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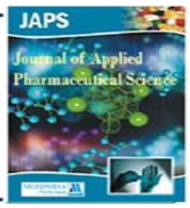

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Thrombolytic Activity and Preliminary Cytotoxicity of Five Different Fractions of Methanol Extract of *Allamanda cathartica* Leaf

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ABSTRACT

Investigation with the crude methanol extract of *Allamanda cathartica* leaves and its different fractions were carried out to evaluate its possible thrombolysis and cytotoxic activities. A quick & rapid methodology (*In-vitro* Thrombolytic model) was applied to find out their thrombolytic potential where streptokinase and water were employed as a positive and negative controls, respectively. Among the extractives, the chloroform (CSF) and hexane (HSF) soluble fractions showed 34.51±0.669% and 32.179±0.581% clot lysis activity respectively compared to standard streptokinase which exhibited 61.5% lysis of clot. In brine shrimp lethality bioassay method, the LC_{50} values of the test samples of *A. cathartica* leaves were assayed where DMSO and Vincristine sulphate were used as solvent & as positive control respectively. The chloroform, hexane and carbon tetrachloride soluble fractions showed significant cytotoxic activity against brine shrimp nauplii and LC_{50} values were 1.45, 5.00 and 5.24 µg/ml respectively.

Keywords: *Allamanda cathartica*, Clot lysis, *In-vitro* Thrombolytic model, Streptokinase, Cytotoxic activity.

INTRODUCTION

The subject of phytochemistry or plant chemistry has developed in recent years as a distinct discipline, somewhere between natural product organic chemistry and plant biotechnology and is closely related to both. It is concerned with the enormous variety of organic substances that are elaborated and accumulated by plants and deals with chemical structures of these substances; their biosynthesis; turnover and metabolism; their natural distribution and biological functions. The range and number of discrete molecular structures produced by plants is huge, therefore much current research devoted to the phytochemical investigation of higher plants should be done for different types of biological activities (Harborne JB, 1998). *A. cathartica* (Synonym- *Echites verticillata* Sessé & Moç, *Orelia grandiflora* Aublet, *Allamanda grandiflora*(Aublet) Poiret in Lam, *Allamanda hendersonii* W.

Bull ex Dombrain.) commonly known as Golden Trumpet, Yellow Bell or Buttercup Flower is a perennial shrub that can grow up to a height of 15 feet tall or more, is native to Brazil but widely cultivated throughout the tropics. *A. cathartica* is primarily used as an ornamental plant. The plant is used to relieve coughs and to clear the nasal passages. The leaves were also made into decoctions for use as a purgative. This plant has anti-bacterial and anti-cancerous properties. It was also widely used in the treatment of jaundice. The root and stem of this plant contain two rare lactones which are active against polio virus and pathogenic fungi. Root is also used in various formulations to treat malarial symptoms. Sap was used to eliminate intestinal worms. The plant is also used as laxative and emetic (David WN, 1997). The leaves, stem and branches of this plant are used against snake bite. (Gomes etal., 2010). The object of this research work was to investigate whether the leaves extract of *A. cathartica* and it's hexane, carbon tetrachloride, chloroform and aqueous soluble partitionates possess thrombolytic activity or not by using an *in-vitro* procedure and also to see their cytotoxic activity.

MATERIALS & METHODS

Collection and Extraction of *A. cathartica* Leaves

The leaves of *A. cathartica* were collected at their fully mature form in April 2011 from Mirpur Botanical Garden and a voucher specimen (DACB – 36081) has been deposited in Bangladesh National Herbarium for future reference. After cleaning, the collected plant materials were chopped, dried and powdered and about 400gm of the powdered material was soaked in 2 litres of methanol at room temperature for 7 days. The extract was filtered through Wattman filter paper (No. 1) and concentrated in a rotary evaporator at reduced temperature and pressure. The concentrated methanol extract was separately partitioned by the modified Kupchan method (Van Wagenen *et al.,* 1993) using hexane, carbon tetrachloride, chloroform and the subsequent evaporation of solvents yielded from leaves: hexane-1.5gm, carbon tetrachloride-1.0 gm, chloroform-850 mg and aqueous-500mg soluble materials. The residues were then stored in a refrigerator for further experimental purposes.

In Vitro Thrombolytic Study

The thrombolytic activity of all extractives was evaluated by the method developed by Prasad (2007) using streptokinase (SK) as a standard.

Streptokinase (SK) Solution Preparation

Commercially available lyophilized Altepase (Streptokinase) vial (Beacon pharmaceutical Ltd) 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 stock from which 100µl (30,000 I.U) was used for *in vitro* thrombolysis.

Specimen

Whole blood (5 ml) was drawn from healthy human volunteers (n = 10) without a history of oral contraceptive or anticoagulant therapy. 500 µl of blood was transferred to each of the ten previously weighed alpine tubes to form clots.

Herbal preparation for thrombolytic activity

100 mg extract 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 micron syringe filter. 100 µl of this aqueous preparation of herbs was added to the alpine tube containing the clots to check thrombolytic activity.

Thrombolytic Study

Experiments for clot lysis were carried as reported earlier (Prasad *et al*., 2007). Venous blood was drawn from healthy volunteers (n = 10) and transferred in different pre-weighed sterile alpine tube (500 µl/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 alpine tube containing clot was properly labeled and 100 µl of plant extract was added to the tubes. As a positive control, 100 µl of SK and as a negative non thrombolytic control, 100 µl of distilled water were separately added to the numbered control 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 of clot lysis. The test was repeated three times.

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

Statistical Analysis

The significance between % clot lysis by herbal extract by means of weight difference was tested by the paired t-test analysis. Data are expressed as mean ± standard deviation.

Brine Shrimp Lethality Bioassay (Mayer *et al*., 1982; Goldstein *et al*., 1974)

Hatching of Brine Shrimp

For the preparation of sea water 38gm of sodium chloride was weighed, dissolved in distilled water to make 1 liter solution and then filtered off to be a clear solution. This simulated sea water was used for a hatching of brine shrimp. The shrimps were allowed for two days to hatch and mature as nauplii (larvae).

Preparation of Sample

All the test samples were taken in vials and dissolved in 200 µl of pure dimethyl sulfoxide (DMSO) to get stock solutions.

Application of Test Sample to the Test Tube Containing Brine Shrimp Nauplii

10 test tubes for each test sample were taken where each contained 5ml of seawater and 10 nauplii. These test tubes were marked from 1 to 10. 100 µl of each stock solution was taken in the

Table. 1: Effect of methanolic extract and its different partitionatesleaves of*A. cathartica*& Streptokinase (Positive control) on *invitro* clot lysis.

Sample	Mean ± S.D. (Clot lysis %)	t-value	P value (Two-tailed) when compared to negative control (water)
ME	23.22±0.889	18.4770	less than 0.0001
HXSF	32.179±0.581	44.5745	less than 0.0001
CTCSF	17.58±0.534	18.7359	less than 0.0001
CSF	34.51±0.669	39.1533	less than 0.0001
AQSF	30.73±0.884	26.2557	less than 0.0001
SK	61.5±0.542	101.4194	less than 0.0001

The average values of three calculations are presented as mean ± S.D. (standard); ME= Methanolic crude extract; HXSF= Hexane soluble fraction; CTCSF= Carbon tetrachloride soluble fraction; CSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction and SK= Streptokinase.

Table. 2: Brine Shrimp Lethality Bio-assay of methanolic extract and its different fractionates of*A. cathartica*leaves

Sample	Brine shrimp lethality bioassay LC_{50} (µg/ml)
ME	7.2±0.28
HXSF	5.00±1.12
CTCSF	5.24±0.74
CSF	1.45±0.49
AQSF	7.9±1.18
VS	0.451±0.03

The average values of three calculations are presented as mean ± S.D. (standard); ME= Methanolic crude extract; HXSF= Hexane soluble fraction; CTCSF= Carbon tetrachloride soluble fraction; CSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction and VS= Vincristine sulfate

first test tube containing 5ml of simulated seawater and 10 shrimp nauplii. Thus, final concentration of the prepared solution in the first test tube was 400µg/ml. Then a series of solutions of varying concentrations were prepared from the stock solution by serial dilution method.

In every case, 100 µl sample solution was added to test tube and fresh 100µl DMSO was added to the vial. Thus different concentrations were found in the different test tubes. Then the samples were subjected to brine shrimp lethality bioassay (Mayer *et al.*, 1982; Goldstein *et al.*, 1974)

Preparation of Control Solution

Two control groups were used in cytotoxicity study, to validate the test method and results obtained due to the activity of the test agent.

Negative Control Test

In this case, only 100µl DMSO was added in 5ml sea water containing 10 nauplii. No extract was added to prepare control solution.

Positive Control Test

Vincristine Sulphate was used as the positive control. Measured amount of the Vincristine Sulphate was dissolved in DMSO to get an initial concentration of 20 µg/ml from which serial dilutions were made using DMSO to get 10, 5, 2.5, 1.25, 0.625,0.3125, 0.15625, 0.078125 and 0.0390 µg/ml. Then the positive control solutions were added to the remarked vials containing ten living brine shrimp nauplii in 5 ml simulated sea water to get the positive control groups.

Counting of Nauplii

After 24 hours, test tubes were observed and the numbers of dead nauplii were counted and the LC_{50} values were calculated.

RESULT AND DISCUSSION

In the clot lysis study, addition of 100µl SK, a positive control (30,000 I.U.) to the clots along with 90 minutes of incubation at 37°C, showed 61.5± 0.542 % clot lysis. The *in vitro* thrombolytic activity study revealed that the chloroform soluble fractionates showed 34.51±0.669 % clot lysis and the hexane soluble fractionate showed 32.179±0.581 % clot lysis activity. Statistical representation of the effective clot lysis percentage by test preparation and positive thrombolytic control (Streptokinase) is tabulated in Table 1.

In brine shrimp lethality bioassay using brine shrimp Nauplii, methanolic extract and its different fractionates of *A. cathartica* leaves showed positive result in comparison with the positive control Vincristine Sulphate and that's why it can be assumed that these test samples are pharmacologically active. By plotting the log of concentration (log C) versus percent (%) mortality for all test samples showed an approximate linear correlation. From the graph, the median lethal concentrations ($LC_{50,}$ the concentration at which 50% mortality of brine shrimp nauplii occurred) were determined. The chloroform soluble fraction showed significant cytotoxic activity against brine shrimp nauplii and LC_{50} value was 1.45±0.49µg/ml. The hexane and carbon tetrachloride soluble fractions also exhibited significant cytotoxicity with LC_{50} values 5.00±1.12µg/ml and 5.24±0.74µg/ml respectively. Moreover, this significant lethality is indicative of the presence of potent cytotoxic and probably insecticidal compounds which warrants further investigation.

CONCLUSION

From this experiment, it can be concluded that methanolic extract and its different partitionates of *A. cathartica* leaves have got good potential as candidates for future thrombolytic and cytotoxic agents and also they can be investigated as a possible

sources of cardio protective drugs. This is only a preliminary study and to make final comment the extract should be thoroughly investigated phytochemically and pharmacologically to exploit their medicinal and pharmaceutical potentialities.

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