In Vitro Membrane Stabilizing and Thrombolytic Activities of *Ophirrhiza mungos, Mussaenda macrophylla, Gmelina philippensis* and *Synedrella nodiflora* Growing in Bangladesh

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Abstract: The methanol extracts and their pet-ether, carbon tetrachloride, chloroform and aqueous soluble partitionates of *Ophirrhiza mungos, Mussaenda macrophylla, Gmelina philippensis* and *Synedrella nodiflora* were subjected to assays for membrane stabilizing and thrombolytic activities. The extractives inhibited heat- as well as hypotonic solution-induced haemolysis of human erythrocytes *in vitro*. The pet-ether soluble fraction of *O. mungos, M. macrophylla* and *S. nodiflora* demonstrated 61.16 % & 24.75%, 52.55% & 23.35% and 60.24% & 22.85% inhibition of hemolysis of RBC caused by hypotonic solution and heat, whereas the carbon tetrachloride soluble fraction of *G. philippensis* showed 49.05% and 21.25% inhibition of hypotonic and heat induced hemolysis of RBC, respectively. Here, acetyl salicylic acid was used as reference standard at 0.10 mg/mL. Among the four plants, the carbon tetrachloride soluble fraction of *O. mungos*, methanol extract of *M. macrophylla*, carbon tetrachloride soluble fraction of *G. philippensis* and chloroform soluble fraction of *S. nodiflora* revealed highest thrombolytic activity with clot lysis value of 50.09%, 49.50%, 47.14% and 46.37%, respectively. Standard streptokinase and water were used as positive and negative controls which showed 65.00% and 3.84% lysis of clot, respectively.

Keywords: Hypotonic solution, haemolysis, acetyl salicylic acid, streptokinase, clot lysis.

1. INTRODUCTION

Ophiorrhiza mungos (L) (Bengali name- Ronjonkali) belonging to the family Rubiaceae is a flowering plant, adapted to many environments. *O. mungos* is an annual herb attaining a height of 30 cm and is distributed all over Bangladesh. Traditionally, this plant is used in wound healing [1] and snake bites [2].

Mussaenda macrophylla Wall. (Local name- Magballi, Dhobi tree, Family- Rubiaceae) is a flowering shrub which is distributed in central and eastern Nepal to about 1800 m in moist places in association with herbs and other shrubs. It is also found to occur in northern India, southern China and Myanmar [3]. Traditionally the bark of this plant is used in snake bite [4]. Previous studies with *M. macrophylla* revealed antibacterial, anticoagulant, anti-inflammatory and hepatoprotective activities [5]. The plant is also active against oral pathogens [6].

Gmelina philippensis Cham. (synonym- *Gmelina hystrix*, Bengali name- Badhara, Korobi, Family- Verbenaceae) is a small tree with pendant branches. It is native to Philippine islands, India and south-east Asia and also distributed in United States, Australia, Vietnam, Thailand, Malaysia, Indonesia, Myanmar and Bangladesh [7]. In the Philippines, the fruit-juice is applied to eczema of the feet while root-juice is used as a purgative and in treating fatigue in Indo-China. In Peninsular Malaysia, the fruit pounded with lime is applied as a poultice to the throat as a remedy for coughs. The extract of the roots is used internally as a stimulant, resolvent and in treating diseases of the joints and nerves. Likewise, an extract of the leaves is employed externally [8].

Synedrella nodiflora (L) (synonym- *Verbesina nodiflora*, Local name- Sinderella weed, Pig grass, Node weed) is a flowering herb which belongs to the family Asteraceae. *S. nodiflora* grows well in different environments and mainly found in Bangladesh, India, Japan, Spain, China and England [9]. The whole plant is diuretic and laxative [10]. The anti-inflammatory [11], insecticidal [12] and analgesic [13] activities of the plant have also been reported.

As a part of our continuing investigation of medicinal plants of Bangladesh [14, 15] we investigated *O. mungos, M. macrophylla, G. philippensis* and *S. nodifloara* and we, here in, report the results of our preliminary experiments.

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2. MATERIALS AND METHODS

2.1. Plant Materials

The whole plant of *O. mungos* and *S. nodiflora* and leaves of *M. macrophylla* and *G. philippensis* were collected from Dhaka and voucher specimen (DACB 35632, 35546, 35633 and 35547 respectively) for each of the plant sample has been deposited in Bangladesh National Herbarium for future reference.

2.2. Extraction

The collected pant parts were sun dried for several days and then oven dried for 24 hours at 40°C to facilitate grinding. The powdered materials (500 gm each) were separately macerated in 2.5 L of methanol for 7 days and then filtered through a cotton plug followed by Whatman filter paper number 1. The extracts were concentrated with a rotary evaporator at low temperature (40-45 ºC) and reduced pressure. The concentrated methanol extracts were partitioned by the modified Kupchan's protocol [16] and the resultant partitionates i.e. pet-ether (PESF), carbon tetrachloride (CTCSF), chloroform (CSF) and aqueous (AQSF) soluble fractions (Table **1**) were used for the biological screenings.

2.3. Membrane Stabilizing Activity

The membrane stabilizing activity of the extractives was assessed by using hypotonic solution and heat-induced haemolysis of human erythrocyte by the method developed by Shinde [17] with slight modification by Sikder [18] by using acetyl salicylic acid as standard.

In hypotonic solution-induced haemolysis, the test sample consisted 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-

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

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

In heat-induced haemolysis, isotonic buffer containing aliquots (5 ml) of the 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 gently by inversion. A pair of the tubes was incubated at 54ºC for 20 min in a water bath. The other pair was maintained at 0-5ºC 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 test samples was calculated according to the following equation-

$$\% \text{ Inhibition of hemolysis} = 100 \times [1\text{-} (OD_2\text{-}OD_1/ OD_3\text{-}OD_1)]$$

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

2.4. Thrombolytic Activity

The thombolytic activity of all extractives was evaluated by the method developed by Prasad *et al*. [19] using streptokinase (SK) as positive control. Aliquots (5 ml) of venous blood were drawn from healthy volunteers that were distributed in five different

Table 1: Kupchan Partitionates of *O. mungos, M. macrophylla, G. philippensis* and *S. nodiflora* Obtained from 5 gm of Crude Extract

Fraction/Crude extract	*O. mungos* (gm)	*M. macrophylla* (gm)	*G. phillippensis* (gm)	*S. nodiflora* (gm)
Crude	5.0	5.0	5.0	5.0
PESF	2.0	1.75	1.5	1.0
CTCSF	1.0	1.2	1.0	1.0
CSF	0.5	1.0	0.5	0.5
AQSF	0.3	0.25	0.5	0.5

pre-weighed sterile micro-centrifuge tubes (500 µl/tube) and incubated at 37°C for 45 minutes. 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 preweighed clot, 100 µl aqueous solution of different partitionates and the crude extract was added separately. As a positive control, 100 µl of streptokinase (SK) and as a negative nonthrombolytic control, 100 µl of distilled water were separately added to the control tubes. All tubes were then incubated at 37°C for 90 minutes and observed for clot lysis. After incubation, the released 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 released clot /clot wt) × 100

2.5. Statistical Analysis

Three replicates of each sample were used for statistical analysis and the values are reported as mean ± SD.

3. RESULTS AND DISCUSSION

The extractives of *O. mungos, M. macrophylla, G. philippensis* and *S. nodifloara* at concentration 2.0 mg/mL significantly protected the lysis of erythrocyte membrane induced by hypotonic solution and heat as compared to the standard, acetyl salicylic acid (0.10 mg/mL). In hypotonic solution and heat- induced conditions, the pet-ether soluble fraction (PESF) of *O. mungos* inhibited 61.16 % and 24.75% haemolysis of RBC, as compared to 73.19 and 30.23% demonstrated by acetyl salicylic acid (0.10 mg/mL), respectively. On the other hand, the pet-ether (PESF) and carbon tetrachloride (CTCSF) soluble partitionates of methanol extract of *M. macrophylla* revealed 52.55% and 45.79 % inhibition of haemolysis of RBC induced by hypotonic solution, respectively. The carbon tetrachloride (CTCSF) and pet-ether (PESF) soluble partitionates of methanol extract of *G. philippensis* exhibited 49.05 % and 44.16% inhibition of haemolysis of RBC induced by hypotonic solution, respectively. The pet-ether (PESF) and methanol soluble fractions of *S. nodiflora* exhibited 60.24 % and 55.42% inhibition of haemolysis of RBC induced by hypotonic solution, respectively (Table **2**).

Besides, the pet-ether soluble partitionate of methanol extract of leaves of *M. macrophylla* protected 23.35 % lysis of human erythrocyte membrane whereas the carbon tetrachcloride and pet-ether soluble partitionates of methanol extract of leaves of *G. philippensis* and whole plant of *S. nodifloara* protected 21.25% and 22.85 % lysis of human erythrocyte membrane induced by heat, as compared to the standard acetyl salicylic acid (30.23% at concentration 0.10 mg/mL). On the other hand, the carbon tetrachloride soluble fraction of methanol extract of whole plant of *O. mungos* and leaves of *M. macrophylla* revealed 21.08% and 22.29 % inhibition of haemolysis of RBC. At the same time, the pet-ether soluble fraction of methanol extract of leaves of *G. philippensis* and methanol extract of whole plant of *S. nodifloara* exhibited 17.23% and 20.55 % inhibition of haemolysis of RBC.

Table 2: Percentage Inhibition of Hypotonic Solution and Heat-Induced Haemolysis of Erythrocyte Membrane by *O. mungos, M. macrophylla, G. philippensis* and *S. nodiflora* Extractives (2 mg/ml) and ASA (0.10mg/ml)

Test Sample	*O. mungos*		*M. macrophylla*		*G. philippensis*		*S. nodiflora*	
	Heat induced (%)	Hypotonic solution induced (%)	Heat induced (%)	Hypotonic solution induced (%)	Heat induced (%)	Hypotonic solution induced (%)	Heat induced (%)	Hypotonic solution induced (%)
ME	17.55±0.66	39.42±0.24	18.31±0.44	35.29±0.35	14.45±0.54	36.42±0.44	20.55±0.85	55.42±0.45
PESF	24.75±0.25	61.16±0.85	23.35±0.21	52.55±0.62	17.23±0.31	44.16±0.55	22.85±0.75	60.24±0.51
CTCSF	21.08±0.44	48.05±0.55	22.29±0.16	45.79±0.75	21.25±0.22	49.05±0.35	19.08±0.22	49.05±0.65
CSF	19.25±0.84	41.65±0.22	20.03±0.31	43.63±0.55	16.63±0.84	39.65±0.61	17.93±0.54	41.65±0.35
AQSF	9.05±0.21	31.25±0.16	8.45±0.81	26.22±0.25	10.39±0.25	29.25±0.16	8.05±0.61	30.25±0.58
ASA	30.23±1.02	73.19±0.88	30.23±1.02	73.19±0.88	30.23±1.02	73.19±0.88	30.23±1.02	73.19±0.88

ME = Methanolic crude extract; PESF = Pet-ether soluble fraction of methanolic extract; CTCSF = Carbon tetrachloride soluble fraction of methanolic extract; CSF = Chloroform soluble fraction of methanolic extract; AQSF = Aqueous soluble fraction of methanolic extract, ASA= Acetyl salicylic acid.

Table 3: Thrombolytic Activity (in Terms of % of Clot Lysis) of *O. mungos, M. macrophylla, G. philippensis* and *S. nodifloara* Extractives

O. mungos						
Test samples	**W1**	**W2**	**W3**	**W4**	**W5**	**Lysis of clot (%)**
ME	5.21±0.004	5.84±.012	5.6±0.005	0.626±0.008	0.398±0.001	36.39±0.882
PESF	4.66±.002	5.1154±0.010	4.92±0.003	0.451±0.008	0.257±0.001	42.89±0.665
CTCSF	4.84±.001	5.356±0.005	5.09±0.006	0.515±0.004	0.257±0.005	50.09±0.982
CSF	4.71±.003	5.492±0.011	5.19±0.006	0.789±.008	0.483±0.003	38.74±0.356
AQSF	4.70±.006	5.2971±0.012	5.09±0.008	0.594±0.006	0.386±0.002	34.84±0.881
Water	4.78±.005	5.04±0.006	5.03±0.008	0.26±0.001	0.25±0.003	3.846±0.565
SK	4.65±0.003	5.05±0.007	4.79±0.009	0.4±0.004	0.14±0.006	65±0.663
M. macrophylla						
Test samples	**W1**	**W2**	**W3**	**W4**	**W5**	**% lysis**
ME	5.03±0.009	5.74±0.011	5.39±0.012	0.707±0.002	0.357±0.003	49.5±0.352
PESF	4.77±0.010	5.22±0.012	5.06±0.014	0.443±0.002	0.287±0.004	35.1±0.912
CTCSF	4.79±0.002	5.36±0.003	5.11±0.009	0.569±0.001	0.322±0.007	43.53±0.454
CSF	4.79±0.008	5.26±0.012	5.09±0.010	0.466±0.004	0.298±0.002	36.05±0.245
AQSF	4.84±0.005	5.391±0.008	5.18±0.010	0.589±0.003	0.342±0.005	38.16±0.981
Water	4.78±.005	5.04±0.006	5.03±0.008	0.26±0.001	0.25±0.003	3.846±0.565
SK	4.65±0.003	5.05±0.007	4.79±0.009	0.4±0.004	0.14±0.006	65±0.663
G. philippensis						
Test samples	**W1**	**W2**	**W3**	**W4**	**W5**	**% lysis**
ME	4.98±0.001	5.25±0.004	5.16±0.006	0.271±0.003	0.179±0.005	33.94±0.342
PESF	4.82±0.002	5.29±0.005	5.12±0.009	0.474±0.003	0.302±0.004	36.28±0.652
CTCSF	4.63±0.006	5.39±0.012	5.03±0.008	0.765±0.006	0.405±0.002	47.14±0.752
CSF	4.83±0.009	5.31±0.011	5.12±0.014	0.477±0.02	0.288±0.005	39.52±0.446
AQSF	4.82±0.003	5.35±0.004	5.28±0.007	0.525±0.001	0.449±0.004	38.29±0.815
Water	4.78±.005	5.04±0.006	5.03±0.008	0.26±0.001	0.25±0.003	3.846±0.565
SK	4.65±0.003	5.05±0.007	4.79±0.009	0.4±0.004	0.14±0.006	65±0.663
S. nodifloara						
Test samples	**W1**	**W2**	**W3**	**W4**	**W5**	**% lysis**
ME	5.10±0.002	5.81±0.005	5.58±0.009	0.709±0.003	0.471±0.007	33.58±0.446
PESF	4.57±0.003	5.11±0.006	4.92±0.008	0.533±0.003	0.347±0.005	34.87±0.624
CTCSF	4.81±0.005	5.26±0.009	5.09±0.012	0.448±0.004	0.283±0.007	36.64±0.982
CSF	4.69±0.001	5.31±0.009	5.02±0.005	0.615±0.008	0.329±0.004	46.37±0.908
AQSF	4.61±0.009	5.39±.013	5.06±0.012	0.777±0.004	0.459±0.003	40.82±1.002
Water	4.78±.005	5.04±0.006	5.03±0.008	0.26±0.001	0.25±0.003	3.846±0.565

ME = Methanolic crude extract; PESF = Pet-ether soluble fraction of methanolic extract; CTCSF = Carbon tetrachloride soluble fraction of methanolic extract; CSF = Chloroform soluble fraction of methanolic extract; AQSF = Aqueous soluble fraction of methanolic extract; W1= Weight of empty vial (gm); W2 = Weight of clot containing vial before clot disruption (gm); W3 = Weight of clot containing vial after clot disruption (gm); W4= W2-W1= Weight of clot before clot disruption; W5=W3-W1= Weight of clot after clot disruption ; SK =Streptokinase.

In order to discover cardio-protective drugs from natural resources the extractives of *O. mungos, M. macrophylla, G. philippensis* and *S. nodiflora* were assessed for thrombolytic activity and the results are presented in Table **3**. Addition of 100 µl SK, a positive control (30,000 I.U.) to the clots of human blood and subsequent incubation for 90 minutes at 37°C, showed 65.00% lysis of clot. On the other hand, distilled water when treated as negative control showed negligible percentages of lysis of clot (3.846%). The mean difference in percentage of clot lysis between positive and negative control was found to be statistically significant. In this study, the carbon tetrachloride soluble fraction of methanolic crude extract of *O.*

mungos and methanol extract, i.e. crude of *M. macrophylla* revealed highest thrombolytic activity (50.09% and 49.50% respectively). However, significant thrombolytic activity was also demonstrated by carbon tetrachloride soluble fraction of *G. philippensis* (47.14%) and chcloroform soluble fraction of *S. nodiflora* (46.37%), respectively.

CONCLUSION

The extractives of *O. mungos*, *M. macrophylla, G. philippensis* and *S. nodifloara* demonstrated varying degrees of inhibition of heat and hypotonic solution-induced haemolysis of human erythrocyte membrane. The erythrocyte membrane resembles lysosomal membrane and as such, the effect of drugs on the stabilization of erythrocyte could be extrapolated to the stabilization of lysosomal membrane [20]. Therefore, as membrane stabilizes that interfere in the release and action of mediators like histamine, serotonin, prostaglandins, leukotrienes etc. [17]. A possible explanation for the stabilizing activity of the extractives might be due to an increase in the surface area/volume ratio of the cells which could be brought by an expansion of membrane or shrinkage of the cell and an interaction with membrane proteins [21]. In addition, from the experiments it can be also stated that these plants have got good potential as candidate for future thrombolytic agents and can be investigated for possible sources of cardio-protective drugs. Further bioassay guided chemical investigations are warranted for the discovery of new drug candidates.

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