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EVALUATION OF *IN VITRO* MEMBRANE STABILIZING ACTIVITY AND THROMBOLYTIC ACTIVITY OF *AVERRHOA BILIMBI* LEAF EXTRACTS

Mohammad Mooneem Mannan, Rumana Akhter, Mohammad Shahriar* and Mohiuddin Ahmed Bhuiyan

Phytochemistry Research Laboratory, Department of Pharmacy, University of Asia Pacific, Dhaka, Bangladesh

***Corresponding author e-mail:** shahriar@uap-bd.edu

ABSTRACT

Averrhoa bilimbi L. belonging to the family Oxalidiaceae has local name include belembuis an important multipurpose tropical tree under-recognized for its nutritional medicinal properties. In this present study, the leaf extracts of *A. bilimbi* were subjected to a comparative evaluation of the membrane stabilization and thrombolytic activity. The thrombolytic and membrane stabilizing activities were assessed by using human erythrocyte and the results were compared with standard streptokinase (SK) and standard anti-inflammatory drug, acetyl salicylic acid (ASA) respectively. The crude chloroform extract of the leaves of *A. bilimbi* demonstrated better membrane stabilizing activity, whereas its methanol and ethanol soluble fractions revealed moderate membrane stabilizing properties compared with standard. The crude extracts were found to have thrombolytic activity with a maximum effect in ethanolic fraction comparable with streptokinase as a positive control and water as a negative control.

Keywords: *Averrhoa bilimbi*, membrane stabilization, thrombolytic activity, streptokinase (SK).

INTRODUCTION

The term of medicinal plants consist of a various types of plants used in herbalism with medicinal activities. These plants are considered as rich resources of ingredients which can be used as complementary and alternative medicines and, also in drug developments and synthesis [1]. In atherothrombotic diseases includes acute myocardial infarction or cerebral infarction, a thrombus is developed in the circulatory system due to the failure of hemostasis causes vascular blockage and sometimes leads to death. For treatment purposes thrombolytic agents like alteplase, anistreplase, streptokinase, urokinase and tissue plasminogen activator (TPA) are commonly used to dissolve the clots [2]. But these thrombolytic agents have limitation to less availability, large doses needed for maximal effect, limited fibrin specificity and bleeding tendency. Since ancient times, herbal preparations have been used for the treatment of thrombotic diseases. Herbal products are often perceived as safe because they are "natural" [3]. Herbal anti-inflammatory agents imparts an implausible effect on the stabilization of erythrocyte membrane undergo hypotonic solution induced and heat induced condition has been studied extensively. 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 [4]. *A. bilimbi* is a multipurpose, long-lived tropical plant locally known as belembu, belemburi; In English, this is also known as- bilimbi, cucumber tree, tree sorrel etc belonging to family Oxalidaceae [5]. *A. bilimbi* is used as traditional medicine for treating cough, cold, itches, boils, rheumatism, syphilis, diabetes, whooping cough, and hypertension in Asia [6,7]. *A. bilimbi* is medicinally utilized as a folk therapy for many symptoms. It is employed for the treatment of fever, mumps, pimples, inflammation of the rectum and diabetes, itches, boils, rheumatism, syphilis, bilious colic, whooping cough, hypertension, stomach ache, ulcer and as a cooling

drink [8]. Earlier studies showed that leaf extract of *A. bilimbi* and its semi–purified fractions possesses hypoglycemic and hypolipidemic properties in Type I diabetic rats [9,10]. A survey of the published literature shows that *A. bilimbi* also includes thrombolytic activity, antifertility activity, cytotoxic activity, antioxidant activity and anti-microbial activity. However there is no research work for the assessment of membrane stabilizing activity and thrombolytic activity of *A. bilimbi* leaf using its different fractions. So our present study is aimed to investigate membrane stabilizing activity and thrombolytic activity of *A. bilimbi* leaf extracts.

MATERIALS AND METHODS

Plant materials collection and identification: The whole plants of *A. bilimbi* were collected during June, 2014 from Sunamgonj, Sylhet, Bangladesh. Then the plant sample was submitted to the National Herbarium of Bangladesh, Mirpur, Dhaka. One week later its voucher specimen was collected after its identification (Accession No. 39642).

Preparation of extracts: The sun dried and powdered leaves (20 gm) of *A. bilimbi* was successively extracted in a Soxhlet extractor at elevated temperature using 200 ml of ethanol (40-60)°C which was followed by chloroform and methanol. All extracts were filtered individually through filter paper and poured on Petri dishes to evaporate the liquid solvents from the extract to get dry extracts. The dry crude extracts were weighed and stored in air-tight container with necessary markings for identification and kept in refrigerator (0-4)°C for future investigation.

Thrombolytic Activity

Blood sample: Blood was drawn from healthy human volunteers (n=5) without a history of oral contraceptive and anticoagulant therapy. 500 µl of blood was transferred to previously weighted micro-centrifuge tubes and was allowed to form clots.

Streptokinase (SK): Commercially available lyophilized Altepase (Streptokinase) vial (Trade name: S-Kinase from Popular Pharmaceuticals Ltd.) of 15,00,000 I.U. was collected and 5ml of 0.9% NaCl was added and mixed properly to prepare the concentration 3,00,000 I.U. This suspension was used as stock from which 100 µl was used for *in-vitro* thrombolysis.

The thrombolytic activity of all extracts were evaluated by using *in-vitro* clot lysis method developed by Prasad *et al.*, (2006) and Ratnasooriya *et al.*, (2008) [11,12] with slight modification, using streptokinase (SK) as the standard substance. Human blood was allowed to clot and different extracts of *Averrhoa bilimbi* leaf were added to the clotted blood. Thrombolytic activity was evaluated by determining % clot lysis using the following equation.

$$\% \text{ of clot lysis} = (W_2\text{-}W_3/W_2\text{-}W_1) \times 100$$

Where, W_1= Weight of microcentrifuge tube alone, W_2= Weight of microcentrifuge tube with blood clot and W_3= Weight of microcentrifuge tube after clot lysis.

Membrane Stabilizing Activity: The membrane stabilization by hypotonic solution and heat-induced haemolysis method was used to assess anti-inflammatory activity of the plant extracts by following standard protocol [13, 14]. Since 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 human erythrocyte haemolysis. To prepare the erythrocyte suspension, whole blood was obtained from healthy human volunteer and was taken in syringes (containing anticoagulant 3.1% Na-citrate). The blood was centrifuged and blood cells were washed three times with solution (154mM NaCl) in 10mM sodium phosphate buffer (pH 7.4) through centrifugation for 10min at 3000g [16,17].

Hypotonic solution-induced haemolysis: The test sample consisted of stock erythrocyte (RBC) suspension (0.5mL) mixed with 5mL of hypotonic solution (50mM NaCl) in 10mM sodium phosphate buffered saline (pH 7.4) containing either the extract (1.0mg/mL) or acetyl salicylic acid (ASA) (0.1mg/mL). The control sample consisted of 0.5mL of RBCs mixed with hypotonic-buffered saline alone. The mixture was incubated for 10min at room temperature, centrifuged for 10min at 3000g and the absorbance of the supernatant was measured at 540nm. 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)

OD_2= optical density of test sample in hypotonic solution

Heat-induced haemolysis: Isotonic buffer containing aliquots (5ml) 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 was added to each tube and mixed gently by inversion. One pair of the tubes was incubated at 56°C for 30min in a water bath, while the other pair was maintained at (0-5)°C in an ice bath. The reaction mixture was centrifuged for 5min at 2500g and the absorbance of the supernatant was measured at 560 nm. The percentage inhibition or acceleration of hemolysis in tests and was calculated according to the equation:

% Inhibition of hemolysis = 100 x [1- $(OD_1-OD_2)/(OD_3-OD_1)$]

Where, OD_1= optical density of unheated test sample

OD_2= optical density of heated test sample

OD_3= optical density of heated control sample

RESULTS AND DISCUSSION

Thrombolytic activity: As a part of discovery of anti-thromolytic drugs from natural sources the extractives of *A. bilimbi* were assessed for thrombolytic activity and the results are presented in table 1. Addition of 100 µl Streptokinase (30,000 I.U.), standard to the clots along with 90 minutes of incubation at 37°C, showed 73.17% clot lysis. Clots when treated with 100 µl sterile distilled water (control) showed only negligible clot lysis (3.54%). In this study, the ethanolic extract of *A. bilimbi* revealed highest thrombolytic activity 47.23%, whereas methanolic, chloroform extract of *A. bilimbi* (15.82%, 11.30%) displayed moderate thrombolytic activities.

Membrane stabilizing activity: The leaf extracts of *A. bilimbi* at concentration of 1.0 mg/mL, were tested against lysis of human erythrocyte membrane induced by hypotonic solution as well as heat, and compared with the standard acetyl salicylic acid (ASA) (0.10 mg/ml) (table 2). For hypotonic solution induced haemolysis, at a concentration of 1.0 mg/ mL, the chloroform extract inhibited 54.55% haemolysis of RBCs as compared to 76.79% produced by acetyl salicylic acid (0.10 mg/mL). On the other hand, during heat induced condition different organic soluble materials of *A. bilimbi* like methanol, ethanol and chloroform extracts demonstrated 67.91%, 57.06%, 43.82% and 57.10% inhibition of hemolysis of RBCs, respectively whereas ASA inhibited 83.75%.

CONCLUSION

On the Basis of above result and available reports, all three leaf extracts of *A. bilimbi* had anti-thrombolytic as well as membrane stabilizing effects. The effects of drugs on dissolution of clots prepared from blood of normal individuals reveal that maximum clot lysis was observed in clot treated with streptokinase. On the basis of the result obtained in this present study we can say that the ethanolic extracts of *A. bilimbi* leaves have better thrombolytic activity compared to standard. The present study also suggests that the membrane stabilizing activity of *A. bilimbi* may be playing a significant role due to its anti-inflammatory activity.

Table 1: % Clot lysis by different extracts of *A. bilimbi*

Samples	% of Clot Lysis
Methanolic Extract	15.82 ± 1.02
Ethanolic Extract	47.23 ± 1.56
Chloroform Extract	11.30 ± 2.25
Control	3.54 ± 0.25
Streptokinase	73.17 ± 2.87

Table 2: Effect of extractives of *A. bilimbi* on hypotonic solution & heat induced hemolysis of erythrocyte

Samples	Concentration (mg/mL)	% Inhibition of Haemolysis	
		hypotonic solution induced	heat induced
Control	50 mM	—	—
Acetyl salicylic acid	0.1	76.79 ± 0.008	83.75 ± 0.76
Methanol Extract	1	44.49 ± 0.07	67.91 ± 0.66
Ethanol Extract	1	19.88 ± 0.07	43.82 ± 0.78
Chloroform Extract	1	54.55 ± 0.09	57.10 ± 0.73

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