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BIOASSAY OF BRINE SHRIMP LETHALITY, CARDIOPROTECTIVE AND ANTI-INFLAMMATORY ACTIVITIES OF METHANOLIC LEAF EXTRACT OF *FICUS BENJAMINA*

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ABSTRACT

Brine shrimp lethality bioassay, clot lysis and hypotonicity induced membrane lysismethod was used to evaluate the cytotoxic,cardioprotective and anti-inflammatory activity of methanolicextract of *Ficus benjamnia*. The extract showed moderate cytotoxic activity. LC_{50} value of the extract was 232.71µg/ml, which was compared with vincristine sulphate (12.59µg/ml). Methanolic leaf extract of *Ficus benjamina* was treated with human blood to evaluate Cardioprotective effect. It showed promising Cardioprotective activity which was about (34.95± 1.98)% compared to streptokinase which was used as standard (63.54 ± 2.61)%. In the case of anti-inflammatory study, methanolic leaf extract showed (20.79±1.07)% and (58.06±1.03)%of membrane stabilization activity at 31.25µg/ml and 1000 µg/ml concentration respectively.

KEYWORDS: *Ficus benjamina*, Cardioprotective, anti-inflammatory, HRBC, brine shrimp, cytotoxicity, clot lysis.

INTRODUCTION

To find out medicament from the herb sauce is one of the human nature from the ancient period of time. This practice is now becoming prominent throughout the world. There is evidence that plants are still extensively used in ethno medicine in all of the continents. There are around 250,000 to 500,000 species of plants are available on Earth [1]. Only a small amount (1-10%) of them are used as food by both humans and animals. Therefore, it is easily anticipatedthat usage of plants in therapeutic practice and remedy purpose is very significant.In Bangladesh, thousands of plant species are known to have medicinal value[2] and 90% of medicinal plants are wild sourced[3].*Ficus benjamina* is a huge tree growing to 60 feet tall and 60-70 feet wide. The thick, shiny, 2-5 inch-long, evergreen leaves generously clothe the long branches, and the tiny figs eventually turn a deep red. Branches will weep to the ground forming a canopy so dense that nothing grows beneath it[4]. The Weeping fig of *Ficus benjamina*, also known as the Benjamin's fig and often sold in stores as just ficus, is a species of flowering plant in the family Moraceae, native to Asia and Australia.In Bangladesh, it is extensively found in Chittagong, Cox's Bazar and northern districts. In Malabar, a decoction of the leaves mixed with oil is applied to ulcers. The milky juice is used against whitening of the cornea by the mundas of Chota Nagpur. When a baby's eyes get white, they mix the juice with mother's milk and instill about two drops of the mixture in its eyes described by Yusuf *et al*. The objective of this present study is to evaluate the

cytotoxic, cardioprotective and anti-inflammatory activity of methanolic leaf extract of *Ficus benjamnia*.

MATERIALS AND METHODS

Plant collection and identification: Leaf of *Ficus benjamina* was collected from Chittagong, Bangladesh. The plantwas identified and authenticated by Dr. Shaikh Bokhtear Uddin, taxonomist and Associate Professor, Department of Botany, University of Chittagong.

Chemicals and drugs: Durakinase, DongkookPhama. Co. Ltd, South Korea provided the lyophilized streptokinase vial (1500000 IU) andsterile distilled water (5 ml) were added and mixed properly.Mixture of distilled water and lyophilized streptokinase was usedas a stock. From this mixture, 100 µL (30 000 IU) was used fordetermination of cardioprotective activity which means *in vitro*thrombolysis. Two more chemicals were purchased from Sigma-Aldrich (Munich, Germany), which were vincristine sulfate and99.5% absolute methanol. Besides,the chemicals used for the anti-inflammatory activity is Sodium Dihydrogen phosphate, Disodium hydrogen phosphate, Sodium Chloride, Dextrose, sodium citrate, citric acid was purchased from Sigma-Aldrich. All chemicals in this investigation were of analytical reagent grade.

Preparation of extracts: Leaves of *Ficus benjamina* was dried and ground into powder (40-80 mesh, 500 g) by using Moulinex Blender AK-241. Then powder was soaked in 2 L of methanol at room temperature [(23.0 ± 0.5) °C] for a week. Some filtrate was obtained by using Whatman filter paper No. 1 and cheese cloth. By using a rotary evaporator filtrated solution was concentrated under reduced pressure. In this filtration process less than 50°C was maintained. To keep the extract glass petri dishes were used. Each of the extracts (100 mg) was suspended in 10 mldistilled water. The suspension was shaken vigorously through a vortex mixer. Suspension of the extract and distilled water was kept overnight and gradually poured through a syringe having 0.22 µmfor the filtration. In this way, soluble supernatant was removed. Then 100 µL of this filtrated aqueous preparation was added to microcentrifuge tubes which contained the clots to check the *ex-vivo* cytotoxic activity. The same concentration (10 mg/ml) of plant extracts was prepared for the *ex-vivo* screening of cardioprotective effects as well as anti-inflammatory effects.

Cytotoxicity screening: To assess the cytotoxic effect of methanolic extract was performed by brine shrimp lethality bioassay, which is widelyused for bioactive compound screening[5]. Cytotoxicity of the methanol extract of *Ficus benjamina* was evaluated by the brine shrimp lethalitybioassay, which iswidely used for screening bioactive compounds. In this study, a simple zoological organism(*Artemiasalina*) was used as a convenient monitor for theexperiment. The eggs of the brine shrimp were collected from an aquarium shop (Dhaka, Bangladesh) and hatched in artificialseawater (3.8% NaCl solution) for 48 h to develop into larval shrimpcalled nauplii. The cytotoxicity assay was performed on the brine shrimp nauplii using the Meyer method.The testsamples (extract) were prepared by dissolving them inDMSO (not more than 50 µl in 5 ml solution) plus seawater (3.8%NaCl in water) to attain concentrations of 10, 50, 100, 200,300 and 500µg/ml. A vial containing 50 µl DMSO diluted to 5 ml was usedas a control.Standard vincristine sulfate was used as a positivecontrol. Mature shrimps were placed into each of the experimentalvials. After 24 h, the vials were inspected using amagnifying glass,and the number of surviving nauplii in each vial was counted. Fromthese data, the percentage lethality of the brine shrimp nauplii was calculated for each concentration using the following formula:

$$\% \text{ Mortality} = \frac{Nt}{No} \times 100\%$$

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

N_0 = Number of total nauplii transferred

The LC_{50} (median lethal concentration) was determined from the log concentration versus % mortality curve [6].

Cardioprotective activity

Blood sample: About 2ml of blood was drawn from six healthy human volunteers. They had no history of taking any types of contraceptive or anticoagulant therapy. Total 500 µL of blood was transferred to each of the six previously weighed microcentrifuge tubes to form clots.

Clot lysis: At first, six different sterile microcentrifuge tubes (0.5 ml/tube) were taken and weighed. Then 2 ml venous blood from human volunteers was added in pre-weighed sterile microcentrifuge tubes. The tubes were incubated at 37°C for 45 min. In this process,serum was totally eliminated after the formation of clots without disturbing the clots. Each tube containing clot was again weighed to know the weight of clot. For the

determination of clot weight, weight of the tube alone were excluded from the weight of clot and the tube. For each microcentrifuge tube containing pre-weighed clot, 100 µL of methanol extracts of plants were added separately. About 100 µL of streptokinase was used as a positive control and 100 µL of distilled water was used as a negative control. At last, all the tubes were incubated at 37°C for 90 min. Inthis way, clot lysis was observed. After incubation,released fluid was removed and tubes were again weighed to observe the difference in weight after clot disruption. The difference in weight before and after clot lysis was expressed as percentage of clot lysis[7,8]. Evaluation of thrombolytic effects of methanolic extracts was performed by the formula below:

Percent (%) of clot lysis = (Weight of releasing clot /clot weight) ×100

Anti-inflammatory activity

The human red blood cell (HRBC) membrane stabilization method: The principle concerned with this method is stabilization of human redblood cell membrane by hypotonicity induced membrane lysis. Theblood was collected (2 ml) from healthy human volunteer who hadnot taken any NSAIDS for 2 weeks prior to the experiment andmixed with equal volume of Alsever solution (2%dextrose, 0.8%sodium citrate, 0.5% citric acid and0.42% NaCl) and centrifuged at3,000 rpm. The packed cells were washed with iso saline and a 10%v/v suspension was made and kept at 4 °C undistributed before use.Various concentrations (31.25, 62.5, 125, 250, 500, 1000 µg/ml) of extracts were prepared in normal saline, diclofenac sodium asstandard with different concentrations (31.25, 62.5, 125, 250, 500,1000 µg/ml) and blood control (distilled water instead of hyposaline to produce 100% hemolysis) were separately mixed with 1 ml(0.15M) of sodium phosphate buffer, 2 ml of hyposaline and 0.5 mlof 10% HRBC suspension was added to prepared. Erythrocytesuspension was absent in drug control while drugs were omitted inblood control. All the assay mixture was incubated at 37^0C for 30min and centrifuged at 3000rpm for 20 min and hemoglobin contentof the supernatant solution was estimated spectrophotometrically at560 nm [9]. The percentage of HRBC membrane stabilization orprotection was calculated by using the following formula:

$$\% \text{ of membrane stabilization value} = \frac{100 - [(\text{Drug test value} - \text{Drug control value}) \times 100]}{\text{Blood control value}}$$

Where, the blood control represented 100% lysis.

Statistical analysis: Statistical significance between the percentage of clot lysis by streptokinase and plant extract was evaluated by paired *t*-test analysis. Expression data was expressed as mean ± SD. The mean difference between positive and negative controls was considered significant at $P < 0.05$ and $P < 0.001$. In the case of anti-inflammatory activity, the results were expressed as mean of the three repetitions and standard deviations were calculated. Statistical comparisons were made using the Independent t-test and $P<0.01$ and $P<0.05$ was considered as significant. The test was performed by using the software SPSS version 20.0 (SPSS for Windows, IBM Corporation, New York, USA).

RESULTS

Brine shrimp lethality bioassay: Following the procedure of Meyer, the lethality of methanolic crude extract of *Ficus benjamina* leaf was 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 effects of the extract. This technique was applied to determine the general toxicity of the plant extract. Percent mortality of brine shrimp at six different concentrations(10, 50, 100, 200, 300, 500) µg/ml of the extract has been presented in Table 1**.** From Table 1it is clear that the percentage of mortality is directly proportional to the extract concentrations. LC_{50} values of methanol extract of *Ficus benjamina* obtained in the present experiment were232.71µg/ml.The LC_{50} value for the standard drug vincristine sulfate was 12.59 µg/ml. However, no mortality was obtained for the negative control group.

Cardioprotective Activity: The addition of 100 µl streptokinase (positive control) to the slits along with 90 minutes of incubation at 37°C, showed 63.54 ± 2.61 clot lysis. However, distilled water (negative control) treated-clots showed only negligible clot lysis (4.92 ± 0.98%). The mean difference in clot lysis percentage between positive and negative control was very significant (p value < 0.05). Treatment of clots with*Ficus benjamina* extract provided the clot lysis34.95±1.98. The mean percentage of clot lysis by *Ficus benjamina* was statistically significant (p value<0.001). *Ficus benjamina* exihibited the values which were significant (p value<0.001) compared to both positive control (streptokinase) and negative control (water). Percent clot lysis obtained after treating the clots with

methanolic extract and appropriate controls is shown in table 2.

Anti-inflammatory activity: Methanolic extract of *Ficus benjamina* was studied for *in vitro* anti-inflammatory activity by HRBC membrane stabilization method which is reported in Table 3. The *in vitro* anti-inflammatory activity of the extract was concentration dependent, with the increasing concentration, the activity is also increased. Here, the methanol extract of *Ficus benjamina* showed 58.06% of membrane stabilization at 1000 µg/ml concentration and 20.79% at 31.25µg/ml. All the results were compared with standard Diclofenac sodium, which showed 86.72% and 51.32% at 1000µg/ml and 31.25µg/ml respectively.

DISCUSSION

The toxicity profile of plant materials is mainly an important criterionto experts and medical practitioners[10-12] and cytotoxic brineshrimp lethality (LC_{50}, 24 h) test was conducted in this experiment toknow about the toxicity of the plant extract. Derived equation from Parra[13], showed a great correlation ($r = 0.85$; $P < 0.05$) betweenthe LC_{50} of brine shrimp lethality test and the severe oral toxicityassay in mice. Based on the derivation of the correlation, a cutoff value forcytotoxicity is determined by LC_{50} which should be less than 10 µg/ml [14].In this experiment,the modest cytotoxicity was found inthe methanolic leaf extract, compared withthe standard drug vincristine sulfate.

In the case of thrombolytic study, methanolic leaf extract demonstrated promising cardioprotective activitywhich was compared with the positive control (streptokinase) as well as negative control (distilled water). By comparing the clot lysis percentage obtained through streptokinase and distilled water, a promisingly significant($P < 0.05$) thrombolytic effect was seen after the clots were treatedwith *Ficus benjamina* extract. It is establishedfrom the previous experiment that there are some bacterial pollutantson plants that have plasminogen receptors which are specificfor plasminogen. Certain plasminogen on the cell surface is rapidlyactivated to plasmin that could lead to fibrinolysis[15]. Bacterialplasminogen activator which also acts as cofactor molecules,such as staphylokinase, and streptokinase, can cause formationof exosite and increase the substrate activity towards the enzyme.Staphylokinase activates plasminogen to be in a position to breakdown clots, and also damages the extracellular molecules secretedby cells and fibrin particles that keep cells organized[16-18].

Ficus benjamina leaf extract exhibited moderate membrane stabilization effect by inhibiting hypotonicity induced lysis of the erythrocyte membrane. The erythrocyte membrane is similar to the lysosomal membrane [19] and its stabilization implies that the extract may as well stabilize lysosomal membranes. It is importantto stabilize the lysosomal membrane to limit the inflammatoryresponse by preventing the release of lysosomal constituents ofactivated neutrophil such as bactericidal enzymes and proteases,which cause further tissue inflammation and damage upon extracellular release [20]. Some of the NSAIDs are known to possessmembrane stabilizing properties which may contribute to thepotential of their anti-inflammatory effect. Though the exactmechanism of membrane stabilization by the extract is notknownyet; hypotonicity-induced hemolysis may arise fromshrinkage of the cells due to osmotic loss of intracellular electrolyteand fluid components. The process may stimulate or enhance theefflux of these intracellular components which can be prevented by the extract [21]. Methanolic extract of *Ficus benjamina* showed significant ($P<0.01$) anti-inflammatory activity 58.06%±1.03 at the concentration of 1000 µg/ml. On thebasis of the above results it can be concluded that *Ficus benjamina* has moderate anti-inflammatory activity.

CONCLUSIONS

Methanolic leaf extract of *Ficus Benjamina* possesses moderate cytotoxic and anti-inflammatory activities and significant cardioprotective activity ex-vivo. It wouldbe fascinating to investigate the mechanismunderlying percentage of inhibition ofhemolysis, cytotoxic activity and clot lytic effects demonstrated by *Ficus benjamina*.

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Conflict of Interest

Authors have none to declare.

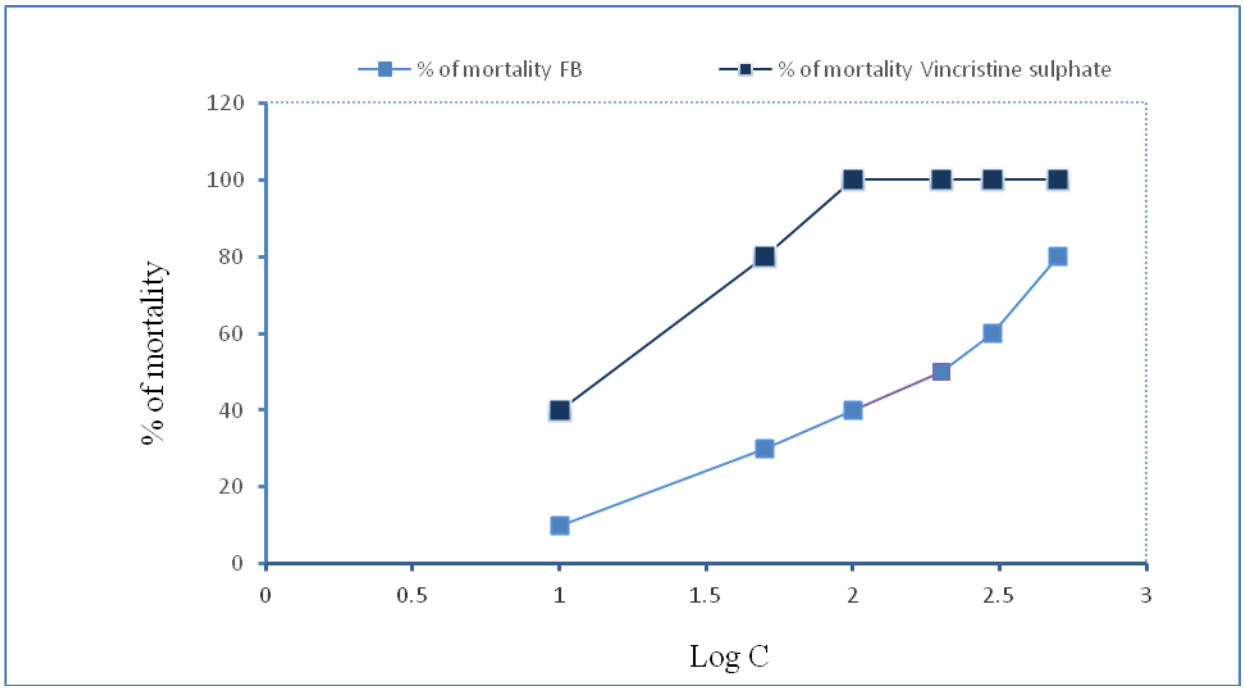

Figure 1: Brine shrimp lethality bioassay. Determination of LC_{50} values for methanol extract of *Ficus benjamina* leaf from linear correlation between log concentrations versus percentage of mortality.

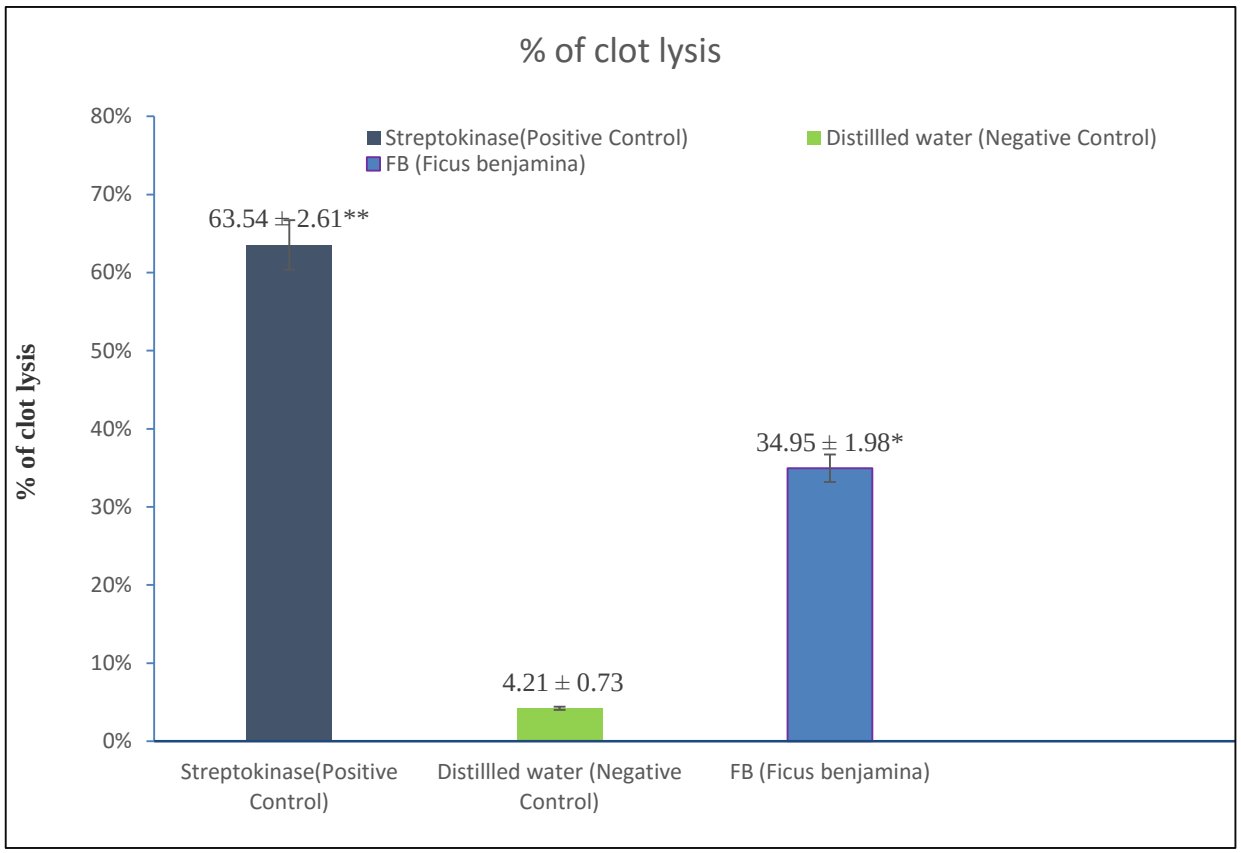

Figure 2: Clot lysis by streptokinase, water and methanolic extracts of *Ficus benjamina*. Effects of drugs on dissolution of clots prepared from blood of normal individuals.

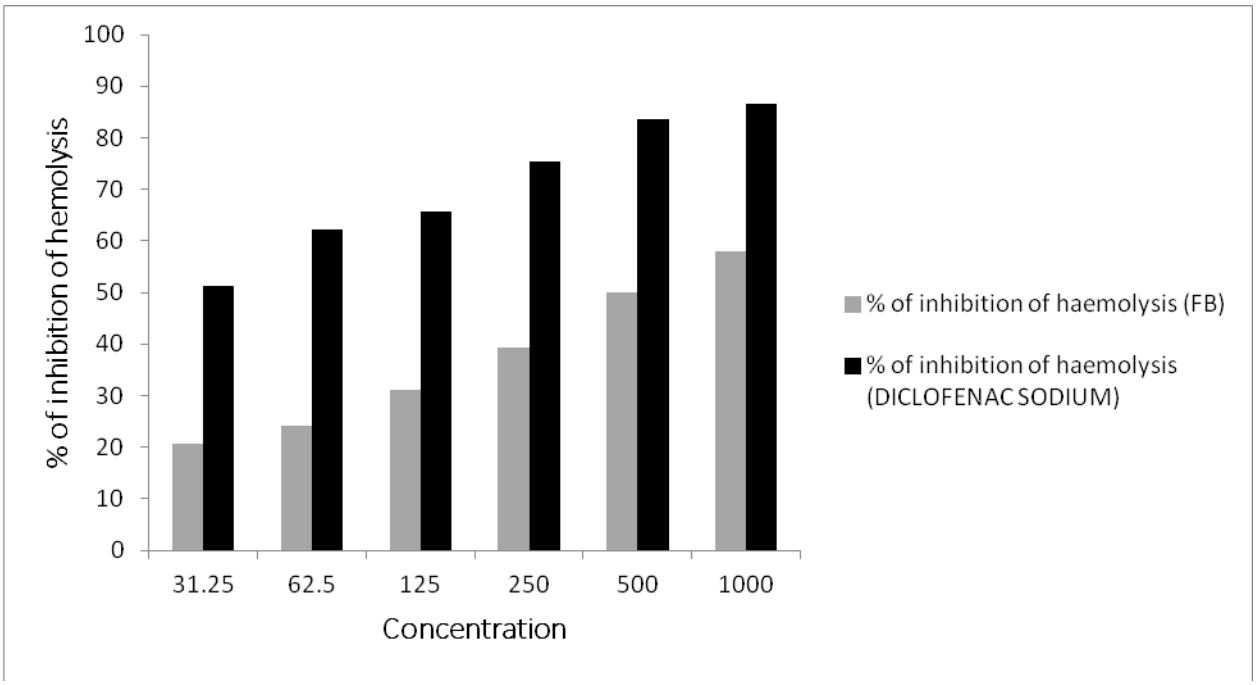

Figure 3: Inhibition of hemolysis (%) of *Ficus benjamina* and diclofenac sodium.

Table 1. Percentage of mortality of the extract at six concentrations

Concentration (ug/ml)	LogC	% of mortality	
		FB	Vincristine sulphate
10	1	10	40
50	1.699	30	80
100	2	50	100
200	2.301	50	100
300	2.477	60	100
500	2.699	70	100
LC_{50}		232.71	12.59

*FB= *Ficus benjamina*

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

Treatment	% of clot lysis (Mean ± S. D.)
Streptokinase(Positive Control)	63.54 ± 2.61**
Distillled water (Negative Control)	4.21 ± 0.73
FB (*Ficus benjamina*)	34.95± 1.98*

*FB= *Ficus benjamina*

Table 3. Percent stabilization of membrane of *Ficus benjamina*

Concentration(µg/ml)	Percent of membrane stabilization	
	Ficus benjamina	Diclofenac sodium
31.25	20.79% ±1.07**	51.32% ±0.97
62.5	24.25% ±0.97*	62.29% ±1.03
125	31.13% ±1.01*	65.58% ±0.66
250	39.42% ±1.26*	75.52% ±0.93
500	49.97% ±1.01*	83.58% ± 0.95
1000	58.06% ±1.03*	86.72% ±0.93

Values are expressed as mean±SEM of three replicate (n=3). **P<0.01, *P<0.05

REFERENCES

1. Borris, R.P. J.Ethnopharmacol, 1996; 51: 29–38.
2. Mollika S, Islam N, Parvin N, Kabir A, Sayem MW, Luthfunnesa and Saha R. Global J Pharmacol, 2014; 8(1): 39-46.
3. Ghani A. Medicinal Plants of Bangladesh: Chemical Constituents and Uses. Asiatic Society of Bangladesh, Dhaka, 1998.
4. Gilman EF and Watson DG. *Ficus benjamina*. Fact Sheet ST-251, November 1993.
5. Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE, McLaughlin JL. Planta Med, 1982; 45(1): 31-4.
6. Alam MN, Biozid MS, Chowdhury AI, Mazumdar MMU, Chowdhury S and Chowdury MIA. Journal of Medicinal Plants Research, 2015; 9(25): 719-23.
7. Prasad S, Kashyap RS, Deopujari JY, Purohit HJ, Taori GM, Daginawala HF. Thromb J, 2006; 4: 14.
8. Alam MN, Biozid MS, Islam MR, Abedin MJ, Chowdhury S. Journal of Coastal Life Medicine, 2015; 3(9): 724-7.
9. Kumar V, Bhat ZA, Kumar D, Bohra P, Sheela S. Int J Drug Dev Res, 2011; 3(2):176-9.
10. Singh A, Singh SK. Fitoterapia, 2005; 76(7-8): 747-51.
11. Fowles RG, Mootoo BS, Ramsewak RS, Khan A. Pharm Biol, 2012; 50(2): 264-67.
12. Okwuosa VN, Molta BS, Ebele S. ApplParasitol, 1993; 34(2): 89-94.
13. Parra AL, Yhebra RS, Sardiñas IG, Buela LI. Phytomedicine, 2001; 8(5): 395-400.
14. Chew AL, Jessica JA, Sasidharan S. Asian Pac J Trop Biomed, 2012; 2(3):176-80.
15. Pantzar M, Ljungh A, Wadström T. Infect Immun, 1998; 66(10): 4976-80.
16. Parry MAA, Zhang XC, Bode W. Trends BiochemSci, 2000; 25(2): 53-9.
17.Collen D. Ann Intern Med, 1990; 112(7):529-38.
18. Rahman MA, Imran T, Islam S. Saudi J BiolSci, 2013; 20(3): 213-25.
19. Chou CT. Phytother Res, 1997; 11:152-54.
20.Murugasan N, Vember S, Damodharan C. ToxicolLett, 1981; 8:33-8.
21. Vadivu R, Lakshmi KS. Bangladesh J Pharmacol, 2008; 3:121-24.