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Md. Hossan sakib
Department of Pharmacy, University of Science and Technology Chittagong, Bangladesh.

Muhammad Sazzad Hossain
Department of Pharmacy, University of Science and Technology Chittagong, Bangladesh.

Sadequr Rahman
Department of Pharmacy, International Islamic University Chittagong, Bangladesh.

Ahsan Ullah
Department of Pharmacy, International Islamic University Chittagong, Bangladesh.

Mohammad Shahin Alam
Department of Pharmacy, University of Science and Technology Chittagong, Bangladesh.

Limon Kanti Shill
Department of Pharmacy, International Islamic University Chittagong, Bangladesh.

Md. Yasin Sarkar
Department of Pharmacy, International Islamic University Chittagong, Bangladesh.

Correspondence
Md. Hossan sakib
Department of Pharmacy, University of Science and Technology Chittagong, Bangladesh.

Investigation of *in-vitro* Cytotoxicity and Thrombolytic activity of methanolic extract of *Sida acuta* (flower)

Md. Hossan sakib, Muhammad Sazzad Hossain, Sadequr Rahman, Ahsan Ullah, Mohammad Shahin Alam, Limon Kanti Shill, Md. Yasin Sarkar

Abstract
The present study is to investigate in-vitro Cytotoxicity, Thrombolytic activity, of methanolic extract of *Sida acuta* (flower).Method: Methanol extract of *Sida acuta* (flower), was used to evaluate its cytotoxicity in Brine shrimp lethality bioassay where vincristine sulphate was used as standard drug. Thrombolytic effect of the extract was investigated in clot lysis experiment.. Result: In Brine shrimp lethality bioassay, LC50 value of the extract was 67.5µg/ml and vincristine sulphate served as the positive control showed LC50 value 14.55µg/ml. The extract exerted 24.786% lysis of the blood clot in thrombolytic activity test while 80.22%. And 7.5% lyses were obtained for positive control (streptokinase) and negative control respectively. Conclusion: Compared to vincristine sulphate, it is evident that the methanol extract of leaves of *Sida acuta were* cytotoxic. The extract again possessed considerable thrombolytic activity.

Keywords: *Sida acuta,* Cytotoxic Activity, Thrombolytic Activity, Vincristine, Streptokinase (SK),

Introduction
The traditional medicine has been the focus for wider coverage of primary health care delivery all over the world [1]. World Health Organization (WHO, 1978) defined traditional medicine as the sum total of knowledge or practices whether explicable or inexplicable used in diagnosing, preventing or eliminating a physical, mental or social disease, which may rely exclusively on past experience or observation handed down from generation to generation, verbally or in writing. Cytotoxicity is the nature of being harmful to cells. Cells presented to a cytotoxic compound can react in various ways. The cells may undergo necrosis, in which they lose membrane integrity and die rapidly as a result of cell lysis; they can stop growing and dividing; or they can activate a genetic program of controlled cell death, termed apoptosis [2]. Cells undergoing necrosis typically exhibit rapid swelling, lose membrane integrity, shut down metabolism, and release their contents into the environment upon lysis. Apoptosis is characterized by well-defined cytological and molecular events, including a change in the refractive index of the cell, cytoplasmic shrinkage, nuclear condensation, and cleavage of DNA [3]. Cytotoxicity assays are used widely in drug discovery research to help predict which lead compounds might have safety concerns in humans before significant time and expense are incurred in their development. Other researchers study mechanisms of cytotoxicity as a way to gain a better understanding of the normal and abnormal biological processes that control cell growth, division, and death [4].

A blood clot (thrombus) developed in the circulatory system due to failure of hemostasis causes vascular blockage and while recovering leads to serious consequences in atherothrombotic diseases such as myocardial or cerebral infarction, at times leading to death [5]. Tissue plasminogen activator (t-PA), Urokinase (UK), streptokinase (SK) etc. is used as thrombolytic agents for the treatment of these diseases. SK and UK are widely used due to lower cost, [6, 7]. as compared to other thrombolytic drugs; their use is related with hyper risk of hemorrhage, severe anaphylactic reaction and lacks specificity [8]. Again, as a result of immunogenicity multiple treatments with SK in a given patient are restricted [9]. Herbal preparations have been used since ancient times for the treatment of several diseases. Herbs and their components possessing antithrombotic activity have been reported before. Herbal products are often perceived as safe because they are "natural" [10]. In this research investigation of thrombolytic activity of *Sida acuta* has included.

2. Materials and Methods

2.1 Preparation of extract

The fresh flowers of *Sida acuta* plant was washed with fresh water after collection. The collected flowers were chopped into small pieces; air dried at room temperature for about 15 days and ground into powder form with the help of grinder and stored in air tight container or vessel and keep in cool and dry place [11]. One hundred gram of the dried powder was taken in two washed jar or conical flax. After that solvent (which extract to be prepared) (95%) (500 ml) was poured into the jars up to 1 inch height above the sample surface as it can sufficiently cover the sample surface. The plastic cover was closed properly with aluminum foil to resist the entrance of air into the jars. The extraction was carried out for ten days with occasional stirring and shaking. Then the plant extract was filtered by a piece of clean white cotton material two times and then through Whitman paper. The filtrate was collected in a beaker. After filtration, the residue was taken for re-extraction in jar and it was extracted with 250ml 95% of that solvent for 10 days. The jar was shaken several times during the process to get better extraction. Then filtration was performed in the same way as described earlier. The filtrate thus obtained was evaporated under reduced pressure within 50-55C through rotatory vacuum evaporator. The concentrated extracts were collected in a petri dish and allow to air dry for complete evaporation of solvents. The whole process was repeated a few times. Finally a respective amount of solvent extract was prepared for experiment [12].

2.2 In-vitro Cytotoxic Study

Brine shrimp lethality bioassay is widely used in the bioassay for the bioactive compounds. Here simple zoological organism (*Artemia salina*) was used as a convenient monitor for the screening. The dried cyst of the brine shrimp were collected from an aquarium shop (Chittagong, Bangladesh) and hatched in artificial seawater (3.8% NaCl solution) with strong aeration for 48 hours day/dark cycles to mature shrimp called naupli. The cytotoxicity assay was performed on brine shrimp naupli using Meyer method [13].

2.3 Materials

Artemia salina Leach (brine shrimp eggs),Sea salt non ionized NaCl, Small tank with perforated dividing dam to hatch the shrimp, Lamp to attract the naupli, Pipette (1 ml and 5 ml), Micropipette (1-10 micro liter), Glass vials (5ml), Magnifying glass, Test sample for experimental plants [13].

2.4 Hatching of Brine Shrimp Eggs

Artemia salina Leach (brine shrimp eggs) collected from the pet shop was used as the test organism. Simulated sea water was taken in the small tank and the shrimp eggs (1.5gm/L) were added to one side of the tank and this side was covered. The shrimps were allowed to one side of tank and this side was covered. The shrimp were allowed for two days to hatch and mature as naupli (larvae). Constant oxygen supply was carried out during the hatching time. The hatched shrimps were attracted to the lamp on the other side of the divided tank through the perforated dam. These naupli were taken for this bioassay [14].

2.5 Preparation of the Simulated Sea Water

38 grams sea salt was weighted accurately, dissolved in 1 liter of sterilized distilled water and then filtered to get clear solution. The p^H of the sea water was maintained between 8-8.5 using 1N Na0H solution [15].

2.6 Preparation of Sample Solution

At first take 19ml distilled water in beaker add 1ml DMSO (dimethyl sulfoxide) thus prepares stock solution. Clean test tubes were taken. These test tubes were used for different concentration (one test tube for each concentration) of test samples. 4 mg methanolic extracts of *Sida acuta* were accurately weighed and dissolved in 4ml stock solution. Thus a concentration of 1000 µg/ml was obtained which used as an extract solution. Then taking 1ml extract solution from beaker & add 9ml stock solution In vials thus prepared final extract solution. From this extract solution 2.5 µg/ml, 5µg/ml, 10µg/ml, 20µ/ml, 40µg/ml, 60µg/ml, 30µg/ml, and 80µg/ml were taken in ten test tubes respectively and adjusted volume 5 ml sea water. Finally 10 naupli are then applied in each test tube [15].

2.7 Preparation of Control group

Control groups are used in cytotoxicity study to validate the test method and ensure that the results obtained are only due to the activity of the test agent and the effects of the other possible factors are nullified. Usually two types of control groups are used- i) Positive control ii) Negative control [16].

2.8 Preparation of Positive Control group

Positive control in cytotoxicity study is a widely accepted cytotoxic agent and the result of the test agent is compared with the result obtained for the positive control. In the present study, vincristine sulphate was used as the positive control. 3 mg of vincristine sulphate was dissolved in 1.8 ml of distilled water to get a concentration of 5 mg/ml. This was used as stock solution of vincristine sulphate. With the help of a micropipette 500,300,100, 50 and 10 µl of the stock solution were transferred in 6 different vials. NaCl solution (brine water) was added to each vial making the volume up to 5 ml. The final concentration of vincristine sulphate in the vials became 500µg/ml, 300µg/ml, 100µg/ml, 50µg/ml and 10µg/ml respectively. The experiment was repeated three times [16].

2.9 Preparation of negative control

100 µl of distilled water, DMSO and ethanol was added to each of the three remarked glass vials containing 5 ml of simulated sea water and 10 shrimp naupli to use as control groups. If the brine shrimp naupli in these vials show a rapid mortality rate, then the test is considered as in valid as the naupli died due to some reason other than the cytotoxicity of the samples [16].

2.10 Application of Brine shrimp Naupli

With the help of the Pasteur pipette 15 living naupli were added to each of the vials containing 5 ml of simulated sea water. A magnifying glass was used for convenient count of naupli. If the counting of the 15 naupli was not be possible accurately [17].

2.11 Counting of the Naupli

After 24 hours, the vials are observed using a magnifying glass and the number of survival nauplii in each vial were counted and recorded. From this data, the percentage of mortality of nauplii was calculated for each concentration of the sample. The median lethal concentration (LC_{50}) of the test samples was obtained by a plot of percentage of the shrimps killed against the logarithm of the sample concentration [17].

2.12 In-Vitro Thrombolytic Study
Thrombolysis is the breakdown (*lysis*) of blood clots by pharmacological means. It is colloquially referred to as *clot busting* for this reason. It works by stimulating fibrinolysis by plasmin through infusion of analogs of tissue plasminogen activator (tPA), the protein that normally activates plasmin [18].

2.13 Preparation of Extract Solution for Thrombolytic Test
10 mg of the extract was suspended in 10ml distilled water and shaken vigorously on a vortex mixer. Then the suspension was kept overnight and decanted to remove the soluble supernatant, which was filtered through a filter paper (Whatman No. 1) the solution, was then ready for *in vitro* evaluation of clot lysis activity [19].

2.14 Preparation of Streptokinase (SK) Solution
To the commercially available lyophilized SK vial (Polamin Werk GmbH, Herdecke, Germany) of 1,500,000 I.U. 5 ml sterile distilled water was added and mixed properly. This suspension was used as a stock from which 100 µl (30,000 I.U) was used for *in vitro* thrombolysis [19].

2.15 Specimen of Thrombolytic Test
3ml blood was drawn from healthy human volunteers without a history of oral contraceptive or anticoagulant therapy (using a protocol approved by the Institutional Ethics Committee of Central India Institute of Medical Sciences, Nagpur). 500 µl of blood was transferred to each of the ten previously weighed alpine tubes to form clots [19].

2.16 Test Procedure for Thrombolytic test
Experiments for clot lysis were carried as reported earlier [19]. Venous blood drawn from healthy volunteers was transferred in different pre-weighed sterile Epen drop tube (500µl/tube) and incubated at 37°C for 45 minutes. After clot formation, serum was completely removed (aspirated out without disturbing the clot formed).Each tube having clot was again weighed to determine the clot weight (Clot weight = weight of clot containing tube – weight of tube alone).Each Epen drop tube containing clot was properly labeled and 100 µl of plant extract was added to the tubes. All the tubes were then incubated at 37°C for 90 minutes and observed for clot lysis. After incubation, fluid obtained was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage. Thrombolytic Activity of methanolic extract of *Sida acuta* flowers clot lysis. Streptokinase and water were used as a positive and negative (non-thrombolytic) control respectively. The experiment was repeated several times with the blood samples of different volunteers.

3. Results and Discussions

Brine Shrimp Lethality Bioassay
Brine shrimp lethality results of the fraction of *Sida acuta* flowers are shown in Figure 1 and LC_{50} calculated value is recorded in Table 1. The fraction showed potential cytotoxic activity with LC_{50} value of 67.5µg/ml. Vincristin sulphate served as the positive control for this brine shrimp lethality assay and its LC_{50} value was 14.55µg/ml.
Toxicity of plant materials is a major concern to scientists and medical practitioners and therefore cytotoxic assay was conducted in this study to determine the toxicity profile of the plant extracts through the Brine Shrimp Lethality (LC_{50}, 24 h) test. Lagarto demonstrated a good correlation (r^2 = 0.91) between the LC_{50} of the brine shrimp lethality test and the acute oral toxicity assay in mice. Based on that correlation, brine shrimp lethality LC_{50} < 10 µg/ml (LD_{50} between 100 and 1000 mg/kg) is considered as the cut off value of cytotoxicity. According to the measured LC_{50} values of the extracts no one was found severely lethal or toxic to be processed as pharmaceutical products in thrombolytic uses. However, the extremely significant effect of *Sida acuta* demonstrates it to be the best cytotoxic component for further processing.

Thrombolytic Activity
The methanolic extract of *Sida acuta* flowers is exerted 24.79% lysis of the blood clot in thrombolytic activity test while 80.22% were obtained for positive control (streptokinase) and 7.5% were obtained for negative control respectively which showed in table 2. So, the extract possessed considerable thrombolytic activity.
In our thrombolytic assay, the comparison of positive control with negative control clearly demonstrated that clot dissolution does not occur when water was added to the clot. When compared with the clot lysis percentage obtained through SK and water, an extremely significant thrombolytic activity was observed after treating the clots with *Sida acuta,* methanol fraction. Cell surface bound plasminogen is easily activated to plasmin, which could lead to fibrinolysis. Bacterial plasminogen activator: staphylokinase, streptokinase, act as cofactor molecules that contribute to exosite formation and enhance the substrate presentation to the enzyme. Staphylokinase activates plasminogen to dissolve clots, also destroys the extracellular matrix and fibrin fibers that hold cells together.

3.1 Tables & Figures

Table 1: Cytotoxic activity of *Sida acuta*

Concentration µg/ml	Naupli no	Live naupli	Log C	% of Mortality	LC 50
2.5	10	10	0.397	0	
5	10	9	0.698	10	
10	10	9	1	10	
20	10	8	1.301	20	**67.5**
40	10	7	1.602	30	
60	10	5	1.778	50	
80	10	3	1.903	70	

Table 2: Thrombolytic activity of Sida *acuta flowers* extract

Group	Percent of lysis
Streptokinase	80.22%
Sida acuta	24.786%
water	7.5%

Fig 1: Determination of LC_{50} value for extract of *sida acuta* flowers from linear correlation between logs of concentrations (Log C) versus percent (%) of mortality.

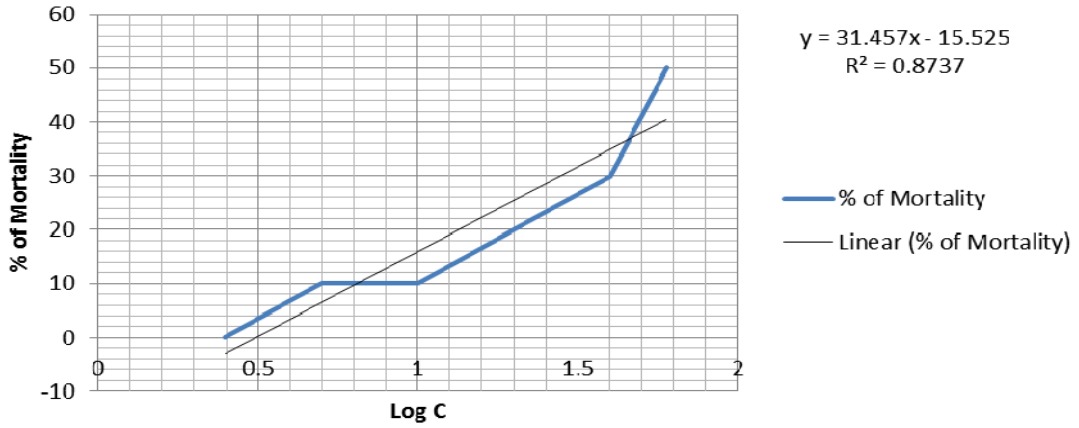
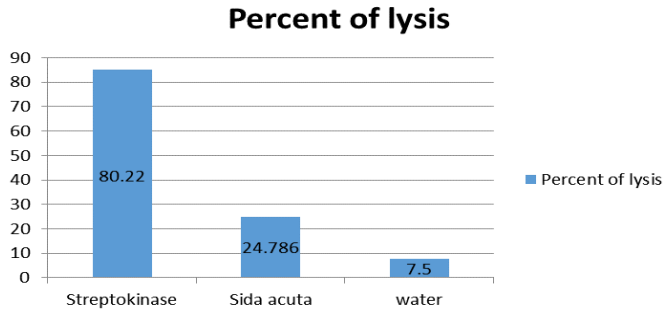


Fig 2: Thrombolytic activity of Sida *acuta flowers* extract



4. Conclusion

From the above study it can be concluded that the methanolic extract of *Sida acuta* may be a potential candidate for future thrombolytic agent. Furthermore study and isolation is needed to obtain site specific and more potent agent that causing this effect. The test was made under full concentration to develop a new compound. I found that the extract I choose, was quite good in use. At the conclusion I can recommend that this plant part is useful for further use and isolation. The thrombolytic and cytotoxic study was close to the standard used. The thrombolytic potency of *Sida acuta* is found 48.87% and the standard have 80.22%. It seems good result or may be said significant as the extract was the mixture of many phytochemical, it shows nearby percent of clot lysis. The cytotoxic result obtained 67.5µg/ml (LC_{50}) it so good, but proper isolation can make it more potent and useful. So, it could be suggested to modify for site specific use.

Here experimental studies of flowers extract exhibited considerable thrombolytic and cytotoxic activity and moderate activity. So, further comprehensive pharmacological and phytochemical investigations are needed to elucidate the specific chemical compounds responsible for cytotoxic and thrombolytic activities and their mode of actions. The long term toxic effect and its protective effects should also be elucidated.

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