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Farhina Rahman Laboni
Department of Pharmacy,
World University of Bangladesh,
Dhaka-1205, Bangladesh.

Faria Afsari
Department of Pharmacy,
World University of Bangladesh,
Dhaka-1205, Bangladesh.

Md. Sariful Islam Howlader
Department of Pharmacy,
World University of Bangladesh,
Dhaka-1205, Bangladesh.

Zubair Khalid Labu
Department of Pharmacy,
World University of Bangladesh,
Dhaka-1205, Bangladesh.

Azima Sultana Julie
Department of Pharmacy,
World University of Bangladesh,
Dhaka-1205, Bangladesh.

Correspondence:
Farhina Rahman Laboni
farhinlaboni@gmail.com
Department of Pharmacy,
World University of Bangladesh,
Dhaka-1205, Bangladesh.

Thrombolytic and membrane stabilizing activities of ethanolic extract of local medicinal plant *Murraya paniculata*. (Family: Rutaceae)

Farhina Rahman Laboni, Faria Afsari, Md. Sariful Islam Howlader, Zubair Khalid Labu, Azima Sultana Julie

Abstract
Murraya paniculata (*M. paniculata*), has a history of ethnomedicinal properties. The crude ethanolic extract and its different partitionates of the leaves of *M. paniculata* was evaluated for its possible thrombolytic and membrane stabilizing activity. The activities of leaf extract were evaluated by using different drugs; Streptokinase (SK) was used as standard drug for thrombolytic activity, and acetyl salicylic acid (ASA) as standard drug for membrane stabilizing activity. In this study, standard drug SK showed 65% against thrombosis, the ethanolic soluble fraction of leaves (ESF) 43.28%, n-Hexane soluble fraction (HXSF) 58.06%, whereas chloroform soluble fraction (CSF) 27.66% and aqueous soluble fraction (AQSF) 22.81%, and for against unstable membrane, standard drug ASA exhibited 71.90±29% inhibition of haemolysis of human red blood cell (RBC) membrane in normal conditions, whereas 62.12±0.26% in heat induced conditions. In normal conditions the AQSF inhibited 33.49±0.51%, ESF inhibited 40.23±0.64%, CSF inhibited 42.66±0.77% and HXSF 60.35±0.50% while in heat induced conditions AQSF inhibited 30.34± 0.42%, ESF inhibited 41.53±0.61%, CSF inhibited 36.22±0.57%, HXSF inhibited 44.11±0.69% respectively. Hence, the results of the present study revealed that the traditional uses of this plant leaves is a remedy for thrombosis and unstable lysosomal membrane.

Keywords: *Murraya paniculata*, streptokinase, acetyl salicylic acid, thrombolytic activity, membrane stabilizing activity.

1. Introduction
Medicinal plants are one of the important contributors to the most of the medicinal preparations as raw plant materials, refined crude extracts and mixtures etc. Several thousands of plants have been identified containing medicinal values and are used to treat different ailments in various cultures worldwide (Farnsworth NR, Soejarto, 1991) [1]. Even in this modern world, majority of the people are still relying on the traditional medicine for their primary health care. (Anthony S. Fauci, 1998) [2]. *Murraya paniculata* (Family: Rutaceae), a well-known medicinal plant commonly known as *Kamini* in Bengali, is found everywhere in Bangladesh (Perry L.M., 1980) [3], grown by the farmers on the plain land, roadside and lower regions, from about a 1000 meters above sea level. It grows even as a mixed crop in the rubber and the tea gardens. It is distributed in Indonesia, Malaya, Southeast Asia, India and Pakistan (Gani A, 2003) [4]. The plant of *M.paniculata* has been most frequently used as traditional and ayurvedic medicines; for cough, bronchitis, asthma, inflammation, hemorrhage, hemorrhoids, eye diseases, diarrhea, leprosy, blood disorders, heart troubles, thirst, fever, vomiting, loss of memory, leucoderma, jaundice, tumors, mouth troubles and gonorrhea in local traditional procedures (Silva LB., 1980) [5]. It is found that 70% of the pregnant women in many countries use the leaves of *M. paniculata* to induce labor (But.P.P, *et al.* 1986) [6]. The macerated roots of the *M.paniculata* are applied on the pubic region and the vagina to help parturition (Kirtikar and Basu, 1975) [7]. Pushpangadan *et al.* (1995) stated the use of the whole plant of *M.paniculata* for treating impotence, sexual disorders, whereas its leaves are used for checking postpartum haemorrhage. In Bangladesh, traditionally the decoction of the *Murraya* leaves are used as a remedy for cough and other symptoms of cold, the soothing and expectorant action of this plant which is used to soothe irritation in the throat and loosen phlegm, deposited in the air-passage, is common in the village of Nagorpur, Tangail. A poultice of the leaves of *M.paniculata* may be applied to wounds for their antibacterial and anti-inflammatory properties. It also is also helpful in relieving rheumatic symptoms when applied to joints.

The plant has been used to control both internal and external bleeding such as peptic ulcers, piles and bleeding gums. It exhibits antispasmodic and blood purifying qualities (Salawu SO, 2011) [8]. The crude ethanolic extract of its leaves has antidiarrhoeal, antinociceptive and anti-inflammatory activities (Jack, Rutaceae, 2010&Rahman M.A., 2010) [9-10]. But not much evidence is found regarding the investigation of thrombolytic and membrane stabilizing activities of *M.paniculata* plant in Bangladesh. Therefore, this investigation was conducted to find out these lacking.

2. Materials and Methods

2.1 Plant materials

For this present investigation the plant *M. paniculata* was collected from, Dhaka, Bangladesh in August 2014 and was identified by Bangladesh National Herbarium, Mirpur, Dhaka

2.2. Preparation of extract

The collected plant leaves were shade dried for several days and then oven dried for 24 hours at 40^0c to facilitate grinding and stored in a tight container. The dried powder material (400gm) was soaked in 1000ml of 90% ethanol for two weeks. The whole mixture was then filter by a piece of clean, white cotton materials. Then the filtrate was filtered again through Whatman filter paper, total filtrate was concentrated using a rotatory evaporator to get the crude extract of *M. paniculata.* The concentrated aqueous ethanol extract was partitioned by the Kupchan method (Van Wagenen *et al.*, 1993) and the resultant partitionates, i.e. Chloroform, hexane and aqueous soluble materials were used for our current investigation.

2.3 *In vitro* thrombolytic activity study

2.3.1 Sample preparation

The crude extract was suspended in 10ml of distilled water and shaken vigorously on a sonicator, then the suspension was kept overnight and decanted to remove soluble supernatant, which was filtered through a filter paper. The solution was then ready for *in vitro* evaluation of clot lysis activity**.**

2.3.2 Thrombolytic assay

The thrombolytic activity of all extractives was evaluated by the method developed by Daginawala, H.F. 2006) [11], using streptokinase (SK) as standard substances**.** Whole blood (5 ml) were drawn from healthy volunteers, and transferred in different pre-weighed sterile tubes (1 ml/tube) to form clots and incubated at 37 °C for 45 minutes. After clot formation, the serum was completely removed without disturbing the clot formed and each tube having Clot, was again weighed to determine the clot weight (clot weight = weight of clot containing tube – weight of tube alone) (Kawasaki T., 2002) [12]. The tubes containing pre-weighed clot was properly labelled, 100 µl crude extract and aqueous solutions of different fractionates were added to the tubes separately. As a positive control, 100 µl of SK and as a negative non thrombolytic control, 100 µl of isotonic solution was separately added to the clot containing tubes. All the tubes were then incubated at 37 °C for 90 minutes and observed for clot lysis. After incubation, the released fluid was removed and vials were again weighed to observe the difference in weight after clot disruption. Differences obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis as shown below:

% of clot lysis = (wt. of released clot /clot wt.) × 100 [13-14].

2.3.3 *In vitro* membrane stabilizing activity study

The membrane stabilizing activity of the extractives was determined with human erythrocytes by following the method developed by (Omale *et al*. (2008) [15].

The erythrocyte membrane resembles to lysosomal membrane and as such, the effect of extracts on the stabilization of erythrocyte could be extrapolated to the stabilization of lysosomal membrane. The membrane stabilizing activity of the extracts was assessed by using hypotonic solution-induced and heat-induced erythrocyte haemolysis. To prepare the erythrocyte suspension, 5 ml of whole blood was withdrawn from healthy human volunteer by hypodermal syringes (containing anticoagulant 3.1% Na-citrate). The blood was centrifuged and blood cells were washed three times with solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH7.4) through centrifugation for 10min at 3000 rpm, [16-17].

2.3.4 Hypotonic solution Induced hemolysis

The experiment was carried out with hypotonic solution. The test sample consists of stock erythrocyte (RBC) suspension(0.5ml) with 5ml of hypotonic solution(50mM NaCl) in 10mM sodium phosphate buffer saline (pH 7.4) containing different fraction of ethanolic extract (2mg/ml) and acetyl salicylic acid (0.1mg/ml).The acetyl salicylic acid was used as reference standard. The mixtures were centrifuge for 10 min at 3000 rpm, and incubated for 10 min at a room temperature. The absorbance of supernatant portion was measured at 540nm using UV spectrophotometer. The percentage inhibition of either haemolysis or membrane stabilization was calculated using the following equation.

% inhibition of haemolysis $=100\times \{(OD_1-OD_2)/OD_1\}$

Where, OD_1= optical density of hypotonic buffered saline solution alone (control) and OD_2= optical density of the test sample in hypotonic solution.

2.3.5 Heat induced haemolysis

Isotonic buffer solution containing 2mg/ml of different fractions of *M. paniculata* were put into two duplicates of centrifuging tube (shinde *et al.* 1999). The vehicle in the same amount was added to another tube as control. Erythrocyte suspension (30ul) was added to each tube and mixed gently by inversion. One pair of tubes was incubated at 54^0C for 20 min in a water bath. The other pair was maintained of 0-5^0 C in an ice bath. The reaction mixture was centrifuged for 10 min at 3000 rpm and the absorbance of the supernatant portion was measured at 540nm. The percentage inhibition or acceleration of hemolysis was calculated according to the equation.

$$\% \text{ inhibition of haemolysis} = 100\times \{1-100\times\frac{OD2-OD1}{OD3-OD1}\}$$

$$=100\times \{\frac{OD3-OD1-OD2+0D1}{OD3-OD1}\}$$

$$=100\times\frac{OD3-OD2}{OD3-)D1}$$

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

3. Result and Discussion

3.1 Thrombolytic Activity

Several thrombolytic drugs obtained from various sources are used for the treatment of thrombosis. Thrombolytic agents are

used to disrupt already formed blood clots in clinical settings where ischemia may be fatal (acute myocardial infarction, pulmonary embolism, ischemic stroke, and arterial thrombosis).Thrombolytic drugs dissolve blood clots by activating plasminogen, which forms a cleaved product called plasmin. Plasmin is a proteolytic enzyme that is capable of breaking cross-links between fibrin molecules, which provide the structural integrity of blood clots. Because of these actions, thrombolytic drugs are also called plasminogen activators and fibrinolytic drugs. There are three major classes of fibrinolytic drugs: tissue plasminogen activator (tPA), streptokinase (SK), and urokinase (UK).While drugs in these three classes all have the ability to effective dissolve blood clots**.** Addition of 100µl streptokinase (SK) for fibrinolytic drugs as a positive control (30,000IU) to the clots and subsequent incubation for 90 minutes at 37°C showed 65% lysis of clot on the other hand sterile distilled water was treated as negative control which exhibited a negligible percentage of lysis of clot 5% between positive and negative control was found very significant (p value<0.01). From *In vitro* thrombolytic activity study revealed that ethanolic soluble fraction (ESF) 43.28%, hexane soluble fraction (HXSF) 58.06%, chloroform extract (CSF) 27.66%, and aqueous soluble fraction (AQSF) 22.81% showed respectively. In this study revealed that the hexane soluble fraction of *M. paniculata* showed highest thrombolytic activity.

3.2 Statistical analysis

The significance between percentages of clot lysis by crude extract by means of weight difference was tested by the paired t-test analysis**.** Statistical representation of the effective clot lysis percentage by different fractionate of crude leave extract results were compared, positive thrombolytic control (streptokinase) and negative control (sterile water), p values < 0.01 were considered to be statistically significant (p indicates probability), has been shown in table and figure 1.

Table 1: Thrombolytic Activity of different crude extract of *M.paniculata*.

Fractions	Weight of empty vial (A) g	Weight of vial with clot (B) g	Weight of clot (B-A)g	Weight of vial with clot after lysis (D) g	Weight of clot lysis (B-D)g	%of clot lysis
ESF	5.14	5.91	0.67	5.62	0.5	43.28*
HXSF	5.29	6.22	0.93	5.68	0.54	58.06*
CSF	5.28	5.75	0.47	5.62	0.13	27.66*
Blank	5.20	5.73	0.53	5.70	0.03	5*
SK	4.65	5.05	0.4	4.79	0.14	65*
AQSF	5.47	6.04	0.57	5.91	0.13	22.81*

*P<0.01, crude extract are significant as compared to positive control and negative control.

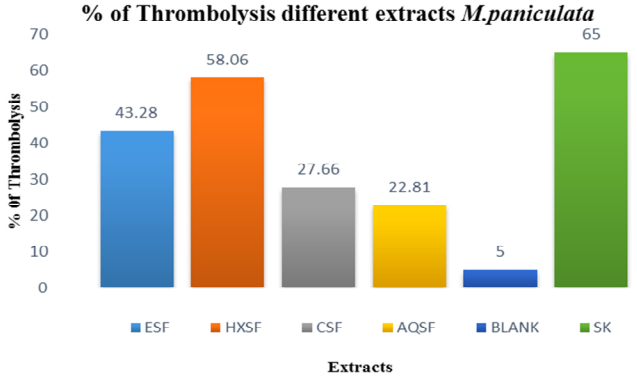

Fig 1: Thrombolytic Activity (in terms of % of clot lysis) of different crude extracts of *M. paniculata*.

3.3 Membrane Stabilizing Activity

At 2mg/ml, different partitionates of crude extracts of *M. paniculata* protected the haemolysis of RBC induced by hypnotic solution and heat as compared to the standard ASA. Therefore, ASA inhibited the haemolysis of RBC 71.90±0.29%, AQSF inhibited 33.49%±0.51, ESF inhibited 40.23%±0.64, CSF inhibited 42.66±0.77% and HSF inhibited 60.35±0.50% respectively by hypnotic solution and Whereas in heat as compared to standard ASA inhibited 62.12±0.26, AQSF inhibited 30.34%±0.42, ESF inhibited 41.53%±0.61, CSF inhibited 36.22±0.57%, HXSF inhibited protected 44.11±0.69% respectively, all data were demonstrated in the figure 2, 3 and table 2.

3.4 Statistical analysis

Three replicates of each sample were used for each test to facilitate statistical analysis and the Data were presented as mean ±standard deviation (SD)**.**

Table 2: Percentage (%) inhibition of heat and hypotonic solution induced haemolysis of erythrocyte membrane by standard and different fractions of *M. paniculata*.

Samples	% inhibition of haemolysis	Hypotonic solution induced
	Heat induced	
Hypotonic medium	-	-
ESF	41.53±0.61	40.23±0.64
HXSF	44.11±0.69	60.35±0.50
CSF	36.22±0.57	42.66±0.77
AQSF	30.34±0.42	33.49±0.51
ASA	62.12±0.26	71.90±0.29

Values are expressed as mean ± SD (standard deviation).
ESF=ethyl soluble fraction, HXSF= hexane soluble fraction, CSF= chloroform soluble fraction, AQSE=aqueous soluble fraction, ASA=acetyl acetic acid

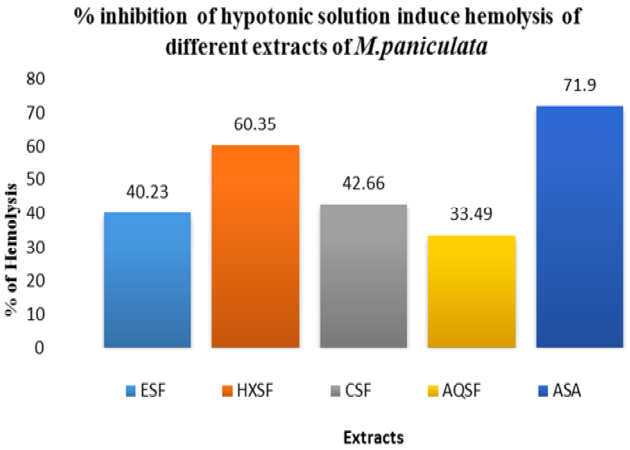

Fig 2: Inhibition of Hypnotic solution induced hemolysis of different crude extracts of *M. paniculata*.

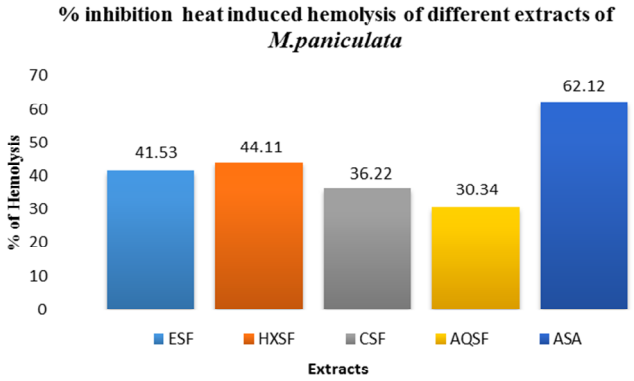

Fig 3: Inhibition of heat induced hemolysis of different crude extracts of *M. paniculata*.

In this study hexane soluble fraction of *M. paniculata* showed 60.35±0.35% by hypnotic solution and in heat 44.11 ± 0.69 respectively, highest protection of haemolysis of RBC membrane. It can be postulated from the observed results that the membrane stabilizing action and inhibition of erythrocyte lysis property of *M. paniculata* may be the possible mechanism of action of its anti-inflammatory activity

4. Conclusion

An *In vitro* thrombolytic and membrane stabilizing study, it is clear that ethanolic crude extract of *M. paniculata* has significant thrombolytic and membrane stabilizing activities. It may be assumed that these extracts can be considered as good source of thrombolytic and membrane stabilizing agents. However, more detailed phytochemical analysis will be necessary to isolate and characterize the active compounds responsible for the thrombolytic and membrane stabilizing activities as well as to understand the exact mechanisms of action of these activities.

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