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In vitro Antimicrobial Effectiveness of Selected Medicinal Plants Extract against Pathogenic Organisms

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A B S T R A C T

Global prevalence of infectious diseases caused by microorganism is a major public health problem. Resistance against antibiotics of abundant bacteria is gradually acquiring. Therefore, investigation for new inventive plant materials with antimicrobial activity has become an insistent necessity. The present study aimed to investigate the antimicrobial potential of ethanol and aqueous aerial extracts of *Mikania scandens, Croton bonplandianum Baill and Eupatorium triplinerve* against gram positive, gram negative bacteria and fungus strains by using agar well diffusion assays and their activities were further determined by Minimum inhibitory concentration (MIC), Minimum bactericidal concentration (MBC), Minimum fungal concentration (MFC) assays. The selected plants were found to possess antimicrobial activity against selected pathogenic microorganisms. Comparative study revealed that the alcoholic extracts of all plants exhibited higher broad spectrum antimicrobial activity than aqueous extracts.The inhibitory property of the ethanol extract of *C. bonplandianum* (EECB) was observed within range of conc. from 2 to 1024 µg/ml. Ethanol extract of *C. bonplandianum* (EECB) was showed significant antibacterial activity with MIC of 128 µg/ml against both gram (+ve & -ve) and antifungal activity with the same MFC value, MBC of 256 µg/ml against gram +ve and fungal strains. The overall results indicates ethanol aerial extracts of *C. bonplandianum* (EECB) can serve as most effective potential source of antimicrobial activities than other plants.

Introduction

Natural products, either as pure compounds or as standardized plant extracts, contribute enormous opportunities for new drug leads because of the unrivalled accessibility of chemical diversity. Usually wild plants have provided mankind with medicine to alleviate suffering from different infectious diseases since ancient times. They are novel source of medicines as they have assortment of chemical agents with potential therapeutic properties. Different aerial part of plant has been used since ancient time either extracted raw compound or a paste. Although, several plant species have been evaluated as a choice for

antimicrobial activity, still there is a need for more research in this field. Plants, which are found to possess *in-vitro* antimicrobial properties, are generally affluent in a variety of phytochemicals including alkaloids, flavonoids, terpenoids, tannins.

M. scandens C. bonplandianum, E. triplinerve, have been commonly used for this study. *M.scandens,E.triplinerve* belonging to the same family Asteraceae. *M. scandens,* herbaceous climbing vine utilised for the treatment of stomach ulcers (Herz *et al.,* 1970; Hasan *et al.,* 2009). *In-vitro* experiments showed that the *M. scandens* flowers displayed marked anti-inflammatory properties. The leaves are used for analgesic and *in vitro* antioxidant and antidiabetic activities.

The leaves of exotic plant *C. bonplandianum* (Euphorbiaceae) used for controlling high blood pressure, for the treatment of skin diseases and cuts wounds and also used as antiseptic and antidote. The seeds have the efficacy to cure jaundice, acute constipation, abdominal dropsy and internal abscesses. The leaf extract has been proved to have wound healing effect and external application has shown to cure the ringworm infection. The seed of *C. bonplandianum* contains diterpines, phorbol ester, including 12-orthotrideconeoly-phorbol-13-acetate (TPA) and myristoyl phorbol acetate (MPA).

E. triplinerve, perennial plants known as ayapana used for control bleeding from open wounds and blood clotting. The essential oil from the flowers of ayapana was reported to possess antiparasitic and anthelmintic actions. The flower essential oil injected into mice was reported to have CNS depressant, analgesic, and sedative effects.

In the present study, we investigated the potential of three wild Indian plants species for antimicrobial property against the both gram positive and gram negative as well as fungal organisms.

Materials and Methods

Collection of plant materials and extract preparation

The aerial parts of *Mikania scandens*, *Croton bonplandianum, Eupatorium triplinerve,* were collected from various regions of Midnapore district of West Bengal, India. Collection of plant materials was independent of season. All species were taxonomically established and authenticated by Central National Herbarium, Botanical Garden, Howrah. *C. bonplandianum* and *E. Triplinerve* were identified with Reference No. CNH/2017/Tech.II/22 and *M. scandens* was identified with Reference No. CNH/57/2014/Tech.II/278.

After authentification the fresh aerial parts collected in bulk. All plant materials were collected with deionised water, shade dried, and grinded mechanically into coarse powder. The powder plant materials were sequentially extracted with ethanol and water (1200 ml) according to their increasing polarity by using Soxhlet apparatus for 24 h at a temperature not exceeding the boiling point of the respective solvent.

The obtained extracts were concentrated under vacuum by using rotary evaporator. Both extracts were collected separately and stored in a freezer at 8^{o}c temperature until further use.

Phytochemical studies

Preliminary phytochemical exploration of the both extracts for the presence of different secondary metabolites such as glycosides, alkaloids, flavonoids, saponins, steroids, tannins were carried out.

Table.1 List of plant species used in the study

Sl.No.	Species	Family	Vernacular name	Plant materials
i)	*Mikania scandens*	Asteraceae	Climbing hempvine	Screened leaf, stem, flowers, fruits
ii)	*Croton bonplandianum*	Euphorbiaceae	Bantulsi	Screened leaf, stem, flowers, fruits
iii)	*Eupatorium triplinerve/ Ayapana triplinerve*	Asteraceae	Ayapana	Screened leaf, stem, flowers

Test strains

Two gram positive bacteria *Bacillus sutbilis* (MTCC No.441), *Staphylococcus aureus* (MTCC No. 3160), two gram negative bacteria *Escherichia coli* (MTCC No.1652),*Salmonella typhi* (MTCC No. 733) and two fungal strains *Candida albicans* (MTCC No.227) *Asperigillus niger* (MTCC No.282) were obtained from Microbiology department which were kept at 4ºc on agar slant and subculture at 37ºc for 24 hrs on nutrient agar before any susceptibility test.

Antimicrobial susceptibility

Culture media

Nutrient agar was used for bacteria and savoured dextrose broth for fungi. For the agar well diffusion experiments savoured dextrose agar was employed. The Muller Hinton agar (MHA) medium was used for the minimal inhibition Concentration (MIC) and minimum bactericidal concentration (MBC) determination.

Standard drugs used for antimicrobial agents

Ciprofloxacin and Fluconazole (Micro Lab, India) were used as reference antibiotics against bacteria and fungi correspondingly.

Preparation of inocula

For the preparation of the inoculate 24h culture was emulsified in 3 ml sterile saline following the McFarland turbidity to obtain a concentration of 10^8 cells/ml. The suspension was standardized by adjusting the optical density to 0.1 at 600 nm (ELICO, SL-244 spectrophotometer). One hundred micro litres (100 µl) of cell suspension with approximately 10^6 -10^8 bacteria per millilitre was placed in petridishes and dispersed over agar.

Zone of inhibition determination by agar well diffusion assay:

Antibacterial assay

Antimicrobial activities of the crude extracts were first screened for their zone of inhibition by the agar well-diffusion method. Shortly, crude extracts were prepared concentration of 50 mg/ml and 100 mg/ml with dimethyl sulphoxide (DMSO) as solvent. The Mueller Hinton Agar (MHA) medium (Hi Media) was prepared and sterilised at 121°C 15 lb/sq for 20 min the autoclave. Thirty millilitres of this sterilised agar medium (MHA) were poured into each 9 cm sterile petridishes under aseptic conditions and allowed to settle. In the following, a well was made in the plates with the help of a sterile stainless steel-borer (6 mm diameter) two holes per plates were made into the set agar containing the bacterial culture. Each well 100 µl of the plant extracts at the various concentration. For each bacterial strain controls were maintained where pure solvents, instead of extract as negative control. Ethanol and Aqueous extracts (50 mg/ml and 100mg/ml) and reference drug (Ciprofloxacin100µg/ml) were

allowed to diffuse for 1 h into the plates and then incubated at 37°C for 18h in inverted position. The results were recorded by measuring the zone of growth inhibition in mm surrounding the wells. Each assay was performed in triplicates and repeated twice.

Antifungal activity

Both the fungal species was cultured in Potato Dextrose broth for 48h at 27°C and Savoured Dextrose Agar (SDA) was employed for the agar well diffusion experiments. Fungal suspensions was adjusted to 10^7 cells/ml. The zone of Inhibition was determined after incubation for 48h at 27°C.Specified test drug ethanol and aqueous extracts (50mg/ml) and (100mg/ml) and standard drug fluconazole (100 µg/ml) were used respectively. All tests were performed in triplicates and repeated twice.

Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) is defined as the lowest concentration able to inhibit any visible bacterial growth on the culture plates. Sensitivity of the microorganisms of both ethanol and aqueous extracts of selected plants can be measured by using tube dilution method where it can show the bactericidal or bacteriostatic. Each tube contained an inoculums density of $5x10^5$ CFU/mL of each of the test organisms. All organisms were grown in Muller Hinton broth. Then the suspension of all the four cultures was added into tubes containing diluted sample of *C. bonplandianum*, *E. triplinerve*, *M. scandens* extracts 2-1024 µg/mL. The dilution of the samples was done with Mueller Hinton broth. Finally, the tubes containing diluted sample of and bacteria was then incubated overnight at 37°C with constant shaking on the shaker. The growth of the microorganisms was determined by turbidity. Clear tubes indicated absence of bacterial growth. For every experiment, a sterility check (ethanol, medium) negative control (ethanol, medium, inoculums) and different standard antibiotics individually were included. The MIC of the samples was the lowest concentration in the medium that completely inhibited the visible growth. The solvent value was deducted accordingly to get the final results of activity.

Minimum Bactericidal concentration (MBC) and Minimum Fungicidal Concentration (MFC) assessment

The minimal bactericidal concentration (MBC) was determined by using the method of *Vila et al.* To determine the MBC and minimal fungicidal concentration (MFC) of the plant extracts against the microorganisms, the plates of the MIC that showed no growth of the microbes were sub-cultured by striping using wire loop on sterile Muller Hinton agar plates. The plates were incubated at 37°C for 18-24 h and at 25°C for 48 h respectively for bacteria and fungi. The MBC and MFC were taken as the lowest concentration of the extract that exhibited not microbial growth on the agar plates.

Evaluation of bactericidal and bacteriostatic capacity

The action of an antibacterial on the bacterial strains can be characterized at two parameters as MIC and MBC. Accordingly to the ratio MBC/MIC, we can apperceive antibacterial activity. If the ratio MBC/MIC=1 or 2, effect is bactericidal but if the ratio MBC/MIC=4 or 16, effect is bacteriostatic.

Results and Discussion

Phytochemical evaluation

The preliminary phytochemical analysis of ethanol and aqueous extracts of *M. scandens,*

C. bonplandianum, and *E. triplinerve* revealed that these plants content flavonoids, alkaloids, tannins, glycosides. Flavonoids were present in both extracts of all selected plants. Alkaloids were present in both extracts of *M. scandens* and aqueous extracts of *C. bonplandianum* and *E. Triplinerve.* Tannins were present in both extracts of selected plants except ethanol extract of *M. scandens* (Table 2).

Antimicrobial susceptibility

In this study, *in-vitro* antimicrobial activity of *M. scandens*, *C. bonplandianum*, and *E. triplinerve* ethanol and aqueous extracts of 2 gram positive, 2 gram negative bacterial strains and 2 fungal strains showed antimicrobial activity (Table 3) followed by the agar-well diffusion assay compared with standard antibiotics such as ciprofloxacin and fluconazole which were used as positive controls. The results showed that selected medicinal plant extracts possess antimicrobial activities against all pathogenic microorganisms (*B.subtilis, S.aureus, E.coli, S.typhi, C.albicans, A.niger)* in dose dependent manner. The highest inhibition activities were observed with the ethanol extract of *C. bonplandianum* on both gram negative bacterial strains *E.coli* and *S.typhi* at the dose of 100 mg/ml than comparatively *E.triplinerve* and *M.scandens.* The gram positive strains also showed significant sensitivity of all plants.

The selected plants showed also potent sensitivity antifungal activities against both the fungal strains (Table 3).

The agar well diffusion assay is a qualitative, non standardised method useful only for the screening of large numbers of samples. Activities revealed with well diffusion assay were confirmed using the micro dilution broth method. Accordingly both the methods, the antimicrobial activities could be qualified and quantified by inhibition zone diameter, MIC and minimum bactericidal or fungicidal concentration(MBC/MFC) of the extracts. The MIC and MBC/MFC values were used to compare the antimicrobial activity of extracts. The results of MIC,MBC and MFC values showed in Table 4 and 5. The data indicate that the extracts exhibited variable levels of antimicrobial activity against the invested microorganisms. The inhibitory property of the ethanol and aqueous extracts of selected plants were observed within a range of concentration from 2 to 1024 µg/ml.The ethanol extract of *M.scandens* showed a significant antibacterial activity with MIC of 128 µg/ml *S.aureus, S.typhi*, MFC of 128µg/ml obtained for the *A.niger* and aqueous extract of *M.scandens* with MIC of 128 µg/ml *S.aureus, S.typhi,* MFC of 128 µg/ml found for the *C.albicans*. The other two plant extracts values were given the same table 4. The bactericidal and bacteriostatic effect was determined using the ratio MBC/MIC and MFC/MIC.

Contagious diseases are the primary cause of morbidity and mortality worldwide. The number of multidrug resistant microbial strains and the emergence of strains which alleviate susceptibility to antibiotics are continually increasing. Such effect has been attributed to indiscriminate use of broad spectrum antibiotics, immunosuppressive agents and ongoing epidermis of human immunodeficiency virus (HIV) infections. This condition provided the impetus to the finding for new antimicrobial substances from various source such medicinal plants.

The plants have traditionally provided a source of hope for novel drug compounds, as plant herbal mixtures have made large contributions to human health and well being. The use of plant extracts with known antimicrobial properties can be of great significance for therapeutic treatment.

Table.2 Phytochemical analysis of selected plant samples

Sl. No.	Constituent	*M.scandens*		*C.bonplandianum*		*E.triplinerve*	
		Ethanol extract	Aqueous extract	Ethanol extract	Aqueous extract	Ethanol extract	Aqueous extract
1.	Flavonoids	+	+	+	+	+	+
2.	Alkaloids	+	+	+	+	+	-
3.	Saponin	-	+	-	+	-	+
4.	Tannins	-	+	+	+	+	+
5.	Steroid	+	-	-	-	-	-
6.	Glycosides	+	-	-	-	-	+

(+) sign indicates presence and (-) sign indicates absence of phytoconstituent.

Table.3 Results of zone of inhibition (mm) in antimicrobial activities

Sl no.	Groups	Antibacterial activity (gram+ve)				Antibacterial activity (gram-ve)				Antifungal activity			
		B. subtilis		*S. aureus*		*E. coli*		*S. typhi*		*C. albicans*		*A. niger*	
		50 mg/ml	100 mg/ml	50 mg/ml	100 mg/ml	50 mg/ml	100 mg/ml	50 mg/ml	100 mg/ml	50 mg/ml	100 mg/ml	50 mg/ml	100 mg/ml
1	EEMS	13.3 ± 0.55	18.7 ± 0.18	17.5 ± 0.83	23.6 ± 0.33	19.2 ± 0.81	24.5 ± 0.93	13.4 ± 0.38	16.9 ± 0.43	23.7 ± 0.53	25.6 ± 0.81	19.5 ± 0.39	24.9 ± 0.33
2	AEMS	10.3 ± 0.54	14.9 ± 0.32	13.4 ± 0.93	16.6 ± 0.81	12.5 ± 0.83	16.9 ± 0.13	10.9 ± 0.39	14.5 ± 0.73	17.5 ± 0.23	21.4 ± 0.63	16.8 ± 0.23	21.5 ± 0.53
3	EECB	19.3 ± 0.81	22.8 ± 1.24	18.3 ± 0.81	24.4 ± 1.24	19.6 ± 0.47	25.3 ± 1.63	16.6 ± 0.47	23.3 ± 1.24	25.3 ± 0.31	28.1 ± 0.18	19.9 ± 0.38	24.3 ± 0.22
4	AECB	16.6 ± 1.94	18.2 ± 1.69	14.6 ± 1.49	18.6 ± 1.09	13.3 ± 1.24	21.3 ± 0.94	14.6 ± 1.24	22.7 ± 1.16	17.3 ± 0.31	21.5 ± 0.23	16.2 ± 0.21	18.5 ± 0.39
5	EEET	18.3 ± 1.94	22.7 ± 1.24	19.6 ± 1.63	23.3 ± 1.62	21.6 ± 1.24	23.8 ± 2.18	22.3 ± 2.05	25.0 ± 1.63	23.2 ± 0.61	24.8 ± 0.13	20.8 ± 0.31	24.0 ± 0.32
6	AEET	15.7 ± 2.16	20.6 ± 1.24	16.1 ± 2.05	19.4 ± 2.16	17.6 ± 2.86	18.3 ± 0.47	17.6 ± 2.05	21.3 ± 2.5	18.0 ± 0.31	24.5 ± 0.23	15.0 ± 0.21	22.0 ± 0.31
7	CPF	23.9±0.51		25.6±0.25		27.2±0.62		28.4±0.56					
8	FLZ									32 ±0.35		29.3±0.55	
9	CNT	-		-		-		-		-		-	

EEMS-Ethanol extract of *M. scandens*, AEMS-Aqueous extract of *M.scandens*, EECB-Ethanol extract of *C.bonplandianus*, AECB-Aqueous extract of *C.bonplandianum*, EEET-ethanol extract of *E.triplinerve*, AEAT-Aqueous extract of *E.triplinerve*, CPF-Ciprofloxacin (100µg/ml), FLZ-Fluconazole (100 µg/ml), CNT-Control

Table.4 MIC, MBC and MFC determination, bactericidal (+) and bactriostatic (-) effect of the ethanol extracts of selected plants

Sl. No	M.O	*M.scandens*				*C.bonplandianus*				*E.triplinerve*			
		MIC	MBC or MFC	MBC /MIC	Effect	MIC	MBC or MFC	MBC /MIC	Effect	MIC	MBC or MFC	MBC /MIC	Effect
1	SA	128	512	4	_	128	256	2	+	256	512	>4	Nd
2	BS	1024	NA	NA	_	128	256	2	+	1024	NA	-	-
3	EC	256	512	2	+	128	512	4	-	256	1024	4	_
4	ST	128	512	4	-	256	512	2	+	128	512	4	_
5	CA	256	512	2	+	128	256	2	+	128	512	4	_
6	AN	128	1024	4	-	256	512	2	+	256	1024	4	_

SA- *Staphylococcus aureus*, BS - *Bacillus sutbilis*, EC - *Escherichia coli*, ST- *Salmonella typhi*, CA - *Candida albicans*, AN - *Asperigillus niger*,NA-No Activity, Nd-No detected activity

Table.5 MIC, MBC and MFC determination, bactericidal (+) and bactriostatic (-) effect of the aqueous extracts of selected plants

Sl.No	M.O	*M.scandens*				*C.bonplandianus*				*E.triplinerve*			
		MIC	MBC	MBC/ MIC	Effect	MIC	MBC	MBC/ MIC	Effect	MIC	MBC	MBC/ MIC	Effect
1	SA	128	256	2	+	128	256	2	+	128	512	4	-
2	BS	256	1024	4	-	128	512	4	-	256	1024	4	-
3	EC	512	1024	2	+	128	512	4	-	1024	NA	-	_
4	ST	128	512	4	-	256	512	2	+	128	512	4	-
5	CA	128	512	<2	-	256	1024	4	_	512	1024	2	+
6	AN	256	NA	NA	-	512	1024	2	+	512	1024	2	+

In the present study, ethanol and aqueous extract of *M. scandens, C. bonplandianum, E. Triplinerve* exhibited dose dependent activity against all the tested pathogenic microbial strains with inhibition activity varied from one plant to another. But comparatively alcoholic extracts of all plants exhibits higher antimicrobial activity due to nature of biological active components which may be enhanced in the presence of ethanol than the aqueous extract. This is due to high polarity of alcoholic solvents which naturally has ability to extracting high quantity of phytochemicals. Among 3 plants extracts *C. bonplandianum* aerial parts extract exhibited maximum zone of inhibition both gram positive as well as gram negative bacteria and also the fungal species. *M. scandens, E. Triplinerve* aerial parts extract showed significant activity.

The antimicrobial activity could be due to the presence of single bioactive compound or combined action of many compounds contained in the extract. Plant components with phenolic structures are highly active against the microorganisms. Several studies have shown that various phytochemicals compound like flavonoids, alkaloids, tannins, saponins, reducing sugar, steroids, glycoside are present in *M. scandens, C. bonplandianum, E. Triplinerve*. Polyphenols like flavonoids and tannins (Cowan, 1999) are

important of antimicrobial activity. The highest antimicrobial activity of ethanol extract of *C.bonplandianum* may be attributed to the presence of active ingredients of flavonoids like quercetin and rutin (*Sumahy Arokiasamy and Narendra kumar Singh et al*.,). The stronger antimicrobial activity of flavonoids is due to their ability to complex with extracellular and soluble protein and to complex with bacterial cell wall synthesis in the effected organisms while that of tannins may be related to their ability to inactivate microbial adhesion, enzymes and cell envelop proteins. The bacteriostatic and bacteriocidal activity could be ascribed to the presence of polyphenol compounds.

In conclusion, this study revealed the efficacy of *C. bonplandianum, M. scandens, E. Triplinerve* as antimicrobial agents against all the tested pathogenic microorganisms. Comparative antimicrobial evaluation among the plants under examination showed that *C. bonplandianum* ethanol extracts can serve as most effective antimicrobial agents than other two plants. Further research is required for isolation and identification of active principles present in the extract for showing the infectious ailments.

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