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Study on Cytotoxic Activity of *Clerodendrum inerme* and *Caesalpinia crista* by Brine Shrimp Lethality Bioassay

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ABSTRACT: The science and practice of medicine and its primary source plays an important role in identifying the new molecule of drug through both synthetically and from that of natural origin. Most of the developing countries have adopted traditional medical practice as an integral part of their culture. Historically, all medicinal preparations were derived from plants, whether in the simple form of raw plant materials or in the refined form of crude extracts, mixtures, etc. The aim of this study was to evaluate the Cytotoxic Activity of Clerodendrum inerme (Family: Verbenaceae) and Caesalpinia crista (Family: Leguminosaceae), two widely distributed shrubs of the Sundarbans mangrove forest. A general cytotoxicity of all the extracts of C. inerme and C. crista were determined by Brine Shrimp Lethality Bioassay where the chloroformic extract of bark of C. inerme has the lowest LC_{50} value of 5µg/ml. The ethanolic and chloroformic extracts of leaf of C. inerme and methanolic extracts of bark of C. crista also exhibited a significant cytotoxic activity with LC_{50} values of 10µg/ml, 9.10µg/ml and 10µg/ml, respectively. In this investigation 0.5% chloramphenicol was used as the reference which LC_{50} value was 10µg/ml.

KEYWORDS: Cytotoxic Activity, Brine Shrimp, Traditional, Sundarbans, Shrubs and Lethality.

1 INTRODUCTION

Brine Shrimp Lethality Bioassay is a recent development in the assay procedure for the bioactive compounds and natural product extracts, which indicates cytotoxicity as well as a wide range of pharmacological activities e.g. anticancer, antiviral, pesticidal etc. (Anderson *et al.*, 1988). Bioactive compounds are almost always toxic in high doses. Pharmacology is simply toxicology at a lower dose or toxicology is simply pharmacology at a higher dose. Thus, *in vivo* lethality of a simple zoological organism (Brine shrimp napulii) can be used as a convenient monitor for screening and fractionation in the discovery of new bioactive natural products (Hui *et al*.,1990). Natural product extracts, fractions or pure compounds can be tested for their bioactivity by this method. This bioassay is indicative of cytotoxicity and a wide range of pharmacological activity of natural products. The brine shrimp lethality bioassay was proposed by Michael et al. in 1956 and modified by others. Since its introduction, this *in vivo* lethality test has been successively employed for providing a frontline screen that can be backed up by more specific and more sophisticated bioassays once the active compounds have been isolated. The brine shrimp lethality bioassay is rapid (24 h), simple (e.g., no aseptic techniques are required), easily mastered, inexpensive, and requires small amounts of test material (Ghisalberti, *et al.,* 1993). Thus, this research work was designed to evaluate the cytotoxic activity of *C. inerme* and *C. crista* by Brine Shrimp Lethality Bioassay for further exploration of their bioactive constituents.

2 MATERIALS AND METHOD

TEST MATERIALS

The Selected plant *Clerodendrum inerme* and *Caesalpinia crista* were collected from Ghagramaree, Chadpai range, Eastern Forest Department of the Sundarbans on 25th March, 2011 and collected plants samples were identified by the

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experts of Bangladesh National Herbarium, Mirpur, Dhaka. This research work was carried out from 26th March to 15th September, 2011 in Molecular Biology and Animal Cell Culture Laboratory of Khulna University, Khulna, Bangladesh.

EXPERIMENTAL MATERIALS

Artemia salina Leach (brine shrimp eggs form store), Table salt, Pure NaCl, Small tank (glass jar) to grow shrimp, cover and lamp to attract shrimp, Pipettes (5 ml,1 ml), Micro-pipette (10µl, 200µl adjustable), Test tube (15 ml), Volumetric flask (10ml), Tween-80, Spoon, Electric water blower to produce current, Electric bulb to produce heat, Stand to hold the bulb, Petri dish, Test tube stand, Beaker (1 liter), pH meter, Chloramphenicol, Aluminum foil, Detergent, Digital balance, Vortex machine.

PREPARATION OF MATERIALS

Collected plant samples were washed by distilled water (DW) to remove undesirable materials and excess of water was drained off. The leaves and stems were separated from each other and they were sliced into small pieces. The sliced materials were weighed by balance. The sliced leaves and stems were dried for few days under sunlight with shadow. After that the dried samples were powdered separately by grinding machine and then 5.4 gm powdered leaf of *C. inerme* was taken into clean flat-bottomed glass container and soaked in 200 ml of 95% ethanol, 5.4 gm powdered leaf of *C. inerme* and 12.8 gm powdered bark of *C. crista* was taken into clean flat-bottomed glass container and soaked in 200 ml of absolute methanol and 5.4 gm powdered leaf and 10.72 gm powdered bark of *C. inerme* and 12.8 gm powdered bark of *C. crista* was taken into clean flat-bottomed glass container and soaked in 200 ml of absolute chloroform. The containers with its contents were sealed and kept for a period of 7 days accompanying occasional shaking and stirring. The whole mixture then underwent a coarse filtration by a piece of clean, white cotton material. Then it was filtered through Whatman filter paper. The filtrates obtained were evaporated under ceiling fan and in a water- bath until dried. It rendered concentrates of greenish, brownish, reddish and sometimes blackish in color. The concentrates were designated as crude extract of 95% ethanol, methanol and chloroform of *C. inerme* and *C. crista*.

HATCHING OF BRINE SHRIMP

60gm Sea salt (pure NaCl 20gm and table salt 40gm) was weighed accurately, dissolved in distilled water to make one liter and then filtered off to get a clear solution. Sea water was taken in the small tank and shrimp eggs were added to the one side of the tank and the side was covered. The eggs were allowed for two days (24-36 hrs) to hatch and mature as nauplii (larvae). The hatched shrimps were attracted to the lamp through the perforations in the dam and they were taken for bioassay.

PREPARATION OF STOCK SOLUTION

7mg of dried ethanolic, methanolic and chloroformic extracts of leaves and chloroformic extract of barks of *C. inerme,* methanolic and chloroformic extracts of barks of *C. crista* were taken into 10ml individual volumetric flask. The extracts were dissolved in 1.4 ml sea water and one drop Tween-80 and adjusted to final concentration of 5µg/µl. As standard 0.5% sterile Chloramphenicol eye drop was used which concentration was 5µg/µl.

PROCEDURE

One hundred clean test tubes were taken, 14 of which were for the samples in seven different concentrations (two test tubes for each concentration), 14 for positive control (two test tubes for each concentration) and 2 for negative control. Then 4ml of seawater was given to each of the test tubes. With the help of the micropipette specific volumes (5, 10, 20, 40, 80,160 and 320µl) of samples were transferred from the stock solutions to the test tubes. Different concentration of choloramphenicol (5, 10, 20, 40, 80, 160 and 320µl) were taken in the rest of the 14 test tubes which are used as positive control and finally the volume is maintained at 10ml in each test tube (sample, positive control and negative control) using sea water. So, the concentration of samples and chloramphenicol became 2.5, 5, 10, 20, 40, 80 and 160µg/ml respectively. Finally with the help of a Pasteur pipette, 10 live shrimps nauplii were taken into each of the test tubes (Meyer *et al.,* 1982). After 24 hrs, the test tubes were observed and the numbers of survived nauplii in each test tube were counted and the results were noted. From this, the percentage of lethality of brine shrimp nauplii was calculated at each concentration for each sample.

STATISTICAL ANALYSIS

By using these following equations Arithmetic Mean, Standard Deviation and Standard Error were calculated:

Eq. (1) Arithmetic Mean ($\overline{X}$) = $\frac{\sum X}{n}$

Where, ΣX = Summation of Observed Value

n = No. of Observation

Eq. (2) Standard Deviation (SD) = $\sqrt{\frac{\sum(X-\overline{X})^2}{n}}$

Where, X =Individual Value

$\overline{X}$ = Mean Value

n = No. of Observation

Eq. (3) Standard Error (SE) = $\frac{SD}{\sqrt{(n-1)}}$

Where, SD = Std. Deviation

n = No. of Observation

3 RESULTS AND DISCUSSIONS

In the cytotoxicity assay based on the lethality of brine shrimp, the crude extract of *C.inerme* and *C.crista* showed lethality indicating the biological activity of the compound present in the extract. Results found in this bioassay are tabulated to the next pages.

Table 1. Results for cytotoxic effect of chloramphenicol

Sample Conc. (µg/ml)	Treatment-1	Treatment-2	Avg. no of alive shrimp sample	Negative control (alive)	Std. deviation	Std. error	Mortality (%)	LC_{50} (µg/ml)
2.5	9	9	9	10	0	0	10	10
5	7	8	7.5		0.707107	0.5	25	
10	5	5	5		0	0	50	
20	4	4	4		0	0	60	
40	3	3	3		0	0	70	
80	0	0	0		0	0	100	
160	0	0	0		0	0	100	

Table 2. Results for cytotoxic effect of the methanolic extract of leaf of C. inerme

Sample Conc. (µg/ml)	Treatment-1	Treatment-2	Avg. no of alive shrimp sample	Negative control (alive)	Std. deviation	Std. error	Mortality (%)	LC_{50} (µg/ml)
2.5	8	7	7.5	10	0.707107	0.5	25	36.5
5	7	7	7		0	0	30	
10	7	6	6.5		0.707107	0.5	35	
20	6	5	5.5		0.707107	0.5	45	
40	5	4	4.5		0.707107	0.5	55	
80	4	2	3		1.414214	1	70	
160	2	1	1.5		0.707107	0.5	85	

Table 3. Results for cytotoxic effect of the ethanolic extract of leaf of C. inerme

Sample Conc. (µg/ml)	Treatment-1	Treatment-2	Avg. no of alive shrimp sample	Negative control (alive)	Std. deviation	Std. error	Mortality (%)	LC_{50} (µg/ml)
2.5	6	7	6.5	10	0.707107	0.5	35	10
5	6	6	6		0	0	40	
10	5	5	5		0	0	50	
20	4	6	5		1.414214	1	50	
40	4	3	3.5		0.707107	0.5	65	
80	3	2	2.5		0.707107	0.5	75	
160	1	0	0.5		0.707107	0.5	95	

Table 4. Results for cytotoxic effect of the chloroformic extract of leaf of C. inerme

Sample Conc. (µg/ml)	Treatment-1	Treatment-2	Avg. no of alive shrimp sample	Negative control (alive)	Std. deviation	Std. error	Mortality (%)	LC_{50} (µg/ml)
2.5	6	8	7	10	1.414214	1	30	9.10
5	6	6	6		0	0	40	
10	5	4	4.5		0.707107	0.5	55	
20	4	4	4		0	0	60	
40	3	3	3		0	0	70	
80	1	1	1		0	0	90	
160	0	1	0.5		0.707107	0.5	95	

Table 5. Results for cytotoxic effect of the chloroformic extract of bark of C. inerme

Sample Conc. (µg/ml)	Treatment-1	Treatment-2	Avg. no of alive shrimp sample	Negative control (alive)	Std. deviation	Std. error	Mortality (%)	LC_{50} (µg/ml)
2.5	7	7	7	10	0	0	30	5
5	5	5	5		0	0	50	
10	4	5	4.5		0.707107	0.5	55	
20	4	4	4		0	0	60	
40	4	3	3.5		0.707107	0.5	65	
80	3	3	3		0	0	70	
160	1	2	1.5		0.707107	0.5	85	

Table 6. Results for cytotoxic effect of the methanolic extract of barks of C. crista

Sample Conc. (µg/ml)	Treatment-1	Treatment-2	Avg. no of alive shrimp sample	Negative control (alive)	Std. deviation	Std. error	Mortality (%)	LC_{50} (µg/ml)
2.5	8	7	7.5	10	0.707107	0.5	25	10
5	5	7	6		1.414214	1	40	
10	4	6	5		1.414214	1	50	
20	4	5	4.5		0.707107	0.5	55	
40	3	2	2.5		0.707107	0.5	75	
80	3	1	2		1.414214	1	80	
160	1	1	1		0	0	90	

Table 7. Results for cytotoxic effect of the chloroformic extract of barks of C. crista

Sample Conc. (µg/ml)	Treatment-1	Treatment-2	Avg. no of alive shrimp sample	Negative control (alive)	Std. deviation	Std. error	Mortality (%)	LC_{50} (µg/ml)
2.5	8	8	8	10	0	0	20	18.2
5	6	7	6.5		0.707107	0.5	35	
10	5	6	5.5		0.707107	0.5	45	
20	4	5	4.5		0.707107	0.5	55	
40	3	4	3.5		0.707107	0.5	65	
80	3	1	2		1.414214	1	80	
160	1	1	1		0	0	90	

Test samples are showed different mortality rate at different concentrations. The mortality rate of brine shrimp was found to be increased with the increase in concentration of the sample and if we plot of percent mortality versus different

concentrations of extracts on the graph paper produced an approximate linear correlation between them. The comparative analyses of LC_{50} value of different plant extract are given below:

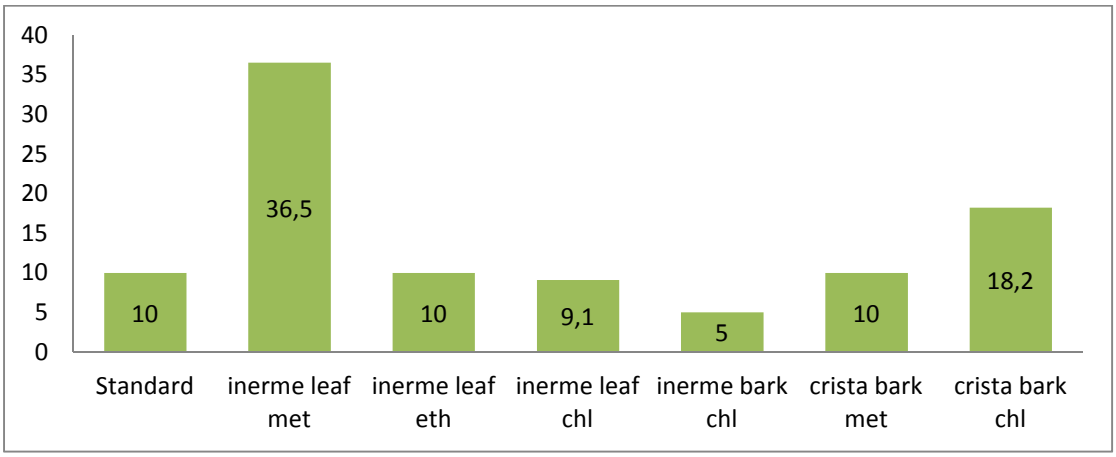

Figure 1. Comparative analysis of LC_{50} values in µg/ml among the plant extracts

LC_{50} = Lowest Concentration where mortality rate is 50%, met= Methanol, eth=Ethanol and chl=Chloroform

The chloroformic extract of *inerme* bark has the lowest LC_{50} value of 5µg/ml. On the other hand methanolic extract of *inerme* leaf has the highest LC_{50} value of 36.5µg/ml. The lower LC_{50} value higher the activity. So, the chloroformic extract of *inerme* bark has higher cytotoxic effect than the others and methanolic extract of *inerme* leaf has lower cytotoxic effect than the others. Also the chloroformic and ethanolic extracts of leaf of *inerme* and methanolic extracts of bark of *crista* shows effective cytotoxicity in contrast of chloroformic extract of bark of *inerme.* Chloroform extracts of both leaf and bark demonstrated the highest potential in cytotoxicity assay indicating the active compound(s) was extracted best with chloroform as solvent system.

4 CONCLUSION

The result shows that the cytotoxic activity exhibited by the solvent fractions was promising and this clearly indicates the presence of potent bioactive compounds and also suggests its possible antitumor activity. In future, further investigation is needed with the hope to search new bioactive compound in *C. inerme* and *C. crista* as well as initiatives are also need to be encouraged to develop any possible medicine in Bangladesh.

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