JOURNAL OF PURE AND APPLIED MICROBIOLOGY, April 2010. Vol. 4(1), p. 373-377

Antimicrobial Activity of *Enicostemma axillare* (Lam.) Raynal

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(Received: 01 October 2010; accepted: 04 November 2010)

The present study shows the *in vitro* antimicrobial activity of leaves of *Enicostemma axillare* (Lam.) Raynal, which was collected from Southern Aravalli hills of Rajasthan. Extraction was done successively in four different solvents i.e. petroleum ether, chloroform, ethyl acetate, methanol and aqueous extract was also prepared separately. The antibacterial and antifungal activity was tested by agar-well diffusion assay. All extracts displayed varied level of antimicrobial activity. The methanolic extract of *E. axillare* leaves exhibited antibacterial effect against all of the bacteria tested and the zone of inhibition varied between 16.66 to 10 mm. The methanolic extract also shows good antifungal activity against *Candida albicans* and *Aspergillus niger*. The results obtained in this study confirm the antimicrobial potential of E. *axillare* leaves, and its usefulness in the treatment of diseases that may be caused as a result of infection.

Key words: Antimicrobial activity, *Enicostemma axillare,* Aravallis, Rajasthan.

Infectious diseases are the leading cause of deaths worldwide. The clinical efficacy of many existing antibiotics is being threatened by the emergence of multidrug resistant pathogen[1]. Bacterial and fungal pathogens have evolved numerous defence mechanisms against antimicrobial agents and resistance to old and newly produced drugs is on the rise. Bacteria and fungi cause several diseases in man, plant and animals. In man bacteria like *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Proteus mirabilis* and *Bacillus subtilis* causes serious infectious diseases. Many fungi like *Aspergillus niger* and *Candida albicans* causes various skin and other diseases in human being.

Enicostemma axillare (Gentianaceae) is a glabrous perennial herb, growing throughout India, known as 'Nagajihva' or 'Mamejava' in Ayurveda. The plant is used in folk medicine to treat diabetes, fever, typhoid, purulent wound, venereal diseases, rheumatism, abdominal ulcer, hernia, swelling, itching and insect poisoning[2,3]. Its anti-inflammatory, hypoglycemic, antimicrobial, antioxidant and anticancer activities have also been reported[4-7]. The plant contains catechins, sterols, saponins, steroids, triterpenoids, alkaloids and volatile oil[8,9].

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Some important chemical constituents include betulin, a secoiridoid glycoside swertiamarine[10-13], monoterpene alkaloids, enicoflavine and gentiocrucine[14,15] and verticilliside, a new flavone C-glucoside[16]. Present study reports the antimicrobial activity of leaves of *E. axillare* against the array of human pathogens.

MATERIAL AND METHODS

Collection of Plant material

The plant material was collected from the Southern Aravalli hills of Rajasthan at the end of the flowering season. The plant was identified from its morphological and microscopical features as mentioned in different standard text and floras[17,18]. The voucher specimen has been deposited in the Department of Botany, M. L. Sukhadia University, Udaipur.

Plant Extracts

The leaves of this plant were washed, shade dried, powdered and extracted (300g) successively and separately with 1.5 X 2 litre each of petroleum ether, chloroform, ethyl acetate and methanol in soxhlet extractor for 6 hrs. The extracts were filtered and filtrate was concentrated to dryness under reduced pressure in rotary vacuum evaporator.

To make stock solution of 10 mg/ml of each extract (crude drug) the appropriate amount is weighed and dissolved in DMSO. The stock solution was passed through 0.2 µm pyrogesic filter to sterilize the solution and further concentrations of 5 mg/ml, 2.5 mg/ml and 1.25mg/ml was made by diluting with Di Methyl Sulfoxide (DMSO).

Fresh plant (30g) material was chopped, divided in three portions, grounded and transferred into suitable bottles and treated with 50ml each of distilled water. The first bottle was autoclaved at 10 lbs for 20 minutes, the second was boiled (100ºC) for 20 minutes and third was mechanically shaken (200 rev. /min) in cold condition for 2 hrs. Filtrate of all three bottles was evaluated for their antibacterial activity.

Microbial strains

The pathological strains of test organisms *i.e. Proteus mirabilis, Klebsiella pneumoniae, Bacillus subtilis, Escherichia coli, Staphylococcus aureus*, *Candida albicans* and *Aspergillus niger* were collected from RNT Medical College, Udaipur.

Antimicrobial susceptibility testing

Well Diffusion Method

The *in - vitro* antimicrobial activity were determined by the agar well diffusion method (19, 20) with some modifications. Cell suspensions containing 10^6 CFU/ml cells for bacteria, 10^6 CFU/ml cells for yeasts, and 10^5 spore/ml of fungi were prepared and 100µl was evenly spread on the surface of the nutrient agar medium for bacteria and Sabouraud dextrose agar medium for yeasts and fungi using glass spreader. Then wells of 6 mm diameter were bored at equidistant. 100µl volumes of each extract of each concentration were dispensed into wells, the plate were incubated at 37º C for 24 hrs for bacterial strains, 48 hrs for yeast and 72hrs for fungi at 28º C. The zone of growth of inhibition was measured. As reference antibiotic Streptomycin (10µg/ml) was used against all the tested bacteria and Amphoteracin -B (30 µg/ml) for yeast and fungi.

The minimum inhibitory concentrations (MICs)

The minimum inhibitory concentrations were determined by the broth dilution method. Proper amount of extract was taken to form the solution of the crude drug sample (25 mg/ml) with DMSO and diluted it serially (1:1) with sterile distilled water up to the concentration of 0.19 mg/ml. As a result, a series of the sample solution in decreasing concentration was obtained by a ratio of 0.5 (final concentration: 25 mg/ml to 0.19 mg/ml). Drew 1 ml of solution from each sample of different concentration and added the solutions into the test tubes that have already been prepared by pouring 9 ml of sterile Liquid Broth medium into them. After that the inoculum of target strains were uniformly added (200µl), put on the lid of tubes and incubated them at 37º for 24 h. The MIC value was defined as the lowest concentration to inhibit visible growth. DMSO, at used concentration in the test, did not interface with the microbial growth.

Minimum bactericidal concentration (MBC)

The content of all the MIC tubes were cultured on the freshly prepared nutrient agar plate, incubated at 37 º C for 24 hours and examined for growth. The MBC was regarded as

Table 1. Showing zone of inhibition of different extracts of *E. axillare* leaves

Extracts	Conc.	Ec**	Kp**	Pm**	Sa**	Bs**	Ca**	An**
PE	10	14 ± 0.0	13± 0.0	10.66±0.46	10± 0.0	10± 0.0	12.66±0.46	12.66±0.46
	05	13.33±0.46	12± 0.0	10± 0.0	-	10± 0.0	11.33±0.46	10± 0.0
	2.5	13± 0.0	-	10± 0.0	-	-	11±0.0	>10± 0.0
	1.25	12± 0.0		10± 0.0	-	-	10.33±0.46	-
In Chlo.	10	13.33±0.46	13± 0.0	10± 0.0	10.66±0.46	10.66±0.46	12.66±0.93	10± 0.0
	05	13± 0.0	12.66±0.46	10± 0.0	10.33±0.46	10.66±0.46	11.66±0.46	>10± 0.0
	2.5	12± 0.0	12± 0.0	10± 0.0	10± 0.0	10± 0.0	11.66±0.46	-
	1.25	11.33±0.46	11.33±0.46	10± 0.0	10± 0.0	10± 0.0	11.33±0.46	-
In EA	10	14± 0.0	12± 0.0	10.66±0.46	-	10± 0.0	11.66±0.46	10± 0.0
	05	13± 0.0	11± 0.0	10.33± 0.0	-	10± 0.0	11±0.0	>10± 0.0
	2.5	11.66±0.46	10± 0.0	10± 0.0	-	10± 0.0	10.66±0.46	-
	1.25	11.33±0.46	10± 0.0	10± 0.0	-	10± 0.0	10.33±0.46	-
In	10	16.66±0.46	15± 0.0	12± 0.0	14.66±0.46	13± 0.0	11.66±0.46	11.66±0.46
MEOH	05	15.66±0.46	14.33±0.46	11.33±0.46	13.33±0.46	12.66±0.46	11.66±0.46	11.33±0.46
	2.5	14.66±0.46	14.33±0.46	11± 0.0	12.33±0.46	12± 0.0	11.33±0.46	10± 0.0
	1.25	14± 0.0	13.33±0.57	10± 0.0	12± 0.0	11.33±0.46	11±0.0	>10± 0.0
Cold water	100%	-	11.66±0.46	NT	11.66±0.46	-	NT	NT
extraction	50%	-	-		-	-		
Hot water	100%	11.66±0.46	1466±0.46	NT	12.66±0.46	-	NT	NT
extraction	50%	-	-		-	-		
Autoclave d	100%	12±0	13.66±0.46	NT	12±00	-	NT	NT
water extraction	50%	-	-		-	-		

the lowest concentration of the extract that prevents the growth of any bacterial colony on solid medium. All tests were performed in triplicate.

RESULTS AND DISCUSSION

The antimicrobial activity of leaves extracts of *E. axillare* was determined against pathological strains of five bacteria and two fungi (Table 1). The antimicrobial activity was observed to be in dose dependent manner *i.e.* 10 mg/ml shown more level of activity than 5 mg/ml against the entire tested microorganism. As shown in Table 1, the extracts from the *E. axillare* leaves displayed antimicrobial activity against the tested microorganism, with the diameter of zone of inhibition ranging between 10mm to 16 mm. Among the four extracts methanolic extract was found to be most active against all the tested organisms, specifically *Escherichia coli* (16mm), *K. pneumoniae* (15mm) and *S. aureus* (14mm). The MIC and MBC of methanolic extract against the entire tested microorganism were observed to be in a range of 0.625 to 0.312 mg/ml (Table 2). The MIC values of the extract were lower than the MBC values, suggesting that the extract is bacteriostatic at lower concentrations but bactericidal at higher concentrations. Furthermore, among the fungi studied, in *C. albicans* maximum inhibition was observed in petroleum ether and chloroform extracts with the diameter of zone of inhibition ranging between 12.66 mm to 10.66 mm. In *A. niger* maximum inhibition was found in petroleum ether but in methanol the zone of inhibition was found at lower concentration (1.25mg/ml) also.

Table 2. Antibacterial activities of *E. axillare* leaves measured in terms of MIC and MBC

Name of the organism	MIC (mg/ml)	MBC (mg/ml)
Klebsiella pneumoniae	0.312	0.625
Escherichia coli	0.312	0.625
Proteus mirabilis	0.625	0.625
Bacillus subtilis	0.625	0.625
Staphylococcus aureus	0.312	0.312

Successive isolation of botanical compounds from plant material is largely dependent on the type of solvent use in the extraction procedure. The present study shows that plant extracts prepared with methanol solvent provided a good zone of inhibition. Petroleum ether, chloroform, ethyl acetate plant extracts, on the other hand found less active against the tested organisms (Table 1). This might have resulted from the less solubility of the active constituents in these solvents.

Hence the present investigation clearly reveals the antimicrobial nature of this plant and suggests that this plant could be exploited in the management of diseases caused by these bacteria in human systems. From the results obtained it supports the folkloric usage of *E. axillare* as a therapeutic agent. In addition, this study form a good basis for selection of the plant for further phytochemical and pharmacological investigation and suggests that the methanol extract contain certain constituents with antibacterial properties that can be used as antimicrobial agents in new drugs for the therapy of infectious diseases caused by pathogens. Since compounds of biological origin are known to posses minimal residual effect. The most active extracts can be further subjected for the isolation of therapeutic antimicrobials and carry out pharmacological evaluation.

ACKNOWLEDGMENTS

One of the authors Dr. (Mrs.) Anita Jain is highly thankful to DST for providing financial assistance.

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