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In vitro Antioxidant, Thrombolytic and Membrane Stabilizing Activities of the *Rourea minor* Leaves

Jannatul Ferdous[1], Mohammad Ashraful Islam[1], S. M. Neamul Kabir Zihad[1], Md. Fazlul Karim Tipu[1], Md. Alamgir kabir[1], Md. Mobinul Haque Adon[1], Jyotimoi Gosh[1] and Jakir Ahmed Chowdhury[2]

[1]Department of Pharmacy, State University of Bangladesh, Dhaka-1205, Bangladesh
[2]Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Dhaka
Dhaka-1000, Bangladesh

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Abstract

Rourea minor is a species which belongs to the family Connaraceae. *Rourea minor* reported previously for antibiotic, antibacterial, hemostatic and wound-healing activities. In this study, we evaluated the antioxidant, thrombolytic and membrane stabilizing activities of the crude methanolic extract of the leaves of *Rourea minor* and its different organic soluble fractions. We measured the total phenolic contents and 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity of the extracts to observe their antioxidant potential. The thrombolytic activity was determined by human blood clot lysis. Anti-inflammatory activity was evaluated by human red blood cell (HRBC) membrane stabilization based on hypotonic solution- and heat-induced haemolysis. The total phenolic content ranged from 9.4 to 57.4 mg GAE/g of the dry extracts and IC_{50} values of the different extracts (Crude methanol, petroleum ether, ethyl acetate and aqueous) were between 20.38 and 269.34 µg/ml. All the extracts showed mild to moderate thrombolytic potential inhibiting the lysis of clot by 3.47% - 18.43%. The extracts also exhibited potential membrane stabilizing activity in both hypotonic solution- and heat-induced haemolysis indicating their anti-inflammatory activity. Thus, it can be concluded that the methanolic extract from the leaves of *Rourea minor* and its different fractions possess mild to moderate antioxidant, thrombolytic activities, and membrane stabilizing potential.

Key words: *Rourea minor*, antioxident, phenolic content, DPPH assay, thrombolytic activity, membrane stabilizing.

Introduction

According to the world health organization, the vast majority of people worldwide rely mostly on traditional medicines for basic healthcare (Hostettmann and Marston, 2002). Previously people were compelled to employ any natural material for the purpose of reducing pain and suffering brought on by illness, physical discomfort and wounds (Sase et al., 2020). This was done in order to preserve health against illness and death. For their own survival, early man began to differentiate between nutrient rich and pharmacologically active plants. These plant based systems have supported clay tablets, parchments, manuscripts, pharmacopoeias, and other works for thousands of years worldwide. Despite the fact that the use of plant derived medicines has significantly decreased since the development of synthetic pharmaceuticals, many of the ingredients used in modern medicine still come from plants (Ahvazi et al., 2012). Scientists worked to isolate various chemical constituents by bioactivity guided isolation and thus were able to identify active compounds, which have been used therapeutically to develop modern medicines.

Rourea minor is a vigorous climbing shrub. There are nine leaves on it, and it is 25 cm long. Its

Corresponding author: Mohammad Ashraful Islam; Email: ashrafulrakib48@gmail.com; Phone: +8801680005854
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genus is Rourea and it is a member of the connaraceae family. It includes 129 varieties and 65 species. It is abundantly available in the Amazon region. Some of these species are found in both Africa and Asia. This species includes some poisonous members. They engage in a limited amount of biological activities (Mai et al., 2022).

Studies on few species of the genus *Rourea* has revealed that isolated comounds and extract derived from this genus exhibited antioxidant, antibacterial, anti-inflammatory, anti-hepatoprotective, and anti-pyretic properties (Osman *et al*., 2019). Chloroform-soluble fraction of methanolic extract of steams of *R. minor* gave glycerol, 1-1-(26-hydroxyhexacosanoyl)-glycerol, 1-O-ß-D-glucopyranosyl--2-N-(20-hydroxypalmitoyl), octadecasphinga-4,8-dienine, rourimin, and 9S, 12S, 13S-trihydroxy-10E-octadecenoic acid . Two novel chemicals, lethedocin 3′-O-β-D-glucopyranoside and 3-O- (6′–O-vanilloyl)-β-D-glucopyranosyl 4-hydroxyphenethyl alcohol as well as phenolic compounds, alkaloids, and fatty acids were discovered in *Rourea minor* tree's chemical composition. Traditionally this plant is used as a tonic for women after childbirth and to treat boils, crimson urine, and urinary incontinence.. In Chinese folk medicine, the leaves of *R. minor's* steams and roots are poisonous and they are frequently used as tying material (He et al., 2006).

Materials and Methods

Collection of plant material and preparation of extracts: The leaves of *Rourea minor* were collected from Rangamati, Bangladesh. The leaves were shed-dried and ground to obtain fine coarse powder. The powdered material was soaked in methanol for 20 days with occasional shaking. Afterward the mixture was filtered through whatman filter paper and the filtrate was then allowed to evaporate until approximately 70% solvent was evaporated. Then partitioning was done by method developed by kupchan and modified by Van Wagenen dissolving the crude extract in 10% aqueous methanol and successive partitioning with petroleum ether and ethyl acetate. The aqueous methanolic fraction was preserved as aqueous fraction. A rotary evaporator was used to dry-out the solvents to obtain dry extracts.

Determination of total phenolic content: The total phenolic contents of the crude methanolic extract of *R. minor* leaves and its different fractions were determined by the Folin-ciocalteu method (Sheikh et al., 2016). At first, 0.5 ml of the extract solutions (2 mg/ml) was mixed with 2.5 ml of 10 times diluted Folin-Ciocalteu reagent and 2 ml of 7.5% Na_2CO_3, and the reaction mixture was incubated at room temperature for 20 min. Afterwards, the absorbance of the reaction mixture was measured at 760 nm using a UV-visible spectrophotometer. The results were calculated from a standard curve constructed with varying concentrations of gallic acid and expressed as mg GAE (gallic acid equivalent)/gm of the extracts.

Determination of DPPH assay: The free radical scavenging capacity of the crude methanolic extract of *R. minor* leaves and its different fractions was determined using 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay according to Brand-Williams et al., 1995. In brief, 3.0 ml of the methanolic solution of DPPH at a concentration of 20 g/ml was mixed with 2.0 ml of the sample (extracts and BHT/standard) solution dissolved in methanol at concentrations ranging from 0.977g/ml to 500g/ml. Then the mixture was left for 30 min at room temperature in a dark place to allow the reactions to be completed and then the absorbance of the solution was measured at 517 nm was measured by UV-visible spectrophotometer against methanol as the blank. The percent inhibition of the DPPH free radical by the samples was determined by the following equation:

$$\%I = (1 - A_{sample}/A_{blank}) \times 100\%$$

Where, A_{sample} is the absorbance of the reaction mixture containing the sample and A_{blank} is the absorbance of the control reaction (containing all reagents except the test material).

Thrombolytic activity: To evaluate the thrombolytic activity of the test extracts, 1 ml fresh blood drawn from healthy volunteers was taken in a

pre-weighed sterile micro centrifuge tube and incubated at 37 °C for 45 min to allow clot formation. Afterwards, the serum was removed from the micro centrifuge tube without rapturing the clot and again weighed to determine the weight of the clot. Then, 100 µl aqueous solution of the sample (crude extracts, different partitions, the standard streptokinase and distilled water as negative control) was added to the micro centrifuge tube. The tube was then incubated again at 37 °C for 90 minutes and following that the released fluid was removed from the tube and weighed again to observe weight difference as the indicator of clot lysis. The results were expressed as percentage of clot lysis calculated from the equation shown below (Sikder et al., 2011):

% of clot lysis = (weight of released clot/weight of clot) × 100%

Membrane stabilizing test

Hypotonic solution-induced haemolysis: The experiments were carried out with hypotonic solution. The test sample consisted of stock erythrocyte (RBC) suspension (0.50 ml) with 5 ml of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffer saline (pH 7.4) containing either the different methanolic extract and its partitionates (1.0 mg/ml) or acetyl salicylic acid (0.10 mg/ml) as a reference standard. The percentage inhibition of either haemolysis or membrane stabilization was calculated using the following equation:

$$\%\ inhibition\ of\ hemolysis = (OD_1 - OD_2)/\ OD_1 \times 100\%$$

Where, OD_1 = Optical density of hypotonic-buffered saline solution alone (control)

OD_2 = Optical density of test sample in hypotonic solution.

Heat induced haemolysis: Heat induced hemolysis was carried out using the method illustrated by Shinde *et al.*, 1999. The percentage inhibition or, acceleration of hemolysis in tests was calculated according to the following equation:

$$\%\ inhibition\ of\ hemolysis = (OD_2 - OD_1)/\ (OD_3 - OD_1) \times 100\%$$

Where, OD_1 = test sample unheated, OD_2 = test sample heated and OD_3 = control sample heated.

Results and Discussion

Antioxidant activity

Determination of total phenolic content: Foline ciocalteu reagent can oxidized phenol and its use in phenolic solution. It is yellow colour but after oxidation process it will turn into blue colour. The intensity of colour change is measured by spectrophotometer at 760 nm. The absorbance value will detect the total phenolic content of the compound (Harbertson and Spayd, 2006).

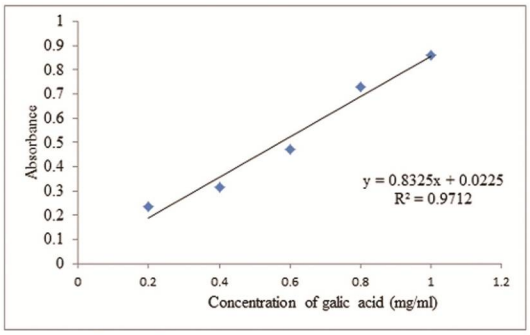

Figure 1. Standard curve of gallic acid for total phenolic determination.

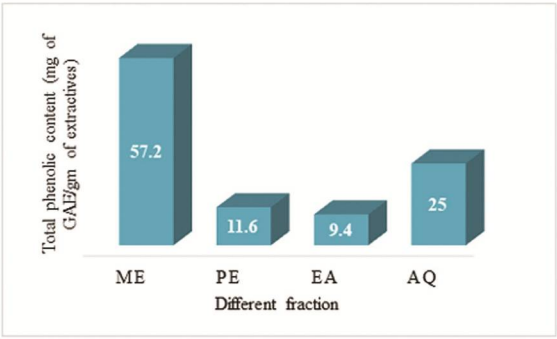

Figure 2. Total phenolic content (mg of GAE / gm of extractives) of different.

Determination of DPPH assay: Different partitionates of the methanolic extract of leaves of *R. minor* i.e. petroleum ether (PE), ethyl acetate (EA) and aqueous (AQ) soluble fractions were subjected to free radical scavenging activity by the method of Brand Willium et al., 1995. Here, *tert*-butyl-1-

hydroxytoluene (BHT) was used as reference standard. In this investigation, methanol extract showed the highest free radical scavenging activity with IC_{50} value of 20.38 µg/ml. The other partitionates exhibited moderate scavenging activity.

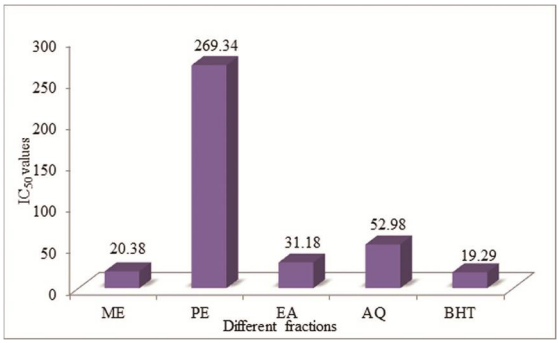

Figure 3. IC_{50} Value of different extract of leaves of *R. minor* .

Thrombolytic activity: Vascular blockage is formed due to blood clot in the circulatory system causes hemostasis. It creates atherothrombotic disease such as acute myocardial or cerebral infection. Alteplase, anistreplase, streptokinase, urokinase and tissue plasminogen activator (TPA) have been known to dissolve clot (Collen, 1990). Streptokinase (SK) was used as standard to determine the thrombolytic activity. 100 µl (3000 IU) SK was added as a positive control to the clots and incubate for 90 minutes at 37°C which showed 13.69% lysis of clot. Moreover, distilled water was used as a negative control which exhibited 3.25% lysis of clot Pet ether soluble fraction (PE) of methanolic extract of *R. minor* exhibited the highest thrombolytic activity (18.43%). The other fractions showed mild to moderate thrombolytic activity.

Table 1. Thrombolytic activity in term of % of clot lysis of different fraction of leaves extract of *R. minor*.

Thrombolytic activity of the leaves of *R. minor*						
Fractions	W1	W2	W3	W4=W2-W1	W5=W2-W3	% of lysis
ME	5133.7	5344.0	5336.7	210.3	7.3	3.47%
PE	4885.5	5209.4	5149.7	323.9	59.7	18.43%
EA	4865.2	5145.4	5094.5	280.2	50.9	18.16%
AQ	4711.0	4911.0	4892.2	200.0	18.8	9.4%
SK	5401.6	5762.4	5713.0	360.8	49.4	13.69%
Blank	4734.9	4941.6	4934.2	206.7	7.4	3.25%

Membrane stabilizing test

Hypotonic solution-induced haemolysis: At a concentration of 1.0 mg/ml and in hypotonic solution induced condition, PE and EA fractions of methanolic extract of *R. minor* leaves showed highest percentage of hemolysis of RBC which were 36.94% and 6.89%, respectively, whereas 65.13% was exhibited by standard acetyl salicylate acid (0.10mg/mL). Other extract of leaves also showed significant inhibition of hemolysis of RBCs.

Heat induced haemolysis: Crude methanol extract (ME) and the aqueous soluble fraction (AQ) of leaves of *R. minor* showed maximum inhibition to heat-induced hemolysis of RBC by 99.46 %, and 99.22%, respectively as compared to 96.31% inhibition caused by the standard acetyl salicylic acid (0.10 mg/ml).

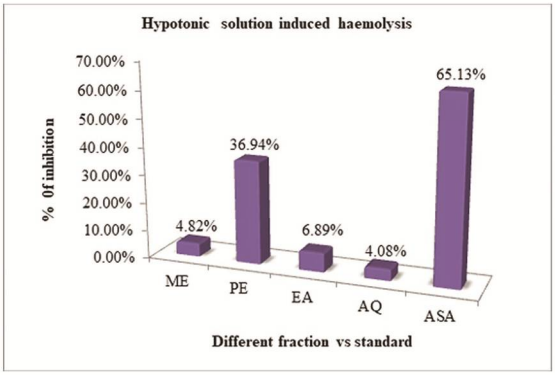

Figure 5. Percent inhibition of hemolysis of different extractives of leaves of *R. minor* on hypotonic solution-induced hemolysis.

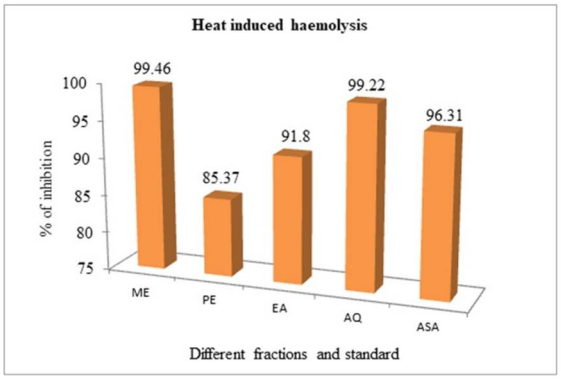

Figure 6. Percent inhibition of haemolysis of different extracts of leaves of *R. minor* on heat induced hemolysis.

Conclusion

The present study disclosed that the leaves extract of *R. minor* exhibited significant antioxidant, thrombolytic activity and membrane stabilizing effect. Further investigations are required to find bioactive compounds responsible for the observed antioxidant, thrombolytic activity and membrane stabilizing effect of the leaves of *R. minor.*

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