ANTIMICROBIAL ACTIVITIES AND CYTOTOXIC PROPERTIES OF THE METHANOLIC EXTRACTS OF *SMILAX ROXBURGHIANA*

Farhad Hossen[1]*, Khondoker Dedarul Alam[1], Umme Bushra[1], Sanjana Safi[1], Sanjida Mahjabeen[2], Rubaida Ahasan[2]

[1]Department of Clinical Pharmacy and Pharmacology, Faculty of Pharmacy, University of Dhaka, Bangladesh
[2]Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Dhaka, Bangladesh

*Corresponding author: Farhad Hossen
Department of Clinical Pharmacy and Pharmacology, Faculty of Pharmacy, University of Dhaka, Bangladesh; Phone: +8801714042244, +8801933166076; E-mail: f.hossen.pr@gmail.com

Summary

The aim of this study was to investigate antimicrobial activities and cytotoxic properties of various solvent fractions of the crude methanolic extract of *Smilax roxburghiana* (Family- Smilacaceae) by using disc-diffusion method and brine shrimp lethality bioassay respectively. The aqueous soluble fraction exhibited the strongest antimicrobial activity in terms of zone of inhibition and spectrum of activity. Average antimicrobial activities were also showed by the n-hexane, carbon tetrachloride and dichloromethane soluble fractions. On the other hand, n-hexane soluble fraction exhibited the most significant cytotoxic property with the LC_{50} value 1.796 µg/ml compared to the positive control, vincristine sulphate (0.3229 µg/ml) and moderate cytotoxicity was showed by the dichloromethane soluble fraction having the LC_{50} value 6.167 µg/ml. These results reveal *in vitro* cytotoxicity and antimicrobial properties of the aerial parts of *S. roxburghiana*.

Keywords: *Smilax roxburghiana,* Smilacaceae, Antimicrobial activity, Disc-diffusion method, Cytotoxicity, Brine shrimp lethality bioassay

Introduction

Smilacaceae, the greenbrier family, is a family of flowering plants. For the past few decades, most botanists have accepted smilacaceae as a distinct family, before which most of the genera of smilacaceae were often assigned to a more broadly defined family liliaceae. One characteristic that

distinguishes smilacaceae from most of the other members of the liliaceae is that it has true vessels in its conducting tissue. Again, the veins of the leaves, between major veins, are reticulate (net-shaped), rather than parallel as in most monocots. The species of this family are found throughout the tropical and warm temperate regions of the world. [1]

Smilax roxburghiana (synonyms: *S. orthoptera*; Family: Smilacaceae; local name: Kumarilata, Katakombor, Kumarika) is a climber having prickly and thorny stems and rhizomatous or tuberous roots. In ayurvedic medicine, the plant is used for the treatment of skin diseases including psoriasis, rheumatoid arthritis, gout, enteritis, urinary tract infections, skin ulcers etc. Previous chemical investigation with the n-hexane extract of whole *S. roxburghiana* plant resulted in the isolation of allylbenzene derivatives and related lignans, phenol having an enone moiety, fatty acids, triterpenoid and steroids and biological investigations exerted significant piscicidal activity and cytotoxicity [2].

As part of our current interest, we carried out the phytochemical and biological investigation of the whole plants of *Smilax roxburghiana*. This paper reports the antimicrobial activities and cytotoxic properties of the methanolic extracts of *S. roxburghiana*.

Material and methods

Collection and identification of the plant materials

Smilax roxburghiana plants were collected from Manikgonj dristrict in February 2010 and identified in the Centre for Biomedical Research, Faculty of Pharmacy, University of Dhaka, Bangladesh. After proper identification, the plants were grinded into coarse powder by using high capacity grinding machine and stored in an airtight container.

Extraction and Fractionation Procedure

500 g of the powdered plant material was soaked into methanol (2.5 L) and the container with the content was sealed by cotton plug and aluminum foil and kept for a period of 15 days accompanying occasional shaking and stirring. The mixture was then filtered through cotton followed by Whatman No.1 filter paper and the filtrate thus obtained was concentrated at 40 °C with a Heidolph rotary evaporator. The concentrated extract was then air dried to solid residue (12.5 g) and treated as crude extract. 5 g of the crude extract of *Smilax roxburghiana* was dissolved in 10% water in methanol (100 ml) and fractionated by the modified Kupchan partitioning protocol [3] into n-hexane (2.81 g), carbon tetrachloride (0.54 g), dichloromethane (0.61 g) and aqueous fractions (13.25 g).

Investigation of antimicrobial activity

Antimicrobial activities of the test samples were carried out by disc-diffusion method [4] using 13 bacterial strains and 3 fungal strains spreaded on nutrient agar (NA) medium.

Test organisms: The microorganisms, bacteria and fungi, used for the experiment were collected as pure cultures from the Institute of Nutrition and Food Science (INFS), University of Dhaka. Here 100 µl suspension of each of the organisms were used containing 10^8 CFU/ml of bacterial strains and 10^4 spore/ml of fungal strains.

Disc-diffusion method: 8 mg of each of the fractions were dissolved in 200 µl of methanol to obtain the sample solutions concentration 40 mg/ml. Sterilized filter paper discs (6 mm in diameter) were soaked with 10 µl (400 µg/disc) of the sample solutions under the laminar hood, dried and placed on the inoculated agar. Kanamycin (30 µg/disc) discs were prepared following procedure and used as the positive control to determine the sensitivity of the microbial species

tested. Negative controls were prepared using the same solvents employed to dissolve the plant extracts. Finally the inoculated plates were incubated at 37 ºC for 24 h for clinical bacterial strains and 48 h for fungal strains. After the completion of incubation period, antimicrobial activities exhibited by the plant samples were evaluated by measuring the zone of inhibition against the test organisms. The experiment was carried out in triplicate and the average zones of inhibition in millimeter were calculated.

Brine shrimp lethality bioassay

Brine shrimp lethality bioassay is widely used for the bioactive compounds [5,6] and it is the simple bioassay useful for screening large number of extracts in the drug discovery process. Here cytotoxicity screening of the samples were carried against a simple zoological organism, brine shrimp nauplii *Artemia salina*.

Brine shrimp hatching: Sea salt solution was prepared by dissolving 38 gm sea salt (NaCl) in 1000 ml noniodized water. The prepared solution was taken in a small glass tank which was divided by a partition having small pores in it. The eggs were placed on one side of the partition and cover with a black carbon paper. On the other side of the tank, an electric lamp was placed for illumination. Approximately after 48 hours the brine shrimp hatched and matured as nauplii and passed through the hole of the partition to the illuminated side of the tank.

Bioassay procedure: 4.00 mg of each fraction was dissolved in 200 µl of DMSO (dimethyl sulfoxide). Then 100 µl of solution was taken in the first test tube containing 10 shrimp nauplii in 5 ml of sea water and thus the final concentration obtained was 400 µg/ml. A series of solutions of varying concentrations were prepared from the stock solution using serial dilution technique. Thus the concentrations of the obtained solutions in each test tubes were 400 µg/ml, 200 µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, 3.125 µg/ml, 1.5625 µg/ml and 0.78125 µg/ml for 10 dilutions. 0.2 mg of Vincristine sulphate (served as the positive control) was dissolved in DMSO to obtain an initial concentration of 20 µg/ml from which serial dilutions were made to acquire 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. The positive control solutions also contained 10 living brine shrimp nauplii in 5 ml sea water. For negative control, 100 µl of DMSO was added to each of the premarked test tubes containing 5 ml of sea water and 10 shrimp nauplii.

Counting nauplii: After 24 hours of incubation, the test tubes were inspected using a magnifying glass and the numbers of surviving larvae were counted. The concentration-mortality data were analyzed statistically by using probit analysis for the determination of LC_{50} values and linear regression for the respective fractions [5,7,8].

Statistical analysis

For each of the extracts, three samples were prepared for the bioassay. The zones of inhibition were calculated as mean ± SD (n=3).

Results

Antimicrobial activity

The result of the antimicrobial screening has been represented in **Table 1**. Previous studies reported that methanol is a better solvent for more consistent extraction of antimicrobial substances from medical plants compared to other solvents [9-11]. This investigation demonstrated promising broad-spectrum antimicrobial activity of aqueous soluble fraction of the methanol extracts of *Smilax roxburghiana* with average zone of inhibition 9 mm to 14.67 mm. On the other

hand, n-hexane soluble fraction and dichloromethane soluble fraction revealed moderate antimicrobial activity (average zone of inhibition 7.67 to 13.33 mm) and carbon tetrachloride soluble fraction exhibited poor antimicrobial activity (7 to 11 mm), compared to the positive control, Vincristine sulphate (21 to 26.67 mm).

Table 1: Antimicrobial activity of *Smilax roxburghiana* extracts by disc-diffusion method

Test microorganisms	Diameter of zone of inhibition (mm)				
	NHSF	CTSF	DMSF	AQSF	Kanamycin
Gram positive bacteria					
Bacillus cereus	8.67 ± 0.58	7.67 ± 0.58	-	10.33 ± 1.15	24.67 ± 0.58
Bacillus megaterium	8.67 ± 1.15	7.67 ± 1.15	8.67 ± 1.15	10.67 ± 1.53	26.67 ± 0.58
Bacillus subtilis	11.67 ± 0.58	7 ± 1	9 ± 1	11.33 ± 2.08	22.33 ± 0.58
Staphylococcus aureus	12.33 ± 1.15	11 ± 1	8.33 ± 1.15	10.33 ± 0.58	23 ± 0.58
Sarcina lutea	9.33 ± 1.15	7 ± 0	-	10.33 ± 0.58	22.33 ± 0.58
Gram negative bacteria					
Escherichia coli	7.67 ± 0.42	-	-	9 ± 0.79	21.67 ± 0.42
Pseudomonas aeruginosa	8 ± 1	7.67 ± 0.58	9.33 ± 1.15	9.67 ± 1.15	21 ± 1
Salmonella paratyphi	8.33 ± 1.15	-	12.67 ± 0.58	11.33 ± 1.15	22.67 ± 0.58
Salmonella typhi	8.67 ± 1.15	-	-	10.67 ± 1.53	25.33 ± 0.58
Shigella boydii	9 ± 1	8.67 ± 0.58	9.67 ± 1.53	9.67 ± 1.15	25 ± 1
Shigella dysenteriae	13.33 ± 0.58	-	12.33 ± 0.58	9.33 ± 1.15	26.33 ± 0.58
Vibrio mimicus	10 ± 1	-	-	11.33 ± 0.58	26.33 ± 1.15
Vibrio parahemolyticus	9 ± 1	7 ± 1	-	9.33 ± 0.58	24.33 ± 0.58
Fungus					
Candida albicans	9.33 ± 0.58	9.67 ± 0.58	9.33 ± 1.53	10 ± 1	23.67 ± 1.15
Aspergillus niger	10.67 ± 0.58	-	13.33 ± 1.15	14.67 ± 0.58	25.67 ± 0.58
Sacharomyces cerevacae	9 ± 1	-	7.67 ± 1.53	10.33 ± 1.15	22.33 ± 1.15

Note: The diameters of zone of inhibition (mm) are expressed as mean ± SD (n=3); a diameter less than 7 mm was considered inactive and denoted by '-'; NHSF: n-hexane soluble fraction, CTSF: carbon tetrachloride soluble fraction, DMSF: dichloromethane soluble fraction and AQSF: aqueous soluble fraction of the methanolic extract

The aqueous soluble fraction, dichloromethane soluble fraction and n-hexane soluble fraction strongly inhibited the growth of *A. niger* (14.67 mm & 13.33 mm) and *S. dysenteriae* (13.33 mm) respectively. Remarkable antimicrobial activity with average zone of inhibition more than 10 mm were shown by the aqueous soluble fraction against *B. cereus* (10.33 mm), *B. megaterium* (10.67 mm), *B. subtilis* (11.33 mm), *S. aureus* (10.33 mm), *S. lutea* (10.33 mm), *S. paratyphi* (11.33 mm), *S. typhi* (10.67 mm), *V. mimicus* (11.33 mm), *S. cerevaceae* (10.33 mm); by the n-hexane soluble fraction against *B. subtilis* (11.67 mm), *S. aureus* (12.33 mm), *S. dysenteriae* (13.33 mm), *A. niger* (10.67 mm); by the carbon tetrachloride soluble fraction against *S. aureus* (11 mm) and by the dichloromethane soluble fraction against *S. paratyphi* (12.67 mm), *S. dysenteriae* (12.33 mm), *A. niger* (13.33 mm).

Brine shrimp lethality bioassay

The LC_{50} values obtained for the *n*-hexane, carbon tetrachloride, dichloromethane and aqueous fractions of the *S. roxburghiana* and that of the positive control, vincristine sulphate, to the brine

shrimp were evaluated and summarized in **Table 2** [5]. In this bioassay, n-hexane soluble fraction of *S. roxburghiana* revealed prominent cytotoxicity with the LC_{50} value of 1.796 µg/ml and dichloromethane soluble fraction showed moderate cytotoxicity with the LC_{50} value of 6.167 µg/ml. Carbon tetrachloride and aqueous soluble fractions exhibited poor cytotoxicity having the LC_{50} values of 61.56 µg/ml and 47.11 µg/ml respectively, compared to that of positive control, Vincristine sulphate, 0.3229 µg/ml. The LC_{50} values of the samples (24 h) were obtained by a plot of percentage of the shrimps killed against the concentrations of the samples and the best-fit line was obtained from the data by means of regression analysis.

Table 2: Cytotoxicity of *Smilax roxburghiana* extracts by Brine shrimp lethality bioassay

Sample	Regression equation	R^2	LC_{50} (µg/ml)
VS	y = 29.799x + 64.634	0.9269	0.3229
NHSF	y = 25.169x + 43.602	0.9376	1.796
CTSF	y = 22.148x + 10.37	0.9752	61.56
DMSF	y = 30.605x + 25.82	0.9670	6.167
AQSF	y = 28.189x + 2.8344	0.9642	47.11

VS: vincristine sulphate

Discussion

Phytoconstituents present in plants, namely, flavonoids, alkaloids, tannins and triterpenoids, have demonstrated exciting potentials for the expansion of the range of modern chemotherapies against a wide spectrum of microorganisms [12,13]. The antimicrobial activities of various plants have been reported by several researchers [14,15].

The results of this study show that all the fractions exhibited varied antimicrobial activities against the tested organisms. The extracts tested were effective antibacterial agents against a group of microorganisms that are implicated in either typhoid fever and/or other gastrointestinal infectious diseases such as dysentery. The aqueous soluble fraction showed the better antimicrobial activity.

Though the brine shrimp lethality assay is inadequate regarding the elucidation of the mechanism of action, it is very useful to assess the bioactivity of the plant extracts. The brine shrimp lethality assay actually has proven to be a convenient system for monitoring biological activities of several plant species that are used in the traditional medicine. The *in vitro* cytotoxicity displayed by the plant extracts tested is an initial indicator of *in vivo* antitumour activity. However, since a wide range of phytocompounds are capable of exhibiting nonspecific cytotoxicity, plant extracts with significant cytotoxic activity should be further assayed using animal models to confirm antitumour activity, and/or a battery of various cell lines to detect specific-cytotoxicity.

Preliminary investigations suggested that there is a chance to get better therapeutic agent against microbial diseases and tumor cells from this plant. Further work especially bioassay-guided fractionation is warranted in order to isolate and characterize the active cytotoxic and antimicrobial constituents.

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