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Research Article

IN VITRO ANTIBACTERIAL ACTIVITIES AND BRINE SHRIMP LETHALITY BIOASSAY OF ETHANOLIC EXTRACT FROM *MORINGA OLEIFERA* LAM. LEAVES

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ABSTRACT
The present work was accomplished to explore the antibacterial activity and cytotoxic potentials of ethanolic extract of *Moringa oleifera* Lam. leaves using disc diffusion method and brine shrimp lethality test respectively. Four Gram-positive and eight Gram-negative human pathogenic bacteria were used for antibacterial screening. Ethanolic extract of leaves showed prominent activity against all the microbes with the zone of inhibition ranging from 10.1 to 26.3 mm. Comparatively higher activity was exhibited by Gram negative bacteria, in that case *Escherichia coli* and *Shigella dysenteriae* showed 26.3 mm and 25.2 mm zone of inhibition. The MIC values for Gram negative bacteria ranged from 62.5 to 125 µg/ml while for Gram positive bacteria ranged from 125-250 µg/ml. Cytotoxic potentials were evaluated from leaves extract against *Artemia salina* Leach at concentrations of 5, 10, 20, 40 and 80 µg/ml and vincristine sulphate was used as positive control. The extracts showed significant brine shrimp lethality having LC_{50} value of 8.12 µg/ml in comparison with the standard vincristine sulphate having LC_{50} value of 6.76µg/ml. The results suggest that ethanol extract of *Moringa oleifera* Lam. leaves can be considered as a source of natural antibacterial and anticancer agent.
Keywords: *Moringa oleifera* Lam., antibacterial activity, disc diffusion method, MIC, cytotoxicity, brine shrimp

INTRODUCTION

The ongoing growing recognition of medicinal plants is due to several reasons, including increasing faith in herbal medicine. Allopathic medicine may cure a wide range of diseases; however its high prices and side effects are causing many people to return to herbal medicines which have fewer side effects[1]. The World Health Organization (WHO) indicates that more than half of the world's populations do not have access to adequate health care services. Therefore, innovative alternative approaches are needed to address this problem. Medicinal plants offer alternative remedies with tremendous opportunities. They not only provide access and affordable medicine to poor people; they can also generate income, employment and foreign exchange for developing countries. Many traditional healing herbs and plant parts have been shown to have medicinal value, especially in the rural areas and that these can be used to prevent, alleviate or cure several human diseases[2,3]. In recent time, the search of potential antimicrobial agents has been shifted to plants. The antimicrobial compounds from plants may inhibit bacteria by different mechanism than the presently used antibiotics and may have clinical value in the treatment of resistant microbial strains[4]. Rapid emergence of multidrug resistant strains of pathogens to current antimicrobial agents has generated an urgent need for new antibiotics from medicinal plants. Many medicinal plants have been screened extensively for their antimicrobial potential worldwide[5,6]. In addition, in the developing countries, synthetic drugs are not only expensive and inadequate for the treatment for disease but are also often with adulteration and side effects. Therefore there is a need to search new infection-fighting strategies to control microbial infections[7,8]. Specimens of *Artemia salina* Leach (brine shrimp), a marine microcrustacean, are used as target organisms to detect bioactive compounds in plants extracts and the toxicity test against these has shown a good correlation with antitumor activity[9]. The significant correlation between the brine shrimp assay and *in vitro* growth inhibition of human solid tumor cell lines demonstrated by the National Cancer Institute (NCI, USA) is significant because it shows the value of this bioassay as a pre-screening tool for antitumor drug research[10,11]. *Moringa oleifera* Lam. belonging to the family Moringaceae is commonly known as Sajna gachh, Sojne (Bengali); Horse-Radish tree, Drumstick tree (English). A small to medium-sized deciduas tree with long strangling branches, large imparipinnate compound leaves of small oblong-obovate leaflets, fragrant pinkish white flowers in loose axillary panicles and long, narrow and ridged cylindrical fruits, planted commonly all over Bangladesh, India, Pakistan, Central America, Afghanistan, and Africa[12,13]. *Moringa oleifera* Lam. have been called a "Miracle tree" for its variety uses of all parts of the tree (seeds, leaves, fruits, bark, roots). Different parts of this plant contain a profile of important minerals and are a good source of protein, vitamins, β-carotene, amino acids and various phenolics[12]. The seeds of *Moringa oleifera* Lam. have been reported to analgesic[14] and antipyretic activities[15]. Its leaves have shown wound healing[15], analgesic[16], hepatoprotective[17], antiulcer[18], hypotensive[19] and diuretic activities[20]. Roots have shown antifertility activity[21] and root bark has shown antiurolithiatic effect[22]. This plant has also been reported to exhibit other diverse activities such as antispasmodic, anti-inflammatory[20], hypolipidemic effects[23]. Consequently, the objective of the present experiment was to investigate the antibacterial and cytotoxic potentials of ethanol extracts of *Moringa oleifera* Lam. leaves.

MATERIALS AND METHODS

Plant material collection

The fresh leaves of *Moringa oleifera* Lam. were collected from Natore city of Bangladesh, in April, 2013 and identified by Mr. Md. Habibur Rahman, taxonomist, National

Herbarium, Mirpur, Dhaka-1216, Bangladesh where a voucher specimen No. DACB32494 has been deposited. The leaves were washed, air dried for 3 days and then oven dried for 24 hours below the temperature 60°C followed by pulverization into coarse powder using a grinding machine.

Plant material extraction

The ground leaves (600 g) were subjected to 95 % ethanol (3 liters) extraction in cold condition in flat bottom glass container through occasional shaking for 7 days[24,25]. The extract was filtered and the solvent was evaporated to dryness in vacuum rotary evaporator at 40°C to 50°C to afford a semisolid mass (80 g) and used for further studies.

Culture media

Nutrient agar media (Difco laboratories) P^H 7.2, nutrient broth media (Difco laboratories) p^H 6.8 and artificial sea water (3.8 % sodium chloride solution) p^H 8.4 were used for antibacterial screening, MIC determination and brine shrimp lethality bioassay respectively[26,27].

Antibacterial activity screening

The antibacterial activity determination was performed by disc diffusion technique[28]. Test organisms were available in Microbiology Laboratory of Pharmacy Department, North South University, Bashundhara, Dhaka, Bangladesh. Pure cultures of these were collected from the Microbiological Laboratory of the Institution of Food and Nutrition Science and Department of Microbiology, University of Dhaka, Bangladesh. The sample of extracts were prepared by dissolving a definite amount of material in appropriate volume of solvent to give the desired concentration and applied on sterile disc (6 mm diameter, filter paper) followed by drying off in an aseptic hood. Thus, such discs contained 500 µg of crude extracts. The activities were compared with standard antibiotic, kanamycin 30 µg/disc. Blank disc impregnated with 10 µl of solvent ethanol followed by drying off was used as negative control. The test discs and standard disc were placed in petridishes seeded with particular bacteria and kept in a refrigerator at 4°C for 12-18 hours to allow maximum diffusion of test material in to the surrounding media. Then the petridishes were incubated overnight at 37°C for growth of bacteria and activities of the plant extracts were expressed by measuring the zone of inhibition in terms of mm.

Determination of Minimum Inhibitory Concentrations

Minimum inhibitory concentration (MIC) was determined by serial tube dilution technique[29] against all the pathogenic bacteria. Inoculums were prepared in the sterile nutrient broth medium so that the suspension contains 10^6 cell/ml. The stock solution was prepared by dissolving 4 mg of the plant extract in 4 ml solvent (DMSO, dimethyl sulfoxide) to obtain concentration 1000 µg/ml and serially diluted to obtain concentrations 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90, 1.9 µg/ml consecutively. 10 µl of properly diluted inoculums were added to each of the nine test tubes. 1 ml of sample solution and 10 µl of inoculums were added to the control test tube CS and CI whereas control test tube CM contained medium only. All the test tubes were incubated for 18 hours at 37°C and evaluated for growth of bacteria.

Brine Shrimp Lethality Bioassay

The *in vitro* lethality in a simple zoological organism such as the brine shrimp lethality, developed by Meyer *et al*[30], was used as a simple tool to guide for cytotoxic activity. Brine shrimp eggs were collected from University of Dhaka, Bangladesh and placed in artificial sea water (3.8 % w/v NaCl in distilled water). The eggs were then incubated at 24-28°C and hatched for 48 hours to provide large number of larvae called nauplii. The test samples were prepared by dissolving the ethanol leaves extracts in DMSO (not more than 50 µl in 5 ml solution) with sea water to obtain concentrations 5, 10, 20, 40, 80 µg/ml. A vial containing 50 µl DMSO diluted to 5 ml was used as a negative control and standard vincristine sulphate was used as a positive control[26]. The matured shrimp (10 nauplii) were applied to each of all experimental vials and control vial. The number of survivors usually swimming was counted with the aid of a magnifying lens for each of the vials at the end of 24 hours. From these data the (%) mortalities were calculated for each concentration of test and control solutions. By using Microsoft Excel the concentration- mortality data were analyzed statistically and LC_{50} values of the plant extracts were determined[27].

RESULTS

The antibacterial activities of crude ethanol extract of *Moringa oleifera* Lam. leaves obtained by disc diffusion method are enlisted in Table 1. In comparison with standard antibiotic kanamycin 30 µg/disc; the leaves extract showed different zones of inhibition at a concentration of 500 µg/disc against four Gram positive and eight Gram negative bacteria. The extracts exhibited greater sensitivity to Gram negative bacteria with the zone of inhibition ranging from 19.3 to 26.3 mm. Highest zone of inhibition was found to be 26.3 mm against *Escherichia coli* followed by *Shigella dysenteriae, Shigella boydii* and *Shigella sonnei* with the zone of inhibition 25.2 mm, 22.3 mm and 22.1 mm respectively. However, comparatively less sensitivity was shown by Gram positive bacteria. Amongst twelve *Sarcina lutea* and *Bacillus subtilis* were two bacteria that had lowest activity with the zone of inhibition 10.1 mm and 11.2 mm. The results of Minimum Inhibitory Concentration (MIC) values of ethanol leaves extracts are also summarized in Table 1. The MIC values for Gram negative bacteria ranged from 62.5 to 125 µg/ml whereas that of Gram positive bacteria ranged from 125-250 µg/ml. In brine shrimp cytotoxicity assay, an approximate linear correlation was observed when logarithm of concentration versus percentage of mortality was plotted on a graph paper (Figure 1). Leaves extracts showed prominent cytotoxic activity in a dose dependent manner and median lethal concentration (LC_{50}) value was found to be 8.12 µg/ml while for standard vincristine sulphate was found to be 6.76 µg/ml (Table 2).

Table 1: *In vitro* antibacterial activities and MIC values of the crude ethanol extracts of *Moringa oleifera* Lam. leaves with standard kanamycin

Tested organisms	Diameter of zone of inhibition (mm)		MIC values (µg/ml)	
	Ethanol extract (500 µg/disc)	Kanamycin (30 µg/disc)	Ethanol extract	Kanamycin
Gram (+ve) bacteria				
Bacillus subtilis	11.2	29.4	250	31.25
Bacillus megaterium	15.1	19.3	125	31.25
Sarcina lutea	10.1	17.2	125	62.50
Staphylococcus aureus	13.2	28.2	250	62.50
Gram (-ve) bacteria				
Escherichia coli	26.3	29.1	62.5	15.62
Salmonella typhii	20.2	28.2	125	31.25
Shigella shiga	21.1	25.2	125	62.50
Shigella sonnei	22.1	25.1	62.5	15.62
Shigella boydii	22.3	24.1	62.5	31.25
Shigella dysenteriae	25.2	28.4	62.5	15.62
Klebsiella species	20.1	24.2	125	62.50
Pseudomonus aeruginosa	19.3	22.1	125	15.62

Table 2: Results of brine shrimp lethality bioassay for ethanol extract of the leaves of *Moringa oleifera* Lam. and for standard vincristine sulphate

Test samples	Conc. µg/ml	Log of conc.	No. of nauplii taken	No. of nauplii dead			Average No. of nauplii dead	Percent (%) of mortality	LC_{50} µg/ml
				Vial1	Vial2	Vial3			
Ethanol extract	5	0.69	10	4	3	5	4.00	40.0	8.12
	10	1.0	10	5	6	6	5.66	56.6	
	20	1.3	10	8	6	8	7.33	73.3	
	40	1.6	10	8	8	8	8.00	80.0	
	80	1.9	10	9	8	10	9.00	90.0	
Vincristine sulphate	5	0.69	10	4	5	3	4.00	40.0	6.76
	10	1.0	10	6	7	7	6.66	66.6	
	20	1.3	10	7	8	8	7.66	76.6	
	40	1.6	10	10	9	10	9.66	96.6	
	80	1.9	10	9	10	10	9.66	96.6	
Control	20 DMSO	00	10	0	0	0	0	0	---

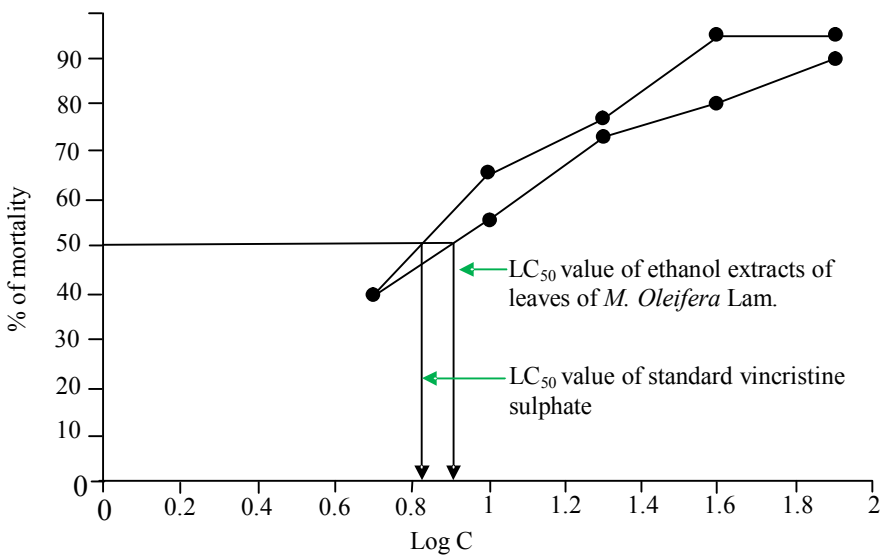

Figure 1: Determination of LC_{50} values for crude ethanol extract of *Moringa oleifera* Lam. leaves and for standard vincristine sulphate from linear correlation between logarithms of concentrations versus percentage of mortalities

DISCUSSION

The results of the present study revealed that the leaves of the plant *Moringa oleifera* Lam. has got profound antibacterial and cytotoxic activity. The Gram negative bacteria are more sensitive to ethanol leaves extract than Gram positive bacteria. The leaves of *Moringa oleifera* Lam. have been known to contain a number of phytochemicals including flavonoids, saponins, tannins and other phenolic compounds having antimicrobial activities[31,32]. The mechanisms of action of these compounds have been proven to be via cell membranes perturbations. Compounds like pterygospermin, benzyl glucosinolate and benzyl isothiocynate have been isolated from *Moringa oleifera* Lam. leaves and these compounds have been reported to have antimicrobial properties against a wide range of bacteria which could partly explain the observed bacteriostatic and bactericidal activity[33]. Thus, the antibacterial activities observed in this experiment could be attributed to such compounds and the results are in good agreement with the previous reports on antibacterial activity of leaves of this plant[34,35]. Antibacterial potency of crude ethanol extracts of leaves of *Moringa oleifera* Lam. indicates that it is more effective against Gram negative bacteria than Gram positive one. However maximum inhibition was obtained with *Escherichia coli, Shigella sonnei, Shigella boydii* and *Shigella dysenteriae* at 62.5 µg/ml concentration. The brine shrimp lethality assay represents a fast, economical and easy bioassay for testing plant extracts bioactivity which in the majority cases correlates reasonably well with cytotoxicity and anti-tumor properties[36]. The degree of lethality shown by the ethanol extracts of leaves was found to be directly proportional to the concentration of the extracts. Maximum mortalities were happened at a concentration 80 µg/ml while least mortalities were at 5 µg/ml. Leaves extracts showed highest cytotoxic activity with LC_{50} value 8.12 µg/ml while standard vincristine sulphate showed LC_{50} value of 6.76 µg/ml. No mortality was observed for negative control group indicating that the results obtained are only due to the activity of the test leaves extracts. Previous reports suggest that leaves of this plant contain the phytochemical niaziminin, which is found to have molecular components that can prevent the development of cancer[37]. Additionally it is an important source of glucosinolate precursors of the isothiocynate group of chemopreventives that can inhibit carcinogenesis[38]. Niazimicin, a compound isolated from *Moringa oleifera* Lam. have also been reported to have potent anti-tumor promoting activity in two stage carcinogenesis in mouse skin using 7, 12-dimethylbenz (a) anthracene [DMBA] as an initiator and 12-O-tetradecanoyl-phorbol-13-acetate [TPA] as a tumor promoter[39]. Thus, significant cytotoxic effects of ethanol extracts of *Moringa oleifera* Lam. leaves exhibited by the present experiment indicates that it can be selected for further cell line assay, as many scientists have shown a correlation between cytotoxicity and activity against brine shrimp nauplii[40].

CONCLUSION

The results of the antibacterial and cytotoxic activity of *Moringa oleifera* Lam. leaves has suggested further potentials of this plant in the area of pharmacology as safe antibacterial and anticancer agent. This extract can be regarded as a promising candidate for other researchers to done more work on *Moringa oleifera* Lam. including phytochemical and biological investigation.

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