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Antibacterial and cytotoxic activity screening of leaf extracts of *Vitex negundo* (Fam: *Verbenaceae*)

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Abstract: The work described in this paper details the biological investigation on *Vitex negundo*, species of *Verbenaceae*. The methanol crude extract of *Vitex negundo* was fractionated with kupchan method and pet-ether and carbon tetrachloride were made for screening the antimicrobial and antitumor potentials using disc diffusion method and brine shrimp lethality bioassay respectively. An established antibiotic (Kanamycin, 30µg/disc) and cytotoxic agent (Vincristine sulphate) were used to compare the results. From the graphs the LC_{50} (50% mortality) values were found as 12.5µg/ml, 1.55µg/ml and 1.56µg/ml for methanolic crude extract, pet-ether and carbon tetrachloride fractions respectively. LC_{90} was also determined from the graph to establish the therapeutic index and the value were found 150.0µg/ml, 50µg/ml and 50µg/ml for methanolic crude extract, pet-ether and carbon tetrachloride fractions respectively. The four fractions were also assayed for antimicrobial screening and all the fractions showed most prominent zone of inhibition against a number of bacterial and fungal strains. Especially in comparison to the standard kanamycin, all fractions gave prominent zone of inhibition against *Bacillus subtilis*, *Bacillus megaterium*, *Salmonella typhi*, *Vibrio mimicus* and a fungal strain, *Aspergillus niger*.
Key words: *Vitex negundo*, antimicrobial activity, antitumor activity, disc diffusion method, brine shrimp lethality bioassay.

Introduction

Plants have a great potential for producing new drugs for human benefit. Plants used in traditional medicine contain a vast array of substances that can be used to treat chronic and even infectious diseases. According to a report of World Health Organization, more than 80% of world's populations depend on traditional medicine for their primary health care needs [1]. The demand for more and more drugs from plant sources is continuously increasing. It is therefore essential for systematic evaluation of plants used in traditional medicine for various ailments. Hence, there is need to screen medicinal plants for promising biological activity. In our study, we choose the leaf portion of *Vitex negundo* (common name: sarsa, samalu, chasta tree, nirgundi) to evaluate its biological activity. *Vitex negundo* (Fam-Verbenaceae) chiefly occurring throughout India [2, 3] is widely distributed in Similipal Biosphere Reserve, Orissa. Though, almost all parts of *V. negundo* are used, the leaves and the barks are the most important in the field of medicine [4]. The decoction of leaves is considered as tonic, vermifuge and is given along with long pepper in catarrhal fever [4]. Water extract of mature fresh leaves exhibited anti-inflammatory, analgesic and antihistamine properties [5]. Leaves of this plant have been shown mosquito repellent effects [6] as well as antiulcerogenic [7], antiparasitic [8], antimicrobial [9] and heaptoprotective [10] potentials. The methanolic root extract possessed potent snake venom (*Viper russellii* and *Naja kaouthia*) neutralizing capacity [11]. The acetone extract of *V. negundo* was found to possess insecticidal, ovicidal, growth inhibition and morphogenetic effects against various life stages of a noxious lepidoteron insect-pest [12]. Some studies have also been done on antimicrobial activity of *V. negundo* along with some other Indian medicinal plants [9, 13, 14, 15]. In the present experiment an attempt has been made to evaluate the antibacterial activity of different extracts (petroleum ether, carbon tetrachloride and methanol crude extract) against thirteen prominent Gram-positive and Gram-negative human pathogenic bacteria and three fungal strains. Besides, cytotoxic activity screening of the extracts was also carried out with view to assess the presence of antitumor activity of different extracts.

Materials and Methods

Plant Materials

Leaf sample (2.0Kg) of *Vitex negundo* was collected from an ayurbadic shop of Dhaka new market on July 2008 and was identified by a taxonomist of Bangladesh National Herbarium (BNH). It was then air-dried and powdered with crushing machine. Then the powdered material was successively extracted with methanol by using cold extraction process [16]. The crude extract was then fractionated into pet-ether and carbon tetrachloride by using kupchan partitioning method [17].

Methods for cytotoxic study

04mg of each fraction (pet-ether, carbon tetrachloride and methanol) of *Vitex negundo* were taken and dissolved in 200µl of pure dimethyl sulfoxide (DMSO) in two vials to get stock solutions. A series of solutions of different concentrations were prepared from the stock solution by serial dilution method and the concentrations were as − 200µg/ml, 100µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml, 6.25µg/ml, 3.125µg/ml, 1.5625µg/ml and 0.78125µg/ml and 0.3905µg/ml. Then the samples were subjected to brine shrimp lethality bioassay [18, 19] for cytotoxic studies. In each test tube, containing different concentrations of test sample, 10 brine shrimp nauplii (*Artemia salina*) were added.

Two control groups were used in cytotoxicity study, to validate the test method and results obtained due to the activity of the test agent. In the study vincristine sulphate was used as the positive control. Measured amount of the vincristine sulphate was dissolved in DMSO to get an initial concentration of 20 µg/ml and serial dilutions were made using DMSO to get 10 µg/ml, 5 µg/ml, 2.5µg/ml, 1.25 µg/ml, 0.625 µg/ml, 0.3125 µg/ml, 0.15625 µg/ml, 0.078125 µg/ml and 0.0390 µg/ml of concentration. 30 µl of DMSO was added to each of three premarked glass vials containing 5 ml of simulated seawater and 10 shrimp nauplii to use as negative control groups. After 24 hours, 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.

Methods for antimicrobial assay

Collected all fractions, i.e. pet-ether, carbon tetrachloride and methanol extracts were tested for antimicrobial study by using standard disc diffusion method [20, 21]. In this study, 16 microorganisms were obtained from the Institute of Nutrition and Food Sciences (INFS), University of Dhaka, Bangladesh. Standard Kanamycin (30 µg/disc) and blank sterile filter paper disc (diameter, 6 mm) were used as positive and negative controls, respectively. Nutrient agar medium (DIFCO) was used in the present study for testing the sensitivity of the organisms to the test materials and to prepare fresh cultures. The sample discs, the standard antibiotic discs and the control discs were placed gently on the previously marked zones in the agar plates, pre-inoculated with test bacteria. The discs were then incubated on the plate aerobically at 37ºC for 24 hours. The diameter of inhibition zone around each disc was measured and recorded at the end of the incubation period.

Results and Discussion

Cytotoxic study

In the present bioactivity study, all of the extracts (pet-ether, carbon tetrachloride and methanol) showed positive results indicating that the test samples are biologically active. Plotting of log of concentration (logC) versus percent mortality (% Mortality) for all test samples showed an approximate linear correlation. From the graphs, the median lethal concentration (LC_{50}, the concentration at which 50% mortality of brine shrimp nauplii occurred) were determined and LC_{90} values were also determined to establish the therapeutic index.

Table 1: Brine shrimp lethality bioassay of *Vitex negundo*

Conc. (µg/ml)	LogC	% Mortality			LC_{50} (µg/ml)			LC_{90} (µg/ml)		
		ME	PE	CTC	ME	PE	CTC	ME	PE	CTC
200	2.301	100	100	100	12.5	1.55	1.56	150	50	50
100	2.000	80	100	90						
50	1.699	70	90	90						
25	1.398	50	80	80						
12.5	1.097	50	70	70						
6.25	0.796	40	70	60						
3.125	0.495	40	60	60						
1.563	0.194	30	50	40						
0.781	− 0.107	20	30	30						
0.3905	− 0.408	10	10	10						

(Here, ME = crude methanol extract, PE = pet-ether fraction, CTC = carbon tetrachloride fraction).

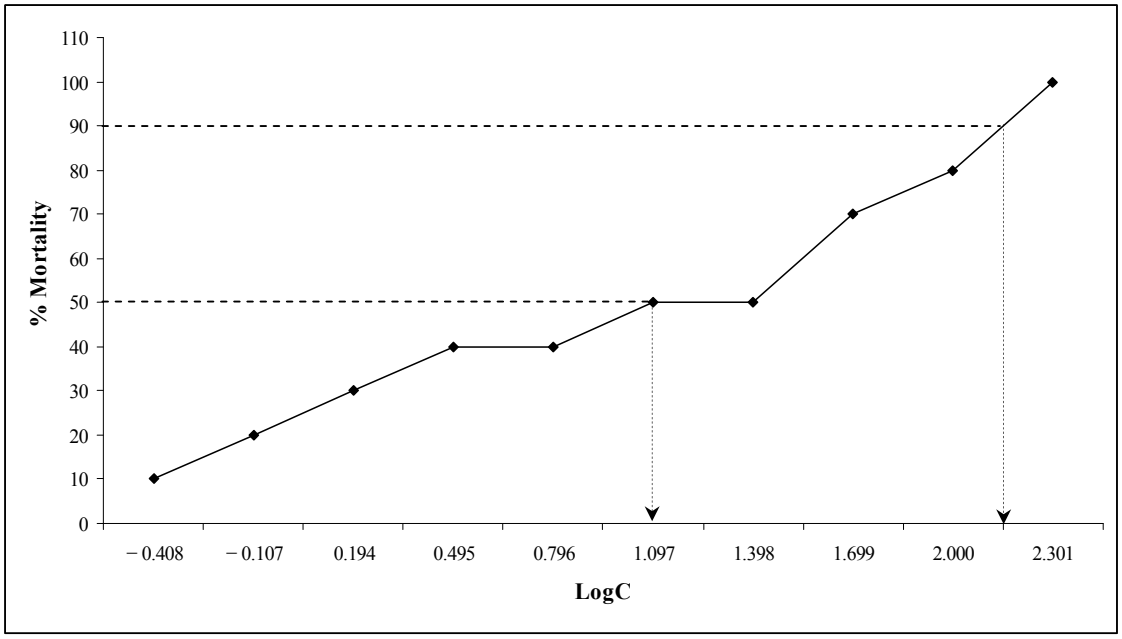

Figure 1: Determination of LC_{50} and LC_{90} of methanolic extract of *Vitex negundo*

The pet-ether and carbon tetrachloride fraction extract of *Vitex negundo* showed significant cytotoxic activity against brine shrimp nauplii and LC_{50} value was found to be 1.55µg/ml and 1.56µg/ml respectively whereas the 12.5µg/ml was found for crude methanolic extract. To get the therapeutic index, LC_{90} (90% mortality) values were calculated and the values were 150µg/ml, 50µg/ml and 50µg/ml for methanol extract, pet-ether fraction and carbon tetrachloride fraction respectively. For the conformity of the result, the test was done for two times. An approximate linear correlation was observed when logarithm of concentration versus percentage of mortality was plotted on the graph paper and the values of LC_{50} and LC_{90} were calculated using Microsoft Excel 2000. All the values were compared with standard cytotoxic agent, vincristine sulphate who's LC_{50} was found to be 0.625µg/ml.

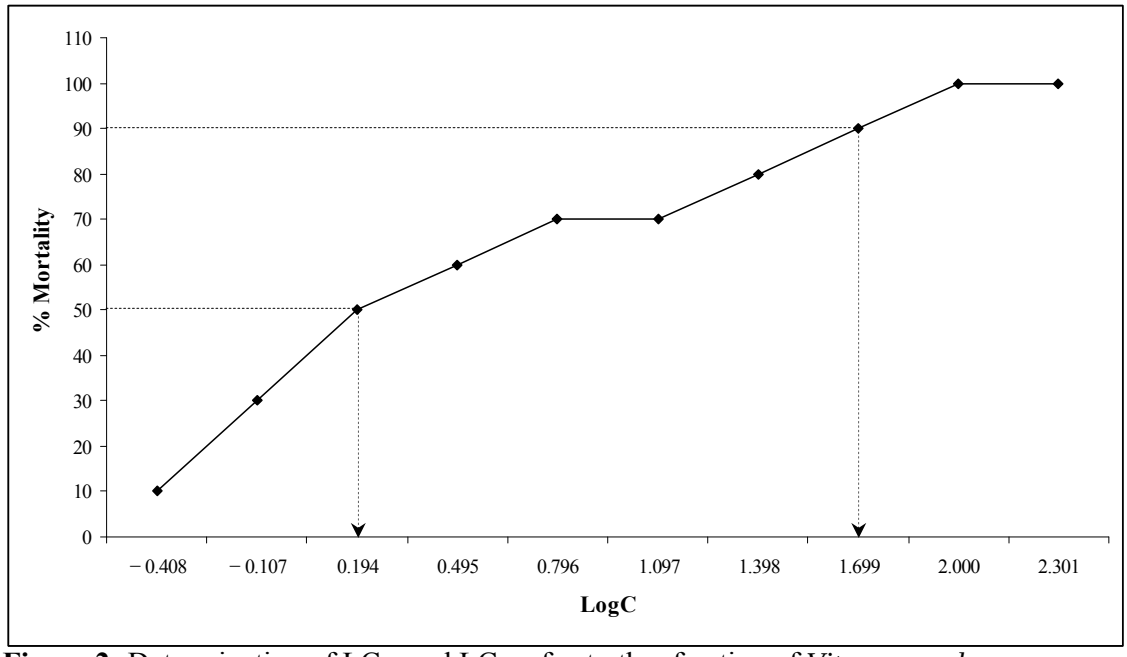

Figure 2: Determination of LC_{50} and LC_{90} of pet-ether fraction of *Vitex negundo*

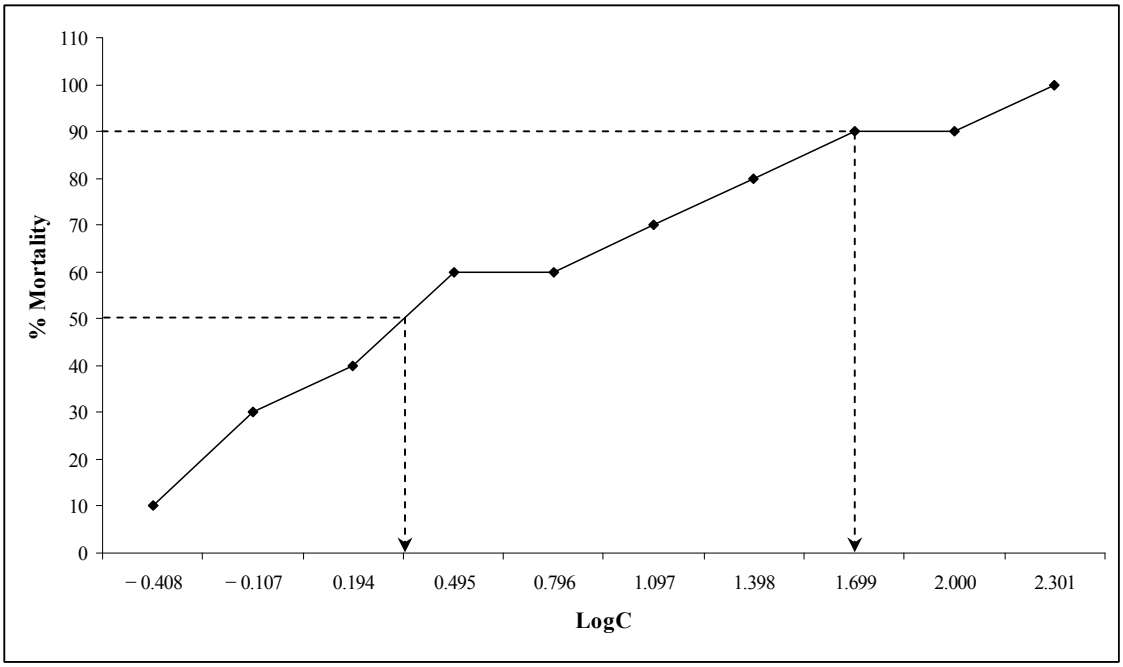

Figure 3: Determination of LC_{50} and LC_{90} of carbon tetrachloride fraction of *Vitex negundo.*

Antimicrobial study

From the study, the zones of inhibition produced by the methanol extract, pet-ether and carbon tetrachloride fractions were found to be 07 − 16 mm, 07 − 11 mm and 06 − 11 mm respectively at a concentration of 200 µg/disc in case of 09 bacterial strains and 02 fungal strains where standard kanamycin (30µg/disc) showed zone of inhibition of 08 – 19 mm. Prominent activity was found against *Bacillus subtilis* (13 – 16 mm) by all of the fractions. Methanol extract showed

Table 2: Antibacterial and antifungal activity of different extracts of *Vitex negundo*

Test organism	Diameter of Zone of Inhibition (mm)			
	Methanol extract	Pet ether fraction	CCl_4 fraction	Kanamycin
	200 µg/disc	200 µg/disc	200 µg/disc	30 µg/disc
Gram positive bacteria				
Bacillus cereus (BTCC-19)	10	08	10	10
Bacillus megaterium (BTCC-18)	08	11	18	19
Bacillus subtilis	16	12	13	12
Staphylococcus aureus (BTCC-43)	09	09	07	10
Sarcina lutea (ATCC-9341)	07	07	09	17
Gram negative bacteria				
Escherichia coli (BTCC-172)	08	07	10	11
Pseudomonas aeruginosa (BTCC-1252)	10	08	06	15
Salmonella paratyphi	09	09	07	10
Salmonella typhi	10	10	11	15
Shigella boydii	09	10	06	11
Shigella dysenteriae	09	09	09	13
Vibrio mimicus	09	11	11	08
Vibrio parahemolyticus	08	08	10	12
Fungi				
Saccharromyces cevevaceae	09	08	07	12
Candida albicans	10	09	07	10
Aspergillus niger	10	11	07	13

significant inhibition (09 – 10 mm) against *Bacillus cereus*, *Pseudomonas aeruginosa*, *Salmonella typhi* Pet-ether and carbon tetrachloride fractions showed most prominent inhibitory action (zone of inhibition ≅ 11 – 18 mm) against *Bacillus megaterium*, *Bacillus subtilis*, *Salmonella typhi* and *Vibrio mimicus* in comparison to standard antibiotic (kanamycin, 30µg/disc).

All the fractions of *Vitex negundo* were also tested for antifungal activity against 03 fungi. The extracts had inhibitory effect against all the test pathogens in different degree. The methanol extract and pet-ether fraction showed profound activity against *Aspergillus niger* and *Candida albicans* respectively.

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