytic, antimicrobial activity.

Introduction

Bioactive compounds were ensuring plant by the presence of alkaloids, anthraquinones, glycosides, saponins, tannins and polyphenols [1]. Abroma augusta Linn f. which is synonymous to Abroma fastuosa (Family-Malvaceae) usually known as Ulat kambal (Bangla) and Devil's cotton (English). It is a shrubs and small tree with horizontal and velvety branches. Leaves are polymorphous about 10-30 cm. long and 6-18 cm. Broad and consists of different alkaloids [2]. Leaves are useful in treating uterine disorders, diabetes, and rheumatic pain of joints, heart disease and headache with sinusitis [3]. Leaves and stem are demulcent and an infusion of fresh leaves and stem in cold water is very efficacious in gonorrhea [4]. Even though previously, researchers have reported on thrombolytic, antimicrobial and antioxidant activities on leaves extract of *A. augusta,* we also work on to improve the all reported data. The antimicrobial compounds from leaves of *Abroma augusta* may inhibit bacterial propagation by different mechanisms than those presently used antimicrobials and may have a significant clinical value in treatment of resistant microbial strains [5]. Thrombosis is the mechanism by which formation of a blood clot, known as a thrombus, happens within a blood vessel. It prevents blood from flowing normally through the circulatory system therefore depriving tissues of normal blood flow and oxygen. These consequences yield necrosis of the tissue in that area leading hypertension, stroke to the heart, anoxia, and so on. Thromboembolic disorders are one of the main causes of morbidity and mortality in Bangladesh [6]. The present study focused on thrombolytic, antimicrobial and antioxidant activities of total phenolics and flavonoids content of abroma *Augusta linn.* leaves extract.

Methods

Chemicals and reagents

Streptokinase (1.5 million unit/vial, Streptase) vial was warmly gifted from Sanofi-Aventis Bangladesh Ltd. Gallic acid (GA), sodium carbonate, 2, 2-Diphenyl-1picrylhydrazyl (DPPH), kanamycin was obtained from Amico laboratories limited, Dhaka, Bangladesh. Methanol, petroleum ether, dichloromethane and chloroform were purchased from a Sigma-Aldrich authorized Bangladesh distributor (Kuri & Company (Pvt) Ltd. 78, Motijheel Commercial Area Dhaka-1000, Bangladesh).

Collection and preparation of plant powder

The whole plant of *A. augusta* was collected from Botanical Garden, Mirpur, Dhaka and identified by taxonomist of National Herbarium, Bangladesh (voucher Specimen No. 12740) and preserved in the Phytochemical Research Laboratory, World University of Bangladesh. The collected whole plant of *A. augusta* was cleaned properly to remove unwanted debris and air dried in shady place for several days. The dried plants were then milled by locally made grinder in order to get uniform coarse powder. The coarse powder (300 g) was further milled and stockpiled in a hermetic glass container till extraction.

Extraction and Fractionation of Plant Materials

The powdered material (25 g) was taken in a cleaned, amber colored reagent bottle (5 L) and soaked in 2.0 L (1:2.5 w/v) of 90% methanol at room temperature (25 ± 1° C). The dark brown color decoction was filtered every 3 days interval up to 2 weeks using filter funnel and a vacuum pump. The filtration output was desiccated applying the rotary evaporator (IKA® RV 10 Basic) at 40°C, low pressure and 125-175 rpm. The retrieved solvent was reused for next consecutive refluxes carried after 24 hours of additional soaking. The crude methanol extract (52 g) was applied to fractionation by Kupchan protocol [7]. The crude extractive was dissolved in 10% aqueous methanol and subsequently extracted with petroleum ether, dichloromethane, and finally with chloroform. The fractionated amounts were PSF= 16.5 g, DCMSF= 12.5 g and AQSF= 8 g respectively. All the organic fractions were applied to evaporation to dryness and used for further analysis.

Phytochemical Screenings

Qualitative experimentation on different Kupchan partitionates was conducted using the standard procedure developed by Sofowara [8].

Preparation of Streptokinase Solution

The commercially available lyophilized streptokinase (Sanofi-Aventis Bangladesh Ltd.) of 1,500,000 I.U per vial were used as positive control.

The lyophilized vial was collected and whole powder was dissolved in 100 ml water to get 1,500,000 I.U. of SK solution. We took 100 µl streptokinase solutions for *in vitro* thrombolysis.

Determination of Total Phenolic Content (TPC)

In the alkaline condition phenols from plant extracts ionize completely. The TPC was determined by using Folin-Ciocalteu reagent [9]. The test was executed by attributing the slight modified method developed by Velioglu [10] 02 mg crude methanolic extract and its different organic soluble fractions were liquefied with 2 ml distilled water to evoke the final concentration of 1 mg/ml. 0.5 mL of extractives (1 mg/mL) was combined with 2 mL Folin-Ciocalteu (diluted 10-fold with deionized water previously) in the test tube. The concoction was allowed to stand still for 5 minutes at room temperature (22 ± 2° C). Each mixture was then added to 2.5 mL 7.5% Na_2CO_3. The combination was gently shaken and allowed for no major jerking for 20 minutes for color development. The intensity of the color change is measured by UV-Vis spectrophotometric (Model: UV-1700 series) method at 760 nm. The absorbance value will reflect the total phenolic content of the compound. Samples of extracts and standard were appraised at a final concentration of 0.1 mg/mL. Total phenolic contents were expressed in terms of Gallic acid equivalent, GAE (Standard curve equation: R^2=0.993), mg of GA/g of dry extract.

Determination of total flavonoid content

The total flavonoid content of crude extract was determined by the aluminum chloride colorimetric method [11]. In brief, 1.5 mL of crude extract (1 mg/mL Methanol) was mixed with 0.1 mL of 10% AlCl3 and then 0.1 mL of 1 M Na-acetate was added to the reaction mixture. The mixture was allowed to stand for 6 min. Then, 1 mL of 1 mol/L NaOH solution was added, and the final volume of the mixture was brought to 5 mL with double-distilled water. The mixture was allowed to stand for 15 min, and absorbance was measured at 415 nm. The total flavonoid content was calculated from a calibration curve, and the result was expressed as mg quercetin equivalent per g dry weight. Total flavonoid content was calculated from the following calibration curve:

Y = 0.0067 X + 0.0132, R2 = 0.973

Where Y is the absorbance of crude extract and X is the quercetin equivalent.

DPPH free radical scavenging activity

The diluted working solution of the flavonoids (10 mg/ml) was prepared using the respective solvents. 10 mg/mL of Ascorbic acid used as reference for compare results of plant extracts. In 3 ml of total reaction solution, 2ml of flavonoids/standard solution (200-1000 µg/mL) and 1.0 mL of DPPH were mixed and allowed to react at 37°C for 30 min. afterward, absorbance value was measured at 520nm and converted into percent antioxidant activity [12] The percentage antioxidant activity was calculated by following formula and calculated as mean value ± SD (n = 3).

In vitro thrombolytic activity

The thrombolytic activity of crude extract and its different organic soluble extractives were evaluated by a method of Daginawala using streptokinase (SK) as standard substance [13] 10 mg of methanolic extracts and its different fractions of whole plant of A. augusta were taken in different vials to which 1 mL distilled water was added. Commercially available lyophilized Streptase (SK) vial (Sanofi-Aventis Bangladesh Ltd.) of 15, 00,000 I.U., was collected and 5 mL sterile distilled water was added and mixed properly. This suspension was used as a stock from which 100 µL (30,000 I.U) was used for the determination of in vitro thrombolytic potentials. Aliquots (5 ml) of venous blood were drawn from healthy volunteers who were distributed in ten different pre weighed sterile vials (1 mL/tube) 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 tube – weight of tube alone). To each vial containing pre-weighed clot, 100 µL aqueous solutions of different organic partitionates of A. augusta along with the crude extracts was added separately. As a positive control, 100 µL of SK and as a negative nonthrombolytic control, 100 µL 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. After incubation, the released blood serum 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:

% of clot lysis = (Weight of clot after 90 minutes /Initial weight of clot) × 100

Justification of streptokinase use

Streptokinase is an enzyme and fibrinolytic drug. As a medication, it is used to dissolve the blood clots [14].

In vitro antimicrobial activity

In this classical method, antibiotics diffuse from a confined source through the nutrient agar gel and create a concentration gradient. Dried and sterilized filter paper discs (7 mm diameter) containing the test samples of known amounts are placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic (Kanamycin) discs and blank discs are used as positive and negative control. These plates are kept at low temperature (4℃) for 24 hours to allow maximum diffusion of the test materials to the surrounding media. The plates are then inverted and incubated at 37℃ for 24 hours for optimum growth of the organisms. The test materials having antimicrobial property inhibit microbial growth in the media surrounding the discs and thereby yield a clear, distinct area defined as zone of inhibition. The antimicrobial activity of the test agent is then determined by measuring the diameter of zone of inhibition expressed in millimetre. In the present study the crude extracts as well as fractions were tested for antimicrobial activity by disc diffusion method.

Justification of kanamycin use

Kanamycin is an antibiotic used to treat severe bacterial infections and tuberculosis. Kanamycin is indicated for short term treatment of bacterial infections caused by gram positive and gram-negative bacteria respectively.

Statistical analysis

Each experiment was independently conducted 3 times. The data were analysed by one-way ANOVA (analysis of variance) followed by Dunnett's test. P-values less than 0.05 were considered as significant.

Results and Discussion

Total Phenol and flavonoid content

The probable antioxidant potentials of *A. augusta* were determined by estimation of total phenolic content of methanolic extractives, pet-ether, dichloromethane and aqueous soluble fractions. In this study, MSF showed the highest phenolic and flavonoid content (62.19 ± 0.26 mg of GAE/gm, (52.09 ± 0.16 mg of QE/gm) of extract respectively while AQSF and DCME displayed the lowest phenolic value (23.44 ± 0.21 mg of GAE/gm of extract, 22.99 ± 0.06 mg of QE/gm) among all partitionists. But other Kupchan fractionates exhibited considerable amount of phenolic and flavonoid content (Table 1).

DPPH free radical scavenging activity

The various organic soluble factions of *A. augusta* were tested for the evaluation of antioxidant capacity using DPPH. The magnitude of IC_{50} of different extractives varied from 36.70 ± 0. 32 µg/mL to 221.31 ± 0.20 µg/mL (Table 2). Among the extractives, methanol soluble fraction, MSF exhibited towering free radical scavenging activity (IC_{50} = 36.70 ± 0. 32 µg/mL) in contrast to IC_{50} = 12.48 ± 0.09 µg/mL of ascorbic acid. Thus, the antioxidant potency of a compound is relative to the loss of DPPH free radicals (DPPH scavenging) that can be quantified through a decrease in the maximum absorption of DPPH at 570 nm. A series of test tube were prepared to evaluate the inhibitory concentration of crude extract. The calibration curve was prepared using with ascorbic acid. The calibration curve (Fig. 1) was prepared using with ascorbic acid. Crude methanolic extract of *A. augusta* leaves showed IC_{50} values of 36.70 ± 0. 32 µg/mL whereas ascorbic acid showed IC_{50} values of 12.48± 0.09 µg/ mL.

Thrombolytic activity

Thrombolytic potentials using streptokinase as positive demonstrated 69.52% lysis of blood clot. On the other conditions, sterile distilled water, a

negative control, explored a negligible percentage of clot break down (4.24%). The level, expressed as percent, of clot lysis by various fractions were observed in the following order, AQSF (46.58%), PSF (40.79%), DCMSF (39.81%) and MSF (13.54%), elucidate in Table 2.

Antimicrobial activity

In-vitro antimicrobial activity of *A. augusta* was shown in (Table 3). Antimicrobic experiment was conducted on four gram positive and five-gram negative bacteria at a conc. of 400 µg/disc. DCMSF exhibited with highest zone of inhibition (19.60 ± 0.12 mm) in *Bacillus subtilis*, a gram-positive bacterium. Other organic soluble fractions such as MSF, PSF and AQSF showed moderate antimicrobial activity ranging from 11.50 ± 0.10 mm to 18.30 ± 0.11 mm respectively compared to the standard antibiotic kanamycin 20 µg/disc ranging from 44.00 ± 0.25 mm to 48.00 ± 0.06 mm of clear zone that characterized the antimicrobial potentials of standard antibiotic. On the other hand, DCMSF faction also displayed the zenithal activity against the growth of *Shigella dysenteriae* (20.00 ± 0.20 mm) which is a gram-negative bacterium. Other Kupchan partitionates of *A. augusta* barred the proliferation of bacteria in a moderate spectrum from (12.00 ± 0.41 mm to 18.15 ± 0.11 mm) subsequently in resemblance of standard antibiotic (42.00 ± 0.23 mm to 50.00 ± 0.19 mm).

Conclusion

The whole plant of *A. augusta* possess significant amount of phenolics and flavonoids content which has potent antioxidant, antimicrobial and thrombolytic activity. More comprehensive investigation should be carried out in view of isolation and characterization of specific chemical compounds responsible therapeutic effect.

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Table 1. DPPH-free radical scavenging activity, total phenolic, flavonoid content of whole plant of *A. augusta*

Sample	Total phenolic content (mg of GAE/gm of extract)	Total flavonoid content (mg of QE/gm of extract)	DPPH Free radical Scavenging activity (IC_{50} µg/ml)
MSF	62.19 ± 0.26	52.09 ± 0.16	36.70 ± 0. 32
PSF	33.44 ± 0.42	42.19 ± 0.26	123.79 ± 0.27
DCMSF	40.31± 0.19	22.99 ± 0.06	86.88 ± 0.71
AQSF	23.44 ± 0.21	32.11 ± 0.16	221.31 ± 0.20
AA (Std.)	-	-	12.48 ± 0.09

Values are means of three biological replicates.

Fig. 1. Dose-response curve of DPPH scavenging activity in the whole plant of *A. Augusta*

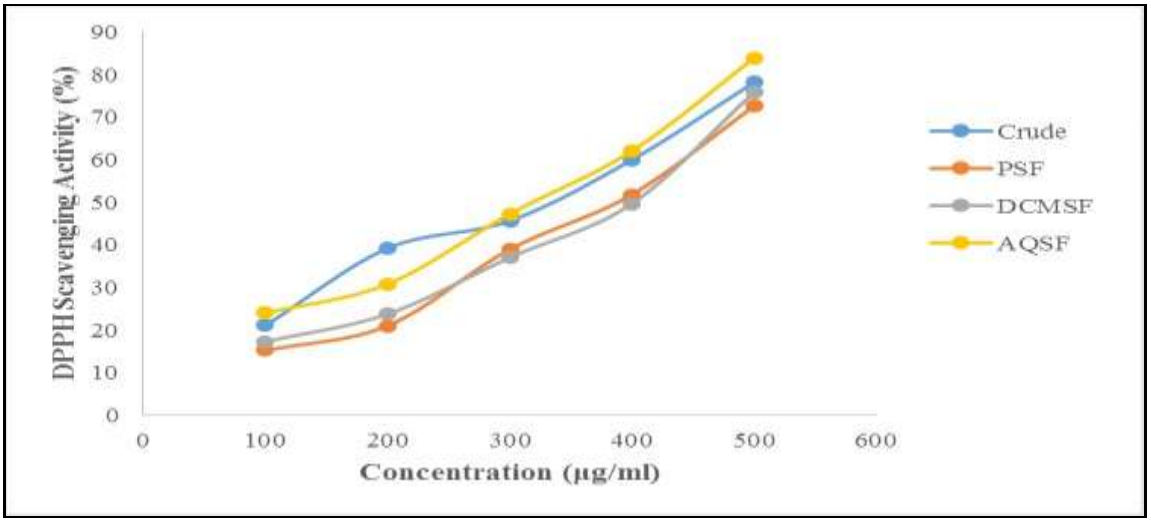

Table 2. Thrombolytic activity of crude extracts and its fractions of whole plant of *A. augusta.*

Fractions	Wt. empty vial W_1 (gm)	Wt. with W_2 vial clot (gm)	Wt. clot W_3= (W_2-W_1) (gm)	Wt. vial after clot lysis W_4 (gm)	Wt. Lysis clot W_5 = W_2.W_4 (gm)	% Clot lysis = (W_5/W_3) × 100
MSF	3.78	4.74	0.96	4.61	0.13	13.54 ± 0.63
PSF	3.81	4.57	0.76	4.26	0.31	40.79 ± 0.44*
DCMSF	3.88	4.91	1.03	4.50	0.41	39.81 ± 0.32*
AQSF	3.71	4.44	0.73	4.10	0.34	46.58 ± 0.21**
Negative control	3.50	4.68	1.18	4.63	0.05	4.24 ± 0.90
Std. (SK)	3.70	4.41	0.71	4.03	0.38	69.52 ± 0.11**

Considering > 20% moderate; **p < 0.01; *p<0.05, the aqueous soluble fraction was found to have significant (**p<0.01) thrombolytic activity at the dose of 100 µg/ml.

Table 3. Antimicrobial activities of different crude extract and fractionates of whole plant of *A. augusta.*

Test Microorganisms	Diameter of zone of inhibition (mm)				
	MSF	PSF	DCMSF	AQSF	Kanamycin
Bacillus cereus	11.50 ± 0.10	-	18.00 ± 0.10	-	44.00 ± 0.25
Bacillus subtilis	16.30 ± 0.04	-	17.40 ± 0.25	-	47.00 ± 0.18
Staphylococcus aureus	17.85 ± 0.21	-	19.60 ± 0.12	18.30 ± 0.11	48.00 ± 0.06
Sarcinalutea	14.00 ± 0.08	-	13.20 ± 0.20	12.65 ± 0.71	45.00 ± 0.31
Escherichia coli	12.00 ± 0.41	-	15.00 ± 0.15	-	46.00 ± 0.17
Salmonella typhi	13.00 ± 0.32	-	15.75 ± 0.10	-	45.00 ± 0.09
Shigella dysenteriae	18.15 ± 0.11	-	20.00 ± 0.20	13.00 ± 0.07	50.00 ± 0.19
Vibrio mimicus	12.56 ± 0.22	-	16.00 ± 0.17	-	45.00 ± 0.20
Vibrio parahaemolyticus	-	-	13.80 ± 0.25	-	42.00 ± 0.23

'-' no measurable zone; values are mean ± SEM of 3 replications.