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In-vitro Evaluation of Cytotoxic and Anthelmintic Activity of *Luffa acutangula*, *Luffa aegyptiaca* and *Momordica cochinchinensis*

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Authors' contributions

This work was carried out in collaboration between all authors. Author MMR designed the study, wrote the protocol, managed the analyses of the study and wrote the first draft of the manuscript. Authors AA and SSS collected and extracted the aerial parts of the plant. Author MSHA managed the literature searches. Authors AF and MSR finalized the final drafting of the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: The study was designed to investigate cytotoxic and anthelmintic activity of aerial parts of *Luffa acutangula* (L.) Roxb. (Family: Cucurbitaceae, locally known as 'Jhinga'), *Luffa aegyptiaca* Mill. (Family: Cucurbitaceae, locally known as 'Dhundul') and *Momordica cochinchinensis* (Lour.) Spreng. (Family: Cucurbitaceae, locally known as 'Kakrol') extracted with various solvents (petroleum ether & methanol).
Study Design: Determination of cytotoxic and anthelmintic activity of aerial parts of three (Cucurbitaceae family) Bangladeshi plants.
Place and Duration of Study: Department of Pharmacy, Jahangirnagar University, Savar, Dhaka-1342. Performed between November 2011- September 2012.
Methodology: The cytotoxic activity was evaluated by Brine Shrimp lethality bioassay and anthelmintic activity by in-vitro test using earth worm *Pheretima posthuma* (Annelida) as test animals.

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Results: In Brine Shrimp lethality bioassay, methanol extract of *M. cochinchinensis* and L. *aegyptiaca* were found to be highly toxic to Brine Shrimp nauplii, having LC_{50} of 1.91±0.79 µg/ml and 3.97±0.61 µg/ml respectively. The three methanol extracts of aerial part of *L. acutangula, L. aegyptiaca* and *M. cochinchinensis* showed moderate anthelmintic activity. 50mg/ml concentration of methanol extract of *M. cochinchinensis* showed maximum activity showing death in test animals at 43±1.3 min which is comparable to the standard (Piperazine Citrate, 10 mg/ml) which killed the test animal at 38 ± 0.63 min.
Conclusion: Further studies are suggested to be undertaken to understand the underlying mechanism of the observed cytotoxic and anthelmintic activity of these three Bangladeshi (Cucurbitaceae family) plants.

Keywords: Brine shrimp lethality bioassay; anthelmintic activity; Pheretima posthuma; L. Acutangula; L. Aegyptiaca; M. Cochinchinensis.

1. INTRODUCTION

Overwhelming evidence has accumulated showing that natural products from plants, microorganisms and marine organisms comprise major portion of the total repertoire of the available anticancer drugs [1,2,3,4]. Research interest on screening of medicinal plants has intensified in recent years with a view to finding out potential cytotoxic principle for cancer chemotherapy [5,6,7,8]. Medicinal plants with a long history of use in treating cancer are overplaying an integral role in cancer chemotherapy in recent years [9,10]. The traditional cytotoxic uses of medicinal plants of Bangladesh are not backed up by enough scientific evidence. Proper scientific evaluation of the pharmacological properties of these plants used in different traditional formulations would carry enormous potential and promise for the 21st century [11]. Helminthiasis is one of the most important groups of parasitic diseases in Indian subcontinent resulting in heavy production losses in livestock. A wide variety of anthelmintics is used for the treatment of helminths in animals. However, the development of resistance in helminths against commonly used anthelmintics has always been a challenge faced by the animal health care professionals. Moreover, synthetic drugs used in helminthiasis treatment have some potential side effects like alopecia and liver dysfunction. These drugs are also contraindicated in pregnancy and liver disease. So, there is a need for development of anthelmintic drugs from natural origin with fewer side effects. Therefore, exploitation of anthelmintic potential of plants indigenous to Indian subcontinent is an area of research interest. This is a new concept in medicinal chemistry and some plants have been found to show considerable anthelmintic activity. In recent years several in vitro anthelmintic studies were done using plant extract. In a study with *Gloriosa superb Lin.* It was found that plant extract show almost equal activity with the standard piperazine citrate. Some other subcontinent plants like *Terminalia arjuna* [12], *Benincasa hispida* [13], *Caesalpinia bonducella* [14], and some common plants like *Allium sativum, Zingiber officinale, Curcurbita mexicana and Ficus religiosa* [15] also have anthelmintic effect. All of these species are available in Bangladesh and other plant species may show more anthelmintic activity. So drugs derived from plant origin have a promising future in helminthiasis treatment. Anthelmintic herbs are herbs that have the ability to destroy intestinal worms and parasites. There are two categories of anthelmintics: i) vermicides, which are agents that destroy worms without necessarily causing their expulsion from the bowels, and should be followed by or combined with laxative or cathartic herbs; and ii) vermifuges, which are agents that expel worms from the bowels and are usually cathartic herbs. The fruit of *Luffa acutangula (cucurbitaceae* family*)* as a potential anticancer agent was studied by examining its

antiproliferative and antiangiogenic activities [16]. The chloroform extracts of *L. aegyptiaca* has wound healing and anti-inflammatory activities that supports the folk medicinal use of the plant [17]. The bioactive antitumor compound in Gac extract is a protein, which is distinct from lycopene, another compound in Gac fruit with potential antitumor activity [18].

2. MATERIALS AND METHODS

The whole plant was collected from Bismile, Savar, Dhaka in July 2011 during rainy season when weed beds were in their maximum densities.The whole plant with leaves was collected and identified by experts in Bangladesh National Herbarium (BNH), Mirpur, Dhaka, where a Voucher specimen (Accession No. 35971, 35972 and 35973) has been deposited for future reference.

2.1 Plant Extraction and Processing

The powdered plant materials (200 gm each of aerial part) were used for extraction by Soxhlet apparatus at elevated temperature (65ºC) using petroleum ether and methanol consecutively (500 ml of each solvent). After each extraction the plant material was dried and used again for the next extraction. Extraction was considered to be complete when the plant materials become exhausted of their constituents that were confirmed from cycles of colourless liquid siphoning in the Soxhlet apparatus. All six extracts of aerial part were filtered individually through fresh cotton bed. The filtrates obtained were dried at temperature of 40±2ºC to have gummy concentrate of the crude extracts. Each extract was kept in suitable container with proper labelling and stored in cold and dry place.

2.2 Phytochemical Screening Method

For alkaloid analysis the extracts were treated with 1%HCl, boiled and filtered. Dragendorff's, Mayer's and Wagner's reagents were used to indicate the presence of alkaloids [19,20]. Libermann Burchard and Salkowski tests for the presence steroids. For the presence of tannins was indicated by ferric chloride and lead acetate test. The presence of protein was determined by Millon's test which was then confirmed by biuret test.

2.3 Cytotoxic Activity Test

Brine shrimp lethality bioassay was used for probable cytotoxic action [21,22]. The eggs of Brine shrimp (Artemia salina Leach) were collected and hatched in a tank at a temperature "around 25ºC" with constant oxygen supply. Two days were allowed to hatch and mature the nauplii. Stock solutions of the samples were prepared by dissolving required amount of extracts in specific volume of pure dimethyl sulfoxide (DMSO). Four ml of seawater was given to each of the vials. Then specific volumes of sample were transferred from the stock solution to the vials to get final sample concentrations of 1, 5, 10, 20, 50, 100, 200 and 500 µg/ml. In the control vials same volumes of DMSO (as in the sample vials) were taken. With the help of a Pasteur pipette 10 living nauplii were put to each of the vials. After 24h the vials were observed and the number of nauplii survived in each vial was counted. From this, the percentage of lethality of Brine Shrimp nauplii was calculated for each concentration of the extract.

2.4 Anthelmintic Assay

The Anthelmintic activity [23] was evaluated in adult earthworm (*Pheretima posthuma*) due to its anatomical and physiological resemblance with the intestinal roundworm parasites of human being. The groups of equal sized earthworms consisting of 3 earthworms in each group were released in 50 ml of sample with desired concentrations 10, 25 and 50 mg/ml. Group of earthworms in 1% Tween 80 was used as control group and group of earthworms in piperazine citrate (10 mg/ml) used as reference. Observations were made for the time taken for paralysis and death of individual worms. Paralysis as said to occur when no movement of any sort could be observed except the worms was shaken vigorously. Death was concluded when the worms neither moved when shaken vigorously nor when dipped in warm water at 50ºC.

2.5 Statistical Analysis

Statistical analysis was performed using One-way analysis of variance (ANOVA) and followed by least significant difference (LSD). All values were expressed as means±SD of three determinations (n=3). The *P* values < 0.05 were considered significant.

3. RESULTS

3.1 Cytotoxic Activity Test

All the extracts were also subjected to Brine Shrimp lethality bioassay for possible cytotoxic action. In this study, methanol extract of *M. cochinchinensis* was found to be the most toxic to Brine Shrimp nauplii, with LC_{50} of 1.35 µg/ml. On the other hand, all the other extracts showed moderate toxicity (Table 1). The high toxicity of methanolic extract of *M. cochinchinensis* probably attributed to the alkaloid that is confirmed in phytochemical screening. The order at which cytotoxic potential of the test samples decreased was as follows: MCO> MAE> MAC> PAE> PCO> PAC.

3.2 Anthelmintic Activity Test

Earthworm used in anthelmintic activity determination of the plant extracts. The three methanol extracts of aerial part of *Luffa acutangula, Luffa aegyptiaca* and *Momordica cochinchinensis* showed moderate anthelmintic activity. Anthelmintic activity increased with concentration of the test sample. 50mg/ml concentration of methanol extract of *Momordica cochinchinensis* (MCO) showed maximum activity which is comparable to the standard (Piperazine Citrate, 10 mg/ml) (Table 2, Fig. 1 & Fig. 2).

Table 1. LC_{50} of the different fractions Brine Shrimp lethality bioassay

Test sample	Concentration (µg/ml)	Log Conc.	% Mortality	Corrected % mortality	LC50 (µg/ml)	LC90 (µg/ml)
MAC	1	0	10±0.00	0±0.00	32.8±1.62	187.17±43.51
	5	0.69897	13.33±4.71	3.7±5.24		
	10	1	26.67±4.71	18.52±5.24		
	20	1.30103	43.33±4.71	37.04±5.24		
	50	1.69897	56.67±4.71	51.85±5.24		
	100	2	80±0.00	77.78±0.00		
	200	2.30103	100±0.00	100±0.00		
	500	2.69897	100±0.00	100±0.00		
PAC	1	0	10±0.00	0±0.00	175.65±10.80	2019.37±377.89
	5	0.69897	10±0.00	0±0.00		
	10	1	13.33±4.71	3.70±5.24		
	20	1.30103	23.33±4.71	14.81±5.24		
	50	1.69897	33.33±4.71	25.93±5.24		
	100	2	43.33±4.71	37.04±5.24		
	200	2.30103	53.33±4.71	48.15±5.24		
	500	2.69897	76.67±4.71	74.07±5.24		
MAE	1	0	36.67±4.71	29.63±5.24	3.97±0.61	67.04±20.00
	5	0.69897	56.67±4.71	51.85±5.24		
	10	1	70±0.00	66.67±0.00		
	20	1.30103	76.67±4.71	74.07±5.24		
	50	1.69897	86.67±4.71	85.19±5.24		
	100	2	96.67±4.71	96.3±5.24		
	200	2.30103	100±0.00	100±0.00		
	500	2.69897	100±0.00	100±0.00		
PAE	1	0	10±0.00	0±0.00	107.22±22.9	1053.97±155.65
	5	0.69897	13.33±4.71	3.70±5.24		
	10	1	16.67±4.71	7.41±5.24		
	20	1.30103	23.33±4.71	14.81±5.24		
	50	1.69897	33.33±4.71	25.93±5.24		
	100	2	53.33±4.71	48.15±5.24		
	200	2.30103	70±0.00	66.67±0.00		
	500	2.69897	83.33±4.71	81.48±5.24		

MCO	1	0	46.67±4.71	40.74±5.24	1.91±0.79	55.27±14.57
	5	0.69897	66.67±4.71	62.96±5.24		
	10	1	76.67±4.71	74.07±5.24		
	20	1.30103	80±0.00	77.78±0.00		
	50	1.69897	90±0.00	88.89±0.00		
	100	2	96.67±4.71	96.3±5.24		
	200	2.30103	100±0.00	100±0.00		
	500	2.69897	100±0.00	100±0.00		
PCO	1	0	10±0.00	0±0.00	139.64±27.77	1475.59±356.25
	5	0.69897	13.33±4.71	3.7±5.24		
	10	1	13.33±4.71	3.7±5.24		
	20	1.30103	23.33±4.71	14.81±5.24		
	50	1.69897	33.33±4.71	25.93±0.00		
	100	2	43.33±4.71	37.04±5.24		
	200	2.30103	60±0.00	55.56±0.00		
	500	2.69897	83.33±4.71	81.48±5.24		
VS	0.06	-1.22185	10±0.00	0±0.00	0.98±0.08	7.27±0.82
	0.125	-0.90309	20±0.00	11.11±0.00		
	0.25	-0.60206	26.67±4.71	18.52±5.24		
	0.5	-0.30103	40±0.00	33.33±0.00		
	1	0	50±0.00	44.44±0.00		
	5	0.69897	86.67±4.71	85.19±5.24		
	10	1	100±0.00	100±0.00		

**MAC= Methanol fraction and PAC= Pet. ether fraction of L. acutangula, MAE= Methanol fraction and PAE= Pet. ether fraction of L. aegyptiaca, MCO= Methanol fraction and PCO= Pet. ether fraction of M. cochinchinensis, VS= Vincristine sulphate (standard).*

Mean ± SD = Mean values ± Standard deviation of three experiments

Table 2. Anthelmintic activity of standard drug and sample

Treatment	Concentration (mg/ml)	Paralysis Time (min)	Death Time (min)
Control (Saline Water)	10	No paralysis	No death observed
Piperazine Citrate (Standard)	10	24 ± 0.87**	38 ± 0.63**
MAC	10	> 90	> 90
	25	> 90	> 90
	50	24±2.4**	45±1.3**
MAE	10	> 90	> 90
	25	34±1.3**	> 90
	50	22±2.1**	52±2.9**
MCO	10	57±1.3**	> 90
	25	22±1.5**	59±2.1**
	50	13±2.7**	43±1.3**

**MAC= Methanol fraction of L. acutangula, MAE= Methanol fraction of L. aegyptiaca, MCO= Methanol fraction of M. cochinchinensis.*
Mean ± SD = Mean values ± Standard deviation of three experiments
** P < 0.05; ** P < 0.001, significantly different form control group.*

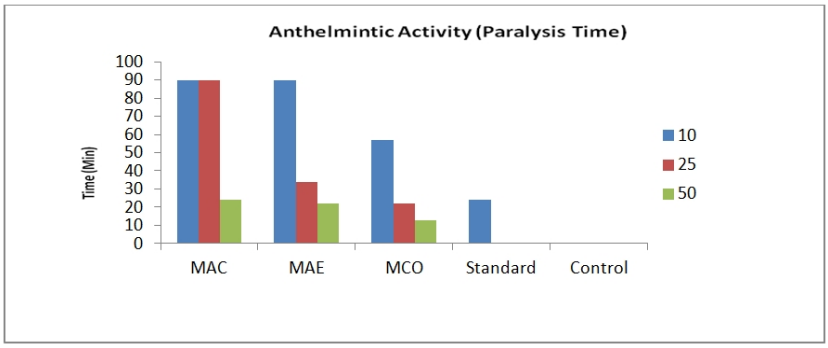

Fig. 1. Anthelmintic Activity of *L. acutangula, L. aegyptiaca* and *M. cochinchinensis* (Paralysis Time)

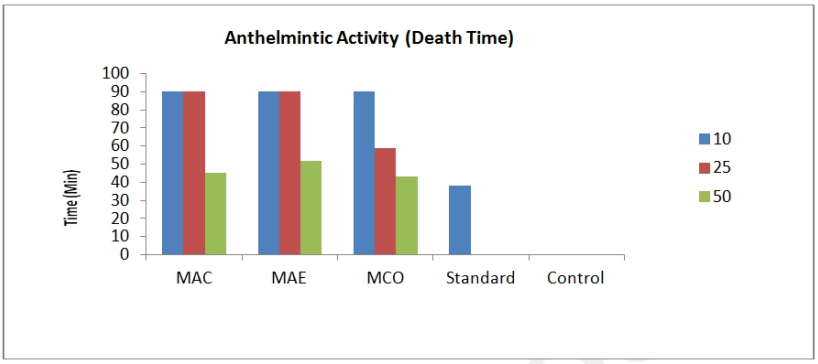

Fig. 2. Anthelmintic activity of *L. acutangula, L. aegyptiaca* and *M. cochinchinensis* (death time)

4. DISCUSSION

The lethality of a test sample in a simple zoological organism such as the shrimp (*Artemia salina*) has been utilized by Meyer et al. [21] in the Brine Shrimp Cytotoxicity Test (BSCT). It is a very useful tool to screen a wide range of chemical compounds for their various bioactivities. It has been well utilized to screen and fractionation of physiologically active plant extracts as well. It has been demonstrated that BSCT correlates reasonably well with cytotoxic and other biological properties [24]. The brine shrimp bioassay has been established as a safe, practical and economic method for determination of bioactivities of synthetic compound [25] as well as plant products [21]. 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. In toxicity evaluation of plant extracts by Brine shrimp lethality bioassay LC_{50} values lower than 1000 µg/ml are considered bioactive [21]. The Brine Shrimp Lethality Bioassay also indicates antifungal effects, pesticidal effects, teratogenic effects, toxicity to environment and many more [26]. Table 1 shows the lethality of different extracts of *L. acutangula, L. aegyptiaca* and *M. cochinchinensis* to the Brine Shrimp nauplii. The degree of lethality shown by the extractives was found to be directly proportional to the concentration of the extractives ranging from the lowest concentration (1µg/ml) to the highest concentration (500 µg/ml). This concentration dependent increment in percent mortality of Brine Shrimp nauplii produced by the *L. acutangula, L. aegyptiaca* and *M. cochinchinensis* indicates the presence of cytotoxic principles in these extractives. Table 3 shows preliminary phytochemical screening revealed the presence of alkaloids and steroids. So the observed cytotoxic action may be due to the presence of such compounds. Again, reports exist on the role of alkaloids and steroids in cytotoxic activity of plant extracts [27,28,29]. However, phenolics and flavonoids are also known to show cytotoxicity in Hoechst 33258 fluorescence assay by inhibiting cellular DNA in a concentration-dependent manner [30]. Piperazine citrate by increasing chloride ion conductance of worm muscle membrane produces hyper polarization and reduced excitability that leads to muscle relaxation and flaccid praralysis [31]. The aerial part extracts not only demonstrated paralysis, but also caused death of worms especially at higher concentration (50 mg/ml). Phytochemical analysis of the crude extract revealed the presence of tannins among other chemical constituents. Tannins were shown to produce anthelmintic activities [32]. Chemically tannins are polyphenolic compounds [33]. It is possible that tannins contained in the extracts of aerial part of *Luffa acutangula, Luffa aegyptiaca* and *Momordica cochinchinensis* produced similar effects. Reported anthelmintic effect of tannins is that they can bind to free proteins in the gastrointestinal tract of host animal [34] or glycoprotein on the cuticle of the parasite [35] and may cause death. Proteinaceous compounds have also been detected in the extracts (Table 3); previously it has been reported that plant cyclotides (small circular peptide) are potent anthelmintic agents. [36]. Anthelmintic activity of aerial part of the plant was good enough to consider it for further investigation.

Table 3. Phytochemical screening results of *L. acutangula, L. aegyptiaca* and *M. cochinchinensis*

Test	MAC	PAC	MAE	PAE	MCO	PCO
Alkaloid	+	+	+	+	+	+
Steroid	+	+	+	+	+	+
Tannin	–	+	–	+	–	+
Protein	+	+	+	+	+	+

+ indicates presence, - indicates absence

5. CONCLUSION

On the basis of the findings of the present study it can be assumed that the methanol extract of *Momordica cochinchinensis*(MCO) was found to be the most toxic to Brine Shrimp nauplii for Cytotoxic activity and also showed maximum anthelmintic activity which is comparable to the standard. However, further studies are suggested to be undertaken to understand the underlying mechanism of the observed activities and to isolate, purify and characterise active phytochemical ingredient(s) responsible for these bioactivities in animal models.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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