CYTOTOXIC (BRINE SHRIMP LETHALITY BIOASSAY) AND ANTIOXIDANT INVESTIGATION OF *BARRINGTONIA ACUTANGULA (L.)*"

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ABSTRACT

Barringtonia acutangula (L.) gaertn. (Family: Barringtoniaceae), a medicinal small to medium evergreen tree known as 'Hijal', is used in diarrhoea, dysentery, colic , flatulence, cooling, aperients expectorant, stimulating emetic, astringents to the bowel, antihelminthic, bronchitis, lumber pain, hallucinations, seminal weakness, gonorrhoea and many other ailments in rural areas of Bangladesh It is also used as traditional medicine in other countries. But till to date, sporadic attempts have been made for the scientific and methodical validation of these traditional claims. In Brine Shrimp Lethality Bioassay, all the extracts produced dose dependent cytotoxicity effect to brine shrimp nauplii with methanol extract of leaf exhibiting highest toxicity having LC50 value 46.24 µg/ml where standard vincristine sulphate had the LC50 value of 0.69 µg/ml. & In antioxidant attempt by reducing power and CUPRAC assays, pet. ether extract of leaf were found to exhibit moderate but concentration dependent reducing power respectively.

Key Word: sporadic; hallucinations; IC_{50} value; ascorbic acid, Vincristine sulphate

Introduction

Barringtonia acutangula (L) Geartn is distributed throughout the country. But it is mainly found in Chittagong, Chittagong Hill Tracts, Tangail, Sylhet and Dhaka. (Ghani A., 2003) Barringtonia acutangula (L) Geartn the plant selected for the current study, has various ethnopharmacological use and a tuberous herb from (Family: Barringtoniaceae). The family of (Family: Barringtoniaceae) has some medicinally valuable species. Barringtonia acutangula is a neglected species on which very few scientific investigations have been conducted. There remains a possibility that the extract of the plant may possess some bioactive compounds. The work described in this article is dedicated to phytochemically and pharmacologically characterize different parts of the specified plant to: (1) Identify the groups of chemical constituents present in the plant parts (2) Rationalize the traditional uses of the selected plant (3) Explore the possible newer medicinal activities of the same plant those are not traditionally claimed

However, in order to develop these medicinal plants as drugs, attempts should be first made to certainly identify them and preclinical studies on them should be carried out to establish their claimed therapeutic properties. These are very important because the biological activity of a plant or its preparation will assist on determining the therapeutic target of its development. Since the chemical constituents and pharmacological actions of most of these plants are known and as they are in current use in traditional medicines, their clinical evaluation can be undertaken.

The present study was designed to identify the groups of chemical constituents that are present in the crude extracts of Barringtonia acutangula (L) Gearth as well as to observe the pharmacological activities of the extracts of the plant.

The present study was designed to identify the groups of chemical constituents that are present in the crude extracts of *Barringtonia acutangula (L) Gearth* as well as to observe the pharmacological activities of extracts of the plant parts of leaves & bark with the solvent of Petroleum ether & Methanol. Brine Shrimp lethality bioassay tests to find out the presence of chemical that has a cytotoxic effects on cell line. Antioxidant activity tests to find out Rerducing power & CUPRAC Assay.

Materials

- *Anemia salina* Leach. (Brine eggs), Sea salt (NaCI)
- Small tank
- Lamp to attract Shrimps
- Pipettes (5, 10ml) and Micropipette (5-50nl), (10-100ul)
- Glass vials, Magnifying glass

Principle

Brine Shrimp lethality bioassay (*Luo et al., 2000; Mclaughlin et al., 1998; Meyer et al., 1982*) is a rapid and comprehensive bioassay for the bioactive compounds of natural and synthetic origin. By this method, natural product extracts, fractions as well as the pure compounds can be tested for their bioactivity. The method utilizes *in vivo* lethality in a simple zoological organism (Brine nauplii) as a convenient monitor for screening and fractionation in the discovery of new bioactive natural products. Brine toxicity is closely correlated with 9KB (human nasopharyngeal carcinoma) cytotoxicity (p=0.036 and kappa = 0.56). ED_{50} values for cytotoxicities are generally about one-tenth the LC_{50} values found in the Brine Shrimp test. Thus, it is possible to detect and then monitor the fractionation of cytotoxic, as well as 3PS (P_{388}) *(in vivo* murine leukaemia) active extracts using the Brine lethality bioassay (*Alkofahi et al., 1988; Mclaughlin et al., 1998; Meyer et al., 1982*). The Brine Shrimp assay has advantages of being rapid (24 hours), inexpensive, and simple (e.g., no aseptic techniques are required). It easily utilizes a large number of organisms for statistical validation and requires no special equipment and a relatively small amount of sample (2-20 mg or less). Furthermore it does not require animal serum as is needed for cytotoxicities (*Mclaughlin et al., 1998*).

Preparation of seawater

38 gm sea salt (without iodine) was weighed, dissolved in one liter of distilled water and filtered off to get clear solution.

Hatching of Brine Shrimp

Artemia salina leach (brine shrimp eggs) collected from pet shops was used as the test organism. Seawater was taken in the small tank and shrimp eggs were added to one side of the tank and then this side was covered. Two days were allowed to hatch the shrimp and to be matured as nauplii. Constant oxygen supply was carried out through the hatching time. The hatched shrimps are attracted to the light (phototaxis) and so nauplii free from egg shell was collected from the illuminated part of the tank. The nauplii was taken from the fish tank by a pipette and diluted in fresh clear sea water to increase visibility and 10 nauplii was taken carefully by micropipette.

Preparation of test solutions with samples of experimental plants

32 mg of each of the test samples were taken and dissolved in 200µl of pure dimethyl sulfoxide (DMSO) and finally the volume was made to 20 ml with sea water. Thus the concentration of the stock solution was 1600µg/ml. Then the solution was serial diluted to 800, 400, 200, 100, 50, 25, 12.5, 6.25 µg/ml with sea water. Then 2.5 ml of plant extract solution was added to 2.5 ml of sea water containing 10 nauplii:

Concentration (µg/ml)	Extract Solution	Sea water containing 10 nauplii	Final volume
800	2.5 ml (1600µg/ml)	2.5 ml	5 ml
400	2.5 ml (800µg/ml)	2.5 ml	5 ml
200	2.5 ml (400µg/ml)	2.5 ml	5 ml
100	2.5 ml (200µg/ml)	2.5 ml	5 ml
50	2.5 ml (100µg/ml)	2.5 ml	5 ml
25	2.5 ml (50µg/ml)	2.5 ml	5 ml
12.5	2.5 ml (25µg/ml)	2.5 ml	5 ml
6.25	2.5 ml (12.5µg/ml)	2.5 ml	5 ml

Preparation of control group

Control groups were used in cytotoxicity study to validate the test method and ensure that the results obtained were only due to the activity of the test agent and the effects of the other possible factors were nullified. Two types of control groups were used

i) Positive control

ii) Negative control

Preparation of the positive control group

Positive control in a 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 sulfate was used. As vincristine is a very cytotoxic alkaloid it was evaluated at very low concentration (10, 5, 1, 0.5, 0.25, 0.125 and 0.06 μg/ml)

Preparation of the negative control group

50 μl of DMSO was added to each of three premarked test tubes containing 4.95 ml of simulated sea water and 10 shrimp nauplii to use as control groups. If the brine shrimps in these vials show a rapid mortality rate, then the test is considered as invalid as the nauplii died due to some reason other than the cytotoxicity of the compounds.

Counting of nauplii

After 24 hours, the test tube were inspected using a magnifying glass against a black background and the number of survived nauplii in each tube was counted. From this data, the percent (%) of lethality of the brine shrimp nauplii was calculated for each concentration. The mortality was corrected using Abott's formula (*Abott W. S., 1925*)

$$P_t = [(P_o - P_c)/ (100 - P_c)] \times 100$$

Where, P_o= Observed mortality

P_c= Control mortality

The effectiveness or the concentration-mortality relationship of plant product is usually expressed as a median lethal concentration (LC_{50}). This represents the concentration of the chemical that produces death in half of the test subjects after a certain exposure time and determined by linear regression method from plotting % mortality against correspondent log of concentration.

Result:

All the extracts were also subjected to Brine Shrimp lethality bioassay for possible cytotoxic action. In this study, methanol extract of aerial part was found to be the most toxic to Brine Shrimp nauplii, with LC_{50} of 46.24 μg/ml whereas anticancer drug vincristine sulphate showed LC_{50} value 0.699 μg/ml. On the other hand, all the other extracts showed moderate to low toxicity (table 4.8). The high toxicity of methanolic extract of leaf probably attributed to the alkaloid that is confirmed in phytochemical screening. The order at which cytotoxic potential of the test samples decreased was as follows: Vincristine sulphate> TBP> > TBM> TLP> TLM (Table 1)

Table 1: LC_{50} of the different fractions Brine Shrimp lethality bioassay

Test Sample	Concentration (μg/ml)	Log Conc.	% Mortality	Corrected % Mortality	LC_{50} (μg/ml)	LC_{90} (μg/ml)
TLM	12.5	1.09691	40	33.33	46.24	363.07
	25	1.39794	40	33.33		
	50	1.69897	50	44.44		
	100	2	60	55.55		
	200	2.30103	80	77.55		
	400	2.60206	100	100		
	800	2.90309	100	100		
TLP	12.5	1.09691	10	0	105.93	598.41
	25	1.39794	20	11.11		
	50	1.69897	20	11.11		
	100	2	60	55.55		
	200	2.30103	70	66.66		
	400	2.60206	70	66.66		
	800	2.90309	100	1000		
	200	2.30103	80	77.77		
	400	2.60206	90	88.88		
	800	2.90309	90	88.88		
TBM	12.5	1.09691	10	0	509.95	5370.31
	25	1.39794	10	0		
	50	1.69897	20	11.11		
	100	2	20	11.11		
	200	2.30103	20	11.11		
	400	2.60206	30	22.22		
	800	2.90309	80	77.77		
TBP	12.5	1.09691	10	0	554.37	4677.35
	25	1.39794	10	0		
	50	1.69897	20	11.11		
	100	2	20	11.11		
	200	2.30103	20	11.11		
	400	2.60206	20	11.11		
	800	2.90309	100	100		
VS	0.06	-1.22185	10	0	.0699	6.33
	0.125	-0.90309	20	11.11		
	0.25	-0.60206	30	22.22		
	0.5	-0.30103	40	33.33		
	1	0	50	44.44		
	5	0.69897	90	88.88		
	10	1	100	100		

[VS= Vincristine Sulfate]

Total Anioxidant Capacity

The phosphomolybdenum method usually detects antioxidants such as ascorbic acid, some phenolics, α-tocopherol, and carotenoids. The phosphomolybdenum method was based on the reduction of Mo(VI) to Mo(V) by the antioxidant compound and subsequent formation of a green phosphate/Mo(V) complex at acid pH.. In essence, it is believed that the molybdenum is easier to be reduced in the complex and electron-transfer reaction occurs between reductants and Mo(VI) and the formation of a green phosphate/Mo(V) complex with a maximal absorption at 695 nm.

Preparation of Reagent solution

3.3 ml of concentrated H_2SO_4 (98%), 0.381 gm sodium phosphate (Na_3PO_4. M.W = 136.09 gm) and 0.494 gm of ammonium molybdate (M.W = 1235.86 gm) were taken into three separate 100 ml volumetric flasks and the volumes were adjusted with distilled water.

Preparation of Standard solution

The stock solution was prepared by taking 0.025 gm ascorbic acid and dissolved into 5 ml of ethanol whose concentration was 5µg/µl. The experimental concentrations from the stock solution were prepared by the following manner:

Concentration (µg/ml)	Solution taken from stock solution (µl)	Solution taken from others	Adjust the volume by distilled water (µl)	Final volume
200	80	-	1920	2.0 ml
100	-	1 ml (200µg/ml)	1.92ml	2.0 ml
50	-	1 ml (100µg/ml)	1 ml	2.0 ml
25	-	1 ml 50/ml)	1 ml	2.0 ml
5	-	1 ml (25µg/ml)	4 ml	5.0 ml

Preparation of Extract solution

0.025 gm of each plant extracts were dissolved into 5 ml of Ethanol to make the concentration of each solution 5µg/µl of plant extract. These solutions were considered as stock solutions. The experimental concentration from these stock solutions was prepared by the following manner:

Concentration (µg/ml)	Solution taken from stock solution	Solution taken from others	Adjust the volume by distilled water (µl))	Final volume
200	40 µl	-	960	1.0 ml

Experimental procedure

- 300µl of each plant extracts or standard of different concentration solutions were taken into different test tubes and 3 ml of reagent solution was added into each of the test tubes.
- The test tubes were incubated at 95^0C for 90 minutes to complete the reaction.
- The absorbances of the solutions were measured at 695 nm using a spectrophotometer against blank after cooling to room temperature.
- A typical blank solution contained 3 ml of reagent solution and the appropriate volume (300µl) of the same solvent used for the sample and incubated under the same conditions as the rest of the samples solution.
- The antioxidant activity is expressed as the number of equivalents of ascorbic acid and was calculated by the following formula equation

$$A = (c \times V)/m,$$ Where;

A = total content of Antioxidant compounds, mg/gm plant extract, in Ascorbic acid Equivalent

c = the concentration of Ascorbic acid established from the calibration curve, mg/ml

V = the volume of extract, ml

m = the weight of crude plant extract, gm

Discussion:

The lethality of a test sample in a simple zoological organism such as the shrimp (*Artemia salina*) has been utilized by Meyer et al. (1982) in the Brine Shrimp Cytotoxicity Test (BSCT). It is a very useful tool to screen a wide range of chemical compounds for their various bioactivities. It has been well utilized to screen and fractionation of physiologically active plant extracts as well. It has been demonstrated that BSCT correlates reasonably well with cytotoxic and other biological properties (*Mclaughlin et al., 1991*). The brine shrimp bioassay has been established as a safe, practical and economic method for determination of bioactivities of synthetic compound (*Almeida et al., 2002*) as well as plant products (*Meyer et al., 1982*). The significant correlation between the Brine shrimp assay and in vitro growth inhibition of human solid tumor cell lines demonstrated by the national Cancer Institute (NCI, USA) is significant because it shows the value of this bioassay as a pre-screening tool for antitumor drug research (*Anderson et al., 1991*). In toxicity evaluation of plant extracts by Brine shrimp lethality bioassay LC_{50} values lower than 1000 µg/ml are considered bioactive (*Meyer et al., 1982*). The Brine Shrimp Lethality Bioassay also indicates antifungal effects, pesticidal effects, teratogenic effects, toxicity to environment and many more (*Vanhaecke P. et. al., 1981*). Table 4.8 shows the lethality of different extracts of B. *acutangula* to the Brine Shrimp nauplii. The degree of lethality shown by the

extractives was found to be directly proportional to the concentration of the extractives ranging from the lowest concentration (12.5 µg/ml) to the highest concentration (800 µg/ml). This concentration dependent increment in percent mortality of Brine Shrimp nauplii produced by the B. *acutangula* indicates the presence of cytotoxic principles in these extractives. Preliminary phytochemical screening revealed the presence of alkaloids and steroids. So the observed cytotoxic action may be due to the presence of such compounds. Again, reports exist on the role of alkaloids and steroids in cytotoxic activity of plant extracts (*Dhar et al., 1973; Vijayan et al., 2004; Badami et al., 2003*). However, phenolics and flavonoids are also known to show cytotoxicity in Hoechst 33258 fluorescence assay by inhibiting cellular DNA in a concentration-

Conclusion

Different crude extracts of *Barringtonia acutangula* (L.) Gaertn. were subjected to cytotoxic vigorous phytochemical and pharmacological investigations to validate the traditional use and to find out any other therapeutic activities. The high toxicity exerted by the extracts of *B. acutangula* in brine shrimp lethality bioassay suggests bioactive principles in the plant. The leaf of the plant exhibited potential antioxidant activity. This part of the plant exerted remarkable analgesic and anti-inflammatory activity.

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