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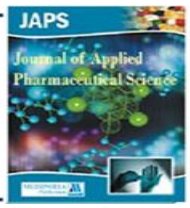

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Antimicrobial and Cytotoxic Activity of the Methanol Extract of *Paederia foetida* Linn. (Rubiaceae)

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ABSTRACT

The antibacterial activities of n-hexane, chloroform, ethyl acetate fractions of methanolic extracts of the whole plants *Paederia foetida* (family Rubiaceae) were screened against various pathogenic bacteria such as *Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis*, *Staphylococcus aureus*, *Sarcina lutea*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella paratyphi* , *Salmonella typhi*, *Shigella boydii*, *Shigella dysenteriae*, *Vibrio mimicus*, *Vibrio parahemolyticus*, *Candida albicans*, *Aspergillus niger*, *Sacharomyces cerevacae* by 'disc diffusion method'. The methanol extract of the whole plants possesses no antimicrobial activity but the ethyl acetate, chloroform and n-hexane fractions exhibited moderate to less activity against some organisms tested compared with the standard antibiotic Kanamycin. Brine shrimp lethality bio-assay was done using brine shrimp Nauplii and dimethyl sulfoxide as a solvent for the methanol plant extracts of Paederia foetida. The LC_{50} value of methanol extract of the plant indicated that the cytotoxicity was very significant.

Keywords: Cytotoxic, *Paederia foetida,* Antimicrobial.

INTRODUCTION

There has been a dominant role playing by the medicinal plants from the beginning of the human civilization (Nostro *et al.*, 2000). Obsession on modern medicinal system leads people to an alternative approach to improve and maintain good health is increased tremendously by using medicinal herb over last centuries. Many important drugs and processed medicines of modern days are from plant origin (Thomas *et al.*, 2008). Almost in all the traditional medicine, the medicinal plants play a major role and constitute the backbone for the same. In order to make sure the safe use of these medicines, a necessary first step is the establishment of standards of quality, safety and efficacy. Keeping this fact in the consideration, the attempts were made to establish physiochemical standards of the plant *Paederia foetida* (Common names- skunk vine, stink vine, Chinese flower plant, Chinese moon etc. Hindi – *Ghandhali*, Assameese – *Bedolilata*,) belonging to family Rubiaceae (Blatter *et al.*, 1981). *Paederia foetida* is one of 30 species in the genus *Paederia* in the family Rubiaceae. It is usually found in Himalayas from Dehradun eastwards upto an altitude of 1800m and also in Assam, Bihar, Orissa, and Bangladesh. It is a slender, perennial herb.

Its stinking and twining branches are 1.5-7 m long. The young stems are purplish or reddish-brown, almost hairless to densely hairy where old stems are yellowish-brown to greyish. The leaf is simply egg-shaped and elliptical-oblong to linear, with sizes about 2-21 cm x 0.7-9 cm. The leaf base is heart-shaped, rounded or sometimes hastate, while the apex is acute to acuminate. The whitish to golden yellow-brown surface is hairless to variably hairy. The petiole size is 0.5-6 cm long. Stipules are present in interpetiolar, rounded or ovate to triangular form in sizes ranging between 1.5-5mm x 2-3 mm. It is usually entire, hairless or hairy. The inflorescence consists of a terminal or axillary cymose panicle that is extremely variable. It grows from widely branched paniculate over 1 m long to rather reduced size, normally 10 cm long. The bracts are either leaf-like or small and linear, with few to numerous flowers, often in lax coiled cymes with peduncle that is 2-30 mm long. The flowers are bisexual, pink or lilac or purplish color and usually 5-merous. The corolla lobes are pinkish to whitish on the inside while the throat is dark purple. The sepal is bell-shaped, with 5 normally smooth triangular-lobed with sizes up to 1 mm x 0.6 mm. The petal is cylindrical to bell-shaped, and sizes 5-17 mm x 2-5 mm. The throat and the inside of the long tube are densely hairy with 5 oblong to triangular lobes and sizes between 1-3 mm x 1.5-3 mm. The margins are wavy and flexed. It has 5 stamens that are inserted in the middle of the tube which includes 2-2.5 mm long anthers. The 2-celled and 2-ovuled ovary is inferior with a small disk and 4-15 mm long style. The stigmas joined the style up to 2 mm of its length. The 2 stigma branches are thread-like and irregularly twisted. The (sub) spherical fruit is a drupe at 4-6 mm in diameter. The fruit walls are thin, dry and brittle. It is crowned by the persistent sepals, shiny pale brown to yellowish or reddish-brown in colour. The 2 semi-orbicular or semi-ellipsoidal kernels are flat on one side and convex or compressed on the other. It is normally slightly smaller than the fruit, without conspicuous wings, black in colour and often conspicuously covered with needle-shaped crystals. The seedling is germinated above the ground, with cotyledons broadly rounded. The veins are prominent while the first pair of leaves form is elliptical and apex is acuminate. It contains bitter taste with having foul smell. It is also reported to be used in gout, vesical calculi, diarrhoea, dysentery, piles, inflammation of the liver and emetic (Indian Materia Medica,2002) (Blatter E, *et al.*,1981). It also enters in to the preparation of Dasmularishta. The major classes of chemical constituent present in this plant are iridoid glycosides, sitosterol, stigmasterol, alkaloids, carbohydrates, protein, amino acid and volatile oil (Steinmetz EF, 1961) (Blatter E, *et al*.,1981). In present study, *Paederia foetida* Linn belonging to the Rubiaceae family has been investigated for chemical and evaluation of biological activities with special emphasis to the antimicrobial screening and cytotoxic study.

MATERIALS AND METHODS

Plant Material

The plant *Paederia foetida* Linn (Family: Rubiaceae) collected from Ishurdi, Pabna and taxonomically identified with the help of the National Herbarium of Bangladesh. Accession number of the plant: 34418. The leaves and the stems cut into small pieces and then dried under the sun for seven days. The dried leaves and stems then grounded into coarse powder with the help of an attrition type of a grinder.

Extracts of Leaves

About 300gm of powdered leaves percolated with 3 liters of methanol in a clean flat-bottomed glass container. The container with its content was sealed and kept for 7 days with occasional shaking and stirring. The mixture was then filtered successively through a piece of clean white cotton. The filtrate thus obtained kept in an open air for the evaporation of the methanol. After 10 to 15 days evaporation of methanol occurred and the extract of methanol remained. Solvent-Solvent partitioning of the crude concentrated methanolic extract was done using the protocol designed by Kupchan. The extract was dissolved in 90% methanol.

Antibacterial assay

The antimicrobial assay was performed by using the disc diffusion method (Bauer *et al.*, 1966; Barry *et al.*, 1980). All collected fractions of the plant like n-hexane, ethyl acetate and chloroform were tested along with the methanol extracts of the whole plants for antimicrobial study by using standard disc diffusion method. 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 were used as positive and negative controls respectively. Nutrient agar medium (DIFCO) was used to prepare fresh cultures for testing the sensitivity of the organisms. The sample discs, the standard antibiotic discs and the control discs were placed gently on the previously marked zones in the agar plates which were previously inoculated with test bacteria. The discs were then incubated on the plate aerobically at 37ºC for 24 hours. The diameter of zone of inhibition around each disc was measured and recorded at the end of the incubation period.

Cytotoxic Activity Test

Brine shrimp lethality bioassay was used for probable cytotoxic activity (Meyer *et al.*, 1982) (Persoone, G., 1988). This bioassay indicates cytotoxicity as well as a wide range of pharmacological activities such as antimicrobial, antiviral, pesticidal & anti-tumor etc. of the compounds (Meyer, 1982; McLaughlin, 1988). Eggs of Brine shrimp, (Artemia salina Leach) collected from pet shop, used as test organism. They were hatched in simulated sea water to get nauplii. Seawater was prepared by dissolving 38 gm of sea salt (NaCl) in one liter of distilled water and after filtering taken in the small tank and then shrimp eggs were added to one side of the tank before the side had been covered. The shrimp were then allowed to hatch for two days to be matured as nauplii. Constant oxygen supply was carried throughout the hatching time. The hatched shrimps were attracted to the lamp through the perforated dam and then they were taken for experiment. With the help of a Pasteur pipette ten living shrimps were added to each of the test tubes containing 5 ml of seawater.

Test samples of desired concentration were prepared by the addition of calculated amount of DMSO. Then samples of different concentrations were added to the pre-marked vials through micropipette and left for 24 hours. Survivors were counted after 24 hours. These data were then processed in a simple program for Probit analysis to estimate LC_{50} values with 95% confidence intervals for statistically significant comparisons of potencies.

Preparation of test solutions with samples of experimental plant

Clean sterilized test tubes were used for ten different concentrations (one test tube for each concentration) of test samples and ten test tubes were taken for standard drug Vincristin for ten concentrations of it and another one test tubes for control test. 4.0 mg of methanolic extracts of *Paederia foetida* were taken and dissolved in 200µl of pure dimethyl sulfoxide (DMSO) to get stock solutions. By using the serial dilution method a series of solutions of different concentrations were prepared from the stock solution and the concentrations were as; 100µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml, 6.25µg/ml, 3.125µg/ml, 1.5625µg/ml and 0.78125µg/ml, 0.3906µg/ml, 0.1953µg/ml.

Preparation of control group

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

i) Positive control

ii) Negative control

Preparation of Positive control group

Positive control in a cytotoxicty study is a widely accepted cytotoxic agent and the result of the test agent is compared with the result obtained for the positive control. In the present study vincristine sulphate is used as the positive control. Vincristine sulphate dissolved in DMSO to get an initial concentration of 20 µg/ml from which 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, 0.0390 µg/ml. Then the positive control solutions were added to the pre-marked vials containing ten living brine shrimp nauplii in 5 ml simulated sea water to get the positive control groups.

Preparation of Negative control group

100 µl of DMSO was added to each of three pre-marked 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 is considered as invalid as the nauplii died due to some reason other than the cytotoxicity of the compounds.

Counting of nauplii

After 24 hours, the vials were inspected using a magnifying glass and the number of survivors were counted. The percentage of mortality calculated for each dilution. The concentration-mortality data were analyzed statistically by SPSS 16.0. The effectiveness or the concentration-mortality relationship of plant product is usually expressed as a median lethal concentration (LC_{50}) value. This represents the concentration of the chemical that produces death in half of the test subjects after a certain exposure period.

RESULT

Antimicrobial study

The study showed that the methanol extract at a concentration of 300µg/disc has no zone of inhibition produced in case of 13 bacterial strains and 03 fungal strains where standard Kanamycin (30µg/disc) showed zone of inhibition of 32-39 mm (Table 1).

The study was done for two times for the confirmation of no inhibitory effect. But, in case of n-hexane fraction of whole plant it shows a moderate antibacterial activity for two gram-positive bacteria like *Bacillus cereus* (12mm) & *Staphylococcus aureus* (14mm) & two gram-negative strains *Escherichia coli* (18mm) and *Vibrio mimicus* (16mm) *where Pseudomonas aeruginosa* (10mm) possess less effect in-contrast to standard Kanamycin. The experiments also revealed that n-hexane extract possess a very less antifungal activity for *Candida albicans* (8mm) & *Sacharomyces cerevacae* (7mm).

The study also showed that the ethyl acetate fractions has no antifungal activity among the three used in experiment along with lower potentiality as antibacterial agent against *Staphylococcus aureus, Escherichia coli, Shigella boydii* &*Vibrio mimicus.* Most likely the chloroform extracts also showed few potentiality against some microorganisms used in the experiment.

Table.1: Antimicrobial activity of different fractions of *Paederia foetida.*

Test microorganisms	Diameter of zone inhibition (mm)				
	HF	**CF**	**EAF**	**MEW**	**Kanamycin**
Gram positive bacteria					
Bacillus cereus	12	10		--	33
Bacillus megaterium	--			--	35
Bacillus subtilis	--	--		--	32
Staphylococcus aureus	14	--	11	--	33
Sarcina lutea	--	--		--	34
Gram negative bacteria					
Escherichia coli	16	14	13	--	32
Pseudomonas aeruginosa	10	9		--	35
Salmonella paratyphi	--	10		--	32
Salmonella typhi	--			--	34
Shigella boydii		13	11	--	37
Shigella dysenteriae	--	--		--	35
Vibrio mimicus	16	12	8	--	32
Vibrio parahemolyticus	--	--		--	33
Fungi					
Candida albicans	8	8	--	--	38
Aspergillus niger	--	--		--	39
Sacharomyces cerevacae	7	--		--	34

MEW = Methanol extracts of Whole Plant leaves.

CF= Chloroform fraction, HF= Hexane fraction, EAF= Ethyl Acetate fraction.

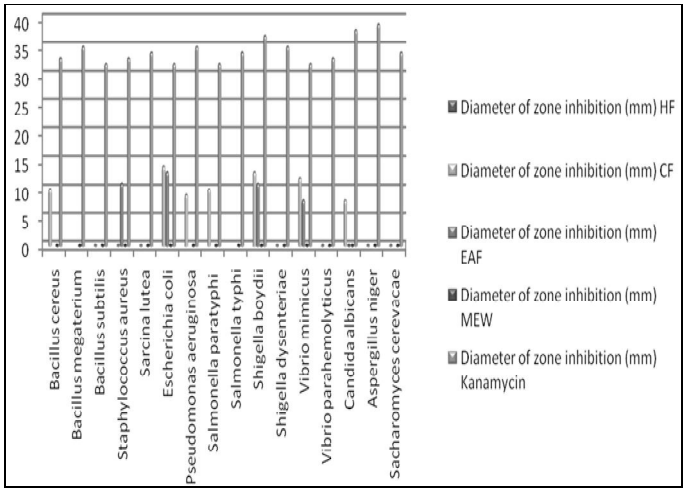

Fig. 1: Antimicrobial Screening Test.

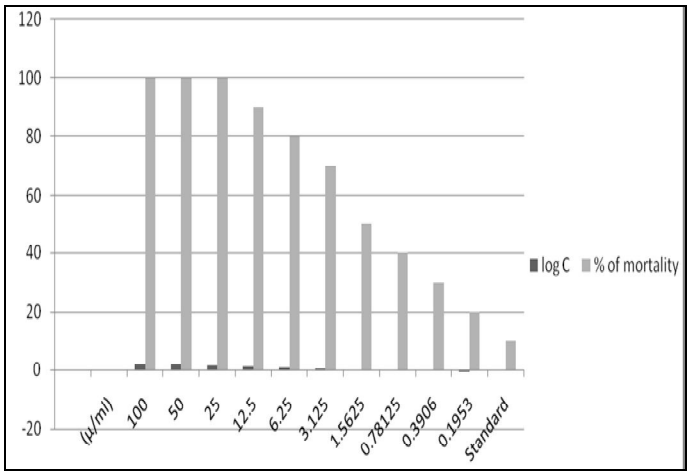

Fig. 2: Brine shrimp lethality bioassay of *Paederia foetida.*

Table. 2: Result of Brine shrimp Lethality Bio-assay of *Paederia foetida.*

Concentration (µ/ml)	log C	Number of nauplii	Number of live	% of mortality	LC_{50}(µ/ml)
100	2	10	00	100	
50	1.6989	10	00	100	
25	1.3979	10	00	100	
12.5	1.0969	10	01	90	
6.25	0.7958	10	02	80	1.5625
3.125	0.4948	10	03	70	
1.5625	0.1938	10	05	50	
0.78125	-0.1072	10	06	40	
0.3906	-0.4082	10	07	30	
0.1953	-0.7092	10	08	20	
Standard		10	09	10	

Brine shrimp lethality bioassay

In the bioassay, the methanol extracts showed lethality indicating the biological activity of the compound present in the extract. Test samples showed different mortality rate at different concentrations. The LC_{50} value for the extract was obtained from the Table 2. Plot of percent of mortality versus log concentration on the graph paper produced an approximate linear correlation between them. From the graph (Figure 2) the concentration at which 50% mortality (LC_{50}) of brine shrimp nauplii occurred can be obtained by extrapolation.

CONCLUSION

In this study the result of the investigation showed that the methanol extracts of whole plants neither possess antibacterial nor have antifungal activity. But, the n-hexane, chloroform & ethyl acetate fraction of the plant have moderate to less antibacterial functions against some strains along with a less potent antifungal activity. As apparent from our results of brine shrimp lethality bioassay it can be revealed that the methanol extracts of *Paederia foetida* have cytotoxic activity. However, to reveal the mechanism behind this effect we need to do further studies. This report can serve to find a new source of medication from this plant.

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