EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 4
Issue: 11
103-108
Year: 2017

EVALUATION OF CYTOTOXIC AND MEMBRANE STABILIZING ACTIVITIES OF *GREWIA ABUTILIFOLIA* LEAVES

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Article Received on 09/09/2017 **Article Revised on 30/09/2017** **Article Accepted on 20/10/2017**

ABSTRACT
The aim of the project was to evaluate the cytotoxic and membrane stabilizing activities of the leaves of *Grewia abutilifolia* (Family-Tiliaceae). The crude methanolic extract of the leaves of *Grewia abutilifolia* and its different Kupchan fraction were screened for cytotoxic and membrane stabilizing activities. In Brine shrimp lethality bioassay the most potent activity was found in Chloroform fraction and showed the higher level of cytotoxicity. The ED50 value of CLF was 90.90mg/ml. Membrane stabilization is the method through which the local anesthetics work. This membrane stabilization was done by using erythrocyte in hypotonic solution which was compared with the acetyl salicylic acid. In hypotonic solution induced conditions, the extract of *Grewia abutilifolia*, were found to inhibit lysis of erythrocyte membrane within the range of 95.67±0.25% to 99.50±0.034%. The Chloroform extract of *Grewia abutilifolia* showed high inhibition (99.50±0.034%) hemolysis of RBC as compared to 96.58±Description:3.56 revealed by acetyl salicylic acid. The Summary of in vitro study represent that *Grewia abutilifolia* exhibits cytotoxicity and membrane stabilizing activity.

KEYWORDS: *Grewia abutilifolia*, cytotoxicity, membrane stabilizing activity.

INTRODUCTION
Grewia abutilifolia is a medicinal plant that growing in Chittagong and Bandarban hills of Bangladesh.[1] The family of this plant is Tiliaceae.[2] Brine shrimp lethality bioassay is a recent development in the assay procedure for the bioactive compounds.[3] The brine shrimp napulii-(Artemia salina) a simple zoological organism is used for convenient monitoring, screening and fractionation.[4,5] Membrane stabilizing agents are the medications that reduce the hyper-excitability of nerves.[6] Beta-blockers are drugs that bind to beta-adrenoceptors and block the binding of norepinephrine and epinephrine to the receptors.[7] This inhibitions of normal sympathetic effects that case beta-blockers are known as sympatholytic drugs.[8] These particular beta-blockers (partial agonists) give theintrinsic sympathomimetic activity (ISA) and some beta-blockers also give membrane stabilizing activity (MSA). The first generation beta-blockers were non-selective.[9] They blocked both beta-1 (β_1) and beta-1 (β_2) adrenoceptors. Second generation beta-blockers are more cardioselective and the third generation beta-blockers are drugs that give vasodilator actions through blockade of vascular alpha-adrenoceptors. In the present study, we investigated and evaluated the cytotoxic and membrane stabilizing activities of methanolic extracts of leaves of *Grewia abutilifolia* to verify the traditional uses of the plant.

MATERIALS AND METHODS
Collection of plant materials
The plant material (leaves) was collected during the month of October, 2016 from Chittagong Hill track, Bangladesh in fresh condition and identified by an expert taxonomist. A voucher specimen was submitted to the National Herbarium Bangladesh (accession number: 41884). Leaves were then washed properly to remove dirty materials and shade dried for several days with occasional sun drying. These were then dried in an oven for 24 hours at considerably low temperature. The dried leaves were ground into coarse powder by a grinding machine in the department of Pharmacy, Southeast University.

Cold extraction of the plant materials
The course powdered plant materials (leaves) that having a weight of about 500 gm. were taken in an amber colored reagent bottle and soaked in 1.5 liter of methanol. The bottle was sealed and kept for a period of about 7 days with occasional shaking and stirring. Then

themixture was filtered through cotton and through Whatman No.1 filters paper. It was concentrated with a rotary evaporator at 50°C temperature under reduced pressure and collect the crude extract (45.39 gm).

Solvent-solvent partitioning of crude extract

An aliquot (20gm) of the concentrated methanolic extract of *Grewia abutilifolia* was fractionated by modified Kupchan method (Van *et al*, 1993). The fractions that was petroleum ether (PEF, 5.89gm), chloroform (CLF, 4.36gm), and aqueous (AQF, 5.89gm), were obtained. Then the fractions was used for the experiment purpose.

Cytotoxicity evaluation

The plant extracts can be tested for their toxicity by Brine shrimp lethality bioassay method.[10] The simple zoological organism; brine shrimp nauplii (*Artemia salina,* Leach) was used for screening in the discovery of new bioactive natural products. The egg of the shrimp was collected from MKA Hatchery in Cox's Bazar.The sea-salt (non-ionized NaCl) 38 grams was dissolved in 1 liter of sterilized distilled water and then filtered off to get the clear solution. The seawater pH was maintained between 8 and 9 using $NaHCO_3$ solution. Brine water was taken in the small tank then the shrimp eggs (1.5 gm/L) were added to one side of the tank and this side was covered. 2 mg sample was dissolved in 400µl dimethyl sulfoxide (DMSO) to get a concentration of 5 µg/µl for each of the sample. The solution was used as stock solution. Five doses (10, 20, 40, 80 and 160 µg) of each sample were used for the cytotoxicity test of brine shrimp nauplii. 2, 4, 8, 16 and 32 µl of each sample were transferred by a micropipette from the stock solution in 5 different vials. Every vial was filled the volume up to 5 ml by adding Brine water. The final concentration of the samples was 10, 20, 40, 80 and 160 µg/ml respectively. The concentration of DMSO in these vials should not exceed 50 µl/5 ml of brine as because above this concentration cytotoxicity due to DMSO may arise. [11] Vincristine sulphate and DMSO were used as the positive and negative control respectively.[12] In the present study, vincristine sulphate was used as the positive control. 2 mg of vincristine sulphate was dissolved in 400 µl of DMSO to get a concentration of 5 µg/µl. This was used as stock solution of vincristine sulphate. With the help of a micropipette 2, 4, 8, 16 and 32µl of the stock solution were transferred in 5 different vials. The brine water was added to each vial making the volume up to 5 ml.[13] The final concentration of vincristine sulphate in the vials became 10, 20, 40, 80 and 160 µg/ml respectively. 2, 4, 8, 16 and 32 µl of DMSO was added to each of the remarked glass vials containing 5 ml of simulated sea water and 10 shrimp nauplii to use as control groups. If the brine shrimp nauplii in these vials show a rapid mortality rate, then the test is considered as in valid as the nauplii died due to some reason other than the cytotoxicity of the samples. 10 shrimpliving naupliiwere transferred to each of the vials. A magnifying glass was used for convenient counting of the nauplii. If the counting of 10 nauplii was not being possible accurately, then a variation in counting from 9-11 might be allowed.

After 24-hours of incubation, the vials were observed using a magnifying glass and the numbers of survivors in each vial were counted and the results were noted. From this, the percentage of viability of the nauplii was calculated at each concentration by the following formula.

$$\% \text{ Nauplii viability} = \frac{N_t}{N_0} \times 100$$

Where, N_t = Number of viable nauplii, N_0 =Number of total nauplii transferred

Evaluation of Membrane stabilizing activity

7ml of blood which was collected from each of the healthy Bangladeshi male human volunteers (aged 20-25 years) without a history of oral contraceptive or anticoagulant therapy and free from diseases (using a protocol approved by Institutional Ethics Committee). The RBC was used for the study.[14] The collected RBCs were kept in a test tube with an anticoagulant EDTA under standard conditions of temperature 23±2°C and relative humidity 55±10%.All the solutions, reagents and buffers were prepared with glass distilled water. To prepare the erythrocyte suspension whole blood (7 ml) was obtained using syringes (containing anticoagulant EDTA) from male volunteers through puncture of the anti-cubital vein.[15] The blood was washed three times using isotonic solution (0.9% saline). The volume of saline was measured and reconstituted as a 40% (v/v) suspension with isotonic buffer solution (pH 7.4) which contained in 1 L of distilled water: $NaH_2PO_4.2H_2O$, 0.26 g; Na_2HPO_4, 1.15 g; NaCl, 9 g (10 mM sodium phosphate buffer). Thus the suspension finally collected was the stock erythrocyte (RBC) suspension. The membrane stabilizing activity of the extract was evaluated by using hypotonic solution induced human erythrocyte hemolysis, designed bywith minor modification.[16] The blood was centrifuged, using centrifugal machine, for 10 min at 3000 g and blood cells were washed three times with solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4). The test sample, consisted of stock erythrocyte (RBC) suspension (0.50 mL), was mixed with 5 mL of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing either the extracts (1.0 mg/mL) or acetyl salicylic acid (0.1 mg/mL). The control sample, consisted of 0.5 mL of RBCs, was mixed with hypotonic-buffered saline alone.[17]The mixture was incubated for 10 min at room temperature, centrifuged for 10 min at 3000 gand the absorbance of the supernatant was measured at 540 nm using UV spectrometer. The anticoagulated blood was poured into clean centrifuge tubes.Then centrifuged at 3000 rpm for 10 min.The supernatants were carefully removed using sterilized Pasteur pipettes. Then the packed erythrocytes were resuspended in fresh isosaline, mixed gently followed by centrifugation for another 5min as above. The supernatants were also removed and this process

was repeated 3 times until clear supernatants were obtained. Then a 2% (v/v) erythrocytes suspension was prepared by diluting 2.0 ml of packed red blood cells with isosaline to 100 ml. The Different fraction of *Grewia abutilifolia* were prepared by weighing appropriate quantity of the extract separately. Then the extracts were dissolved in normal saline to serve as stock solution.The washed blood added with the stock solution.Then centrifuged at 3000 rpm for 10 min at room temperature. The supernatants were collected into test tubes and absorbance (Abs) of the released hemoglobin was taken at 540 nm.

RESULTS AND DISCUSSIONS

Cytotoxicity Studies

Table 1: Effect of vincristine sulphate (Standard) on brine shrimp nauplii at different concentration.

Concentration (µg/ml)	% of viability	ED_{50} (µg/ml)
10	25	20
20	20	
40	15	
80	10	
160	5	

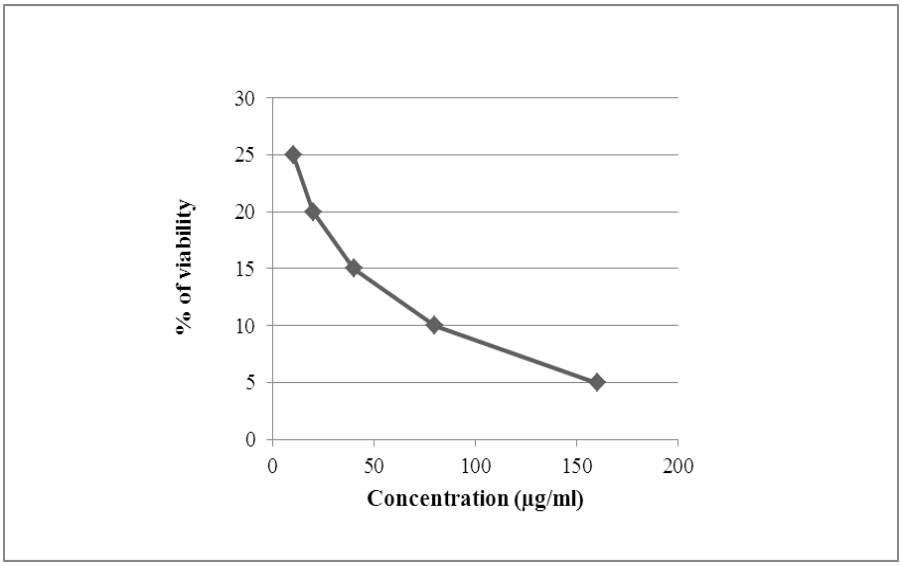

Figure 1: Effect of vincristine sulphate (Standard) on brine shrimp nauplii at different concentration.

Table 2: Effect of *Grewia abutilifolia* and its different fractions on brine shrimp nauplii at different concentrations.

Name of sample	Concentration (µg/ml)	% of viability	ED_{50} (µg/ml)
CME	10	88	90.90
	20	79	
	40	64	
	80	44	
	160	30	
Pet Ether fraction	10	79	81.63
	20	72	
	40	66	
	80	49	
	160	0	
Chloroform fraction	10	80	90.90
	20	67	
	40	50	
	80	44	
	160	0	
Methanol + H_{20} fraction	10	84	42.50
	20	77	
	40	47	
	80	42	
	160	32	

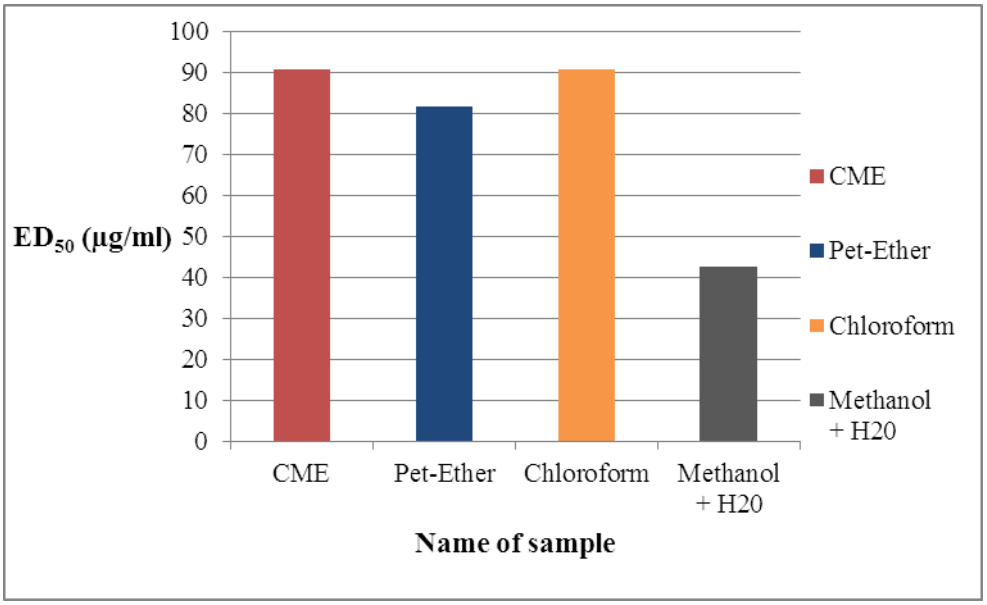

Figure 2: ED_{50}(µg/ml) values of different extractives of *Grewia abutilifolia* on the viability of brine shrimp nauplii after 24 hrs of incubation.

The membrane stabilizingeffect

Table 3: Effect of *Grewia abutilifolia* and its different fractions on membrane stabilizing activity at different concentrations.

Treatment	Concentration	Mean ± SD hypotonic solution	% of inhibition of hemolysis ± SEM
STD (Aspirin)	0.10 mg/ml	0.071± 0.08	96.58± 3.56
Control	1mg/ml	1.913 ± 0.33	--
CME	1mg/ml	0.016±0.008	99.12±0.63
Pet - Ether	1mg/ml	0.083±0.016	95.67±0.25
Chloroform	1mg/ml	0.009±0.001	99.50±0.034
Me+ water	1mg/ml	0.075±0.09	96.28±3.76

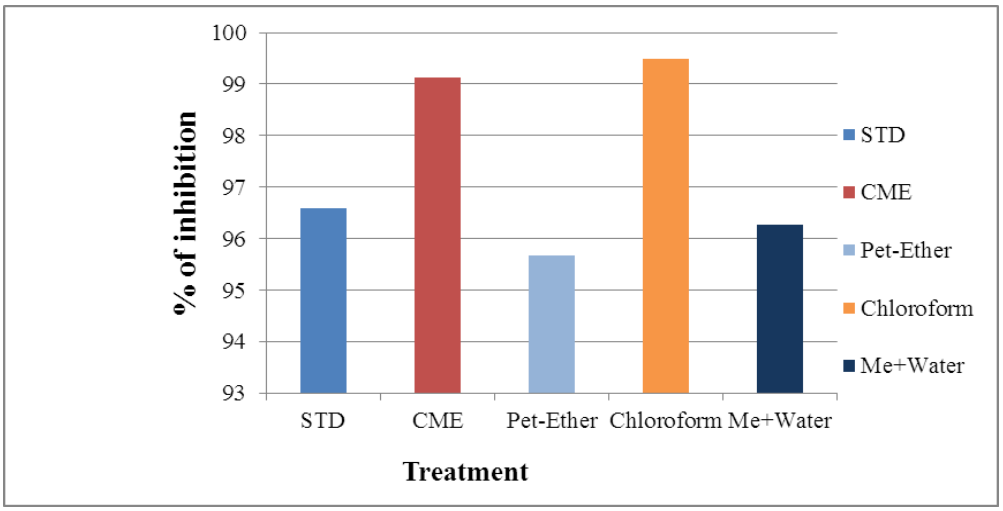

Figure 3: Effects of crude methanol extract and its different fractions on *Grewia abutilifolia* on membrane stabilizing activity.

DISCUSSION

The study was investigate and evaluate of the cytotoxicity and membrane stabilizing effect of *Grewia abutilifolia* in vitro testing.[18] The cytotoxicity of the crude methanol extract and its fractions of *Grewia abutilifolia* was evaluated by Brine shrimp viability bioassay. All the extracts showed cytotoxic effect.[19] The ED_{50} values of crude methanol extract *Grewia abutilifolia* and its three fractions such as petroleum ether, chloroform, and aqueous extracts were found to be 90.90,81.63, 90.90, 42.50 µg/ml respectively (Figure-2). The ED_{50} value of vincristine sulphate (standard) was 20

µg/ml. So among the extractives of *G.abutilifolia* the most potent activity was found in Chloroform fraction and the ED_{50}value of CLF was 90.90µg/ml because the lower ED_{50} means higher toxicity.[20] Membrane stabilizing effects involve the inhibition or total abolishing of action potentials from being propagated across the membrane.[21] In hypotonic solution induced conditions, the extract of *Grewia abutilifolia* were found to inhibit lysis of erythrocyte membrane within the range of 95.67±0.25% to 99.50±0.034%. Chloroform extract of *Grewia abutilifolia* displayed high inhibition (99.50±0.034%) hemolysis of RBC as compared to 96.58± 3.56 demonstrated by acetyl salicylic acid. In the summary of *in vitro* study represent that *Grewia abutilifolia* exhibits cytotoxicity effect and membrane stabilizing activity.

CONCLUSION

In this research *Grewia abutilifolia* was used for cytotoxicity test and membrane stabilizing activity test.The result demonstrate that The cytotoxicity of all the extractives of *Grewia abutilifolia was* studied by using brine shrimp viability bioassay. The lower ED_{50} means higher toxicity. Among the extractives of *Grewia abutilifolia* the most potent activity was found in Chloroform fraction. In hypotonic solution induced conditions, the extract of *Grewia Abutilifolia*, were found to inhibit lysis of erythrocyte membrane, Chloroform extract of *Grewia Abutilifoilia* displayed high inhibition hemolysis of RBC as compared to acetyl salicylic acid. *Grewia Abutilifolia* showed highly cytotoxicity, membrane stabilizing activities. More studies needed to determine the active compounds responsible for these activities and I wish to think that this project work will help to identify the active constituents, structure and their action mechanism responsible for the activity. I also think that this research work will help the new investigator to evaluate and determine the activities in future research.

ACKNOWLEDGMENTS

It's our pleasure and proud privilege to express our heartiest regards and gratitude to our respected teacher Yesmin Begum, Assistant professor, Department of Pharmacy, Southeast University, Dhaka, Bangladesh for making this research possible. Her support, guidance and advice throughout research greatly appreciated. We also thank to our professor and chairman Dr. Md. Siddiqul Islam, Department of Pharmacy, Southeast University, Dhaka, Bangladesh for providing all the facilities, guidance and financial support to perform this project work.

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