Journal of Coastal Life Medicine

journal homepage: www.jclmm.com

Original article doi: 10.12980/JCLM.3.2015J5-50



# Estimation of cytotoxic potency by brine shrimp lethality bioassay application of *Clerodendrum infortunatum* Linn.

Talukdar Muhammad Waliullah[1*Δ], Akter Mst Yeasmin[1Δ], Ashraful Md Alam[2], Wahedul Md Islam[3], Parvez Hassan[3]

[1]*Molecular Cellular Biology Laboratory, Bioscience Department, GSST, Shizuoka University, 836-Oya, Suruga-ku, Shizuoka 422-8529, Japan*

[2]*FIQC Laboratory, Department of Fisheries, Matshya Bhaban, Ramna, Dhaka-1000, Bangladesh*

[3]*Institute of Biological Sciences, University of Rajshahi, Rajshahi-6205, Bangladesh*

ARTICLE INFO

*Article history:*
Received 12 May 2015
Received in revised form 18 May 2015
Accepted 29 Jun 2015
Available online 10 Jul 2015

*Keywords:*
Plant
Ethnobotanical information
Median lethal dose
Cytotoxicity
*Artemia salina*
Standard deviation

ABSTRACT

**Objective:** To learn a scientific and systematic knowledge of anticancer, antimicrobial and pharmacological activities of natural products and estimate cytotoxic potency by using ethanol and chloroform extracts of root, leaf and stem of *Clerodendrum infortunatum* (Verbenaceae) due to its random use in customary and traditional medicine to cure common ailments such as intestinal disorder, diarrhea, tuberculosis and respiratory problems *etc*.

**Methods:** The *in vitro* application was carried out with the bench-top bioassay method by using brine shrimp lethality bioassay.

**Results:** All of the crude extracts were found to be lethal and effective. The $LC_{50}$ value of ethyl alcohol fraction of root was 20.845 mg/L compared to the standard drug tetracycline of 14.675 mg/L to brine shrimp nauplii, indicating that the extracts were biologically active.

**Conclusions:** The cytotoxic study of $LC_{50}$ value showed that a good correlation with the antibiotic tetracycline. From the comparative correlation error bars and percentage, we understood that ethyl alcohol fraction of root extract was very effective. This study serves as a basis for further research to lead compounds to be isolated so that it may be as a template for the implications of these results for bioactivity and drug discovery potential of herbal products.

## 1. Introduction

The use of indigenous knowledge of traditional medical practitioners as leads provides a useful route employed in the search for novel drugs[1]. The antimicrobial potential of different medicinal plants is being extensively studied all over the world, but only a few studies have been carried out in a systematic manner[2]. Medical knowledge derived from traditional societies has motivated searches for new bioactive molecules derived from plants that show potent activity against bacterial pathogens[3]. Nowadays, some researchers are providing laboratory trials to justify for the use of the plants in folk medicine to treat various infectious diseases[4].

In the present study, *Clerodendrum infortunatum* Linn. (Verbenaceae) (*C. infortunatum*) is selected to assess its ethnobotanical importance due to its random use as traditional medicine to cure common ailments such as intestinal disorder, diarrhea, tuberculosis and respiratory problems *etc*. It is reported that Bangladesh has over 5 000 medicinal plants and uses of these plants for medicinal purposes are remarkable[5]. *C. infortunatum* is generally used in folk, hill tracts and rural slums as indigenous medicine, food supplement, decoration of home appliances and firewood[6,7]. *Clerodendrum* is a very large and diverse genus and till now five hundred and eighty species of the genus have been identified and are widely distributed in Asia, Australia, Africa and America[8]. Root and leaf extracts of *Clerodendrum indicum*, *Clerodendrum phlomidis*, *Clerodendrum serratum*, *Clerodendrum trichotomum*, *Clerodendrum chinense* and *Clerodendrum petasites* have been used for the treatment of rheumatism, asthma and other inflammatory diseases[9]. It was also reported that tribes use *Clerodendrum inerme* as an antidote of poisoning from fish, crabs and toads[10]. Roots, leaves and fresh juice of leaves of *C. infortunatum* were used in eliminating ascarids and tumors and also as a laxative[11]. Another study on *Clerodendrum chinense*

*Corresponding author: Talukdar Muhammad Waliullah, Molecular Cellular Biology Laboratory, Bioscience Department, GSST, Shizuoka University, 836-Oya, Suruga-ku, Shizuoka 422-8529, Japan.
Tel: +81-080-9486-2020
E-mail: olymwt@live.com
ΔThe authors equally contributed to this work.

Foundation Project: Supported by Ministry of Science and Information & Communication Technology, Government of the Peoples Republic of Bangladesh under the project of Research and Development (Grant No. Bitojopromo/sha-12/goupro-8/2008/268).

and *Clerodendrum splendens* has been reported that chloroformic extracts of flower and stem were active against *Plasmodium falciparum* and *Trypanosoma cruzi*, suggesting that leaf extracts showed promising activities against *Trypanosoma cruzi*[12].

To explore a scientific idea, this study was designed to undergo an *in vitro* evaluation of the cytotoxic potency carrying on *Artemia salina* by brine shrimp lethality bioassay for identification and determination of chemical structure of the bioactive compounds.

## 2. Materials and methods

### 2.1. Plant collection and identification

The plant specimen was collected from Rajshahi University Campus, Bangladesh. Identification of voucher specimen was confirmed at the Taxonomical Section, Department of Botany, University of Rajshahi, Bangladesh.

### 2.2. Preparation of extracts

Root, leaf and stem were dried in shade, stored in cotton bags and then finely powdered (100 g) separately with the help of a grinder. Each ground material was soaked in 500 mL ethyl acetate and chloroform separately for 24–72 h and filtered (Whatman No. 1). The filtered one was then allowed to vaporize in rotary evaporator until completely dried and kept in a refrigerator. Then dried extract (100 mg and 50 mg), for further study, was weighed and dissolved in 10 mL of respective solvents for dilution. The concentrations of the final extract were 100 μg/10 μL and 50 μg/10 μL.

### 2.3. In-vitro cytotoxicity bioassay

Brine shrimp lethality bioassay is a bench-top bioassay method for evaluating anticancer, anti-microbial and pharmacological activities of natural products[13-15]. Natural product extracts, fractions or pure compounds can be tested for their bioactivity by this method. Here *in vivo* lethality of a simple zoological organism (brine shrimp nauplii) was used as a convenient monitor for screening of a fractionation in the discovery of new bioactive natural products[16]. Generally, the median effective dose value for cytotoxicity is one tenth of median lethal dose value in the brine shrimp test[14]. Bioactive compounds are almost always toxic in high doses. There is a positive correlation between brine shrimp toxicity and cytotoxicity[17,18].

### 2.4. Hatching of brine shrimp

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

### 2.5. Preparation of test sample

Ethyl alcohol and chloroform fractions of *C. infortunatum* root, leaf and stem extracts were applied against brine shrimp nauplii. Initially, 2 mg extracts of each part were separately dissolved in 100 μL of pure dimethyl sulfoxide (DMSO) to make them hydrophilic before adding 19.98 mL of water to get a concentration of 200 mg/L for each which was used as stock solution A. From the stock solution A, 10 mL was taken (with a concentration of 200 mg/L) and diluted up to 20 mL with brine water to obtain a concentration of 100 mg/L and that made a stock solution B; and from the stock solution B, 10 mL was taken (with a concentration of 100 mg/L) and diluted up to 20 mL with brine water to obtain a concentration of 50 mg/L for making stock solution C. This is called the serial dilution method. Then the concentrations were made 200, 100 and 50 mg/L. During experiments, according to the dose-mortality effects, dose ranges were made different for some convenience in achieving a good result since the extracts were active against the brine shrimp nauplii.

### 2.6. Preparation of the control group

For each concentration, one test tube containing the same volume of DMSO diluted up to 10 mL with sea water and 30 brine shrimp nauplii was used as negative control group. It was used to verify the validity of the test. When the nauplii in the control showed a rapid mortality, then the test was considered to be invalid as the nauplii might die due to reasons other than the cytotoxicity of the compounds.

### 2.7. Application of test sample and brine shrimp nauplii to the test tubes

In each of the five test tubes, 10 mL brine shrimp solution (3.8%) was taken, containing 30 brine shrimp nauplii with the help of a micropipette, and specific volumes of each sample were transferred from the stock solution B to the respective vials to get final concentrations of 200, 100 and 50 μg/mL. The volumes of DMSO in these test tubes should not exceed 10 μL/mL of the brine shrimp solution, because once above this concentration, toxicity due to DMSO may arise.

### 2.8. Counting of nauplii

After 24 h, the test tubes were observed. The number of survived nauplii in each test tube was counted and the results were noted. From that, mortality percentage of brine shrimp nauplii was calculated at each concentration for each sample.

### 2.9. Statistical analysis

The dose-mortality data were analyzed statistically by probit analysis[19]. The effectiveness or the dose-mortality relationship (concentration-mortality relationship) of the plant product was usually expressed as a $LC_{50}$ value, which represented the

concentration of the chemical that caused death in half of the test subjects after a certain exposure period.

## 3. Results

The results of the brine shrimp lethality bioassay were presented. The ethyl alcohol and chloroform extracts of root, leaf and stem of *C. infortunatum* were subjected to apply against the brine shrimp (*Artemia salina*) nauplii for the detection of their biological activity. Several doses were selected by a pilot (*ad hoc*) experiment and a final experiment was set up with 3 replications and control doses. From the experiment, it was revealed that each of the test samples showed different mortality rates at different concentrations. The mortality rates of brine shrimp nauplii increased with the rise of the concentration of the tested crude extracts. The $LD_{50}$ values along with 95% confidence limits, regression equations and *Chi*-squared value of the crude extracts (root, leaf and stem) and the tetracycline for 24 h of exposure were determined, using probit analysis[20]. Regression lines drawn by plotting log concentrations of the extracts of *C. infortunatum* against probit mortality revealed linear correlation between log doses and empirical probit mortality (Figures 1-3).

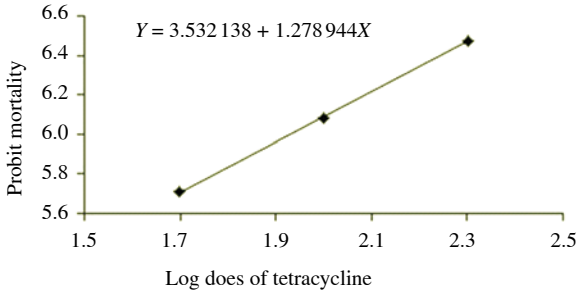


**Figure 1.** Probit regression line between probit mortality of brine shrimp nauplii and log does (+1 mg/cm$^2$) of tetracycline after 24 h exposure.

The $LC_{50}$ value of root, leaf and stem were 20.845, 24.017 and 31.379 mg/L for ethyl alcohol, 30.702, 32.907 and 42.559 mg/L for chloroform respectively and 14.675 mg/L for tetracycline.

The 95% confidence limits of root, leaf and stem were 5.3350 to 81.447, 6.3610 to 90.675 and 11.543 to 85.303 for ethyl alcohol, 11.373 to 82.880, 14.041 to 77.119 and 20.822 to 86.987 for chloroform respectively and 2.1180 to 101.670 for tetracycline.

The regression equations of root, leaf and stem were $Y$ = 3.123 247 + 1.445 058$X$, Y = 3.066 222 + 1.395 237$X$ and $Y$ = 2.783 323 + 1.461 687$X$ for ethyl alcohol (Figure 2), $Y$ = 2.544 355 + 1.611 173$X$, $Y$ = 2.371 780 + 1.710 795$X$ and $Y$ = 2.440 868 + 1.577 916$X$ for chloroform respectively (Figure 3) while $Y$ = 3.532 138 + 1.278 944$X$ for tetracycline (Figure 1).

The $\chi^2$ values (*df*) of root, leaf and stem were 0.111 416, 0.008 432 and 0.008 245 for ethyl alcohol, 0.063 406, 0.042 076 and 0.006 749 for chloroform respectively and 0.021 533 for tetracycline with 1 degree of freedom.

The $LC_{50}$ of cytotoxic bioassay test was compared to the standard antibiotic tetracycline with correlation error bars and percentage (Figure 4).

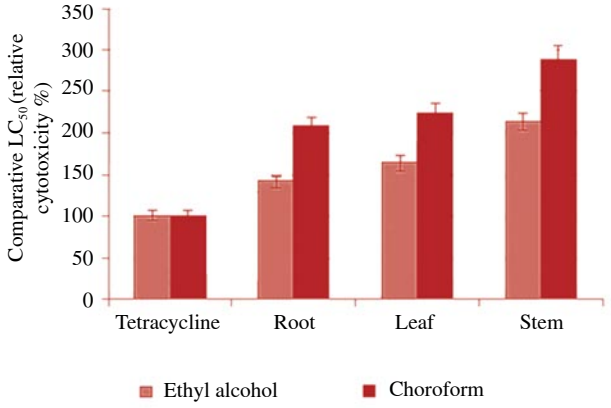


**Figure 4.** The comparative $LC_{50}$ with correlation error bars with percentage.

All of the crude extracts were found to be lethal and appeared significantly effective to brine shrimp nauplii indicating that the extracts were biologically active.

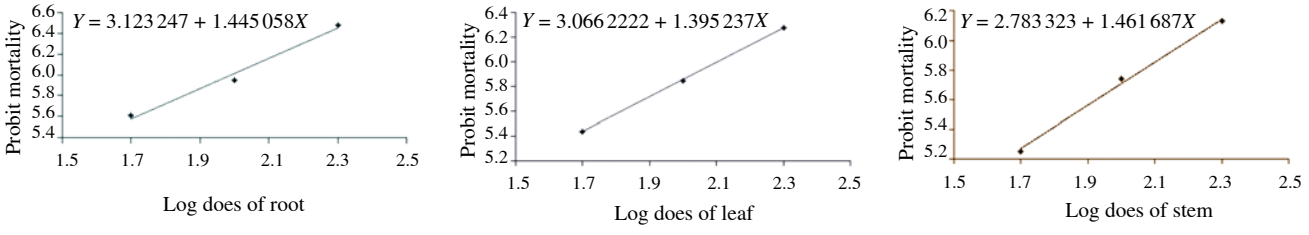


**Figure 2.** Probit regression lines between probit mortality of brine shrimps nauplii and log does (+1 mg/cm$^2$) of root, leaf and stem of ethyl alcohol extracts after 24 h exposure.

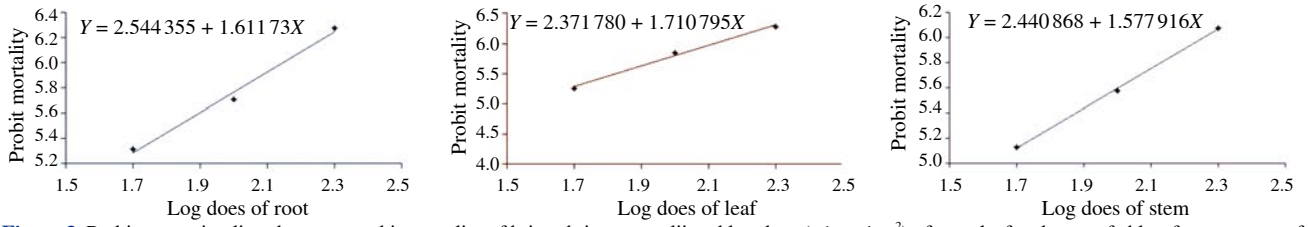


**Figure 3.** Probit regression lines between probit mortality of brine shrimps nauplii and log does (+1 mg/cm$^2$) of root, leaf and stem of chloroform extracts after 24 h exposure.

## 4. Discussion

From the present study, the mortality rates of brine shrimp nauplii increased with the rise of the concentration of the tested crude extracts. The $LC_{50}$ values of the crude extracts of root, leaf and stem were found to be 20.845, 24.017 and 31.379 mg/L for ethyl alcohol, 30.702, 32.907 and 42.559 mg/L for chloroform respectively and 14.675 mg/L for the tetracycline. The 95% confidence limits, regression equation and $\chi^2$ values were also significant. Patil and Magdum studied the lethality test of *Euphorbia hirta*[21,22]. Recorded cytotoxicity observation on brine shrimp nauplii also supports our previous antimicrobial activities of *C. inforunatum* and *Clerodendrum viscosum*[23-25]. Furthermore, consecutive chain of study represents our earlier results of insecticidal, repellent and mortality activity of *C. inforunatum*[26,27].

The cytotoxic study of $LC_{50}$ value showed a good correlation with the antibiotic tetracycline. From the comparative correlation error bars and percentage, we understood that root extract of ethyl alcohol fraction was very effective. Cytotoxic evidence of biologically active nature of the extracts of *C. infortunatum* was emerged from the result of the brine shrimp lethality bioassay. However, the potential chemical components of this plant showing bactericidal, fungicidal or other toxicological activities are yet to be identified.

The above findings indicate that *C. infortunatum* possesses promising biological properties and appears to be an effective material for the development of antimicrobial drugs and eco-friendly biopesticides.

## Conflict of interest statement

We declare that we have no conflict of interest.

## Acknowledgments

Institute of Biological Sciences, University of Rajshahi has provided the lab facility. This study was supported by Ministry of Science and Information & Communication Technology, Government of the Peoples Republic of Bangladesh under the project of Research and Development (Grant No. Bitojopromo/sha-12/goupro-8/2008/268).

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