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Assessment of Cytotoxic Activity Using *Artemia salina* (Brine Shrimp Nauplii) of *Citrus limon* L (Lemon) Seed Extract

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Abstract

For preliminary assessment of toxicity the brine shrimp lethality assay is deliberated a useful implement. To assessment natural marine products for pharmacological activity before they are used as a general bio-assay it is essential to evaluate the appropriateness of the brine shrimp methods. *Citrus limon* L (lemon) is an excellent preventative medicine and in the native medication chest it has a wide range of uses. It can assistance weight loss and reduce the risk of heart disease, anemia, kidney stones, digestive issues, and cancer. The current study was aimed to investigate cytotoxic properties of seed extract of *Citrus limon* L. (lemon) to give an appropriate guide for future investigation. The cytotoxic activity was determined by calculating the LC50 values. The *Citrus limon* L. (lemon) seed extract shows very good cytotoxic activity against the brine shrimp nauplii. Compared with the LC50 value of standard vincristine sulphate 1.25 mg/mL, the LC50 value of seed extract remained 5.55 mg/mL. However, these outcomes recommend that experimental extracts of *Citrus limon* L. (lemon) holds cytotoxic activity which sustenance its used in traditional medicine.

Keywords: *Citrus limon*; Cytotoxic activity; Brine shrimp; Vincristine sulphate

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Introduction

For treatment of ailments the natural products have been used and from natural sources a remarkable number of drugs have been isolated. About 4 billion people in the world depend on plant sources of drugs according to WHO [1]. New chemical compounds such as glycosides, steroids, alkaloids, terpenoids, flavones etc. are mainly accountable for their various therapeutic belongings and pharmacological actions are represented by the plant kingdom [2]. Michael et al. [3] proposed the shrimp lethality assay and later Vanhaecke et al. [4], developed it. This method is based on the capability of killing laboratory cultured Artemia nauplii brine shrimp [5]. Scientists have noticed that human body have developed hardy to drugs which are obtainable in market. Using synthetic drugs source various toxic and side belongings on human body. That is why a curiosity has grown in the world for herbal drugs. So we have selected *Citrus limon* L. plant for study since of its medicinal uses [6].

Citrus limon L belongs to the Rutaceae family and a species of *C. limon* native to South Asia, primarily North eastern India. The plants of most species of Citrus remained large ever green shrubs or small trees, 5 m to 15 m tall. Principally for its juice which has both culinary and cleaning uses the tree's ellipsoidal yellow fruit utilized for culinary and non-culinary resolutions between the world. In cooking and baking the pulp and rind (zest) are recycled. About 5% to 6% citric acid and with a pH of around 2.2 the juice of the lemon used as a sour taste. It is cultivated mainly for alkaloids which show anticancer and antibacterial activities in crude extracts of different parts of lemon against clinical significant in bacterial strain [7-9].

To the best of our knowledge, very few pharmacological studies have been reported so far on *Citrus limon* L (lemon) the medicinal plant. However present study was designed to assessment the role of cytotoxic activity of this plant as a part of the extension of our research on bioactivity screening of Bangladeshi pharmaceutical plants.

Materials and Methods

Chemicals and reagents

Artemia salina leach (Brine shrimp eggs), Small tank with perforated dividing dam to hatch the

Table 1: Cytotoxic activity of different samples.

Treatment	Conc. (µg/ml)	No. of dead nauplii	% Mortality	LC50 (µg/ml)
	400	10	100	
	200	10	100	
	100	9	90	
Seed extract	50	9	90	
	25	7	70	
	12.5	6	60	5.5
	6.25	5	50	
	3.13	4	40	
	1.57	2	20	
	0.78	1	10	
	10	10	100	
Vincristine sulphate	5	10	100	
	2.5	9	90	1.25
	1.25	7	70	
	0.625	6	60	
	0.312	4	40	
	0.156	3	30	
	0.078	2	20	
	0.039	1	10	

shrimp, Magnifying glass, Micropipette, Sea salt (NaCl), Glass vials, Lamp to attract shrimps, Test tubes, Pipettes.

Collection of plant materials

The seeds of the plant of *Citrus limon* L were collected from near Jahangirnagar University fields, Dhaka, Bangladesh.

Preparation of plant extract

The collected plant seeds were detached from undesirable materials or plant parts washed by using tap water. To ensure the active ingredients free from decomposition to evade any photochemical degradation the seeds were dried by shade drying. With the help of an appropriate grinder the seeds stood ground into a coarse powder. Until analysis originated the powder was deposited in an airtight container and reserved in a cool, dark and dry place. 500 gm grinded seeds powder of *Citrus limon* L were soaked accordingly in 500 ml of water in a glass container for 2 days to 3 days accompanying regular shaking and stirring. Then experienced a coarse filtration by a piece of clean, white cotton material for the whole mixture. Whatman No.1 filter paper filtered it. The filtrate was kept in an open space to evaporate the solvent. Thus the desired extract was obtained [10].

Preparation of seawater

Simulated sea-water was prepared by dissolving 76 g of iodine free sodium chloride in 2 liters of distilled water to make a concentration of 3.8%.

Hatching of brine shrimps

Here the test organisms of *Artemia salina* leach (brine shrimp eggs) were collected from pet shops. Seawater was occupied in the small tank and shrimp eggs remained added to one side of the tank and then this side was enclosed. As nauplii it took two days to hatch the shrimp to be matured. Constant oxygen supply was carried out through the hatching time. The hatched shrimps were involved to the lamp through the perforated dam and they were occupied for experiment. Ten living shrimps were used having 5 ml of seawater to each of the test tubes with the assistance of a pasteur pipette [11].

Preparation of the test sample

To get stock solutions vials took all the test samples and dissolved in 200 µl of pure Dimethyl Sulfoxide (DMSO). In the first test tube then 100 µl of solution was taken containing 5 ml of virtual seawater and 10 shrimp nauplii. The final concentration that we prepared the solution in the first test tube remained 400 µg/ml. By serial dilution method a series of solutions of variable concentrations were equipped from the stock solution. 100 µl sample solution remained added to test tube and fresh 100 µl DMSO was added to vial in every case. Thus different concentrations were established in different test tubes [12].

Preparation of the positive control group

The result of the test sample and the result obtained for the positive control were compared with each other. In the study, vincristine sulphate acted as the positive control. Vincristine sulphate measured and dissolved in DMSO to get an original concentration of 10 µg/ml from which serial dilutions were finished by DMSO to get 5 µg/ml, 2.5 µg/ml, 1.25 µg/ml, 0.625 µg/ml, 0.312 µg/ml, 0.156 µg/ml, 0.078 µg/ml and 0.039 µg/ml. The premarked test tubes were used to add the positive control solutions which contained ten living brine shrimp nauplii in 5 ml simulated seawater to acquire the positive control groups [13].

Preparation of the negative control

Each of three premarked glass test tubes were recycled for adding100 µl of DMSO containing 5 ml of simulated seawater and as control groups ten shrimp nauplii was used. Showing a rapid mortality rate is deliberated as invalid as the nauplii have died. Some important reasons are behind this other than the cytotoxicity of the compounds [14].

Counting of nauplii

The number of survivors was counted and the vials were inspected by a magnifying glass afterward 24 h. For each dilution, the percent (%) mortality was intended. By using Microsoft Excel program, we analyzed he concentration- mortality data statistically. As a median Lethal Concentration (LC50) value, the efficiency or the concentration mortality connotation of plant product is usually uttered. The concentration of the chemical is signified by the test that is produced death in half of the test issues after a certain acquaintance era [15].

Results

Cytotoxic activity

After 24 h the lethal concentration (LC50) of the test samples were attained against the logarithm of the sample concentration by a plot of percentage of the shrimps died and the best -fit line was obtained from the curve data by means of regression analysis. The LC50 of the extract is 5.55 µg/ml whereas the LC50 of the standard vincristine sulphateis 1.25 µg/ml (Table 1).

Discussion

At higher doses, bioactive compounds are almost very toxic. Thus, a simple zoological entity can be used as an expedient informant for screening in case of *in vivo* lethality and fractionation in the finding of new bioactive natural products [16]. With the increasing of concentration of the extract and plotting of concentration versus

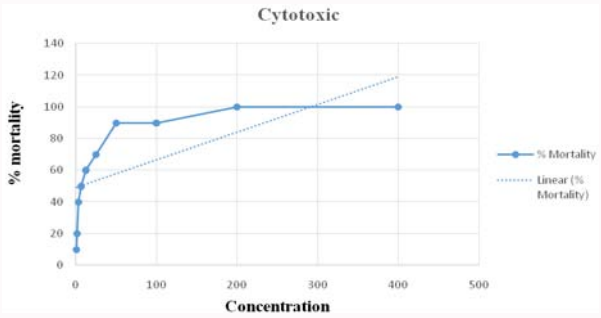

Figure 1: Cytotoxic effect of *Citrus limon* seed extract.

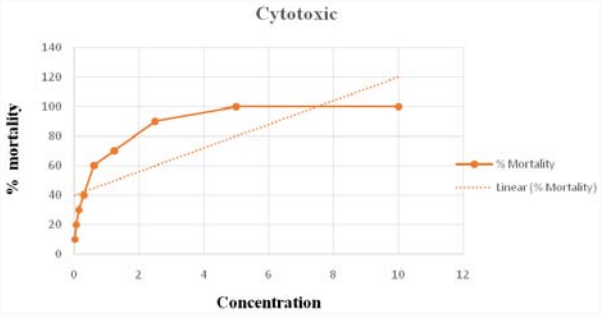

Figure 2: Cytotoxic effect of vincristine sulphate.
Linear equation: y=8.0375x + 39.952; R2=0.6021

response percentage put on the Ldp Line software, the mortality rate of the brine shrimp was found to be increased and produced an approximate linear correlation between them (Figure 1 and 2). At 50% concentration mortality (LC50) of brine shrimp naupliiin stigated by the test extract and it was designed from the graph by extrapolation and was created LC50 in Table 1 [17].

Having cytotoxicity in the seed extract, the brine shrimp lethality is a rapid, simple and convenient method for recognizing biological activity. The seed extract of lemon shows very good activity against the brine shrimp nauplii. The LC50 value of seed extract was 5.5 mg/mL compared with the LC50 value of standard vincristine sulphate was 1.25 mg/mL. Therefore, the extract may comprise cytotoxic compounds we can know it from the response obtained in this assay. However, this can't be confirmed without further higher and specific tests. So, further investigations are needed to get more information about the activities of the plant [18].

Conclusion

The finding of this study supports the view that medicinal plants are promising sources of potential cytotoxic activity that may be effective for cancer therapy. From the thousands of years, nature is giving us medicinal gift which act as natural source of modern drugs. One of those gifts is *Citrus limon* L which contains so many pharmacological activities. So it is clear that our experimental plant possesses cytotoxic activity but we used the same dose in various mice model in another experiments of behavioral studies found healthy. However it requires further investigation to explore these effects in carcinogenesis.

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