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Evaluation of *In vitro* Antimicrobial Activity of Phytochemicals and Extracts of *Enicostemma littorale*

P. Mahajan Rita, M. Bharambe Shailendra, P. Mahulikar Pramod and H. More Dhananjay*

School of Chemical Sciences, North Maharashtra University, Jalgaon - 425 001, India

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In case of surgical operations and retarded immunity, opportunistic fungal and bacterial infections have become common cause for the mobility and mortality. Traditional plants are valuable source of novel antifungal and anti bacterial. The aqueous and ethanol extracts of leaves, whole plant and secondary metabolites of *Enicostemma littorale* showed better antimicrobial activity against three each test bacterial and three fungal species.

Key words: *Enicostemma littorale*, antimicrobial activity, phytochemicals, aqueous extracts, ethanol extracts.

In recent years, the popularity of complementary medicine has increased. Dietary measures and traditional plant therapies as prescribed by ayurvedic and other indigenous systems of medicine are used commonly in India. The World Health Organization (WHO) has also recommended the evaluation of the plants effective in conditions where safe modern drugs are lacking. Recently, an intensive search for medicinal properties has been carried out from numerous plant materials[1]. Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity. There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action for new and re-emerging infectious diseases[2-3].

Enicostemma littorale Blume[4, 5] belongs to family Gentianaceae is a perennial, glaborous, herb, 10-15 cm high, branched from the base; stem erect or procumbent. The plant is pungent and very bitter in taste. The literature survey revels that it possesses anthelmintic, antidiarrheal activity. It is also used for the treatment of weakness and febrifuge. Commonly it is used as stomach tonic and laxative. The crushed plant is also used in the treatment of snake bite[6-7].

Isolation of secondary plant metabolites (phytochemicals), with unknown pharmacological activities, have been extensively investigated as a

* To whom all correspondence should be addressed.
Fax: +91-257-2258403
E-mail: dhmore@rediffmail.com

source of medicinal agents. Thus, it is anticipated that phytochemicals with adequate antimicrobial efficacy will be used for the treatment of microbial infection. Since time immemorial, man has used various parts of plants in the treatment and prevention of various ailments[2].

MATERIAL AND METHODS

Sample Collection and Preparation

Enicostemma littorale was collected from ranges of Satpuda Mountain in Maharashtra state of India. The plants were identified at the School of Life Science, North Maharashtra University, Jalgaon. and used freshly for extraction after grinding (within 6 hrs).

Preparation of Extract

Ethanol extraction

The crushed plant material [100 g and 50 g for whole plant and leaves, respectively] was placed in thimble of Soxhlet apparatus[8] and extraction carried out by using ethanol as solvent for 12-14 hrs. The extracts were filtered; ethanol was distilled off using rotary evaporator to furnish the desired brownish-green residues, with the yield of which was 3.9% and 2.63% for whole plant and leaves, respectively. The obtained residues were then dissolved in ethanol at 1000, 2000, 3000 and 5000 µg/ml concentration[9].

Aqueous extraction

The 25 g of crushed whole plant material was soaked in 25 ml, 50 ml, and 100 ml each in distilled water for 24 hrs. The extract was filtered by using muslin cloth. The final volume were corrected to viz. 25 ml, 50 ml, and 100 ml by washing residue with distilled water[9, 10] and used for antimicrobial testing. Antimicrobial screening was carried out using same procedure adopted for leaves.

Isolation of Phytochemicals

Whole fresh plant of 35g *E. littorale* was ground in a mixure and homogenized by methanol: water mixture 4:1 (10 X Wt.) for 5 minute. Then it was filtered and the filtrate was evaporated. 2M H_2SO_4 was added and extracted with chloroform yielded terpenoids. Aqueous acid layer was made alkaline with NH_4OH and then extracted with chloroform-methanol (3:1, 60 ml) twice , this extract afforded most of the alkaloids whereas remaining aqueous basic layer was evaporated and extracted with methanol yielded quaternary alkaloids[11].

Phytochemical Analysis

The extracts were analyzed for the presence of alkaloids, terpenoids, alkaloids, Quaternary alkaloids tannins, using standard procedure[1,2,12,13].

Dilution of Phytochemicals

The 100,200 and 500 µg/ml concentrations of isolated phytochemicals were prepared by dilution with appropriate solvents.

Antimicrobial studies

The ethanol and aqueous extracts of *Enicostemma littorale* were tested for antimicrobial activity by agar disc diffusion method[1].

Preparation of culture medium and inoculation

For antibacterial activity, 35 g nutrient agar and 10 g agar agar were suspended in distilled water (1000 ml) and dissolved by boiling. Media and Petri dishes were sterilized in autoclave at pressure 15 lbs for 20 minutes. Under aseptic condition, 20 ml of media was dispended into sterilized petri dishes to yield a uniform depth of 6 mm. After solidification of the medium; the bacterial cultures were inoculated by spread plating technique. In this study, the bacteria species such as *Escherichia coli, Agrobacterium tumefaciens* and *Pseudomonas putida* were used as the test strain for ethanol and aqueous extracts of whole plant and leaves. Isolated phytochemicals were tested against other bacteria species such as *Escherichia coli, Agrobacterium tumefaciens* and *Pseudomonas putida*.

Disc application and incubation

Discs of 6 mm diameter were prepared from Whatman No. 1 filter paper, sterilized by autoclaving and subsequently dried at 80^0C for an hour. The sterilized discs were immersed in respective formulations of extracts of Enicostemma littorale and dried for 3-5 min. After drying the discs were placed on nutrient agar surface with flamed forceps and gently press down to ensure contact with the agar surface. The discs were spaced for enough to avoid both reflection waves from the edges of Petri dishes and overlapping rings of inhibition, finally, the Petri dishes were incubated for 24 hrs at 37°C in an inverted position. After 24 hrs the diameter (mm) of the inhibition zone around each spot was

measured. Antibacterial activities were indicated by clear zone of growth inhibition.

Antifungal Activity

Antifungal activity of respective formulations of ethanol and aqueous extracts of *Enicostemma littorale* was screened for the *in vitro* growth inhibitory activity against *P. lilacinus, C.falcatum, and A. awamori* by using agar disc diffusion method. The fungi were cultured in czepadox broth medium. The sterilized medium taken in the sterilized Petri dishes were inoculated with a spore suspension of *P. lilacinus, C. falcatum and A. awamori*. The filter paper discs were immersed in respective formulations of extracts. After drying the discs were placed on the surface of czepadox broth medium with flamed forceps and gently press down to ensure contact with the medium surface. After 48 hrs, the inhibition zone appear around the disc in each plate was measured (diameter in mm). To rule out the activity of solvent used in the preparation of test formulations, solvents (water, ethanol) were used in control plate.

RESULT AND DISCUSSION

The antimicrobial activity of *Enicostemma littorale* extracts were assayed *in vitro* by agar disc diffusion method against three bacterial and three fungal test species. The microbial growth inhibition of both aqueous and ethanol extracts of whole plant and leaves of screened plant are summarized in Table 1-4.

Table 1 summarizes the bacterial growth inhibition of ethanol and aqueous extracts of leaves and whole plant respectively. As compared to leaves, whole plant exhibited maximum inhibition against *Agrobacterius tumefaciam* followed by *Pseudomonas putida*, in ethanol extract. In aqueous extract of leaves maximum growth inhibition observed against *Agrobacterius tumefaciam*. Where as, in case of whole plant maximum inhibition was found against *Pseudomonas putida* and *Agrobacterius tumefaciam*. By comparing these results, the ethanol extracts of leaves and whole plant demonstrated maximum antibacterial activity.

The antifungal activity of ethanol and aqueous extracts, respectively of leaves and whole plant is summarized in Table 2 as compare to whole plant, leaves exhibited maximum inhibition against *Paecilomyces lilacinus* followed by *Aspergillus awamori* in aqueous extracts. In ethanol extracts of leaves maximum inhibition was observed against *Paecilomyces lilacinus*, in case of whole plant maximum inhibition found against *Aspergillus awamori*.

In overall results, whole plant exhibited better antibacterial activity and leaves showed better antifungal activity.

Table 3 summarizes the antibacterial activity of phytochemicals. Compound A and B exhibited maximum inhibition against *Escherichia coli* followed by *Pseudomonas putida* but compound D exhibited maximum inhibition against *pseudomonas putida* followed by

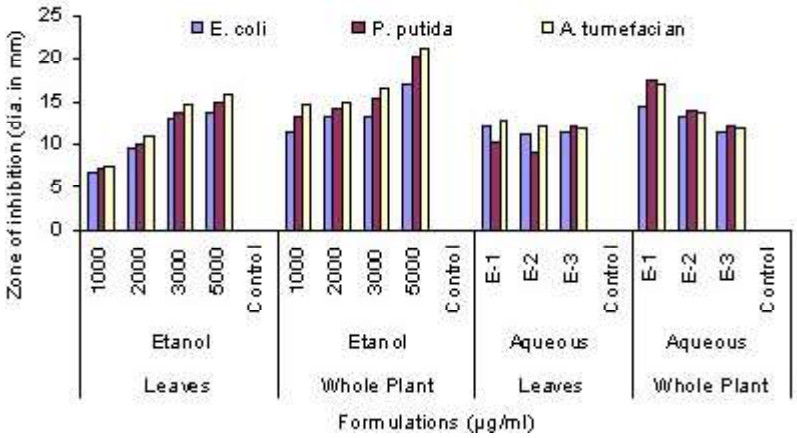

Fig. 1. Antibacterial activity of Ethanol & aqueous extracts of *Enicostemma littorale*

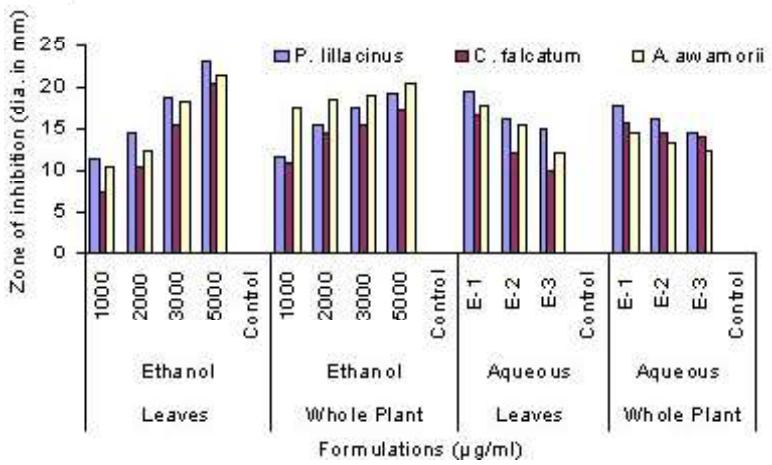

Fig. 2. Antifungal activity of Ethanol & aqueous extracts of *Enicostemma littorale*

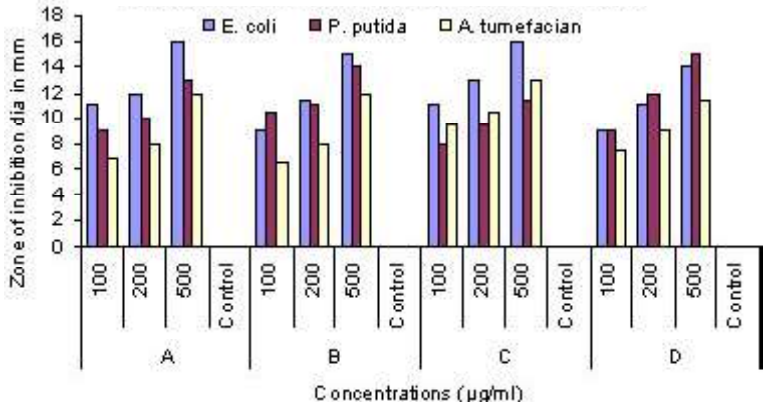

Fig. 3. Antibacterial activity of isolated phytochemicals

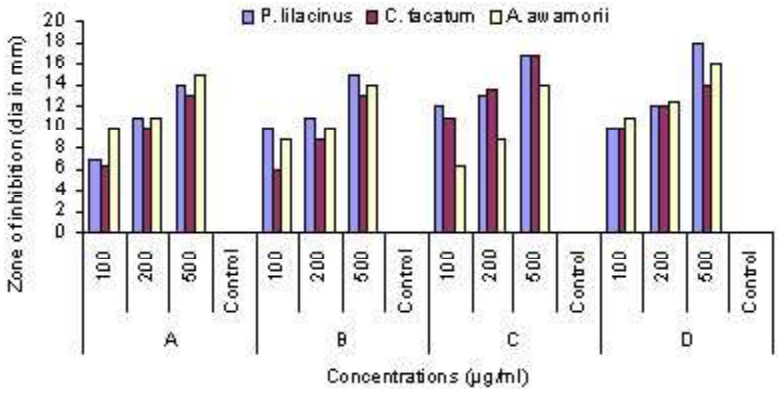

Fig. 4. Antifungal activity of isolated phytochemicals

Table 1. Antibacterial Activity of Ethanol and Aqueous Extracts of *Enicostemma littorale*

Name of Material	Type of Extract	Formulations	Zone of inhibition (diameter in mm)		
			Escherichia coli	*Pseudomonas putida*	*Agrobacterium tumefaciam*
Leaves	Ethanol	1000 µg/ml	6.7	7.2	7.5
		2000 µg/ml	9.5	10.1	10.8
		3000 µg/ml	13.0	13.6	14.7
		5000 µg/ml	13.5	15.0	15.8
		Control	00	00	00
Whole Plant	Ethanol	1000 µg/ml	11.5	13.2	14.7
		2000 µg/ml	13.2	14.2	15.0
		3000 µg/ml	13.3	15.6	16.6
		5000 µg/ml	17.0	20.4	21.2
		Control	00	00	00
Leaves	Aqueous	E-1	12.3	10.3	12.7
		E-2	11.2	9.1	12.0
		E-3	11.5	12.2	11.8
		Control	00	00	00
Whole Plant	Aqueous	E-1	14.5	17.5	17.0
		E-2	13.1	14.0	13.5
		E-3	11.5	12.2	11.8
		Control	00	00	00

E-1: 25 g crushed plant material in 25 ml distilled water.E-2: 25 g crushed plant material in 50 ml distilled water.
E-3: 25 g crushed plant material in 100 ml distilled water.

Table 2. Antifungal Activity of Ethanol and Aqueous Extracts of *Enicostemma littorale*

Name of Material	Type of Extract	Formulations	Zone of inhibition (diameter in mm)		
			Paecilomyces lilacinus	*Colletotrichum falcatum*	*Aspergillus awamori*
Leaves	Ethanol	1000 µg/ml	11.3	7.5	10.6
		2000 µg/ml	14.6	10.5	12.5
		3000 µg/ml	19.0	15.5	18.2
		5000 µg/ml	23.2	20.6	21.4
		Control	00	00	00
Whole Plant	Ethanol	1000 µg/ml	11.7	11.2	17.7
		2000 µg/ml	15.5	14.8	18.6
		3000 µg/ml	17.7	15.5	19.2
		5000 µg/ml	19.4	17.5	20.6
		Control	00	00	00
Leaves	Aqueous	E-1	19.6	16.7	17.8
		E-2	16.2	12.2	15.5
		E-3	15.2	10.0	12.2
		Control	00	00	00
Whole Plant	Aqueous	E-1	18.0	15.7	14.5
		E-2	16.2	14.7	13.5
		E-3	14.5	14.0	12.5
		Control	00	00	00

E-1: 25 g crushed plant material in 25 ml distilled water. E-2: 25 g crushed plant material in 50 ml distilled water.
E-3: 25 g crushed plant material in 100 ml distilled water

Escherichia coli. Compound C exhibited maximum inhibition against *Escherichia coli* followed by *Agrobacterium tumefaciam*. Overall results of antibacterial activity showed that all the compounds reflected maximum zone of inhibition on *Escherichia coli* followed by *Pseudomonas putida*.

Table 3. Antibacterial activity of isolated Phytochemicals

Compound	Concentration (µg/ml)	Zone of Inhibition (dia.in mm)		
		Escherichia coli	*Pseudomonas putida*	*Agrobacterium tumefaciam*
A	100	11	9	7
	200	12	10	8
	500	16	13	12
	Control	0	0	0
B	100	9	10.5	6.5
	200	11.5	11	8
	500	15	14	12
	Control	0	0	0
C	100	11	8	9.5
	200	13	9.5	10.5
	500	16	11.5	13
	Control	0	0	0
D	100	9	9	7.5
	200	11	12	9
	500	14	15	11.5
	Control	0	0	0

A – Tannins, B- Terpenoids, C- Alkaloids (Wagner reagent), D- Alkaloids (Dragendroff reagent).
Standard- Ampiciline (500µg/ml). Activity- E.colli-21.7mm, P.putida-30.7mm A. tumefacian -32.2mm.

Table 4. Antibacterial activity of isolated Phytochemicals

Compound	Concentration (µg/ml)	Zone of Inhibition (dia.in mm)		
		Paecilomyces lilacinus	*Colletotrichum falcatum*	*Aspergillus awamori*
A	100	7	6.5	10
	200	11	10	11
	500	14	13	15
	Control	0	0	0
B	100	10	6	9
	200	11	9	10
	500	15	13	14
	Control	0	0	0
C	100	12	11	6.5
	200	13	13.5	9
	500	17	17	14
	Control	0	0	0
D	100	10	10	11
	200	12	12	12.5
	500	18	14	16
	Control	0	0	0

A - Tannins, B- Terpenoids, C- Alkaloids (Wagner reagent), D- Alkaloids (Dragendroff reagent).
Standard- Flucanazole (500µg/ml). Activity- A.niger- 30.0mm, C.falcatum- 29.5mm, A.awamorii- 22.0mm.

The result of antifungal activity of phytochemicals are given in Table 4. Compound A exhibited maximum inhibition against *Aspergillus awamori* followed by *Paecilomyces lilacinus* but compound B and D exhibited maximum inhibition against *Paecilomyces lilacinus* followed by *Aspergillus awamori.* Compound C exhibited maximum inhibition against both *Paecilomyces lilacinus* and *Colletotrichum falcatum.* Overall results of antifungal activity visualized that all the compounds exhibited maximum zone of inhibition on *Aspergillus niger* followed by *Aspergillus awamori.*

In conclusion, the overall result of present investigation warrants the potential of plant products in integrated test management.

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