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Preliminary Antimicrobial Activity and Cytotoxicity of Leaf Extracts of *Mussaenda roxburghii* Hook. f.

[Actividad antimicrobiana preliminar y citotoxicidad de extractos de hojas de *Mussaenda roxburghii* Hook. f.]

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Abstract
In the present study the *in vitro* antimicrobial activity, along with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), of different extracts of leaves of *Mussaenda roxburghii* Hook. f. were evaluated against 13 pathogenic microorganisms. The methanol extract and its carbon tetrachloride and chloroform soluble fractions showed the highest antimicrobial activity. The chloroform soluble fraction showed the maximum inhibition zone of 16.0 mm against *Bacillus megaterium* with MIC and MBC values of 7.81 µg/ml and 250 µg/ml, respectively. Ciprofloxacin (30 µg/disc) was used as standard antimicrobial agent. In the brine shrimp lethality bioassay, both the petroleum-ether and carbon tetrachloride soluble fraction of crude methanol extract demonstrated strong cytotoxic activity with LC_{50} value of 0.52 and 0.62 µg/ml, respectively compared to that of 0.451 µg/ml exhibited by standard vincristine sulfate.

Keywords: *Mussaenda roxburghii;* extracts; antibacterial activity; cytotoxocity.

Resumen
En el presente estudio, la actividad antimicrobiana in vitro, junto con la concentración inhibitoria mínima (CIM) y la concentración bactericida mínima (CBM), de diferentes extractos de las hojas de Mussaenda roxburghii Hook. f. fueron evaluadas contra 13 microorganismos patógenos. El extracto metanólico y sus fracciones solubles en tetracloruro de carbono y cloroformo mostraron la actividad antimicrobiana más alta. La fracción soluble en cloroformo mostró la zona de inhibición máxima de 16,0 mm en contra de Bacillus megaterium, con valores de MIC y CBM de 7,81 g/ml y 250 mg/ml, respectivamente. Ciprofloxacina (30 µg/disco) se usó como agente antimicrobiano estándar. En el bioensayo de letalidad con Artemia salina, tanto el petróleo-éter y tetracloruro de carbono como la fracción soluble del extracto de metanol crudo demostraron una fuerte actividad citotóxica con valores de LC_{50} de 0,52 y 0,62 mg/ml, respectivamente, en comparación con la de 0,451 mg/ml de sulfato de vincristina utilizado como estándar.

Palabras Clave: *Mussaenda roxburghii*; extracto; actividad antibacterial; citotoxicidad.

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INTRODUCTION

Herbal medicines are also in great demand in the developed world for primary health care because of their efficacy, safety and lesser side effects. Several plants were known to possess medicinal value including antimicrobial, cytotoxic properties. Bacterial and fungal infections were some of the most serious global health issues of the present century (Gomathi *et al.*, 2011). Plants are used in modern medicine where they occupy a very significant place as raw material for important drugs (Audu *et al.*, 2007). Plants specifically herbal medicines have received much attention as source of new antibacterial drugs since they are considered as time-tested and comparatively safe both for human use and for environment (Abdul *et al.*, 2010). The use of herbal medicine has become increasingly popular worldwide and medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effects (Estakhr *et al.*, 2011).

Mussaenda roxburghii Hook. f. (family-Rubiaceae) is a perennial shrub grows in the foothills and moist areas of valley. Roots are used in treatment of jaundice (Saha *et al.*, 2011), skin diseases, cuts, wounds and boils etc (Patil and Joshi, 2011). Leaves are used in the ailments of bone fracture (Das *et al.*, 2009). The paste obtained from leaf of this plant is applied to treat boils (Rahman, 2010). Previous phytochemical investigation led to isolation of a new iridoid, shanzhiol which showed mild antibacterial activity against both *Staphylococcus aureus* and *Escherichia coli* with a MIC of 100 µg/ml by the broth dilution method (Chandra *et al.*, 2012).

As part of our ongoing research with medicinal plant of Bangladesh (Kuddus *et al*., 2010, Kuddus *et al*., 2011) the present study has been undertaken to evaluate the preliminary antimicrobial activity and cytotoxicity of *M. roxburghii* as well as to find out logical evidence for its folk uses and for discovery of new drug candidates.

MATERIALS AND METHODS

Plant Material

The leaves were collected from Rangamati in November, 2011. A voucher specimen (DUSH-9602) for this collection has been deposited in Bangladesh National Herbarium, Mirpur, Dhaka-1216. The samples were then cut into small pieces and sun dried for 7 days followed by oven drying for 24 hours at 40 ºC to facilitate proper grinding.

Reagents and chemicals: All chemicals *i.e.* methanol, petroleum ether (b.p. 60-80 °C), carbon tetrachloride, chloroform and other reagents used in these experiments were of the highest analytical grade.

Extraction and Isolation

The powdered material (500 g) was soaked in 2.0 L of methanol in a large conical flask for 7 days with occasional shaking and stirring. The whole mixture was then filtered off through a cotton plug followed by Whatman filter paper no.1 and the filtrate thus obtained was concentrated by evaporation at room temperature. A portion (5.0 g) of the concentrated methanolic extract was fractionated by the modified Kupchan partitioning protocol (Van Wagenen *et al.*, 1993) which afforded petroleum-ether (520 mg), carbon tetrachloride (850 mg), chloroform (410 mg) and aqueous (2.1 g) soluble materials.

Antimicrobial Screening

The antimicrobial screening was performed by the disc diffusion method (Rahman and Rashid, 2008) against thirteen bacteria and three fungi (Table-1) collected as pure cultures from the Institute of Nutrition and Food Science (INFS), University of Dhaka, Bangladesh. Here, standard ciprofloxacin (30 µg) disc was used as reference.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Determination

Minimum inhibitory concentrations (MIC) are important to monitor the activity of new antimicrobial agents (Jennifer 2001) and are generally regarded as the most basic laboratory measurement of the activity of an antimicrobial agent against an organism (Turnidge *et al.*, 2003). The MIC was determined for crude methanolic extract and its carbon tetrachloride, chloroform and aqueous soluble fractions of the bark extract by serial dilution technique (Jennifer 2001). Thirteen test tubes were taken, ten of which were marked as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and the rest three were assigned as TM (only medium), TMC (Medium + extractive solution) and TMI (medium + inoculum). In all the test tubes containing the calculated amount of broth medium, the sample from the mother solution was added with serial dilution which gave solutions of varying concentrations (1000 µg/ml to 0 µg/ml). Lastly, the inoculum was added to the test tubes, shaken using rotamixer and incubated at 37 °C for 24

hours. The control test tube (TM) was used to ascertain the sterility of the medium. After 24 hours the test tubes were checked for the microbial growth and the clear test tubes compared to McFarland turbidity standards were marked for the MIC determination. The lowest concentration (highest dilution) of antibiotic that prevented the turbidity was considered as the minimal inhibitory concentration (MIC). At this dilution, the antibiotic is bacteriostatic. The minimum bactericidal concentration (MBC) was determined by sub-culturing the broth media into fresh agar medium from each tube in which no growth was visible. The growth of one colony indicated a 99.8% fall in viable count (Brumitt *et al.*, 1984).

Cytotoxicity Screening

DMSO solutions of the extractives were applied against *Artemia salina* in a one-day in *vitro* assay (Martinez *et al*., 1997; Meyer *et al.*, 1982,). For the experiment, 4 mg of each of the Kupchan fractions was dissolved in DMSO and solutions of varying concentrations such as 400, 200, 100, 50, 25, 12.50, 6.25, 3.125, 1.563, 0.78125 µg/ml were obtained by serial dilution technique. Vincristine sulphate and DMSO were used as the positive and negative control, respectively.

RESULTS AND DISCUSSION

In most developing countries of the world, plants are the main medicinal sources used in treating infectious diseases. The various phytochemical compounds detected are known to exhibit medicinal activity as well as physiological activity. Plants are important source of potentially useful structures for the development of new chemotherapeutic agents.

The methanol extract of *M. roxburghii* and its different partitionates *i.e.* petroleum-ether, carbon tetrachloride, chloroform and aqueous soluble fractions were subjected to antimicrobial screening with a concentration of 400 µg/disc in every case. All the test samples except the aqueous soluble fraction showed mild to moderate antimicrobial activities (Table Nº 1) against most of the organisms used in the assay (Zone of inhibition = 8.0-16.0 mm). Among the all samples, the chloroform soluble fraction exhibited moderate antimicrobial activity against *Bacilllus megaterium* (16.0 mm) and *Vibrio parahemolyticus* (14 mm).

Table Nº 1
Antimicrobial activity of *M. roxburghii* extractives at 400 µg/disc.

Test microorganisms	Diameter of zone of inhibition (mm)				
	CME	PESF	CTSF	CSF	Ciprofloxacin
Gram positive bacteria					
Bacillus cereus	9	8	10	13	42
B. megaterium	9	11	10	16	42
B. subtilis	11	11	8	9	41
Staphylococcus aureus	10	10	8	13	42
Sarcina lutea	10	12	13	11	42
Gram negative bacteria					
Escherichia coli	8	9	8	12	41
Pseudomonas aeruginosa	11	9	13	12	41
Salmonella Paratyphi	10	8	13	10	42
Salmonella Typhi	10	12	9	12	48
Shigella boydii	8	12	10	13	42
Shigella dysenteriae	9	8	10	10	42
Vibrio mimicus	13	9	9	13	41
V. parahemolyticus	12	9	9	14	42

Here, CME = Crude methanolic extract; PESF = Petroleum-ether soluble fraction; CTSF = carbon tetrachloride soluble fraction; CSF= chloroform soluble fraction of the methanolic extract of *M. roxburghii*

As the crude methanolic extract; petroleum-ether, carbon tetrachloride and chloroform soluble fraction of the methanolic extract of *M. roxburghii* showed better inhibitory activity against the microorganisms, the minimum inhibitory concentration (MIC) required to inhibit the growth of organisms were measured in this study. The MIC value of chloroform soluble fraction (where no bacterial growth was observed in broth media) was found to be 7.81 μg/ml and the MBC value (where no bacterial growth was observed in agar media) was found to be 250 μg/ml against *B. megaterium* (Table Nº 2). No microbial growth was observed in the test tubes TM (containing medium only) and TMC (medium + test sample, no inoculum) indicating that the medium and the test sample were not contaminated by microorganism and the total investigation was performed properly in the sterile condition. Microbial growth was observed in the test tube TMI (medium + inoculums) revealing the fact that there was no problem with the sub cultured microorganisms.

Table Nº 2
Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the test samples of leaf of *M. roxburghii*

Test microorganisms	CME		CTSF		CSF		Ciprofloxacin	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Bacillus cereus	15.62	500.0	15.62	250.0	31.25	250.0	0.62	1.25
B. megaterium	31.25	500.0	15.62	500.0	7.81	250.0	1.25	2.5
B. subtilis	31.25	500.0	31.25	500.0	15.62	500.0	0.62	2.5
Staphylococcus aureus	15.62	250.0	31.25	500.0	15.62	250.0	1.25	2.5
Sarcina lutea	15.62	500.0	7.81	500.0	31.25	250	0.31	1.25
Escherichia coli	31.25	250.0	15.62	250	15.62	500	0.31	2.5
Pseudomonas aeruginosa	31.25	500.0	7.81	500.0	62.5	500	1.25	2.5
Salmonella Paratyphi	15.62	250.0	15.62	250.0	15.62	250	0.62	2.5
Salmonella Typhi	15.62	500.0	31.25	250.0	31.25	500.0	0.62	1.25
Shigella boydii	15.62	500.0	15.62	500.0	15.62	500.0	1.25	2.5
Shigella dysenteriae	31.25	250.0	15.62	500.0	7.81	500.0	1.25	2.5
Vibrio mimicus	31.25	500.0	31.25	250.0	62.5	250	0.31	1.25
V. parahemolyticus	15.62	500.0	31.25	250.0	7.81	250	1.25	2.5

Among many recent advances in cancer chemotherapy, phytochemicals play an important role in cancer chemotherapeutic drugs. A search for new anti-cancer drugs has taken many different approaches. The brine shrimp test (BST) represents a rapid, inexpensive and simple bioassay for testing plant extract lethality which in most cases correlates reasonably well with cytotoxic and anti-tumour properties (McLaughlin and Rogers, 1998).

The crude methanol extract of leaves and it's different partitionates *i.e.* petroleum-ether, carbon tetrachloride, chloroform and aqueous soluble fraction were tested for brine shrimp lethality bioassay following the procedure of Martinez *et al*., (1997). The lethality of the extractives to brine shrimp was determined and the results are given in Table Nº 2. The lethal concentration LC_{50} of the test samples after 24 hr was obtained by a plot of percentage of the shrimps died against the logarithm of the sample concentration (toxicant concentration) and the best-fit line was obtained from the curve data by means of regression analysis. Among all the samples, both the petroleum-ether and carbon tetrachloride soluble fraction of crude methanol extract demonstrated strong cytotoxic activity with LC_{50} value of 0.52 and 0.62 μg/ml, respectively compared to that of 0.451 μg/ml exhibited by standard vincristine sulfate (Table Nº 3).

Table Nº 3
LC_{50} values of the test samples of *M. roxburghii*

Test samples	Regression line	R^2	Cytotoxic activity (LC_{50} µg/ml)
VS	y = 30.8x + 60.64	0.972	0.451
CME	y = 21.668x+12.138	0.9277	55.89
PESF	y = 15.378x+54.354	0.9499	0.52
CTSF	y = 17.731x+53.655	0.9575	0.62
CSF	y = 22.122x+37.617	0.8736	3.63
AQSF	y = 13.434x+11.586	0.8301	2.86

Here, VS = Vincristine sulfate

CONCLUSIONS

The results of *in vitro* antimicrobial screening of *M. roxburghii* indicated that the methanol extract and its carbon tetrachloride and chloroform soluble partitionates have moderate antimicrobial activity which suggests the presence of a new iridoid, shanzhiol in this plant. In brine shrimp lethality assay, all the samples exhibited significant lethality against shrimp nauplii suggesting the presence of bioactive materials having cytotoxic properties. Thus, this plant could be subjected to extensive chromatographic separation and purification processes to isolate bioactive compounds for the discovery of novel therapeutic agents.

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