



Comparative Study of Cytotoxic and Thrombolytic Effects of *Flemingia stricta* and *Nymphaea nouchali* Leaves

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Abstract To evaluate comparative cytotoxic and thrombolytic activity of crude methanol extract of *F. stricta* and *N. nouchali* leaf. The screening of cytotoxic activity was done using brine shrimp lethality bioassay while the thrombolytic activity was evaluated using the *in vitro* clot lysis model. In a brief, venous blood drawn from five healthy volunteers was allowed to form clots which were weighed and treated with the test plant materials to disrupt the clots. Weight of clot after and before treatment provided a percentage of clot lysis and compared the result with streptokinase as positive control and water as negative control. Moderate cytotoxicity was found for both the methanol extracts and it is compared with the standard drug vincristine sulfate in the brine shrimp bioassay. In the present study, the LC₅₀ values of the methanol crude extract of *F. stricta*, *N. nouchali* and vincristine sulfate were 252.31, 229.94 and 12.59 μg/mL, respectively and in case of thrombolytic model, both the extract *F. stricta* and *N. nouchali*. In thrombolytic study, it is found that *F. stricta* and *N. nouchali* showed 34.78 ± 1.88% and 20.45 ± 2.13% of clot lysis respectively. Among the herbs studied *F. stricta*, showed very significant (p < 0.001) percentage (%) of clot lysis than the *N. nouchali* compared to reference drug streptokinase (63.54 ± 2.61%). The results of the study demonstrated that the leaf of the plants possess promising thrombolytic activity *in vitro* when tested on human blood as well as preliminary cytotoxic activity on Brine shrimp.

Keywords *Flemingia stricta*, *Nymphaea nouchali*, cytotoxicity, Thrombolytic, streptokinase, Brine shrimp

Introduction

Flemingia stricta Roxb. (Fabaceae) is an erect subshrub, distributed in southeast Asian country such as- Bangladesh, Bhutan, China, India, Indonesia, Laos, Myanmar, Philippines, Thailand and Vietnam [1]. In Bangladesh, it is available in Chittagong, Chittagong Hill Tracts and Sylhet. It is known as Charchara (in Bangla) and Krangdunaduepay, SaiKheu (Marma); Keramkana (Tripura); Tamatamaking (Khumi) and Harsanga, Khaskura, Uskura (Chakma) in local tribes of Chittagong, Bangladesh [2]. The species is used to treat bone fracture, cough, goiter and polio, asthma, and polio [3-5]. *Nymphaea nouchali* (Nymphaeaceae) is another important medicinal plant which is also known as water lily (<http://ethnobotanybd.com>), distributed in Bangladesh, Australia, Afghanistan, China, Egypt, India, Indonesia, Malaysia, Myanmar, Nepal, Pakistan, Philippines, South Africa, Sri Lanka, Thailand, Vietnam, Zimbabwe (<http://www.iucnredlist.org>). In Bangladesh it is known as Sapla, Sada Sapla, and Shaluk. Traditionally it is also known as Bibalchak (Mandi), Gechhak-afluk (Mandi), Aphlak (Garo) in local tribes of Bangladesh [6]. Roots and rhizomes of this plant contain protein, tannic and gallic acids, starch, gum, resin, glucosides and the alkaloids, nupharine and nymphaeine. Leaves contain flavone glucoside, myricitrin, tannic acid,



phytosterin, steroids and flavonoids. Flowers contain a cardiac glycoside, nymphalin having digitalis-like action. Flowers and rhizome also yield two alkaloids, both showing sedative action in small doses [7]. Powdered rhizomes are demulcent and diuretic; used in piles, dysentery and dyspepsia. Flowers are astringent, cardiotoxic and refrigerant; alleviative of cough, bile, vomiting, giddiness, worms and burning of the skin. Filaments are astringent and cooling; useful in burning of the body, bleeding piles and menorrhagia. Seeds are used as a cooling medicine in cutaneous diseases [8]. Rhizome paste is used for menstruation. The dried rhizome powder is used for dyspepsia (Garro) [9]. Rhizome paste is used to treat menstruation problem (Mandi) [10]. The literature survey revealed that there are no scientific studies carried out regarding the cytotoxic and thrombolytic activity, thus the experiment was designed to evaluate the comparative *in vitro* cytotoxic, thrombolytic activity of *F. stricta* Roxb and *N. nouchali* leaf extract.

Materials and Methods

Plant collection and identification

Whole plants of *F. stricta* and leaf of *N. nouchali* were collected from different parts of Chittagong region, Bangladesh. The plants were identified by Dr. Shaikh Bokhtear Uddin, Taxonomist and Professor, Department of Botany, University of Chittagong.

Chemicals and drugs

To the commercially available lyophilized Streptokinase (SK) vial (Durakinase, Dongkook Phama. Co. Ltd, South Korea) of 15 00000 I.U., 5 ml sterile distilled water was added and mixed properly. This suspension was used as a stock from which 100 μ l (30,000 I.U) was used for *in vitro* thrombolysis. Absolute ethanol (99.50%) and vincristine sulfate (VS) were purchased from Sigma-Aldrich, Munich, Germany.

Extract preparation

Both of the plant materials were dried and ground (Moulinex Blender AK-241, Moulinex, France) into powder (40-80mesh, 500 g) and soaked for 7 days with 2–3 days interval in 2.0 L of ethanol at room temperature ($23 \pm 0.5^\circ\text{C}$). Filtrate obtained through cheesecloth and Whatman filter paper No. 1 was concentrated under reduced pressure at the temperature below 50°C using rotary evaporator (RE 200, Sterling, UK). The extracts (yield 4.4–5.6% W/W) were all placed in glass Petri dishes (90 X 15 mm, Pyrex, Germany). A 100 mg each of the extracts was suspended in 10 ml distilled water and the suspension was shaken vigorously on a vortex mixer. The suspension was kept overnight and decanted to remove the soluble supernatant, which was filtered through a 0.22- μ m syringe filter. A 100 μ l of this aqueous preparation was added to the microcentrifuge tubes containing the clots to check thrombolytic activity. The same concentration (10 mg/ml) of extracts was prepared for screening the cytotoxic properties.

Cytotoxicity screening

Cytotoxicity of the methanol extracts were evaluated by the brine shrimp lethality bioassay, which is widely used for screening bioactive compounds [11-12]. In this study, a simple zoological organism (*Artemia salina*) was used as a convenient monitor for the screening. The eggs of the brine shrimp were collected from an aquarium shop (Dhaka, Bangladesh) and hatched in artificial seawater (3.8% NaCl solution) for 48 h to develop into larval shrimp called nauplii. The cytotoxicity assay was performed on the brine shrimp nauplii using the Meyer method. The test samples (extract) were prepared by dissolving them in DMSO (not more than 50 μ L in 5 mL solution) plus seawater (3.8% NaCl in water) to attain concentrations of 10, 50, 100, 150, 200 and 300 μ g/mL-1. A vial containing 50 μ L DMSO diluted to 5 mL was used as a control. Standard vincristine sulfate was used as a positive control. Mature shrimps were placed into each of the experimental vials. After 24 h, the vials were inspected using a magnifying glass, and the number of surviving nauplii in each vial was counted. From these data, the percent (%) of lethality of the brine shrimp nauplii was calculated for each concentration using the following formula:

$$\% \text{ Mortality} = \frac{N_t}{N_o} \times 100\%$$

Where N_t = Number of dead nauplii after a 24-h incubation;



N_0 = Number of total nauplii transferred i.e., 10.

The LC_{50} (median lethal concentration) was determined from the log concentration versus % mortality curve.

Thrombolytic activity

Blood specimen

Whole blood (2 ml) was drawn from healthy human volunteers ($n = 5$) without a history of oral contraceptive or anticoagulant therapy. A 500 μ l of blood was transferred to each of the three previously weighed microcentrifuge tubes to form clots.

Clot lysis

Experiments for clot lysis were carried as reported earlier [13]. Briefly, 2 ml venous blood drawn from the healthy volunteers was distributed in three different pre weighed sterile microcentrifuge tube (0.5 ml/tube) and incubated at 37°C for 45 min. After clot formation, 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 microcentrifuge tube containing pre-weighed clot, 100 μ l of organic extracts of both plants (*F. stricta* and *N. nouchali*) were added separately. As a positive control, 100 μ l of SK and as a negative non-thrombolytic control, 100 μ l of distilled water were separately added to the control tubes numbered. All the tubes were then incubated at 37°C for 90 min and observed for clot lysis. After incubation, fluid released was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference in weight before and after clot lysis was expressed as percentage of clot lysis as shown below:

$$\% \text{ of clot lysis} = (\text{weight of released clot} / \text{clot weight}) \times 100$$

The experiment was repeated with the blood samples of the 5 volunteers.

Statistical analysis

The significance between % clot lysis by SK and plant extracts was tested by the paired t-test analysis using the software SPSS, version 20.0 (SPSS for Windows, Version 20.0, IBM Corporation, New York, USA). Data are expressed as mean \pm standard deviation. The mean difference between positive and negative control was considered significant at $P < 0.05$.

Results

Brine shrimp lethality bioassay

Following the procedure of Meyer, the lethality of the methanol crude extract of *F. stricta* and *N. nouchali* leaves were determined on *Artemia salina* after sample exposure for 24 h. The negative control (vehicle only) and vincristine sulfate (positive control) were also used to compare the toxic activities of the extracts. This technique was applied to determine the general toxicity of the plant extract. Percent mortality of brine shrimp at six different concentrations (10 to 500 μ g/mL) of the extracts has been presented in Table 1. From Figure 1, it is clear that the % mortality is directly proportional to the extract concentrations. LC_{50} values of the methanol extract of *F. stricta* and *N. nouchali* obtained in the present experiment were 252.31 and 229.94 μ g/mL respectively. Therefore, the *N. nouchali* demonstrated greater toxicity compared with *F. stricta*. The LC_{50} value for the standard drug vincristine sulfate was 12.59 μ g/ml. However, no mortality was obtained for the negative control group.

Table 1: % of mortality of both the extract at six concentrations

Concentration(μ g/ml)	LogC	% of mortality		
		FS	NN	Vincristine sulphate
10	1	10	0	40
50	1.699	10	10	80
100	2	30	30	100
200	2.301	40	60	100



300	2.477	60	70	100
500	2.699	90	90	100
LC ₅₀		252.31	229.94	12.59

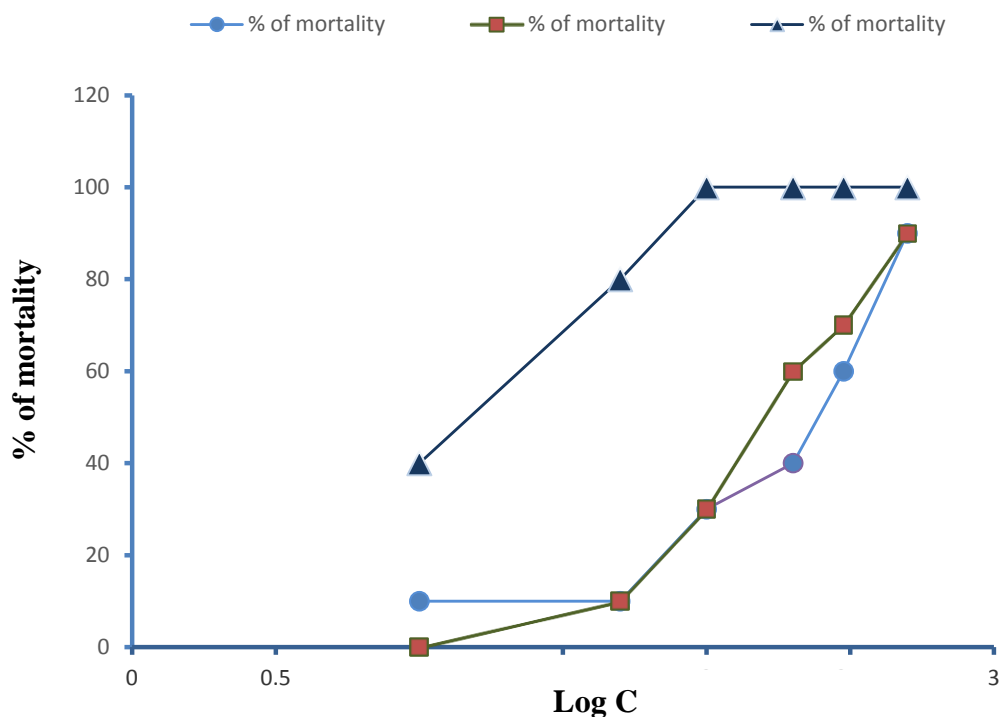


Figure 1: Brine shrimp lethality bioassay. Determination of LC₅₀ values for methanol extract *F. stricta* and *N. nouchali* leaves from linear correlation between log concentrations versus % of mortality.

Thrombolytic activity

Addition of 100 µl SK (positive control) to the clots along with 90 min of incubation at 37°C, showed 63.54 ± 2.61% clot lysis. However, distilled water (negative control) treated-clots showed only negligible clot lysis (4.21 ± 0.73%). The mean difference in clot lysis percentage between positive and negative control was very significant (p value < 0.05). Treatment of clots with *F. stricta* and *N. nouchali* extracts provided the clot lysis 34.78 ± 1.88% and 20.72 ± 2.21%, respectively. The mean percentage of clot lysis by *F. stricta* and *N. nouchali* was statistically very significant (p value < 0.001). *F. stricta* showed relatively higher percentage of clot lysis than the *N. nouchali* although the values were significant (p value < 0.001) compared to those of both positive control SK and negative control water. Percent clot lysis obtained after treating the clots with both organic extracts and appropriate controls is shown in Figure 2.

Table 2: Effect of both extracts (10 mg/ml) on in-vitro clot lysis

Treatment	% of clot lysis (Mean ± S. D.)
Streptokinase(Positive Control)	63.54 ± 2.61*
Distilled water (Negative Control)	4.21 ± 0.73*
FS	34.78 ± 1.88**
NN	20.72 ± 2.21**



Statistical representation of the effective clot lysis percentage by herbal preparations, positive thrombolytic control (Streptokinase) and negative control (sterile distilled water) done by paired t-test analysis; clot lysis% is represented as mean \pm S.D. ** $P < 0.001$, * $P < 0.05$ compared to control.

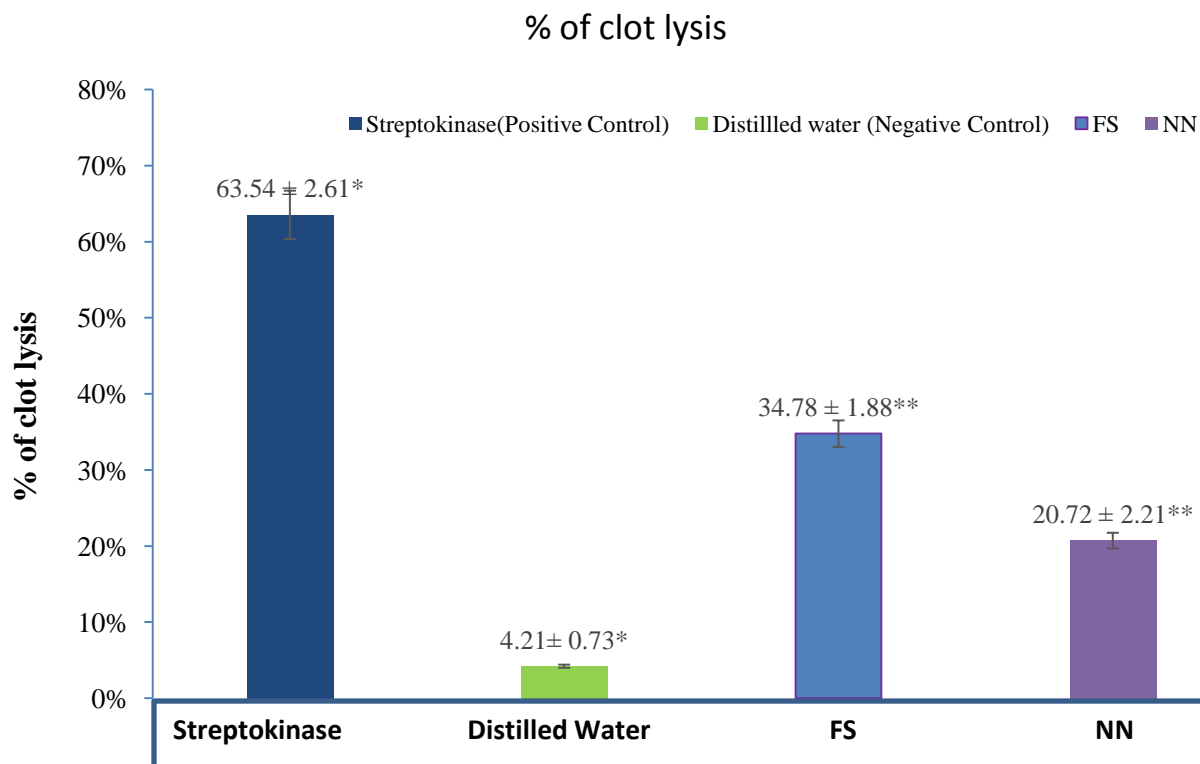


Figure 2: Clot lysis by streptokinase, water and both of organic extracts

Effects of drugs on dissolution of clots prepared from blood of normal individuals. Maximum clot lysis (63.54 \pm 2.61%) was observed in clot treated with streptokinase (SK). Both of the plant extracts show 34.78 \pm 1.88% and 20.72 \pm 2.21% clot lysis, respectively. Water (as a negative control) showed 4.21 \pm 0.73% clot lysis.

Discussion

Toxicity on plant materials is actually a main condition to experts and medical practitioners [14-16] and cytotoxic assay was conducted in this study to learn both the toxicity profile of plant extracts via the Brine Shrimp Lethality (LC_{50} , 24 h) check. Lagarto showed a great correlation ($r = 0.85$; $P < 0.05$) between the LC_{50} of brine shrimp lethality test and the severe oral toxicity assay in mice [17]. Based on that correlation, brine shrimp lethality $LC_{50} < 10$ μ g/ml (LD_{50} between 100 and also 1000 mg/kg) is measured as cutoff value on cytotoxicity [18-19]. In this current investigation, modest brine shrimp cytotoxicities were found for all extracts compared with the standard drug vincristine sulfate. However, these activities might be due to the presence of bioactive or inhibitory compounds or factors in the fractions or synergism by the existence of some compounds.

In thrombolytic assay, both the comparison on positive control to negative control actually demonstrated the clot. Compared to the clot lysis percentage obtained through SK and also water, an extremely significant (p value < 0.05) thrombolytic study was observed after treating the clots to *F. stricta* and *N. nouchali* extract. It is established which there are bacterial pollutants of plants that have plasminogen receptors which certain to plasminogen. Cell surface certain to plasminogen is instantly activated to plasmin that could lead to fibrinolysis [19]. Bacterial plasminogen activator: staphylokinase, streptokinase, act as cofactor molecules that cause exosite formation and improve the substrate performance towards the enzyme. Staphylokinase activates plasminogen to be able to break down clots,



also damages the extracellular matrix and fibrin particles that keep cell together [20-22]. Beneath context of over, it would be fascinating to investigate both the mechanism underlying clot lytic effects demonstrated by *F. stricta* and *N. nouchali* extract

However, these activities might be due to the presence of bioactive or inhibitory compounds or synergism by the existence of some compounds. Because a variety of constituents, such as saponin, tannin, polyphenols, flavonoids, and alkaloids, may be present in the extracts, further extensive investigations are required to determine the active cytotoxic and thrombolytic properties present in the leaf extracts.

Conclusion

In conclusion, both of the plant extract *F. stricta* and *N. nouchali* leaf possess moderate cytotoxic and blood clots lytic activity *in vitro*. However, *in vivo* clot dissolving property and active component(s) of *F. stricta* for clot lysis are yet to be investigated. Further work will find whether or not, phytochemicals derivative from this plant could be incorporated as a thrombolytic agent for the progress of the patients suffering from atherothrombotic diseases.

Acknowledgement

Authors wish to thank Botanist Dr. Shaikh Bokhtear Uddin, Associate Professor, Department of Botany, University of Chittagong, Bangladesh, who helped to identify the plant. The authors are also grateful to the Department of Pharmacy, International Islamic University Chittagong, Chittagong, Bangladesh, for providing research facilities.

Conflict of Interest Statement

We declare that we have no conflict of interest.

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