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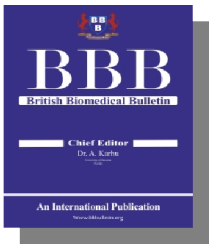

Original

A Comparative Study on Evaluation of *In vitro* Cytotoxic Activity of *Ipomoea pes-caprae and Murraya koenigii*

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A B S T R A C T

The purpose of the research is to find out the comparative cytotoxic potentials of *Ipomoea Pes-Caprae and Murraya koenigii* fractions (crude methanol, Pet ether, Chloroform) by using brine shrimp mortality assay. These two plant extracts were tested at concentration 400, 200, 100, 50, 25, 12.5, 7.25, 3.125 µg/ml. The extracts of both plants showed remarkable cytotoxic activity. Following, the ascertainment of LC_{50} values of the extracts (crude, Chloroform, pet ether) the extracts of *Ipomoea Pes-Caprae (*1.649,5.691, 1.583 µg/ml) and *Murraya koenigii(* 4.365, 10, 5.370 µg/ml) were respectively compared to a reference drug vincristin sulphate with LC_{50} 0.839 µg/ml. *Ipomoea Pes-Caprae* was determined as more cytotoxic than *Murraya koenigii.* Where chloroform extract of *Murraya koenigii* showed low cytotoxicity than all the extract of *Ipomoea Pes-Caprae.*

Introduction

A living body is always sensitive to the compounds with bioactive properties which are also toxic at some higher doses and it upholds the statement that 'Pharmacology is simply toxicology at higher doses and toxicology is simply pharmacology at lower doses'. Brine shrimp lethality bioassay[1] is a rapid and comprehensive bioassay for the bioactive compound of the natural and synthetic origin. Following this method extracts of natural product, fractions as well as the pure compounds can be tested for their bioactivity. In this method for monitoring, screening and fractionating of new bioactive natural products, *In-vivo* lethality in a simple zoological organism (Brine shrimp nauplii) is used. This bioassay not only indicates the cytotoxicity as well as a wide range of pharmacological activities such as antimicrobial, antiviral, pesticidal & antitumor etc. of the compounds[2-4].

Ipomoea pes-caprae (Family-Convolvulaceae) is a pantropical, trailing vine that routinely colonizes sand dunes and commonly referred as railroad vine. It does not tolerate prolonged frost conditions. It is propagated through many tropical beaches including those of Asia, Australia and the Caribbean and commonly found in the outer Himalayas, from the Ravi eastwards, ascending to 5,000 feet, in Assam, Chittagong, Upper and Lower Burma. Its range extends approximately 30° North latitude to 30° South latitude[5]. Leaves are used in rheumatism, and as stomachic and tonic. The extract of the leaves have the astringent, diuretic and laxative properties[6]. It has biological activity like antioxidant, analgesic and anti-inflammatory, anti-spasmodic, anticancer, antinociceptive, antihistaminic, insulogenic and hypo-glycemic[7]. *Murraya koenigii* (Family-Rutaceae). Almost every part of this plant has a strong characteristic odor. The people of the plains, particularly of southern India, use the leaves of this plant as a spice in different curry preparations. The leaves, the bark and the roots of *Murraya koenigii* (L.) Spreng. can be used as a tonic and a stomachic. The bark and the roots are used as a stimulant by the physicians. They are also used externally to cure eruptions and the bites of poisonous animals. The green leaves are stated to be eaten raw for curing dysentery, and the infusion of the washed leaves stops vomiting[8]. The present study has been designed to evaluate cytotoxic activity of the methanol extracts of both plants and compared their cytotoxic effects which can help in authenticating the sample in future study.

General introductions of these two plants are given through the following table.

Materials and Methods

Collection and Identification of the Plant

Plant samples (whole plant) of Ipomoea Pes-Caprae & Murraya koenigii were collected from Cox's bazaar in July 2012. Then the plant was identified by the Bangladesh National Herbarium and collects an accession number- DACB: 35979 & 35780.

Processing of plants

Collected Plant materials were dried in shade and grinded into fine powder using a mechanical grinder, 50 gm of powder was measured for preparing solvent extract. By using standard procedure[9]. Three extract namely crude methanol, chloroform, and pet ether were prepared for both species.

Brine shrimp eggs were hatched in simulated sea water to get nauplii. By the addition of calculated amount of dimethyl-lsulphoxide (DMSO), desired concentration of the test sample was prepared[2]. The nauplii were counted by visual inspection and are taken in vials containing 5 ml of simulated sea water. Then samples of different

concentrations were added to the pre-marked vials through micropipette. Then the vials were left for 24 hours and Survivors are counted.

Materials that were needed in this study

Artemis saliva leach (brine shrimp eggs), Sea salt (NaCl), Vials, Small tank with perforated dividing dam to hatch the shrimp, Lamp to attract shrimps, Filter paper, DMSO (Dimethyl Sulfoxide) as neutral solvent, Weighing machine, Air pump, Pipettes and Micropipette, Vincristine sulphate as standard, Sample of experimental plant, Magnifying glass.

Preparation of seawater

38 gm sea salt (pure NaCl) was measured, dissolved in one liter of distilled water and filtered off to get clear solution.

Hatching of brine shrimps

Artemia salina leach (brine shrimp eggs) collected from pet shops was used as the test organism. Seawater was taken in the small tank and shrimp eggs were added to one side of the tank and then this side was covered. One day was allowed to hatch the shrimp and to be matured as nauplii. Constant oxygen supply was carried out through the hatching time. The hatched shrimps were attracted to the lamp through the perforated dam and they were taken for experiment[1,5,13].

With the help of a Pasteur pipette 10 living shrimps were added to each of the test tubes containing 5 ml of seawater.

Preparation of test samples of the experimental plant

All the test samples were taken in vials and dissolved in 100 µl of pure dimethyl sulfoxide (DMSO) to get stock solution[12]. Then 50 µl of solution was taken in the first test tube containing 5ml of simulated seawater and 10 shrimp nauplii. Thus, final concentration of the prepared solution in the first test tube was 400µg/ml. Further, a series of solutions of varying concentrations were prepared from the stock solution by serial dilution method. In every case, 50 µl samples were added to test tube and fresh 50µl DMSO was added to vial. Thus different concentrations were found in the different test tubes (Table 3).

Preparation of control group

Control groups are used in cytotoxicity study to validate the test method and ensure that the results obtained are only due to the activity of the test agent and the effects of the other possible factors are nullified. Usually two types of control groups are used.

(i) Positive control (ii) Negative control

Preparation of the positive control group

Positive control in a cytotoxicty study is a widely accepted cytotoxic agent. The result of the test agent is compared with the result obtained for the positive control. In this research vincristine sulphate was used as a positive control[2,3]. Measured amount of the vincristine sulphate was dissolved in DMSO to get an initial concentration of 20µg/ml from which serial dilutions were made using DMSO to get 10µg/ml, 5µg/ml, 2.5µg/ml, 1.25µg/ml, 0.625 µg/ml, 0.3125µg/ml. Then the positive control solutions were added to the premarked vials containing ten living brine shrimp nauplii in 5 ml simulated sea water to get the positive control groups.

Preparation of the negative control group

100 µl of DMSO was added to each of three premarked glass vials containing 5 ml of simulated sea water and 10 shrimp nauplii to use as control groups. If the brine shrimps in these vials show a rapid mortality rate, then the test was considered as invalid

as the nauplii died due to some reason other than the cytotoxicity of the compounds[2,3].

Counting of nauplii

After 24 hours, the vials were inspected using a magnifying glass and the number of survivors were counted. The percent (%) mortality was calculated for each dilution. The concentration-mortality data were analyzed statistically by using linear regression using a simple IBM-PC program. The effectiveness or the concentration-mortality relationship of plant product is usually expressed as a median lethal concentration (LC_{50}) value[2,3]. This represents the concentration of the chemical that produces death in half of the test subjects after a certain exposure period.

Results & Discussion of Brine Shrimp Lethality Bioassay

In our current study all crude extracts showed positive result indicating that test samples were biologically active. Each of the test samples showed different mortality rates at different concentrations. Plotting of log of concentration versus percent 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) was determined for the samples. There was no mortality in the negative control groups indicating the test as a valid one and the results obtained are only due to the activity of the test agents. Brine Shrimp Bioassay is the simplest process which in most cases indicates potential cytotoxic and anti-tumor properties[2]. By using this process, developed by Meyer, we determined LC_{50} of crude methanol extract. The results are given below-

The LC_{50} values of crude extract, chloroform soluble fraction and pet ether found to be 1.649 µg/ml 5.691 and 1.583 µg/ml respectively [Table 4, Figure 1(a), 2(a) & 3(a)]. The positive control vincristine sulphate showed LC_{50} at a concentration of 0.839 µg/ml.

From the results of the brine shrimp lethality bioassay it can be well predicted that both the crude extract and pet ether soluble fractions possess cytotoxic principles .The chloroform extract was found to have considerable cytotoxic activity.

The LC_{50} values of crude extract, pet ether and chloroform soluble fraction found to be 4.365 µg/ml 5.370 and 10.00 µg/ml respectively [Table 6 Figure 1(b), 2(b) & 3(b)]. The positive control vincristine sulphate showed LC_{50} at a concentration of 0.839 µg/ml.

From the results of the brine shrimp lethality bioassay it can be well predicted that both the crude extract and pet ether soluble fractions possess mild cytotoxic principles. The chloroform extract was found to have considerable low cytotoxic activity.

Comparative Result Discussion

From the above result comparing the two plants (Table 5 & Table 7), the LC_{50} of *Ipomoea pes-caprae of* three Crude, Pet ether, Chloroform fractions are 1.649, 1.583, 5.691µg/ml whereas the value of LC_{50} of *Murraya koenigii* three fractions are 4.3655, 5.370, 10 µg/ml it can be claimed that *Ipomoea pes-caprae* is more cytotoxic than *Murraya koenigii*. Moreover, among fractions of both plants the fractions of chloroform posses considerable low toxicity then others. Thus, the cytotoxic potential of Ipomoea pes-caprae & Murraya koenigii ensure a valid scientific basis for the use of these plants in the indigenous system of medicine[14]. There for further details analysis is needed to completely establish this evidence.

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Table 1. Plant Profile (*Murraya koenigii & Ipomoea pes-caprae)*

Murraya koenigii	*Ipomoea pes-caprae*
1. **Domain**: *Eukaryota* 2. **Kingdom:** *Plantae* 3. **Subkingdom:** *Viridaeplantae* 4. **Phylum:** *Tracheophyta* 5. **Subphylum:** *Euphyllophytina* 6. **Infraphylum:** *Radiatopses* 7. **Class:** *Magnoliopsida* 8. **Subclass:** *Rosidae* 9. **Super order:** *Rutanae* 10. **Order:** *Rutales* 11. **Suborder:** *Rutineae* 12. **Family:** *Rutaceae* 13. **Genus:** *Murraya* 14. **Specific epithet:** *koenigii* 15. **Botanical name: -** *Murraya koenigii* (L.) Spreng	1. **Domain:** *Eukaryota* 2. **Kingdom:** *Plantae* 3. **Subkingdom:** Viridaeplantae 4. **Phylum:** Tracheophyta 5. **Subphylum:** Spermatophytina 6. **Infraphylum:** Angiospermae 7. **Class:** Magnoliopsida 8. **Subclass:** Asteridae 9. **Superorder:** Asteranae 10. **Order:** Solanales 11. **Family:** Convolvulaceae 12. **Genus:** Ipomoea L. 13. **Specific epithet:** *pes-caprae R. Br* 14. **Botanical name:** *Ipomoea pes-caprae (L.) R. Br*

Table 2. Test samples of experimental plants

Plant part	Test Sample	Calculated amount (mg)
Whole plant of *Ipomoea pes-caprae* Whole plant of *Murraya koenigii*	Extract of *Ipomoea pes-caprae* & *Murraya koenigii*	7.0

Table 3. Test samples with concentration values after serial dilution

S. No. of Test tubes	Concentration (µg/ml)
1	400
2	200
3	100
4	50
5	25
6	12.5
7	7.25
8	3.125

Table 4. Results of the test sample of *Ipomoea pes-caprae*

Sample	LC_{50} (µg/ml)	Regression Equation	R^2
Vincristine Sulphate (positive Control)	0.839	y = 30.05x + 56.01	R^2 = 0.916
Crude methanol extract	1.649	y = 30.45x + 6.598	R^2 = 0.842
Chloroform fraction	5.691	y = 27.27x + 24.03	R^2 = 0.948
Petroleum ether fraction	1.583	y = 19.76x + 56.91	R^2 = 0.850

Table 5. % of mortality of *Ipomoea pes-caprae and* Vincristine Sulphate

Ipomoea pes-caprae								Vincristin Sulphate			
Con (C) (µg/ml)	Log C	% Mortality			LC50 (µg/ml) Based on log C			Con (C) (µg/ml)	Log C	% Mortality	LC50 (µg/ml) Based on log C
		Crude	CF	Pet Ether	Crude	CLF	Pet Ether				
400	2.602	100	100	100	1.649	1.198	1.583	40	1.60206	100	0.839
200	2.301	100	90	100				20	1.30103	90	
100	2	100	70	100				10	1	90	
50	1.698	100	70	100				5	0.69897	80	
25	1.397	90	60	90				2.5	0.39794	70	
12.5	1.096	90	50	80				1.25	0.09691	70	
6.25	0.795	90	50	70				0.625	-0.20412	50	
3.125	0.494	80	40	60				0.3125	-0.50515	30	

Table 6. Results of the test samples of *Murraya koenigii*

Sample	LC_{50} (µg/ml)	Regression equation	R^2
Vincristine sulphate (positive control)	0.839	y = 30.05x + 56.01	R^2 = 0.916
Crude ext	4.365	y = 28.474x + 30.91	0.9643
Pet ether soluble fraction	5.370	y = 24.124x + 31.396	0.9683
Chloroform soluble fraction	10.00	y = 24.519x + 24.533	0.9736

Table 7. Effect of crude extract, pet ether &chloroform soluble fraction of *Murraya koenigii* on shrimp nauplii

Murraya koenigii								Vincristine Sulfate			
Conc (C) (µg/ml)	Log C	% Mortality			LC_{50} (µg/ml)			Conc (C) (µg/ml)	Log C	% Mortality	LC_{50} (µg/ml)
		Crude	Petether	CF	Crude	Petether	CF				
400	2.602	100	90	90	4.365	5.370	10.0	40	1.60206	100	0.839
200	2.301	100	90	80				20	1.30103	90	
100	2	90	80	70				10	1	90	
50	1.699	80	70	70				5	0.69897	80	
25	1.398	70	70	60				2.5	0.39794	70	
12.5	1.097	60	60	50				1.25	0.09691	70	
6.25	0.796	60	50	40				0.625	-0.20412	50	
3.125	0.495	40	40	40				0.3125	-0.50515	30	

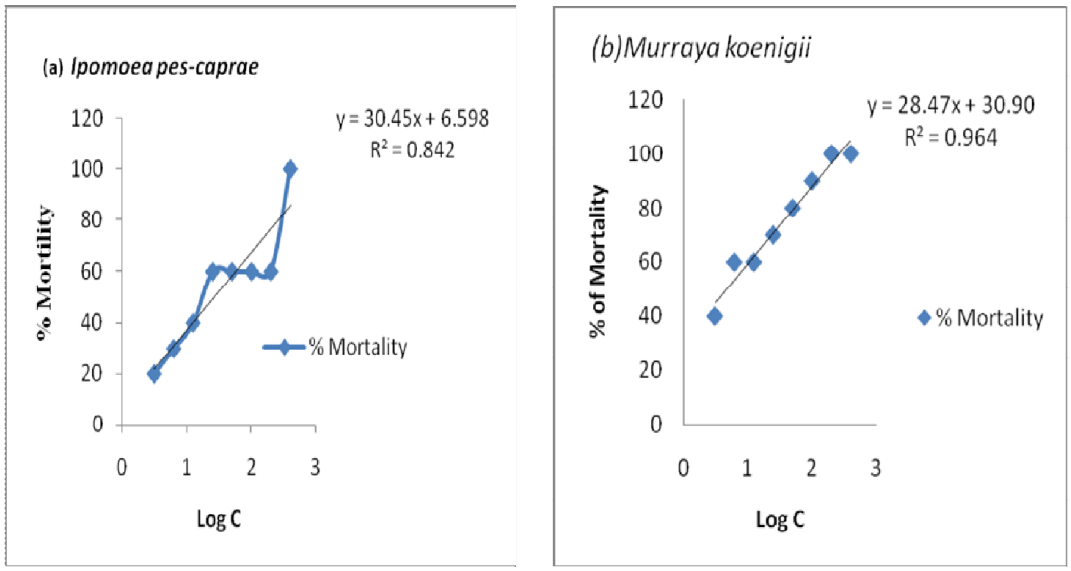

Figure 1. Effect of Crude Methanol Extract of *Ipomoea pes-caprae (a) and Murraya koenigii* (b) on Brine Shrimp Nauplii

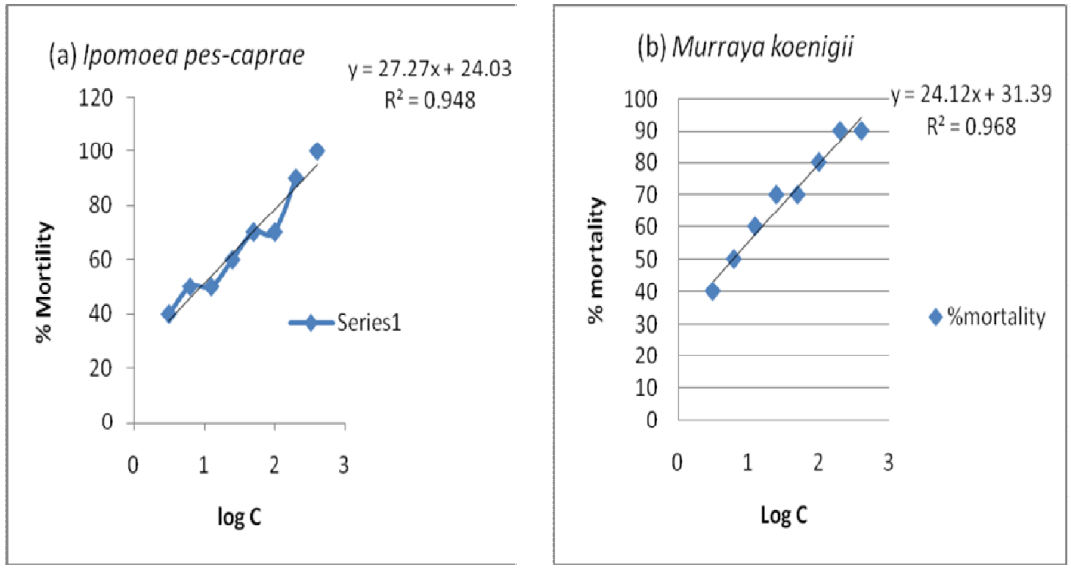

Figure 2. Effect of chloroform Extract of *Ipomoea pes-caprae* (a) and *Murraya koenigii* (b) on Brine Shrimp Nauplii

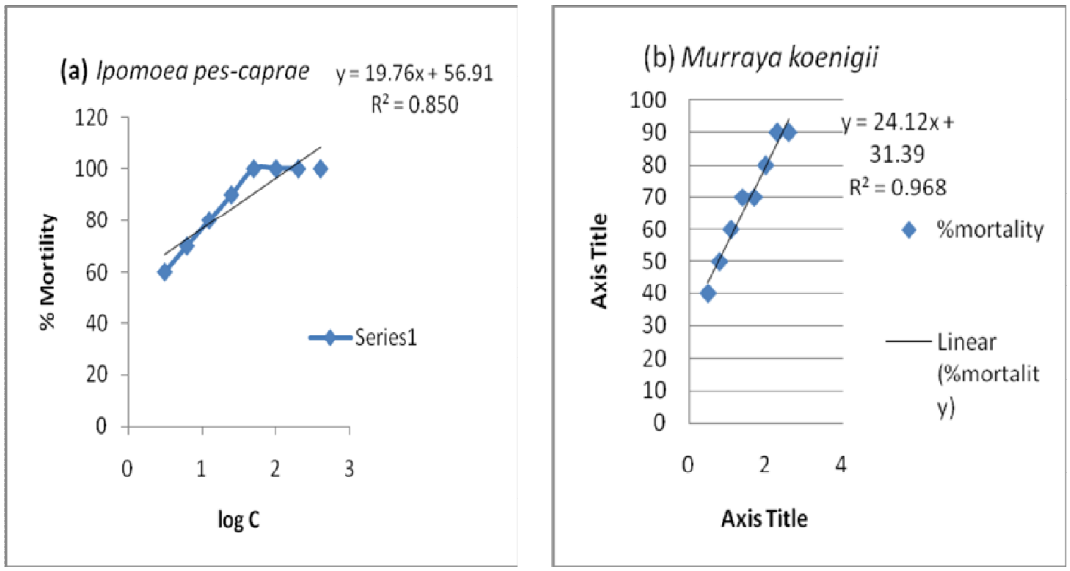

Figure 3. Effect of Pet Extract of *Ipomoea pes-caprae* (a) *and Murraya koenigii* on Brine Shrimp Nauplii

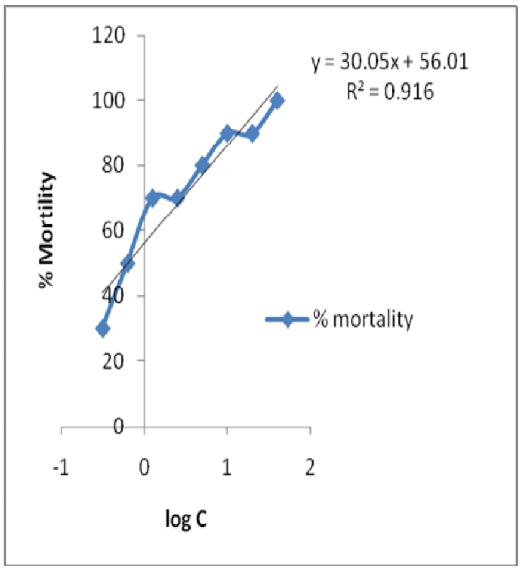

Figure 4. Effect of Vincristine Sulphate on Brine Shrimp Nauplii