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Research Article

IN-VITRO THROMBOLYTIC AND CYTOTOXIC ACTIVITY OF METHANOLIC EXTRACT OF FLEMINGIA MACROPHYLLA LEAVES

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ABSTRACT

An investigation was made to find out the cytotoxic and thrombolytic activity of methanolic extract of leaves of *Flamingia macrophylla.* In-vitro test to observe thrombolytic and cytotoxic potential was done with the methanolic extract of *F. macrophylla* in this purpose. Streptokinase was used as positive control and water as negative control in the methodology of thrombolytic testing. In case of cytotoxic activity observation, Brine shrimp lethality bioassay testing method was used, 5% DMSO and brine shrimps were used as test materials, where DMSO as solvent for proper solution. The percent of thrombolysis by the extract showed 43.067 ± 3.0601%, whereas percent of thrombolysis by streptokinase was found 68.582 ± 1.7764%. From the cytotxicity study of the crude extract LC_{50} was found 1.981µg/ml with 95% confidence limit [1] was 1.7299-2.2706 µg/ml. The current study refers the plant leaves as impressive thrombolytic and cytotoxic agent for further laboratory study.

Keywords: Flemingia macrophylla, thrombolytic, Cytotoxicity, DMSO, 95% confidence limit.

INTRODUCTION

History says that, natural products are nothing new as the agents for treating various diseases. Naturally plants not only provide us food, shelter but also they provide remedies for many years. Different chemical constituents contained in plant exhibit different activities in alleviation of abnormal health condition of human beings or animals. In case of traditional medicine, the practitioners are appreciated to use different parts of plant because of having several chemical constituents in them which fulfill their wants.

Flemingia macrophylla belonging to the family Fabaceae is a woody leguminous shrub. It is a native plant of sub-humid to humid region, thus it is naturally found in Asia including Bhutan, southern China, Cambodia, India, Indonesia, Laos, Myanmar, Malaysia, Nepal, northern Pakistan, Philippines, Sri Lanka, Thailand Vietnam and Chittagong district of Bangladesh. It has been cultivated and naturalized in Sub-Saharan Africa, Central and South America, and tropical Australia [2-4]. It is a multipurpose plant widely used in agriculture, crop improvement, fodder, dyes and for various therapeutic purposes. Locally this plant is known as charchara (Bengali) [5]; apa apa, hahapaan, pok kepokan (Indonesia); serengan jantan, beringan (Malaysia); tÓp mo'láto, cây dau ma, cai duoi chon (Vietnam) [6].

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. In vitro thrombolytic activity of crude extract was enumerated and was compared with streptokinase, which is a well known anticoagulant used in myocardial infarction. [7] Brine shrimp lethality bioassay is a bench top bioassay method for evaluating anticancer, antimicrobial and other pharmacological activity of natural products. Natural products extracts, fractions or pure compounds can be tested for their bioactivity by this method. *Artemia* found favor as a "standard" organism in toxicological assays of crude extract, despite the recognition that it is too robust an organism to be a sensitive indicator species. [8]

MTERIALS AND METHODS

Plant Collection and Identification

The matured plant leaves was collected from Bandarban, hilly region of Chittagong division of Bangladesh. Then it was identified by taxonomist Dr. Shaikh Bokhtear Uddin, Associate Professor, Department of Botany, The University of Chittagong.

Plant Material Extraction

The extract was prepared by cold extraction process. In this process the coarse powder was submerged in methanol (95%) since methanol is the most common solvent for extracting most of the constituents present in herbal materials. Amber glass bottle were used for this purpose, which were kept at room temperature and allowed to stand for several days (5-7) with occasional shaking and stirring. When the solvent became concentrated the contents were first decanted by using cotton and then filtered through Whatman No.1 filter paper. The filtrate so obtained was then concentrated to dryness through the evaporation of solvent using rotary evaporator. The extracts of the plants obtained was the crude extracts.

IN-VITRO THROMBOLYTIC STUDY

Extract Preparation for Test

10mg methanolic extract of *F. macrophylla* was suspended in 10ml distilled water. The mixture was shaken vigorously on a vortex mixer. The mixture was kept overnight at room temperature to make the crude extract soluble in water. The soluble part of extract in water was separated as supernatant and the insoluble part was found as sediment part. The supernatant was then separated using Whatman filter paper. Then this solution is ready for thrombolysis study.

Sampling

Ten healthy human volunteers were selected for this study. The volunteers shouldn't have recent history of oral contraception or anticoagulant therapy for last 7-10 days at least. Five alpine tubes were weighed for each volunteer and labeled properly. Under

aseptic condition, 5ml of fresh blood was drawn from each human volunteer. Freshly collected blood was then transferred to previously weighed alpine tube, each filled up to 500µl blood. They were then kept to form clot.

Assay of Extract

Each properly labeled blood filled alpine tube was then incubated at 37^{0}C for 45 minutes. After clot formation, serum was withdrawn completely, without disturbing the clot. Each tube was weighed again to get the weight of clot. The equation for calculating weight of clot is given below-

Clot weight = Weight of clot filled tube – Weight of empty tube

To each alpine tube containing clot, 100µl of aqueous extract of *F. macrophylla* was added separately. All the tubes were then incubated at 37^{0}C for 90 minutes and observed for clot lysis.

After incubation, supernatant fluid released was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference between previous weight and at now was plot as ratio to obtain the percent of clot lysis.

% of clot lysis=
(Weight of lysis/weight of clot before lysis)×100 [9]

IN-VITRO CYTOTOXICITY BIOASSAY

The most recent development in the bioassay for testing crude plants cytotoxicity is the Brine Shrimp lethality testing. Bioactive compounds are almost always toxic in high doses. Pharmacologically potent drugs are simply toxic if at a higher dose. Here in-vitro lethality of a simple zoological organism (brine shrimp nauplii) is used as a convenient monitor for screening a fractionation in the discovery of new bioactive natural products. Generally the median effective dose (ED_{50}) values for cytotoxicity are one tenth (1/10) of median lethal dose (LD_{50}) values in the brine shrimp test. [10]

Hatching of Brine Shrimp

Small amount of brine shrimp eggs were hatched at simulated sea water. About 38gm pure NaCl (also known as sea salt) was dissolved in 1L of distilled water to make simulated sea water. The pH should have at 8.3-8.7. Then a little number of collected shrimp eggs was liberated into water and continuous ventilation for oxygen supply was attached to the tank of hatching. A continuous lighting system was also assured.

Preparation of Test Sample

9 test tubes were taken, then cleaned and dried well. The stock solution was prepared by using 10mg methanolic extract of *F. macrophylla* with 10mL of solvent. Distilled water and DMSO was used as solvent. The stock solution was prepared in such a way to have the ratio 1:1 (i.e., 1mg/ml crude solution). This stock solution was further diluted in different concentrations as 10 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml and 500µg/ml. A negative control was prepared using only solvent alone. In each concentration 10 nauplii was discharged and the whole solution was made up to 5mL adding sea water.

Observation of nauplii

The naupliis with drug, in various conc., was kept in enlightened and well ventilated condition for 24 hours. After 24 hours naupliis were counted, using magnifying glass. The number of dead was projected by deducting the living from initial number.

Statistical Analysis for Cytotoxic Action [11]

From the percent of lethality probit data for each concentration was calculated using "BioStat-2009", and then they were plotted against log concentration of corresponding for calculation of LC_{50}, which was obtained 1.9818µg/ml (Table 2 and Figure 2).

RESULTS

Table 1: This table shows the percent of lysis caused by methanol extract of F. macrophylla leaves at the concentration of 1mg/ml

Sample	% of thrombolysis*	t-test value**	p-value**
Streptokinase	68.582 ± 1.7764%	86.258	<0.0001
F. macrophylla	43.067 ± 3.0601%	44.505	<0.0001

*% of thrombolysis ± SD
** One sample *t-test* value
** p< 0.0001 is considered statistically significant

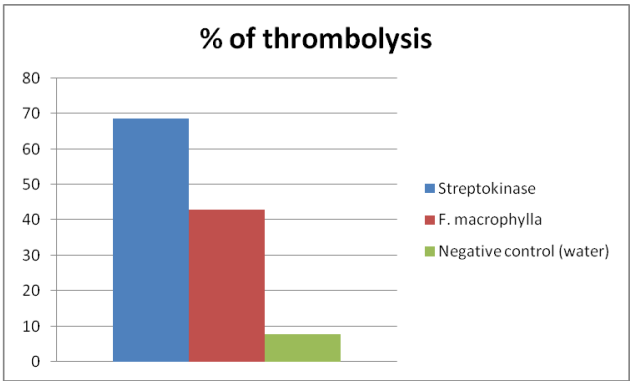

Figure 1: The above figure shows percentage of thrombolysis by *F. macrophylla* in comparison with Streptokinase and negative control (water).

Table 2: This table emphasizes the percent of lethality caused by *F. macrophylla* methanolic extract at different concentration

SL. No.	Concentration µg/ml	Log C	No. of nauplii	No. of living	% of mortality
1	10	1.00	10	9	10
2	50	1.70	10	8	20
3	100	2.00	10	4	60
4	200	2.30	10	4	60
5	300	2.48	10	1	90
6	400	2.60	10	0	100
7	500	2.70	10	0	100

Table 3: LC_{50}, 95% confidence limit, Chi-square

$Log_{10} LC_{50}$	LC_{50}	95% confidence limit	Chi-square
0.2971	1.9819	1.7299-2.27706	0.8734

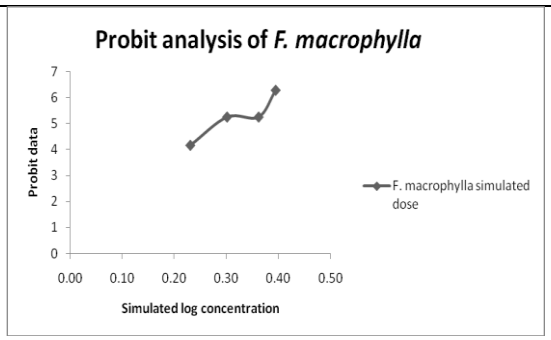

Figure 2: Figure above shows probit analysis of F. macrophylla for 50% lethal concentration data

DISCUSSION

A huge numbers of thrombolytic drugs are obtained from different sources and then further modified, using recombinant technology. This is performed to make them more site-specific and to reduce

their side-effect. Sometime patient may dies due to bleeding or internal hemorrhage, so direct use of herbal thrombolytic drug is not expected. Their further modification is required.

The observed thrombolytic percentage shows quite good effect of thrombolysis of the methanolic extract of *F. macrophylla* leaves . It possesses a significant percent of thrombolysis comparing with streptokinase (positive control) and makes a difference from negative control (water). The percentage of thrombolysis of extract of *F. macrophylla* leaves is 43.067 ± 3.0601% and that of streptokinase is 68.582 ± 1.7764%. So it can be concluded as significant anti-coagulant agent.

The cytotoxic drugs are tested because they are useful in anti-cancer drugs discovery. At present time anti-cancer drugs are matter of concern for scientists. They are developing newer drugs on regular basis. Considering this fact it can be said that if any crude drugs, especially from natural source, having cytotoxic effect can be prior for further isolation and modification.

It would be sounds of hope that the obtained LC_{50} of methanolic extract of *F. macrophylla* is 1.9819µg/ml. it can be considered for further analysis as a lead candidate.

CONCLUSION

Since the methanolic extract of leaves of *F. macrophylla* shows significant thrombolytic and cytotoxic properties, the further laboratory study and chemical isolation of this plant might confirm an effective drug in pharmacologic aspects as anti-coagulant and in anti-cancer therapy. Methanolic extract of *F. macrophylla* has most promising data in both assays that suggest its effectivity in both types of pharmaceutical arena.

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