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Full Length Article

Antimicrobial Activity of *Clerodendrum viscosum* (Verbenaceae)

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ABSTRACT

The crude extracts of *Clerodendrum viscosum* Vent. (Verbenaceae) were tested in different solvent systems against six gram positive bacterial strains, viz., *Staphylococcus aureus*, *Sercinia lutea*, *Bacillus subtilis*, *B. megaterium*, *B. cereus* and *Streptococcus-β–haemolyticus*, nine gram negative bacterial strains, viz., *Salmonellae typhi*, *Shigella dysenteriae*, *Escherichia coli*, *S. shiga*, *S. boydii*, *S. sonnei*, *Proteus* sp., *Klebsiella pneumoniae* and *Pseudomonus aeruginosa* and seven fungal strains, viz., *Aspergillus niger*, *A. fumigatus*, *A. flavus*, *Candida albicans*, *Vasin factum*, *Mucor* sp. and *Fusarium oxysporum* by using disc diffusion and micro broth dilution techniques. All the extracts possessed antimicrobial activity with different potency against a variety of microorganisms pathogenic to human beings. Some extracts were bacteriostatic and fungistatic, while rest of the extracts showed bactericidal and fungicidal potential. The MIC values (64-128 μg/mL) of ethyl alcohol extract were determined against each gram positive and gram negative bacterial strains; *S. aureus*, *B. subtilis*, *S.-β–haemolyticus*, *S. typhi*, *E. coli* and *Klebsiella* sp. For cytotoxic experiment, ethyl alcohol root extract was more toxic (LC_{50} 20.845 ppm) than other extracts analyzed in Brine shrimp test. The results of the potential of *C. viscosum* will be creative for developing a broad spectrum antimicrobial formulation.

Key Words: Antimicrobial activity; Vascular plants; *Clerodendrum viscosum*; Zone of inhibition; Cytotoxic; Gram positive; Gram negative

INTRODUCTION

Tracheophytes (vascular plant) form a unique division in the plant kingdom having hostility to the attack of insects, snails and slugs. The genus *Clerodendrum* (Verbenaceae) is a very large and diverse genus and till now 580 species of the genus have been identified and are widely distributed in Asia, Australia, Africa and America.

The genus is taxonomically characterized by its entire or toothed, oppositely arranged leaves, terete stems, terminally or axillary cymose inflorescence, hypogynous bisexual flowers and exalbumenous seeds (Kirtikar & Basu, 1991; Steane *et al*., 1999). The genus exhibits a wide spectrum of folk and indigenous medicinal uses (Moldenke, 1985; Rueda, 1993; Hsiao & Lin, 1995; Steane *et al.*, 1999). Microorganisms are often a cause of existing diseases, regarding a solemn public health issue in a major part of the population as revealed by either personal or authorized health care systems. The economic crisis, high cost of industrialized medicines, inefficient public access to medical and pharmaceutical care, in addition to the side effects caused by synthetic drugs are some of the factors contributing to the central role of medicinal plants in health care (Johann *et al*., 2007).

Plants are always surrounded by an enormous number of potential enemies such as bacteria, viruses, fungi, insect etc. (Van Wyk & Gericke, 2000). It is logical to expect biologically active compounds to be produced by plants as a chemical defense measure against their enemies. Natural products have been a consistently successful source in drug discovery and offer more opportunities to find antimicrobial drugs or lead compounds (Wang *et al.,* 2006).

Plants synthesize very complex molecules with specific stereochemistry and can show biological activity with novel modes of action (Houghton, 1996). Plants, being a rich source of therapeutic agents, have contributed to the drug industry for a long time. More than 70% of all medicinal compounds have been derived from a small fraction of the World's biodiversity. There is a great potential to exploit further World's genetic resources by exploring the huge amount of diversity present in plant world (Glime & Saxena, 1991). Keeping this in view, the present study was undertaken to screen out *in vitro* antimicrobial activity of *C. viscosum* to use as a possible source for new antimicrobial substances against important pathogens of agricultural and veterinary importance.

MATERIALS AND METHODS

Plant collection and identification: The plant specimen

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was collected from Rajshahi University in 2009 and identification of voucher specimen was confirmed at the taxonomical section, Department of Botany, University of Rajshahi, Bangladesh.

Preparation of extracts: Root, leaf and stem were dried in shade and stored in cotton bags and then finely powdered (100 g) separately with the help of a grinder. Each ground material was soaked in 500 mL ethyl alcohol, chloroform and ethyl acetate separately for 24-72 h and filtered (Whatman No. 1). The filtrate was then allowed to evaporate in rotary evaporator until completely dry and kept in a refrigerator. Then (100 mg & 50 mg) dried extract for further study, was weighed and dissolved in 10 ml of respective solvents for dilution. The concentration of the final extract was 100 µg/10 µL and 50 µg/10 µL.

Microorganisms: Six gram positive bacterial strains, viz., *Staphylococcus aureus, Sercinia lutea, Bacillus subtilis, B. megaterium, B. cereus* and *Streptococcus-β–haemolyticus,* nine gram negative bacterial strains, viz., *Salmonellae typhi, Shigella dysenteriae, Escherichia coli, S. shiga, S. boydii, S. sonnei, Proteus* sp., *Klebsiella pneumoniae* and *Pseudomonus aeruginosa* and seven species of fungal strains, viz., *Aspergillus niger, A. fumigatus, A. flavus, Candida albicans, Vasin factum, Mucor* sp. and *Fusarium oxysporum* were employed in this test. These species were obtained from the mother stock of the Molecular Biology Laboratory, Institute of Biological Sciences, University of Rajshahi, Bangladesh.

Media: Nutrient broth, Nutrient agar and Sabouraud dextrose agar (Mast Diagnostics, Mast Group Ltd. Merseyside, UK) were used.

Disc diffusion assay: Antimicrobial activity was determined as diameter of inhibition zone using disc diffusion method (Bauer *et al.*, 1966). Nutrient agar (NA) and Sabouraud Dextrose agar (SDA), were distributed in sterilized petridishes for bacteria and fungi, respectively. This was accomplished by placing 10 µL of the extract on a small paper disc. This disc was placed on an agar growth medium containing a confluent lawn of microorganism. The concentration of the organism was also 10 µL/petridish. Minimum Inhibitory Concentrations (MIC) were determined by serial dilution technique.

Preparation of the test discs: Sterile test discs were prepared by punching and saturating filter paper (Whatman no 1) discs (each disc is 6 mm in diameter) in plant extracts solution using spirit flame sterilized forceps. These discs were dried in the sterilized petridishes. The name (code) of the plant extract was written at the bottom of the petri dishes.

Preparation of the standard: Inhibition zone of all extracts was compared to the standard antibiotic (Ciprofloxacin 05 µg/disc) and antifungal (Fluconazole 10 µg/disc).

Culture media and inoculums: Solid media of Nutrient and Sabouraud Dextrose agar were prepared by dissolving 2.8 g and 6.2 g powder of agar respectively in 100 mL water. About 25 mL of media was poured into a petridish. The inoculum was prepared by culturing a large number of organisms in a tube containing 10 mL liquid media for bacterial strains and incubating over night at 37°C. On the other hand, the inoculum of fungal strains was transferred directly into petridish and incubated at 37°C for 72 h. The agar plates for the assay were prepared by labeling them with the date, the name of the microorganism and the name (code) of the discs. The inoculi of bacteria were transferred into Petri dish containing solid nutrient media of agar using a sterile swab. The swab was used to spread the bacteria on the media in a confluent lawn. It was done by rotating the Petri dish at 90°C and continuing the spread of bacteria. One swab was used for one species of bacteria.

Placing test discs: Dried test discs were transferred on bacterial lawn under aseptic conditions using spirit-flame sterilized forceps each time. Each disc was placed gently on the agar surface and plated with the forceps so that it sticks. The Petri dish was incubated upside down at 37°C for 24 h. Resulting zones of inhibition were observed and measured in millimeters. Tests were repeated in triplicate and were performed to insure reliability of the results.

Brine shrimp test: Brine shrimp lethality test using larvae of *Artemia salina* L. was performed according to Meyer *et al.* (1982). Freshly hatched nauplii were added to vials containing different concentrations of ethyl alcohol, chloroform and ethyl acetate extracts of *C. viscosum* root, leaf and stem. For each sample, 2 mg of extract was initially dissolved in 200 µL of DMSO to make the extracts hydrophilic. Then, 19.98 mL of distilled water was added to get a stock solution of 200 ppm. Sample extracts of 100 and 50 ppm were made from the stock solution using serial dilution technique (Reiner, 1982) and were placed in 15 separate vials. Thirty nauplii were placed in each vial. After 24 h, the number of killed nauplii in each vial was counted and compared to the control. The LC_{50} value was calculated by probit analysis with 95% confidence intervals, regression equations and X^2 value (df). The experiments were replicated thrice along with a standard, ciprofloxacin.

RESULTS AND DISCUSSION

All the crude extracts of root, leaf and stem of *Clerodendrum viscosum* Vent. were subjected to screening against six gram positive bacteria, nine gram negative bacteria and seven species of fungae. It is clear from inhibition zones (Table I) that most of the extracts of root, leave and stem of *C. viscosum* are effective against both organisms; bacteria and fungi. The highest value of inhibition zone was 12 mm in diameter and the lowest value of inhibition zone was 7 mm in diameter, while the inhibition zones of the standard Ciprofloxacin were 19 mm for highest and 14 mm for lowest (Table I). In case of fungi, highest and lowest zones were 11 and 7 mm for plant extracts; while for Fluconazole those were 18 and 13 mm, respectively. Each disc was 6 mm in diameter (Table II).

Table I: Antibacterial activity of *C. viscosum* extracts and standard antibiotic Ciprofloxacin

Test organisms	Diameter of zone of inhibition (in mm)/each disc= 6mm																		
	Ethyl alcohol						Chloroform						Ethyl acetate						Cipro
	Root		Leaf		Stem		Root		Leaf		Stem		Root		Leaf		Stem		
µg/disc →	100	50	100	50	100	50	100	50	100	50	100	50	100	50	100	50	100	50	10
Gram positive bacteria																			
S. aureus	9	-	10	-	8	-	7	-	10	-	-	-	10	-	7	-	-	-	15
S. lutea	10	-	9	-	11	-	10	-	9	-	12	-	9	-	9	-	9	-	18
B. subtilis	10	-	9	-	11	-	9	-	11	-	9	-	11	-	11	-	9	-	15
B. megaterium	11	-	12	-	10	-	12	-	10	-	11	-	12	-	12	-	12	-	16
B. cereus	10	-	10	-	10	-	10	-	11	-	10	-	10	-	10	-	10	-	18
S.-β–haemolyticus	8	-	9	-	9	-	9	-	8	-	9	-	10	-	9	-	10	-	18
Gram negative bacteria																			
S. typhi	12	7	12	-	10	-	12	7	10	-	9	-	11	7	12	7	10	-	19
S. dysenteriae	10	-	12	-	11	-	9	-	9	-	8	-	9	-	9	-	9	-	17
E. coli	10	-	9	-	11	-	10	-	9	-	9	-	11	-	11	-	9	-	19
S. shiga	11	-	12	-	10	-	12	-	10	-	11	-	12	7	12	-	12	-	15
S. boydii	10	-	10	-	10	-	10	-	11	-	10	-	10	-	10	-	10	-	15
S. sonnei	12	-	9	-	12	-	11	-	10	-	9	-	10	-	9	-	10	-	14
Proteus sp.	9	-	8	-	8	-	7	-	9	-	-	-	10	-	7	-	-	-	16
Klebsiella sp.	12	7	12	-	11	-	10	-	11	7	10	-	12	7	11	-	9	-	14
P. aeruginosa	9	-	10	-	9	-	8	-	9	-	8	-	10	-	9	-	7	-	17

Table II: Antifungal activity of *C. viscosum* extracts and the standard antifungal Fluconazole

Test organisms	Diameter of zone of inhibition (in mm)/each disc= 6mm																		
	Ethyl alcohol						Chloroform						Ethyl acetate						Fluco
	Root		Leaf		Stem		Root		Leaf		Stem		Root		Leaf		Stem		
µg/disc →	100	50	100	50	100	50	100	50	100	50	100	50	100	50	100	50	100	50	10
A. niger	11	-	10	-	8	-	7	-	9	-	-	-	10	-	7	-	8	-	15
A. fumigatus	9	-	9	-	9	-	10	-	9	-	8	-	9	-	9	-	9	-	16
A. flavus	10	-	8	-	8	-	9	-	11	-	9	-	9	-	11	-	9	-	14
C. albicans	10	-	11	7	8	-	9	-	10	-	9	-	8	-	10	-	11	-	16
Vasin factum	9	-	10	-	10	-	10	-	9	-	8	-	10	-	9	-	9	-	17
Mucor sp.	10	-	9	-	9	-	9	-	11	-	10	-	10	-	9	-	10	-	18
F. oxysporum	8	-	9	-	8	-	8	-	10	-	9	-	8	-	7	-	8	-	13

The MICs of the ethyl alcohol leaf extracts of *C. viscosum* were determined against three gram positive and three gram negative bacterial strains; *S. aureus, B. subtilis, S.-β–haemolyticus, S. typhi, E. coli* and *Klebsiella* sp. using serial dilution technique (Reiner, 1982) and the results are presented in Table III. The results indicated that MICs of leaf extract ranged from 64-128 µg/mL as those of relatively good antibacterial compounds.

The mortality rates of brine shrimps nauplii increased with the raise of the concentration of the tested crude extracts. The LC_{50} values of the crude extracts of root, leaf and stem and the Ciprofloxacin were determined, using Probit analysis (McLaughlin *et al.*, 1991) and were found to be 20.845, 24.017 and 31.379 ppm for ethyl alcohol, 30.702, 32.907 and 42.559 ppm for chloroform and 33.448, 48.083 and 24.882 ppm for ethyl acetate, respectively. In the Brine Shrimp Test (Table IV), 95% confidence limits, regression equation and χ^2 values were also significant.

All of the crude extracts were found to be lethal to brine shrimp nauplii indicating that the extracts are biologically active. Antibacterial activity of *C. viscosum* has also been reported earlier (Cheng *et al*., 2001; Mahato & Chaudhary, 2005). Some other species of the genus *Clerodendrum* have also been reported for their antibacterial activity. Misra *et al.* (1995) reported hexane extracts of *C. colebrookianum* at concentrations of 1000 and 2000 ppm showed strong antibacterial activities against various Gram positive and Gram negative pathogens such as *S. aureus, S. haemolyticus, E. coli, P. aeruginosa*. Alcoholic extracts of leaves and flowers of *C. inerme* also exhibited antibacterial activity against *E. coli* and *S. aureus* (George & Pandalai, 1949). Pectolinarigenin and chalcone glucoside isolated from leaves of *C. phlomidis* showed antifungal activity (Roy *et al*., 1995). Two phenyl propanoid glycosides (acteoside & acteoside isomer) isolated from *C. trichotomum* showed potent inhibition of HIV-1 integrase with IC_{50} values of 7.8±3.6 and 13.7±6.0 µM (Kim *et al.*, 2001). A new hydroquinone diterpenoid was isolated from *C. uncinatum* and was strongly fungitoxic to the spores of *Cladosporium cucumerinum* (Dorsaz *et al*., 2004).

CONCLUSION

The above findings clearly indicate promising antibacterial and antifungal properties of *C. viscosum* against life threatening pathogens. Thus, *C. viscosum* appears to be an effective material for development of antimicrobial drugs and eco friendly biopesticides.

Table III: Minimum inhibitory concentration (MIC) of ethyl alcohol leaf extract of *C. viscosum* against six pathogenic bacteria

Test tube No.	Nutrient broth medium added (mL)	Diluted solution of Ethyl alcohol extract from leaf (µg/mL)	Inoculums added (µL)	Gram positive bacteria			Gram negative bacteria		
				S. aureus	*B. subtilis*	*S.-β-haemolyticus*	*S. typhi*	*E. coli*	*Klebsiella sp*
1	1	512	10	-	-	-	-	-	-
2	1	256	10	-	-	-	-	-	-
3	1	128	10	+	-	+	-	+	-
4	1	64	10	+	+	+	+	+	+
5	1	32	10	+	+	+	+	+	+
6	1	16	10	+	+	+	+	+	+
7	1	8	10	+	+	+	+	+	+
8	1	4	10	+	+	+	+	+	+
9	1	2	10	+	+	+	+	+	+
10	1	1	10	+	+	+	+	+	+
Cm	1	0	0	-	-	-	-	-	-
Cs	1	1024	0	-	-	-	-	-	-
Cs	1	0	10	-	-	-	-	-	-
Results of MIC (µg/ml)				128	64	128	64	128	64

(Notes: + = indicates growth, - = indicates no growth)

Table IV: Cytotoxic effects of *C. viscosum* extracts against *A. salina* nauplii after 24h of exposure and standard Ciprofloxacin

Samples	LC_{50} value (ppm)	95% confidence limits		Regression equations	X^2 value (df)
		Upper	Lower		
			Ethyl alcohol		
Ciprofloxacin	14.675	101.678	2.118	Y = 3.532138 + 1.278944 X	0.0215 (1)
Root	20.845	81.447	5.335	Y = 3.123247 + 1.445058 X	0.1114 (1)
Leaf	24.017	90.675	6.361	Y = 3.066222 + 1.395237 X	0.0843 (1)
Stem	31.379	85.303	11.543	Y = 2.783323 + 1.461687 X	0.0082 (1)
			Chloroform		
Ciprofloxacin	14.675	101.678	2.118	Y = 3.532138 + 1.278944 X	0.0215 (1)
Root	30.702	82.880	11.373	Y = 2.544355 + 1.611173 X	0.0634 (1)
Leaf	32.907	77.119	14.041	Y = 2.37178 + 1.710795 X	0.0420 (1)
Stem	42.559	86.987	20.822	Y = 2.440868 + 1.577916 X	0.0067 (1)
			Ethyl acetate		
Ciprofloxacin	14.675	101.678	2.118	Y = 3.532138 + 1.278944 X	0.0215 (1)
Root	33.448	79.724	14.033	Y = 2.335113 + 1.710795 X	0.0474 (1)
Leaf	48.083	87.898	26.302	Y = 2.12178 + 1.710795 X	0.0646 (1)
Stem	24.882	75.85641	8.161	Y = 2.760945 + 1.594544 X	0.1517 (1)

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