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Original Article

Antioxidant, thrombolytic and membrane stabilizing activities of *Mussaenda roxburghii* Hook. f.

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Abstract

There is growing interest in exploiting plants for medicinal purposes especially in Asia. Herbal medicine, which uses medicinal plants primarily present as an alternative to such situation. In the present study, the crude methanol extract of leaf of *Mussaenda roxburghii* Hook. f. along with its all Kupchan fractions were investigated for antioxidant, membrane stabilizing and thrombolytic activity. The antioxidant activity was evaluated by using free radical scavenging (DPPH) assay. Here, butylated hydroxytoluene (BHT) was used as standard antioxidant. The total phenolic content was also determined and expressed in gallic acid equivalent. A positive correlation was seen between total phenolic content and free radical scavenging activity of *M. roxburghii* having correlation coefficient (R^2) of 0.58. During assay for thrombolytic activity, the petroleum-ether soluble fraction revealed 37.7% lysis of clot while standard streptokinase (SK) used as positive control, demonstrated 66.8% lysis of clot. The membrane stabilizing activity was assessed by hypotonic solution and heat-induced methods and was compared with standard acetylsalicylic acid.

Keywords: *Mussaenda roxburghii,* extracts, antioxidant, thrombolytic, membrane stabilizing

1. Introduction

Medicinal plants have immensely contributed to the development of human health & welfare. Plants produce a diverse range of bioactive molecules, making them a rich source of different types of medicines [1]. Plants are used in modern medicine where they occupy a very significant place as raw material for important drugs [2]. Plants specifically herbal medicines have received much attention as source of new antibacterial drugs since they are considered as time-tested and comparatively safe both for human use and for environment [3]. Plant based natural constituents can be derived from any part of plant's bark, leaves , flowers, roots, fruits, seeds etc that is any part of the plant may contain active components. Antioxidant based drugs or formulations for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease and cancer have appeared during the last three decades. This has attracted a great deal of research interest in natural antioxidants.

Mussaenda roxburghii Hook. f. (family-Rubiaceae) is a perennial shrub grows in the foothills and moist areas of valley. The paste obtained from leaf of this plant is applied to treat boils [4]. Roots are used in treatment of jaundice [5], skin diseases, cuts, wounds and boils etc [6]. Leaves are used in the ailments of bone fracture [7]. Previous phytochemical investigation led to isolation of a new iridoid, shanzhiol which showed mild antibacterial activity against both *Staphylococcus aureus* and *Escherichia coli* [8].

As part of our ongoing research with medicinal plant of Bangladesh [9, 10] the present study has been undertaken to evaluate the antioxidant, membrane stabilizing and thrombolytic activities of *M. roxburghii* as well as to find out logical evidence for its folk uses and for discovery of new drug candidates.

2. Materials and Methods

Plant material:

The leaves were collected from Rangamati in November, 2011. A voucher specimen for this collection has been deposited in Bangladesh National Herbarium, Mirpur, and Dhaka-1216. The samples were then cut into small pieces and sun dried for 7 days followed by oven drying for 24 hours at 40 ^{o}C to facilitate proper grinding.

Reagents and chemicals:

All chemicals *i.e.* methanol, petroleum-ether (b.p. 60-80 °C), carbon tetrachloride, chloroform and other reagents used in these experiments were of the highest analytical grade.

Extraction and Isolation:

The powdered material (550 g) was soaked in 1.5 L of methanol in a large conical flask for 7 days with occasional shaking and stirring. The whole mixture was then filtered off through a cotton plug followed by Whatman filter paper no.1 and the filtrate thus obtained was concentrated by evaporation at room temperature. A portion (5.0 g) of the concentrated methanol extract was fractionated by the modified Kupchan partitioning protocol [11] which afforded petroleum-ether (520 mg), carbon tetrachloride (850 mg), chloroform (410 mg) and aqueous (2.1 g) soluble materials.

Total phenolics analysis:

The total phenolic content of *M. roxburghii* was measured by employing the method [12] involving Folin-Ciocalteu reagent as an oxidizing agent and gallic acid as standard. To 0.5 ml of extract solution (2.0 mg/ml) in water, 2.5 ml of Folin-Ciocalteu reagent (diluted 10 times with water) and 2.0 ml of sodium carbonate (7.5% w/v) solution were added. After 20 min of incubation at room temperature, the absorbance was measured at 760 nm using a UV-visible spectrophotometer. Total phenolics were quantified with the help of

calibration curve obtained from gallic acid (0-100 µg/ml). The phenolic content of the sample was expressed as mg of GAE (gallic acid equivalent/gm of the dried extract).

Free radical scavenging activity:

The free radical scavenging activity (antioxidant capacity) of the plant extractives on the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was estimated by the method established by Brand-Williams *et al.*, 1995 [13]. Here, 2.0 ml of a methanol solution of the sample (extractive/standard) at different concentration (500 µg/ml to 0.977 µg/ml) were mixed with 3.0 ml of a DPPH methanol solution (20 µg/ml). After 30 min of reaction at room temperature in dark place, the absorbance was measured at 517 nm against methanol as blank by UV spectrophotometer. Inhibition of free radical DPPH in percent (I%) was calculated as follows:

$$(I\%) = (1 - A_{sample}/A_{blank}) \times 100 \text{ --(Equation no. 1)}$$

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

Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted with inhibition percentage against extractive/standard concentration.

Thrombolytic activity:

The thrombolytic activity of all extractives of *M. roxburghii* was evaluated using streptokinase as standard [14]. The dry crude extract (100 mg) was suspended in 10 ml of distilled water and it was kept overnight. Then the soluble supernatant was decanted and filtered. Aliquots (5 ml) of venous blood were drawn from healthy volunteers who were distributed in five different pre-weighed sterile micro-centrifuge tube (1 ml/tube) and incubated at 37°C for 45 min. After clot formation, the serum was completely removed without disturbing the clot and each tube having clot was again weighed to determine the clot weight (Clot weight = weight of clot containing tube – weight of tube alone).

To each micro-centrifuge tube containing pre-weighed clot, 100 µl aqueous solutions of different partitionates along with the crude extract was added separately. As a positive control, 100 µl of streptokinase and as a negative non thrombolytic control, 100 µl of distilled water were separately added to the control tubes. All the tubes were then incubated at 37°C for 90 min and observed for clot lysis. After incubation, the released of fluid was removed and tubes were again weighed to observe the difference in weight after clot disruption. The differences in weights taken before and after clot lysis were expressed as percentage of clot lysis as shown below:

% of clot lysis = (wt of clot after lysis/clot wt) × 100
---------- (Equation no. 2)

Membrane stabilizing activity:

The erythrocyte membrane resembles to lysosomal membrane and as such, the effect of drugs on the stabilization of erythrocyte could be extrapolated to the stabilization of lysosomal membrane [15]. The membrane stabilizing activity of the extractives was assessed by using hypotonic solution-induced and heat-induced mice erythrocyte haemolysis [16]. To prepare the erythrocyte suspension, whole blood was obtained using syringes (containing anticoagulant EDTA) from mice through cardiac puncture. The blood was centrifuged and blood cells were washed three times with 154 mM NaCl in 10 mM sodium phosphate buffer (pH 7.4) through centrifugation for 10 min at 3000 g.

Hypotonic solution-induced haemolysis:

The test sample comprised of stock erythrocyte (RBC) suspension (0.50 ml) mixed with 5 ml of hypotonic solution (50 mM NaCl) in

10 mM sodium phosphate buffered saline (pH 7.4) containing either the extract (1. 0 mg/ml) or acetyl salicylic acid (ASA, 0.1 mg/ml). The control sample consisted of 0.5 ml of RBCs mixed with hypotonic buffered saline alone. The mixture was incubated for 10 min at room temperature, centrifuged for 10 min at 3000 *g* and the absorbance of the supernatant was measured at 540 nm. The percentage inhibition of either haemolysis or membrane stabilization was calculated using the following equation-

% Inhibition of haemolysis = 100 x (OD_1-OD_2/OD_1), ----------(Equation no. 3)

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:

Isotonic buffer containing aliquots (5 ml) of different extractives were put into two duplicate sets of centrifuge tubes. The vehicle, in the same amount, was added to another tube as control. Erythrocyte suspension (30 µl) was added to each tube and mixed by gentle inversion. A pair of the tubes was incubated at 54 ^{o}C for 20 min in a water bath. The other pair was maintained at 0-5 ^{o}C in an ice bath. The reaction mixture was centrifuged for 3 min at 1300 g and the absorbance of the supernatant was measured at 540 nm. The percentage inhibition or acceleration of haemolysis in tests was calculated according to the equation:

% Inhibition of hemolysis = 100 x [1- (OD_2-OD_1/ OD_3-OD_1)] ----------Equation no. 4)

Where, OD_1 = optical density of unheated test sample, OD_2 = optical density of heated test sample and OD_3 = optical density of heated control sample

3. Results and Discussion

The present study was undertaken to evaluate the total phenolic content, free radical scavenging activity, thrombolytic and membrane stabilization activities of the organic soluble materials of a methanol extract of *M. roxburghii* and the results have been summarized in Table 1-2.

The amount of total phenolic content differs in different extractives and ranged from 36.67 to 85.99 mg of GAE/gm of extractives of *M. roxburghii*. Among all extractives, the highest phenolic content was found in crude methanol extract (85.99 mg of GAE/gm of extractives). The aqueous and chloroform soluble fraction have significant amount of phenolic compounds.

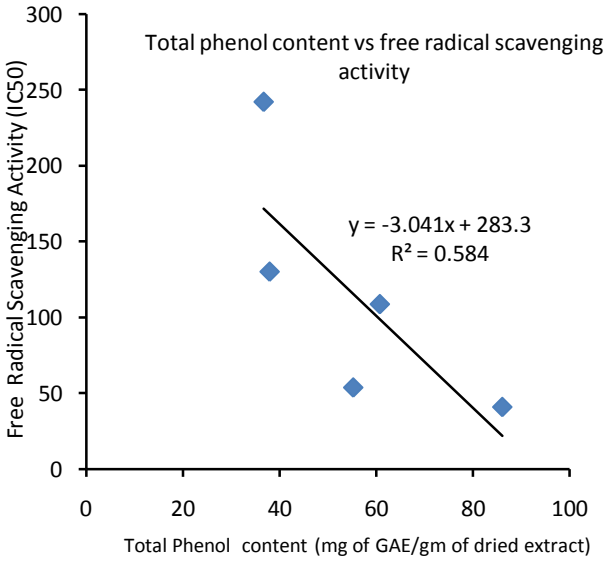

Figure 1: Correlation between the total phenolic content and free radical scavenging activity

The crude methanol extract along with its different Kupchan partitionates *i.e.* petroleum-ether, carbon tetrachloride, chloroform and aqueous soluble partitionates were subjected to free radical scavenging activity by the method of Brand-Williams *et al.,* 1995. Here, tert-butyl-1-hydroxytoluene (BHT) was used as reference standard. In this investigation, crude methanol

extract and its chloroform soluble fraction showed the strong free radical scavenging activity with IC_{50} value 41.0 and 53.84 µg/ml, respectively as compared to IC_{50} value 27.50 µg/ml produced by standard BHT.

Sample	Total Phenolic Content (mg of GAE/gm of dried extract)	Free Radical Scavenging activity (IC_{50} µg/ml)	Thrombolytic activity (% Clot lysis)
BHT	ND	27.50	ND
SK	ND	ND	66.8±1.16
CME	85.99±1.14	41.0±0.78	16.3±1.11
PESF	37.90±0.96	130.19±1.19	37.7±0.82
CTSF	36.67±1.18	242.12±0.67	12.34±1.33
CSF	55.18±1.21	53.84±0.64	20.61±0.66
AQSF	60.68±0.83	108.79±0.97	18.24±0.45

Table 1: Total phenolic content, free radical scavenging activity (IC_{50} µg/ml) and thrombolytic activity (% Clot lysis) of different Kupchan fractions of *M. roxburghii.*

Here, ND=Not determined; CME=Crude methanol extract; PESF=Petroleum-ether soluble fraction; CTSF=Carbon tetrachloride soluble fraction; CSF=Chloroform soluble fraction; AQSF=Aqueous soluble fraction of the methanolic extract of *M. roxburghii*.

The correlation analysis revealed that correlation exists between total phenolic content and free radical scavenging activity. The correlation coefficient (R^2) for the total phenolics and free radical scavenging (Figure 1) was 0.58 indicating a positive relationship between the total phenolics and free radical scavenging activity. This result suggests that 58% of the free radical scavenging activity resulted from the contribution of the phenolic compounds [17]. Different secondary metabolites, such as volatile oils, carotenoids and vitamins may also contribute to the antioxidant capacity, which in this case contributed to approximately 20% of the antioxidant activity [18]. It is well known that phenolic compounds are potential antioxidants and free radical-scavengers; hence, there should be a close correlation between the content of phenolic compounds and antioxidant activity [19].

In order to identify the drugs with the ability to promote lysis of blood clot from natural sources, the extractives of *M. roxburghii* were assessed for thrombolytic activity. Addition of 100 µl streptokinase, a positive control (30,000 I.U.) to the clots of human blood and subsequent incubation for 90 minutes at 37 °C, showed 66.8% lysis of clot. On the other hand, distilled water when treated as negative control, showed negligible lysis of clot (3.62%). The mean difference in percentage of clot lysis between positive and negative control was found to be statistically significant. In the study of thrombolytic activity, the petroleum-ether soluble fraction of methanol extract exhibited highest thrombolytic activity (37.70%).

Sample	Concentration (mg/ml)	Haemolysis inhibition(%)	
		Hypotonic solution induced	Heat induced
Hypotonic medium	50 mM	- -	- -
CME	2.0 mg/ml	54.38	31.62
PESF	2.0 mg/ml	40.22	25.40
CTSF	2.0 mg/ml	21.42	16.19
CSF	2.0 mg/ml	41.34	27.56
AQSF	2.0 mg/ml	12.03	9.68
Acetyl salicylic acid	0.1 mg/ml	71.9	42.12

Table 2: Effect of extractives of leaf of *M. roxburghii* on hypotonic solution and heat-induced haemolysis of erythrocyte membrane.

The methanol extract of *M. roxburghii* and its different partitionates at concentration 1.0 mg/ml were tested to know the activity against lysis of human erythrocyte membrane induced by hypotonic solution as well as heat, as

compared to the standard acetyl salicylic acid (0.10 mg/ml) (Table 2). At 1.0 mg/ml in hypotonic solution induced condition the methanol extract inhibited 54.38% while chloroform and petroleum-ether soluble fraction inhibited 41.34% and 40.22% haemolysis of RBC as compared to 71.9% revealed by acetyl salicylic acid (0.10 mg/ml), respectively. The aqueous and carbon tetrachloride soluble fraction showed mild to moderate inhibition of haemolysis of RBCs. During heat-induced condition the crude methanol extract demonstrated highest 31.62% haemolysis of RBC. The erythrocyte membrane resembles to lysosomal membrane and as such, the effect of drugs on the stabilization of erythrocyte could be extrapolated to the stabilization of lysosomal membrane. Therefore, as membrane stabilizes that interfere in the release and or action of mediators like histamine, serotonin, prostaglandins, leukotrienes, etc [15, 16].

Conclusions

From the above results, it can be concluded that the medicinal plant, *Mussaenda roxburghii* Hook. f. showed moderate antioxidant, membrane stabilizing and thrombolytic activity suggesting the presence of bioactive materials having good biological properties. These could be of particular interest in relation to find out its unexplored efficacy and can be a potential source of chemically interesting and biologically important drug candidates.

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