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THROMBOLYTIC AND MEMBRANE STABILIZING ACTIVITIES OF *LAUNAEA SARMENTOSA*

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ABSTRACT: This study was carried out to evaluate the thrombolytic and membrane stabilizing activities of *Launaea sarmentosa* plants. The crude methanolic extract of *L. sarmentosa* (whole plants) was investigated for *in-vitro* thrombolytic and membrane stabilizing activities at five different concentrations of 2, 4, 6, 8 and 10 mg/ml respectively. In the case of thrombolytic study, it was dose-dependently increased ($p<0.01$), where streptokinase (30,000 I.U.) was used as standard, and distilled water was treated as a negative control. In the *in vitro* clot lysis assay method, 10 mg/ml concentration most significantly showed 22.57% lysis of clot ($p<0.001$). Similarly, crude methanolic extract of *L. sarmentosa* dose-dependently increased the membrane stabilizing activity in comparison to the standard drug, acetylsalicylic acid (0.10 mg/ml; $p<0.01$). 10 mg/ml of the extract exhibited 12.11% and 20.55% inhibition of hemolysis by both hypotonic solution and heat-induced hemolysis, respectively.

INTRODUCTION: Medicinal plants are moving from brink to mainstream use with a greater number of people seeking remedies and health approaches free from side effects caused by synthetic chemicals [1]. Medicinal plants are used by the traditional physicians for treatment and disease-treating formulations from ancient time [2]. In developed countries thromboembolic disorders such as pulmonary emboli, deep vein thrombosis, strokes and heart attacks, *etc*. are the main causes of morbidity and mortality [3].

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Thrombolytic therapy uses drugs called thrombolytic agents, such as alteplase, anistreplase, streptokinase, urokinase, and tissue plasminogen activator (TPA) to dissolve clots. Inflammation is one of the important pathological disorders. It is a part of the non-specific immune response that occurs in reaction to any type of bodily injury, is a complex biological response of vascular tissues to harmful stimuli [4, 5]. The leaves, stems, barks, flowers and underground parts of medicinal plants are most often used for traditional medicines.

Launaea sarmentosa (*L. sarmentosa)* is a coastal plant, which belongs to Asteraceae family. The plant is usually available in Bangladesh, South Africa, Madagascar, Seychelles, Mauritius, India, Srilanka, Indo-China and Java islands [6]. The local name of *L. sarmentosa* is Vortashak.

We previously examined *L. sarmentosa* for the management of pain, pyrexia and inflammation properties in Swiss albino mice and Wister albino rats and observed a dose-dependent analgesic, antipyretic and anti-inflammatory effects on above mentioned [7]. The whole plant is used in rheumatoid arthritis, gout, and the leaf in rheumatism and also to heal skin injuries caused by fish spines while fishing. Preliminary phytochemical studies of *L. sarmentosa* revealed the presence of terpenoid, tannin, glycoside, alkaloid which are generally known to involve in the inhibition of synthesis of prostaglandins, leukotriene's, and other endogenous substances that are key players in triggering pain perception [8]. So, an attempt was made to assess scientifically the methanolic extract of *L. sarmentosa* for *in-vitro* thrombolytic and membrane stabilizing activities.

MATERIALS AND METHODS:

Chemicals: Drugs and chemicals used in the study include streptokinase (SK; Beacon Pharmaceuticals Ltd., Bangladesh), acetylsalicylic acid (ASA; Beximco Pharmaceuticals Ltd., Bangladesh), disodium phosphate anhydrate (Merck, Germany), monosodium phosphate dehydrate (Merck, Germany), EDTA (Merck Millipore, Germany) and sodium chloride (Merck, Germany).

Collection of Sample and Preparation of Extract: The whole plants of *L. sarmentosa* were collected from the Sonadia deep of Cox's Bazar. The plant was taxonomically identified by Bangladesh National Herbarium, Mirpur, Dhaka and its voucher specimen number were DACB-38312.

The whole plants were first were washed thrice with speedy running tape water and twice with distilled water to remove the adhered salts, soils, and other associated animals, parasites and then dried in the shade at a temperature between 21-30 °C for 15 days. After the process of drying, these were grounded in a blender into fine powders. Then 500 gm of the fine powdered crude plant was soaked in 1.5 liter of methanol at room temperature for two weeks. Then the solution was filtered using filter cloth and Whatman's filter paper. The total filtrate was concentrated with a rotary evaporator to render the methanolic extract of brownish red color [9].

Preparation of Standard Streptokinase Suspension: For thrombolytic activity, commercially available lyophilized SK vial of 15, 00,000 I.U., was collected and 5 ml sterile distilled water was added and mixed properly. This suspension was used as a standard, from which 100µl (30,000 I.U.) was utilized for *in-vitro* thrombolytic test.

Preparation of Test Sample for Thrombolytic Activity: Five different test solutions were used to evaluate the *in-vitro* thrombolytic activity of the plant extract. The plant extract was dissolved in methanol and shaken vigorously on a vortex mixer to prepare 2, 4, 6, 8 and 10 mg/ml of the test samples, respectively. The suspension was kept overnight and decanted to remove the soluble supernatant, which was filtered through a 0.22-micron syringe filter. 100 µl of methanolic extract was added to the microcentrifuge tube containing the clots to check thrombolytic activity [10].

Thrombolytic Potential Measurement: The thrombolytic activity of *L. sarmentosa* was carried out by *in-vitro* clot lysis assay method. 5 ml of venous blood was drawn from healthy volunteers without a history of oral contraceptive or anticoagulant therapy, which were then distributed in five different pre-weighed sterile micro-centrifuge tubes (0.5 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 – the weight of tube alone). Each micro-centrifuge tube containing clot was properly labeled, and 100 µl of the test sample from various concentrations (2, 4, 6, 8 and 10 mg/ml) was added to the tubes accordingly. As a positive control, 100 µl of SK and as a negative control, 100 µl of distilled water were separately added to the control tubes.

All the tubes were then incubated at 37 °C for 90 minutes and observed for clot lysis. After incubation, the fluid was removed and tubes were again weighed to observe the difference in weight after clot disruption. The difference obtained in weight taken before and after clot lysis was expressed as the percentage of clot lysis which was computed using the following formula:

% of clot lysis = (weight of released clot/weight of clot before treatment) × 100

***In-vitro* Membrane Stabilizing Activity: Preparation of Test Sample for Membrane Stabilizing Activity:** Different concentrations of the methanolic extract of *L. sarmentosa* (2, 4, 6, 8 and 10 mg/ml respectively) were prepared as the test samples for membrane stabilizing activity.

Red Blood Cells (RBC) Collection: 5 ml of whole blood was collected from healthy human volunteers in a test tube containing an anticoagulant (EDTA 2.2 mg/ml of blood) under standard conditions of temperature 23 ± 2 °C and relative humidity 55 ± 10%.

Preparation of Phosphate Buffer Solution: A pH of about 7.4 with buffer strength of 10 mM was obtained using 0.0352% monosodium phosphate dehydrate and 0.1099% disodium phosphate anhydrate. The buffer was made by adding 0.352 gm monosodium phosphate dehydrate and 1.099 gm disodium phosphate anhydrate to 1000 ml water.

Preparation of Isotonic Solution: A solution that has a concentration of electrolytes, non-electrolytes or a combination of the two that will exert equivalent osmotic pressure as that solution with which it is being compared. Either 0.16 M sodium chloride (NaCl) solution (approximately 0.95% salt in water) or 0.3 M non-electrolyte solution is approximately isotonic with human red blood cells. For the preparation of 500 ml isotonic solution of 154 mM strength, 4.5045 gm NaCl was added and mixed.

Preparation of Hypotonic Solution: A solution of lower osmotic pressure than that of a reference solution or an isotonic solution is called hypotonic solution. For the preparation of 500 ml hypotonic solution, having the strength of 50 mM, 1.4625 gm NaCl was added and mixed.

Erythrocyte Suspension: For the preparation of erythrocyte suspension, the collected RBC was centrifuged, supernatant was removed, and the blood cells were 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 rpm using the same volume as supernatant. Finally, it was resuspended in the same volume of this isotonic buffer solution.

Assessment of Membrane Stabilizing Activity: The *in-vitro* membrane stabilizing activity was assessed by two well-known methods [11, 12].

Hypotonic solution-induced haemolysis: 0.5 µl of erythrocyte suspension was mixed with 5 ml hypotonic solution (50mM NaCl) in 10 mM sodium phosphate buffer saline (pH 7.4) containing either the different concentrations of methanolic extract (2, 4, 6, 8 and 10 mg/ml respectively) or acetylsalicylic acid (0.10 mg/ml). Here, ASA was used as a reference standard. 0.5 ml of RBC was mixed with hypotonic buffered saline alone to consist of the control sample. The mixture was incubated for 10 min at room temperature, then centrifuged for 10 min at 3000 rpm and finally the optical density of supernatant was measured at 540 nm. The percentage (%) inhibition of hemolysis was calculated by the following equation:

$$\% \text{ inhibition of haemolysis} = \{(OD_{control} - OD_{test\ sample})/OD_{control}\} \times 100$$

Heat Induced-Hemolysis: Aliquots (5 ml) of the isotonic buffer, containing a different concentration of the methanolic extract 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 tubes were incubated at 54 °C for 20 min in a water bath. The other pair was maintained at 0-5°C in an ice bath. The reaction mixture was centrifuged for 3min at 3000rpm and the absorbance of the supernatant was measured at 540 nm using UV spectrophotometer. The percentage inhibition of hemolysis was computed using the following equation:

$$\% \text{ of haemolysis inhibition} = \{(OD_2 - OD_1)/ (OD_3 - OD_1)\} \times 100$$

Where, OD1 = test sample unheated; D2 = test sample heated and OD_3 = control sample heated.

Statistical Analysis: All the values in the test were expressed as mean ± standard deviation (SD). Each result were statistically analyzed for statistical significance using one-way analysis of variance (ANOVA) followed by Dunnett's t-test with the

Statistical Package for Social Sciences (SPSS 16.0, IBM, USA).

RESULTS AND DISCUSSION: The effects of *L. sarmentosa* on *in-vitro* clot lysis are tabulated in **Table 1**. It is observed that the percentage of clot lysis was 45.49% when 100 µl of SK (30,000 I.U.) was used as a positive control. On the other hand, compared to water as the negative control, the percentage of clot lysis was negligible (6.06%). So, the percentage of clot lysis was dose dependant, and 10mg/ml concentration showed the most significant ($p<0.001$) lysis of clot (22.57%).

TABLE 1: *IN-VITRO* THROMBOLYTIC ACTIVITY OF METHANOLIC EXTRACT OF *L. SARMENTOSA* IN CLOT LYSIS

Treatment	The weight of vial alone	Weight of clot containing a vial	Weight of clot containing vial after clot disruption	% of clot lysis (mean ± S.D)	P- value
ME (2mg/ml)	0.8268	0.1305	0.1249	4.29 ± 0.046	< 0.16
ME (4mg/ml)	0.8318	0.1498	0.1344	10.28 ± 0.074	< 0.08
ME (6mg/ml)	0.8259	0.1640	0.1396	14.87 ± 0.086	< 0.07
ME (8mg/ml)	0.8134	0.1579	0.1293	18.11 ± 0.006**	< 0.01
ME (10mg/ml)	0.8267	0.1639	0.1269	22.57 ± 0.009**	< 0.01
Blank (water) and Streptokinase					
Treatment	**W1**	**W2**	**W3**	**% of lysis**	**P- Value**
SK	0.8275	0.3833	0.2089	45.49 ± 0.068***	< 0.001
Blank	0.8167	0.3263	0.3066	6.03 ± 0.077	

All values are expressed as mean ± SD, (n=6). *** P<0.001and ** P<0.01 significant compared to control (ANOVA followed by Dunnett's t-test).

The membrane stabilizing activities of the methanolic extract of *L. sarmentosa* are presented in **Table 2** and **3**. The higher concentration (10 mg/ml) of methanolic extract produced the most significant % inhibition of hemolysis respectively, by hypotonic solution (12.11 ± 0.037) and heat (20.55 ± 0.87). In contrast, ASA used as standard (0.10 mg/ml) in membrane stabilization, revealed 70.01 ± 0.017% inhibition of hypotonic solution-induced hemolysis and 56.32 ± 0.228% inhibition of heat-induced hemolysis correspondingly.

TABLE 2: HYPOTONIC SOLUTION-INDUCED HAEMOLYSIS OF METHANOLIC EXTRACT OF *L. SARMENTOSA*

Treatment	Concentration	The optical density of samples in hypotonic solution (Mean ± SD)	% inhibition of hemolysis
Control	---	3.698 ± 0.0053	
ME	2 mg/ml	3.396 ± 0.0037	8.16 ± 0.049
ME	4 mg/ml	3.357 ±0.004	9.22 ± 0.036
ME	6 mg/ml	3.325 ± 0.0019	10.08 ± 0.044
ME	8 mg/ml	3.287 ± 0.0027*	11.11 ± 0.029
ME	10 mg/ml	3.250 ± 0.019**	12.11 ± 0.037
ASA	0.10 mg/ml	1.109 ± 0.0045***	70.01 ± 0.017

All values are expressed as mean ± SD, (n=6). *** P < 0.001, ** P < 0.01 and * P < 0.05 significant compared to control (ANOVA followed by Dunnett's t-test).

TABLE 3: HEAT INDUCED-HAEMOLYSIS EFFECT OF METHANOLIC EXTRACT OF *L. SARMENTOSA*

Treatment	Concentration	OD of sample ± SD		% inhibition of hemolysis
		Heated solution	Unheated solution	
Control	- - -	1.093 ± 0.035		
ME	2 mg/ml	0.879 ± 0.107	0.856 ± 0.011	9.7 ±.069%
ME	4 mg/ml	0.780 ± 0.004	0.743 ± 0.017	10.57 ±.043%
ME	6 mg/ml	0.715 ± 0.014	0.650 ± 0.022	14.67 ±.062%
ME	8 mg/ml	0.587 ± 0.082	0.461 ± 0.342*	19.93 ±.036%
ME	10 mg/ml	0.405 ± 0.008	0.227 ± 0.021**	20.55 ±.087%
ASA	0.10mg/ml	0.672 ± 0.025	0.129 ± 0.029***	56.32 ± 0.228%

All values are expressed as mean ± SD, (n=6). *** P < 0.001, ** P < 0.01and * P < 0.05 significant compared to control (ANOVA followed by Dunnett's t-test).

Flavonoids and terpenoids are compounds effective in treating acute inflammation whereas steroids and glycosides are the constituents that are effective in the chronic inflammation [13]. These phyto-compounds are well recognized for anti-inflammatory possessions as well as inhibition of pain perception which has previously reported in the extract of *L. sarmentosa*. Flavonoids are a well-known compound for the analgesic, anti-inflammatory and antipyretic activities [14]. So, it is anticipated that the present studies will stimulate further efforts towards the development of new, safe, more effective and urgently needed medications with lesser side effects for the treatment of fever, pain, and inflammatory diseases.

CONCLUSION: This study corroborated that the methanolic extracts of *L. sarmentosa* at higher concentration have moderate *in-vitro* thrombolytic and membrane stabilizing activities. Further investigations are required to know their detailed underlying mechanism.

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CONFLICT OF INTEREST: Nil

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