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Research Article

BRINE SHRIMP LETHALITY BIOASSAY OF METHANOLIC EXTRACT OF CENTELLA ASIATICA LEAVES

Rishikesh [1] *,Drishti Rani Ghosh [1], MD. Asraful Islam [1,] S.M. Siddiqul Islam[2], Md. Moshfiqur Rahman[2].
Department of Pharmacy, The University of Asia Pacific [1], Dhaka-1209, Bangladesh.
Department of Pharmacy, Manarat International University [2], Dhaka-1216, Bangladesh.
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Corresponding Author's email: rishibd@gmail.com

Abstract: The methanol extract of *Centella asiatica* was evaluated for cytotoxic effect. Cytotoxic activity was investigated by brine shrimp lethality bio-assay (BSLB). The extract showed potent cytotoxic effect in Brine Shrimp Lethality Bioassay where the extract shows the value of LC50 and LC 90 is 1.905µg/ml and 3.350µg/ml after 18hr & 24hr repetitively. After 18hr & 24hr for n-haxane value of LC50 and LC 90 are 1.831µg/ml and 3.217 µg/ml and after 21hr & 24hr for CCl_4 value of LC50 and LC 90 is 1.152 µg/ml and 2.195 µg/ml the study gave a mild indication to the use of the plant extract as a potential source for cytototoxic compounds. The mortality rate of brine shrimp was found to be increased with the increased concentrations of sample, and a plot of log of concentration versus percent mortality on the graph produced an approximate linear correlation. Toxicological activity, indicate that have potent anti-mutagenic activity, clot lytic property and anti-oxidative activity. The obtained results support for the uses of this plant as traditional medicine.

Key words: Floating Brine shrimp, Cytotoxicity, Methanolic Extract, *Centella asiatica.*

INTRODUCTION

Medicinal plants play a dominant role in the treatment of varieties of human diseases from the twilight of the human civilization[1]. Obsession on modern medicinal system leads people to an alternative approach to improve and maintain good health is increased tremendously by using medicinal herb over last centuries. Many of the modern day's important drugs and processed medicines are of plant origin. Medicinal plants contain different therapeutic agents which may have thrombolytic activity, antimicrobial activity,cytotoxic effect etc.

Fig.1: ***Centella asiatica***

Centella asiatica have been of medical interest due to their good therapeutic value in folk medicine [3]. Working with different medicinal plants extract showed that they can lyses thrombus as streptokinase [2, 4]. The plant sap can act against microorganisms by preventing the growth of microbial colony [5]. Some of the plant extract also increase lethality of the cell due to their known cytotoxic effect. Brine shrimp lethality bioassay is performed for evaluating the level of toxicity [6, 7].
Brine shrimp lethality bioassay is a recent development in the assay procedure for the bioactive compounds [11, 15] and natural product extracts, which indicates cytotoxicity as well as a wide range of pharmacological activities e.g. anticancer, antiviral, and pharmacological activities of natural products etc.[8].

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, a simple zoological organism(brine shrimp napulii- *Artemia salina*) can be used as a convenient monitoring for screening and fractionation in the discover of new bioactive natural products [9].

Natural product extracts, fractions or pure compounds can be tested for there bioactivity by this method. This bioassay is indicative of cytotoxicity and a wide range of pharmacological activity of natural products. Brine shrimp lethalityis

a general bioassay, which is an indication of cytotoxicity,antibacterial activities, pesticidal effects and various pharmacologic actions [10].

MATERIALS AND METHODS

Collection and identification of the plant sample:

The plant centella asiatica (Family: Umbelliferae) was collected from Dhaka and was taxonomically identified with the help of National Herbarium of Bangladesh, Dhaka. The leave and the steam were cut into small pieces and the sun dried for seven dayes.The leaves and stems were ground into coarse powder with the help of an attrition type of a grinder.

Extraction of leaves:

About 250 gm of powdered leaves was taken in a clean flat –bottomed glass container and percolated with 3 liters of Methanol. The container with its content was sealed and kept for 7 days with occasional shaking and stirring .the mixture was the filtered successively through a piece of clean white cotton .The filtrate thus obtained are kept in a open air for the evaporation of the methanol. After 10 to 15 days all the methanol are evaporated and I got the extract of methanol.

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Brine shrimp:

Brine shrimp is the English name of the genus *Artemia* of aquatic crustaceans. *Artemia*, the only genus in the family Artemiidae, have evolved little since the Triassicperiod. The historical record of existence of *Artemia* dates back to 982, more than one thousand years ago, from Lake Urmia, Iran, while Schlösser was the first person to give drawings of *Artemia* in 1756. *Artemia* are found worldwide in inland saltwater lakes, but not in oceans[12].

Life cycle:

Brine shrimp eggs are metabolically inactive and can remain in total stasis for two years while in dry oxygen-free conditions, even at temperatures below freezing. This characteristic is called cryptobiosis meaning "hidden life" (also called diapauses). While in cryptobiosis, brine shrimp eggs can survive temperatures of liquid air (−190 °C, −310 °F) and a small percentage can survive above boiling temperature (105 °C, 221 °F) for up to two hours. Once placed in brine (salt) water, the cyst-like eggs hatch within a few hours. The nauplii, or larvae, are less than 0.5 mm in length when they first hatch. Brine shrimp have a biological life cycle of one year, during which they grow to a mature length of around one centimeter on average. This short life span, along with other characteristics such as their ability to remain dormant for long periods, has made them invaluable in scientific research, including space experiments[13].

Tolerance to salinity:

Brine shrimp can tolerate varying levels of salinity. A common biology experiment in school is to investigate the effect of salinity levels on the growth of these creatures. The preferred level of salinity is about 120ppt.

Nutritional benefit:

The nutritional properties of newly hatched brine shrimp make them particularly suitable to be sold as aquarium food as they are high in lipids and unsaturated fatty acids (but low in calcium).

Preparation of stock solution:

4 mg of dried crude extract was taken in 10 ml volumetric flask and volume was adjusted by 5ml water. The concentration of this solution was 400 µg/µl and added by 100 µl DMSO solution. Similarly we made concentration of 400 µg/µl like as n-hexane, CCl4, and Fr-1.

Fig.2: Brine shrimp nauplii.

Preparation of simulated sea water:
20g of NaCl and 18g of table salt were weighed accurately, dissolved in distilled water to make one liter and then filtered off to get a clear solution.

Hatching of brine shrimp:
Sea water was taken in the small tank and shrimp eggs were added to the one side to the divided tank and the side was covered. The shrimps were allowed for 24 hours 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. In hatching time was 22 hours.

Application of test solution and brine shrimp nauplii to the test tubes:
For crude extract, Seven (07) clean test tubes were taken, six (06) of test tube contain different samples concentration and one (1) for negative control test. Then we taken ½(half) portion of stock solution in fist test tube and added 100 µl DMSO solution and added 2.5ml of sea water was given of this test tubes. Similarly, this process contain next test tube and negative control test tube contain only 5ml of sea water. This six test tube contain concentration respectively 200 µg/µl, 100 µg/µl, 50 µg/µl, 25 µg/µl, 12.5 µg/µl, 6.75 µg/µl, 3.125 µg/µl. Finally with the help of a Pasteur pipette 15 living shrimps were kept to each of the test tubes [11].For n-hexane, CCl4, and Fr-1 solution prepared by the following above this procedure.

Counting of Nauplii:
After 18hrs the test tubes were observed and the number of survived nauplii in each test tube was counted and the results were noted. Form this, the percentage of lethality of brine shrimp nauplii was calculated at each concentration for each sample. Like above procedure after 24 hour's the percentage of lethality of brine shrimp nauplii was calculated at each concentration for each sample.

RESULT AND DISCUSSION
Toxicity means adverse or poisonous effects of drugs, toxins or their metabolites. Though the extracts of the plant *Centella asiatica* possess potent cytotoxic activity, hence it may say that they may show some anti microbial activity. Brine Shrimp Lethality Bioassay, a bench top bioassay method for evaluating anticancer, antimicrobial and other pharmacological activities of natural products are a recent development in the bioassay for the plant extracts of the plant, *Centella asiatica* were examined on the larvae of brine shrimp, *Artemia salina* Leanh. This bioassay method is indicative of cytotoxicity and a wide range of pharmacological activities of the compound. In cytotoxicity, the LC50 value of the extract was found significant (250 µg/ml) which indicates that the extract of *Centella asiatica* has high pharmacological actions. It also indicates that the plant might have the potentiality to kill cancer cells [10]. In the bioassay, the crude extract showed lethality indicating the biological activity of the compound present in the extract Tests sample showed different mortality rate at different concentrations. Plot of percent of mortality versus log concentration on the graph paper produced an approximate linear correlation between them. From the graph (figure) the concentration at which 50% & 90% mortality (LC50, LC90) of brine shrimp nauplii occurred can be obtained by extrapolation.

Test sample 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 plot of percent mortality versus concentration on the graph paper produced an approximate linear correlation between them. From the Table-1,Table-2, Table-3, the concentration at which 50% mortality (LC_{50}) of brine shrimp nauplii occurred were obtained by extrapolation, the values were found 1.905 µg/ml, 1.831 µg/ml, and 1.152µg/ml, after 18hours, and 24 hours respectively for the crude extract, n-hexane, and CCl_4. The 90% mortality (LC_{90}) values were found 3.35 µg/ml, 3.217 µg/ml, and 2.195µg/ml, after 18hours and 24 hours respectively for the crude extract, n-hexane, and CCl_4.So all fraction are less potent lethality against the brine shrimp nauplii. Brine shrimp lethality is a general bioassay, which is an indication of cytotoxicity, antibacterial activities, pesticidal effects and various pharmacologic actions [10].

A general bioassay that appears capable of detecting a broad spectrum of bioactivity present in crude extracts is the brine shrimp lethality bioassay (BSLB). The technique is easily mastered, of little cost, and utilizes small amount of test material. The aim of this method is to provide a front-line screen that can be backed up by more specific and more expensive bioassays once the active compounds have been isolated. It appears that BSLB is predictive of cytotoxicity activity [15]. The result obtained from the brine shrimp lethality bioassay of *Centella asiatica* can be used as a guide for the isolation of cytotoxic compounds from the methanolic extract of the leaves of this plant.

CONCLUSION
This report may serve as a footstep to use this plant as a new source of medication. From this experiment, in summary, pharmacological evaluation of methanol extract of *Centella asiatica* has got the very good potential as a candidate for future thrombolytic agent and also it can be investigated as a possible source of antitumour drugs. This is only a

preliminary study and to make final comment the extract should thoroughly investigated phytochemically and pharmacologically to exploit their medicinal and pharmaceutical potentialities.

Table 1: Brine shrimp bioassay of Crude extract of *Centella asiatica*

Conc. C µg/ml	Log C	No. of Brine shrimp added	No. of alive Brine shrimp after 18 hours	No. of alive after24 hours	Mortality	%of mortality	LC_{50} µg/ml	LC_{90} µg/ml
200	2.301	15	6	5	9.5	63.333	1.905	3.35
100	2	15	8	7	7.5	50		
50	1.699	15	9	8	6.5	43.333		
25	1.398	15	10	9	5.5	36.66		
12.5	1.097	15	12	11	3.5	23.333		
6.25	0.796	15	12	11	3.5	23.33		
Blank		15	14	13	1.5	10		

Table 2: Brine shrimp bio-assay of n-hexane fraction of *Centella asiatica.*

Conc. C µg/ml	Log C	No. of Brine shrimp added	No. of alive Brine shrimp after 18 hours	No. of alive after24 hours	Mortality	%of mortality	LC50 µg/ml	LC_{90} µg/ml
200	2.301	15	6	5	9.5	63.333	1.831	3.217
100	2	15	8	7	7.5	50		
50	1.699	15	9	8	6.5	43.333		
25	1.398	15	10	8	6	40		
12.5	1.097	15	12	10	4	26.666		
6.25	0.796	15	12	11	3.5	23.333		
Blank		15	14	12	2	13.333		

Table 3: Brine shrimp bioassay of CCl4 fraction of *Centella asiatica.*

Conc. C µg/ml	Log C	No. of Brine shrimp added	No. of alive Brine shrimp after 18 hours	No. of alive after24 hours	Mortality	%of mortality	LC_{50} µg/ml	LC_{90} µg/ml
200	2.301	15	1	0	14.5	96.666	1.152	2.195
100	2	15	3	1	13	86.666		
50	1.699	15	5	3	11	73.333		
25	1.398	15	7	5	9	60		
12.5	1.097	15	9	8	6.5	43.333		
6.25	0.796	15	10	9	5.5	36.66		
Blank		15	13	11	3	20		

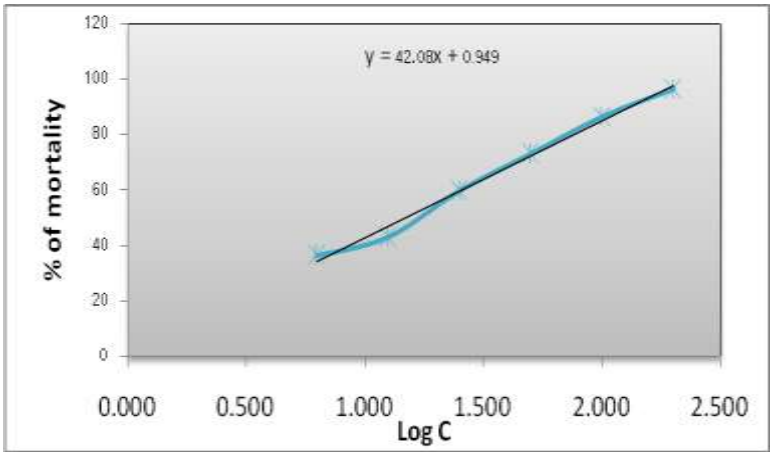

Fig.3: Graphical representation of Brine shrimp lethality bioassay of the *Centella asiatica*

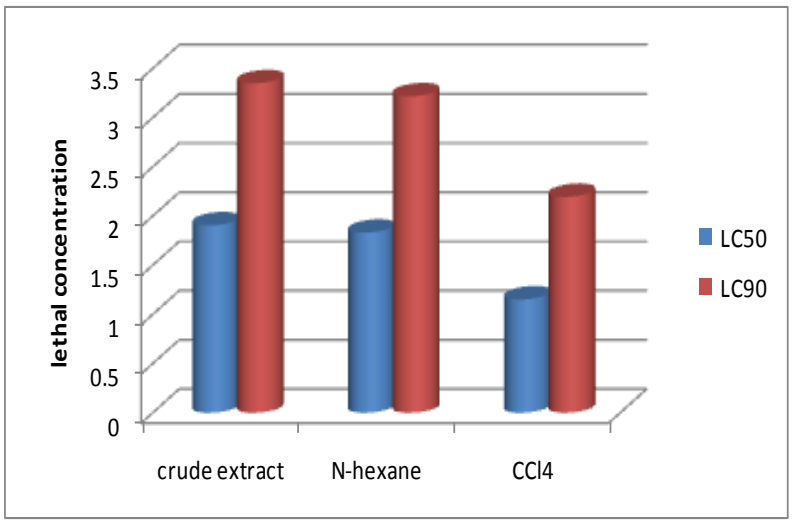

Fig.4: Comparison among LC50 & LC90 concentration

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