



Evaluation of membrane stabilizing, cytotoxic and anti-diarrheal activities of leaves of *Calotropis Gigantea* R.BR.

Muhammed Mahfuzur Rahman^{1*}, Md. Yeunus Mian¹, Abdullah Al Faysal², S. M. Ashikur Rahman¹, Ridwan Islam³, Rabeya Moni¹

¹Department of Pharmacy, State University of Bangladesh, Dhaka, Bangladesh

²Department of Pharmacy, East West University, Dhaka, Bangladesh

³Department of Pharmacy, BRAC University, Dhaka, Bangladesh

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ABSTRACT

Different partitionates of leaves of *Calotropis gigantea* were evaluated for membrane stabilizing activity by reduction of hemolysis in hypotonic solution and heated solution along with cytotoxic and anti-diarrheal activities. The first activity was assessed by using erythrocyte in hypotonic solution and heat in which acetyl salicylic acid, the reference standard, gave $71.9 \pm 0.004\%$ and $42.2 \pm 0.005\%$ inhibition of hemolysis in both conditions, respectively. Among the partitionates of the leave extracts maximum membrane stabilizing activity was revealed by aqueous soluble fraction ($63.77 \pm 0.022\%$) in hypotonic solution and petroleum ether soluble fraction ($32.62 \pm 0.052\%$) in heat induced condition. In determining cytotoxic activity using brine shrimp nauplii, crude methanol extract showed the most cytotoxicity (LC₅₀ value of $1.79 \pm 0.004 \mu\text{g/ml}$) among the partitionates whereas the standard vincristine sulphate gave LC₅₀ value of $0.45 \pm 0.008 \mu\text{g/ml}$. The anti-diarrheal activity of the crude methanol extract was determined on mice using loperamide as standard. The extract exhibited significant anti-diarrheal activity at both doses (200 mg/kg and 400 mg/kg).

Key words: *Calotropis gigantea*, Membrane stabilizing activity, Cytotoxic activity, Anti-diarrheal activity, Acetyl salicylic acid, Loperamide, Vincristine sulphate.

INTRODUCTION

Inflammatory cells produce a complex mixture of growth and differentiation of cytokines as well as physiologically active arachidonate metabolites. In addition they possess the ability to generate reactive oxygen species (ROS) that can damage cellular biomolecules which in turn augment the state of inflammation [1]. 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 [2]. Therefore, as membrane stabilizes that interfere in the release and or action of mediators like histamine, serotonin, prostaglandins, leukotrienes etc [3]. Two types of experiments are done to determine the membrane stabilizing activity. One of them is hypotonic solution induced hemolysis and another is heat induced hemolysis. Brine shrimp lethality bioassay has been suggested for screening pharmacological activities in plant extracts. It is considered as a useful tool for

preliminary assessment of toxicity and is a rapid and comprehensive bioassay for the bioactive compounds of natural and synthetic origin. A simple zoological organism (Brine shrimp nauplii) is utilized in this method to conveniently monitor *in vivo* lethality for screening and fractionation in the discovery of new bioactive natural products. This bioassay indicates cytotoxicity as well as a wide range of pharmacological activities such as antimicrobial, antiviral, pesticidal & anti-tumor etc. of the compounds [4-5].

Diarrhea is characterized by increased frequency of bowel movement, wet stool and abdominal pain [6]. It is a leading cause of malnutrition and death among children in the developing countries of the world today. The anti-diarrheal activity can be evaluated using the method of castor oil induced diarrhea in mice [7].

Calotropis gigantea R.Br. (Bengali: *Akanda*) is an evergreen tree that grows open woodland at elevations < 2,800 m either as shrub, or as a small

*Corresponding Author Address: Muhammed Mahfuzur Rahman, Lecturer, Department of Pharmacy, State University of Bangladesh, Dhaka-1205, Bangladesh, Email: mahfuz.du.phr@gmail.com

tree which grows in Chittagong, Chittagong Hill Tracts, Sylhet, on the edges of the forest in Bangladesh [8].

Extracts of different plant parts viz. root, stem, leaf, and stem+leaf of *Calotropis* affect germination and seedling vigor of many agricultural crops have been reported [9-11]. In the present study, the organic soluble materials of a methanol extract of the leaves and its different organic soluble partitionates were evaluated for the membrane stabilizing activity in terms of hypotonic solution-induced hemolysis and heat-induced hemolysis, anti-diarrheal activity and lethality bioassay of *C. gigantea* for the first time.

MATERIALS AND METHODS

Plant materials: The leaves of *C. gigantea* were collected from Sher-e-bangla Agriculture University, Dhaka. A voucher specimen for this plant has been maintained in Bangladesh National Herbarium, Dhaka, Bangladesh (Accession no. 35903). The leaves were sun dried for several days for better grinding. The dried leaves were then ground to a coarse powder using high capacity grinding machine. Four hundred grams (250 g) of the powdered material was soaked in a cleaned, ambered color reagent bottle (2.5 liters) and soaked in 1.5 L of methanol. The container with its content was sealed by bottle cap and kept for a period of 20 days accompanying occasional shaking and stirring. The whole mixtures were then filtered through a fresh cotton plug and finally with a Whatman No. 1 filter paper. The extract was concentrated with a rotary evaporator at low temperature (40-45°C) and reduced pressure. The concentrated methanolic extract was partitioned by modified Kupchan method [12] and the resultant partitionates i.e., pet-ether (PESF), carbon tetrachloride (CTCSF), chloroform (CSF), and aqueous (AQSF) soluble fractions were used for the experimental processes.

Membrane stabilizing activity: The membrane stabilizing activity of the extractives was assessed by using hypotonic solution induced erythrocyte hemolysis designed by Shinde *et al.*, 1999. To prepare the erythrocyte suspension, blood was drawn from healthy human volunteers (n=10) and 2ml of blood was transferred to the previously weighed centrifuge tubes and EDTA was used to prevent clotting. The blood was washed three times with isotonic solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4) through centrifuge action for 10 min at 3000 g.

Hypotonic solution-induced hemolysis: The test sample consisted of stock erythrocyte (RBC)

suspension (0.50 mL) mixed with 5mL of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing either the extracts (1.0 mg/mL) or acetyl salicylic acid (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 hemolysis or membrane stabilization was calculated using the following equation-

$$\% \text{ inhibition of hemolysis} = 100 \times (\text{OD}_1 - \text{OD}_2 / \text{OD}_1)$$

Where, OD₁ = optical density of hypotonic-buffered saline solution alone (control) and OD₂ = optical density of test sample in hypotonic solution.

Heat-induced hemolysis: Isotonic buffer containing aliquots (5 ml) of the different extracts 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. One pair of the tubes was incubated at 54°C for 20 min in a water bath, while the other pair was maintained at (0-5)°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 hemolysis in tests and was calculated according to the equation:

$$\% \text{ Inhibition of hemolysis} = 100 \times [1 - (\text{OD}_2 - \text{OD}_1 / \text{OD}_3 - \text{OD}_1)]$$

Where, OD₁ = optical density of unheated test sample, OD₂ = optical density of heated test sample and OD₃ = optical density of heated control sample.

Brine shrimp lethality bioassay: Brine shrimp lethality bioassay [4-5] technique was applied for the determination of general toxic property of the plant extractives. DMSO solutions of the samples were applied against *Artemia salina* in an *in vivo* assay. For the experiment, 4 mg of each of the pet-ether, carbon tetrachloride and chloroform soluble fractions were dissolved in DMSO and solutions of varying concentrations (400, 200, 100, 50, 25, 12.50, 6.25, 3.125, 1.563, 0.781 µg/ml) were obtained by serial dilution technique using DMSO. Vincristine sulphate was used as positive control. The nauplii are counted by visual inspection and are taken in test tubes containing 5 ml of simulated sea water. Then samples of different concentrations are added to the premarked test tubes through micropipette. The test tubes are then left for 24 hours. Survivors are counted after 24 hours.

Evaluation of anti-diarrheal activity: The anti-diarrheal activity of the methanolic extract of *C.*

gigantea leaves was evaluated using the method of castor oil induced diarrhea in mice [7]. The test animals (Swiss albino mice) were divided into control, positive control and test groups containing five mice in each group. Control group received vehicle (1% Tween 80 in normal saline) at dose of 10 mg/kg orally. The positive control group received loperamide at the dose of 50mg/kg orally. The two test group received methanolic extract of *C. gigantea* leaves at the doses of 200 mg/kg & 400 mg/kg body weight respectively. Each animal was placed in an individual cage; the floor lining was changed at every hour. Diarrhea was induced by oral administration of castor oil to each mouse after the above treatment. During an observation period of 5 hours; the number of diarrheic feces excreted by the animals was recorded.

Statistical analysis: Three replicates of each sample were used for statistical analysis and the values are reported as mean \pm SD.

RESULTS AND DISCUSSION

The present study was undertaken to evaluate the membrane stabilizing activity of different organic soluble materials of the methanol extract of leaves of *C. gigantea*. The partitionates of *C. gigantea* at concentration 2.0 mg/mL significantly protected the lysis of erythrocyte membrane induced by hypotonic solution & heat induced, as compared to the standard acetyl salicylic acid (0.10 mg/mL) (Table 1). The aqueous soluble extract demonstrated 63.77 \pm 0.022% inhibition of hemolysis of RBC as compared to 71.9 \pm 0.004% produced by acetyl salicylic acid (0.10 mg/mL) in the hypotonic solution induced hemolysis. The chloroform soluble extract and the pet ether soluble extract also exhibited moderate inhibition of

hemolysis of RBC. In the heat induced hemolysis, the pet ether soluble fraction revealed 32.62 \pm 0.032% inhibition of hemolysis as compared to 42.2 \pm 0.005% inhibition of hemolysis by acetyl salicylic acid (0.10 mg/mL). The methanolic extract also showed moderate inhibition of hemolysis of RBC. In case of brine shrimp lethality bioassay, the lethality of the methanol extract and its pet-ether, carbon tetrachloride, chloroform and aqueous soluble fractions were evaluated against *A. salina*. The results of the brine shrimp lethality testing after 24 hours of exposure to the samples and the positive control, vincristine sulphate is given in Table 1. The methanol extract showed the maximum cytotoxic activity having LC₅₀ of 1.79 \pm 0.004 μ g/ml as compared to 0.45 \pm 0.008 μ g/ml for vincristine sulphate. Other partitionates showed mild cytotoxicity.

In determining the anti-diarrheal activity, the methanolic extracts of *C. gigantea* can reduce the number of diarrheic feces to 36.84% and 52.24% at the dose of 200 mg/kg & 400 mg/kg body weight respectively compared to 50.03% reduction by standard (loperamide). Reduction of diarrheal activity was evaluated statistically and found significant (Table 2).

CONCLUSION

It can be concluded that the different partitionates of methanolic extracts of *C. gigantea* exhibited moderate membrane stabilizing activity and mild cytotoxic activity. Methanolic extracts of *C. gigantea* at two different doses also demonstrated significant anti-diarrheal activity. Further work is needed to isolate the secondary metabolites and study thoroughly for more precise and accurate activities.

Table 1: The membrane stabilizing & cytotoxic activities of different partitionates of *C. gigantea*

Sample code	Membrane stabilizing activity (% inhibition of hemolysis)		Cytotoxic activity (LC ₅₀ μ g/ml)
	Hypotonic solution induced	Heat induced	
MEF	54.19 \pm 0.011	29.45 \pm 0.071	1.79 \pm 0.004
PESF	58.86 \pm 0.042	32.62 \pm 0.032	7.03 \pm 0.007
CTCSF	26.34 \pm 0.078	22.07 \pm 0.036	16.63 \pm 0.008
CSF	60.47 \pm 0.072	16.07 \pm 0.011	9.93 \pm 0.004
AQSF	63.77 \pm 0.022	11.07 \pm 0.012	8.7 \pm 0.011
ASA	71.9 \pm 0.004	42.02 \pm 0.005	-
VS	-	-	0.45 \pm 0.008

The average values of three calculations are presented as mean \pm S.D. (standard deviation); VS = Vincristine sulphate; ASA = Acetyl Salicylic Acid; MEF = Methanolic extract; PESF = Pet-ether soluble fraction; CTCSF = Carbon tetrachloride soluble fraction; CSF= chloroform soluble fraction; AQSF = Aqueous soluble fraction of the methanolic extract of *C. gigantea*.

Table 2: The anti-diarrheal activity of methanolic extract of *C. gigantea*

Sample code	Number of diarrheal feces (mean)	% reduction of diarrhea	t-test value	Standard error	P value	Level of significance
Control	12.6	-				-
Standard	6.2	50.79	8.26	0.775	0.0001	Statistically significant
ME ₁	8.4	33.33	5.82	0.721	0.0004	Statistically significant
ME ₂	6.8	46.03	9.87	0.632	0.0001	Statistically significant

Here, ME₁= Methanolic extracts of *C. gigantea* at the dose of 200mg/kg body weight, ME₂= Methanolic extracts of *C. gigantea* at the dose of 400mg/kg body weight.

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