Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 4, Suppl 1, 2012

Research Article

# ANTIMICROBIAL ACTIVITY AND PHYTOCHEMICAL SCREENING OF METHANOL EXTRACT OF *ENICOSTEMMA AXILLARE*

**G.LEELAPRAKASH[1*], S.MOHAN DASS[2]**

[1.]Department of Biochemistry, Administrative Management College, 18th km, Bannerghatta road, Kalkere, Bangalore 560083, [2.]Kaamadhenu Arts and Science College, Sathyamangalam, Tamil Nadu 638503. Email: lpleelaprakash@gmail.com

*Received: 14 Oct 2011, Revised and Accepted: 20 Nov 2011*

**ABSTRACT**

The present study attempt to evaluate the antibacterial, antifungal activity and also to screen the phytochemicals present in the *Enicostemma axillare* methanol extract (EAME). Disk diffusion method was used to study the antibacterial activity against *Corynebacterium*, *Bacillus subtilis, Rhodospirillum, Salmonella paratypi, Klebsiella pneumoniae, Micrococcus luteus, Staphylococcus albus, Vibrio cholera, Escherichia coli, Pseudomonas aureginosa* and antifungal activity against *Aspergilus niger, Aspergilus fumigates, Microsporum gypseum, Candida albicans, Monoscus ruber.* The phytochemicals was screened by different chemical test and Rf values were calculated by Thin layer chromatography (TLC) in different solvent systems. In this study, we observed that the EAME had antibacterial and antifungal activities by formation of inhibitory zone and it was compared with standard antibiotics such as Ciprofloxacin and Fluconazole respectively. Qualitative phytochemical analysis of EAME confirms the presence of various phytochemicals like Alkaloids, Glycosides, Tannins, Carbohydrates, Proteins and amino acids, Saponins and Flavonoids. For TLC analysis different solvent systems were used to develop best separation of the phytoconstituents present in the EAME. Maximum number of solvent systems gave good results except for Toluene: Ethyl acetate: Glacial acetic acid (12.5:7.5:0.5), Ethyl acetate: Butanol: Formic acid: Water (3:2:0.5:2), Ethyl acetate: Formic acid: Glacial acetic acid: H2O (100:11:11:26), Ethyl acetate: Ethanol: Acetic acid: H2O (8:1:1:8). In this study we concluded that the methanol extract of *Enicostemma axillare* showed good antibacterial and antifungal activity, and it may be attributed due to the presence of phytochemicals it may be used as antimicrobial agents.

**Keywords:** Antibacterial activity, Antifungal activity, *Enicostemma axillare,* Thin layer chromatography, Zone of inhibition.

## INTRODUCTION

Plants produce a variety of secondary metabolites that have been the basis of treatment and cure for various bacterial infections and diseases in human population. In recent years these metabolites are being used, either directly as precursors or as lead compounds in the pharmaceutical industry to make drugs against microorganisms.

*Enicostemma axillare* belongs to family Gentianaceae. It is also called as Vellarugu in Tamil, Chota chirayata in Hindi, Mamejavo in Gujarati and Nagajivha in Bengal. It is a glabrous perennial herb attaining height of 15-20 inch with sessile lanceolate leaves and is found throughout India up to a height of 1500ft. The plant is used in folk medicine to treat diabetes mellitus, rheumatisum, abdominal ulcers, hernia, swelling, itching and insect poisoning [1], invivo anti-inflammatory [2], invitro anti-inflammatory [3], hypoglycemic [4, 5, 6] and anticancer [5] activities have been reported.

The whole plant is used in medicine as digestive, anti-inflammatory, liver tonic, antimalarial, antipyretic and as a laxative [1, 7]. According to ayurvedic literature survey, the fresh juice of leaves has been used as a bitter tonic, to control arthritis, in typhoid fever and as cooling agent. The plant is traditionally used in the treatment of hepatic diseases and as a blood purifier. It also acts as ethnomedicine for snakebite [8]. The plant paste is applied on boils. The leaves are fed to cattle to increase appetite.

In addition to a bitter secoiridoid glycoside, swertiamarin [9, 10], steroids, alkaloids, saponins, flavonoids, triterpenoids, phenolic acides and xanthones [5] were isolated from *Enicostemma axillare*. Swertimarin is a representative constituent of many crude drugs and formulations, which are marketed in Japan and other countries and these drugs are normally evaluated by their high Swertimarin content [11]. Many such compounds have protective effects due to their antioxidant properties [12].

Qualitative analysis of the ash content of aerial parts of the plant revealed the presence of minerals like iron, sodium, potassium, calcium, magnesium, silica, phosphate, sulphate, chloride and carbonate. Monoterpene alkaloids like enicoflavin and gentiocrucine were also isolated. In addition to the steroids, triterpenoids including catechins, betulin, saponins were also isolated [13]. These reported activities and many of the ethnobotanical used of the plant related to its hepatoprotective activity. Many such compounds have protective effects due to their pharmacological activities [12].

Present study was carried out to screen the phytochemical compound and evaluate its antimicrobial activities. Ten bacterial species and 7 fungal strains were used for antimicrobial study. The antimicrobial activities of the extracts were determined by the disc diffusion method.

## MATERIALS AND METHODS

### Plant material

The whole plant of *Enicostemma axillare* was collected in fresh condition from Coimbatore region of Western Ghats, Tamilnadu. Further it was identified at Botanical Survey of India (southern circle), Coimbatore and voucher specimens were deposited. The plant were dried under shade then ground into a uniform powder using a blender and stored in polythene bags at room temperature.

### Preparation of extracts

The extraction of soluble compounds from *Enicostemma axillare* by the soxhlet method was performed using methanol as solvents. 25 g of dried powdered plant sample were taken in a paper cone and placed into soxhlet apparatus. 100ml of methanol, a polar solvent was taken in the round bottom flask attached to the soxhlet apparatus. A condenser was attached to this setup. Then the whole setup was placed on a heating mantle. The temperature was set in the range of 25-30$^0$C. Methanol gets vaporized and rises up to the condenser where it condenses back into liquid. This liquid falls into the plant sample in the cone and extracts certain compounds and falls back into the round bottom flask. This process was continued till all the compounds get extracted from the plant. The Dark redish brown semisolid extracts obtained from

the above process was evaporated over night and stored in screw cap vials. The percentage yield was 15.2.

**Preliminary Phytochemical screening**

Preliminary phytochemical screening was performed to identify phytochemicals present in the EAME. There are several sophisticated techniques e.g. ultra violet and infrared spectroscopy, nuclear magnetic resonance and high-performance liquid chromatography for identification of various groups of phytochemical compounds in plant extract; however, in the present work, the phytochemicals were detected by colour tests and thin layer chromatography.

**Colour test** [14, 15, 16]

The Methanolic extracts of the whole plants were subjected to various preliminary phytochemical tests for detecting different classes of Phytocompounds.

**Thin Layer Chromatography (TLC)**

EAME were subjected to thin layer chromatographic analysis, to find the presence of number of chemical constituents to support the chemical test. Analytical TLC plates were prepared by pouring the silica gel G and GF slurry on the glass plates. Prepared chromo plates were dried in air for 30 minutes and then in an oven at $110^0$C for another 30 minutes. Precoated TLC plates also used for some specific phytoconstituents. A line was drawn on the TLC plate at a distance 2cm from the base. Marks were made on the line for sample application. The sample was spotted on the line with the help of capillary tube and it was allowed to dry. The plate was placed in the developing jar with the different mobile phase such as Toluene : Etheylacetate: Diethylamine (7:2:1), Toluene: Ethyl acetate: Glacial acetic acid (12.5 :7.5 :0.5), $CHCL_3$: Glacial acetic acid: Methanol: $H_2O$( 64:32:12:8), $CHCL_3$: Methanol (9:1), Ethyl acetate: Butanol: Formic acid :Water (3:2:0.5:2), Ethyl acetate: Formic acid: Glacial acetic acid:H2O (100:11:11:26), Ethyl acetate: Ethanol: Acetic acid : H2O (8:1:1:8), Ethyl acetate: Methanol: Water (100: 13.5: 10), and Toluene : CHCL3: Ethanol (4:4:1).When taken out of the jar, the solvent front was drawn. The plates were then kept in the iodine jar for a few seconds, shaken and taken out. They were examined under the UV/Vis lamp and the spots were identified. The spots were labelled and their distances from the baseline were measured. The $R_f$ values were calculated and the best solvent system for separating the constituents was identified.

**Determination of antimicrobial activities** [17, 18, 19]

Antimicrobial activity of the extracts was tested by using filter paper disc method. Zone of inhibition (mm) were formed, which are measured, averaged and mean value were tabulated.

**Microorganisms**

The bacterial and fungal strains used for the screening were *Corynebacterium* (NCIM - 2640), *Bacillus subtilis* (NCIM - 2063), *Rhodospirillum* (NCIM - 5128), *Salmonella paratyphi* (NCIM - 2501) , *Klebsiella pneumoniae* (NCIM - 2707) , *Micrococcus luteus* (NCIM - 2169), *Staphylococcus albus*(NCIM - 2178), *Vibrio cholerae* (MTCC - 1738), *Escherichia coli* (NCIM - 2065), *Pseudomonas aeruginosa* (NCIM - 2200), *Aspergillus niger* (MTCC – 1344), *Aspergillus fumigatus* (MTCC - 1811), *Microsporum gypseum* (MTCC - 2819), *Candida albicans* (NCIM - 3100), and were collected from Kovai Medical Center and Hospital, Coimbatore.

**Inoculum preparation**

The bacteria and fungi cultures were taken from slant culture and applied to agar containing nutrient broth and Sabouraud's Dextrose Broth respectively. The inoculums were standardized by adjusting the turbidity of the culture to McFarland standards. The turbidity of the culture adjusted by the addition of sterile broth (if excessive) or by further incubating to get required turbidity.

**Preparation of impregnated filter paper discs**

Whatmann filter paper No1 was used to prepare discs (6mm in diameter), which are placed in Petri dishes and are sterilized. Using micropipette, 20 µl of antibiotics was applied to each disc. EAME (50µl) were impregnated on the disc in petri dishes and then allowed to dry and the paper discs were placed on to the agar.

**Antibacterial assay**

Antibacterial activity was tested using a modification of the disc diffusion method. The standardized inoculums spread with a sterile cotton swap into petriplates containing 10 ml of Mueller Hinton Agar. Sterile filter paper discs (6mm in diameter) impregnated with the EAME extract were placed on the cultured plates and incubated at 25 or $37^0$C, depending on the bacteria. The solvent without extracts served as negative control. Standard antibiotic Ciprofloxacin (5µg) was employed as positive control. After 24 h of incubation, the antibacterial activity was assessed by measuring the inhibition zone. The diameters of the zones of inhibition by the EAME were then compared with the diameters of the zones of inhibition produced by the Ciprofloxacin discs. Each experiment was carried out in triplicate and the mean diameter of the inhibition zones was recorded.

**Antifungal assay**

The standardized fungal inoculums spread evenly on the Sabouraud's Dextrose Agar using sterile cotton swap. Each petri dish was divided into two parts, in 1 part, sample disc (200µg) and another part Fluconazole (20µg) were placed with the help of sterile forceps. Then the plates were incubated at room temperature for 24-48 hours. After incubation, the antifungal activity was evaluated by measuring zones of inhibition. The diameters of the zone of inhibition by the EAME were then compared with the Fluconazole. Each experiment was carried out in triplicate and the mean diameter (mm) of the inhibition zones was recorded.

**Statistical Analysis**

The experimental results were expressed as the mean±SD

**RESULTS**

**Preliminary phytochemical screening**

Table 1 shows the physical properties of EAME. It is dark redish brown in colour with characteristic taste. Table 2, represents the various phytochemicals present in the extract. EAME contains Alkaloids, Glycosides, Tannins, Carbohydrates, Proteins and amino acids, Saponins and Flavonoids. TLC analysis of extract under various solvent systems shows the presence of solutes and Rf value was calculated. In that solvent system Toluene: Etheylacetate: Diethylamine (7:2:1), $CHCL_3$: Glacial acetic acid: Methanol: $H_2O$ (64:32:12:8), $CHCL_3$: Methanol (9:1), Ethyl acetate: Methanol: Water (100: 13.5: 10) and Toluene: CHCL3: Ethanol (4:4:1) shows good separation (Figure 1) and given many numbers of compounds when compared to the rest of the solvent system. There was no spots produced in Toluene: Ethyl acetate: Glacial acetic acid (12.5:7.5:0.5), Ethyl acetate: Butanol: Formic acid: Water (3:2:0.5:2), Ethyl acetate: Formic acid: Glacial acetic acid: H2O (100:11:11:26), Ethyl acetate: Ethanol: Acetic acid: H2O (8:1:1:8) (Table3).

**Table 1: Physical characteristic of EAME**

| Physical characteristic | EAME |
|---|---|

| | |
|---|---|
| Colour | Dark redish brown |
| Odour | No odour |
| Taste | Characteristic |
| Consistency | Viscous |
| Percentage yield | 15.2% |

**Table 2: Preliminary Phytochemicals screening of EAME**

| Phytochemicals | Test/reagent | EAME |
|---|---|---|
| Alkaloids | i)Mayer's test | + |
| | ii) Dragendorff's test | + |
| | iii) Wagner's test | + |
| | iv) Hager's test | + |
| | v)Tannic acid test | + |
| Glycosides | i)Kedde's test | ++ |
| | ii) Keller Killiani test | ++ |
| Sterols | i)Libermann-Buchard test | - |
| | ii)Salkowski test | - |
| Tannins and Phenolic | i)Ferric chloride test | + |
| | ii)Gelatin test | + |
| | iii)Vanillin Hydrochlotide test | + |
| | iv)Alkaline reagent test | + |
| | v)Mitchell's test | + |
| Carbohydrates | i)Molisch's test | ++ |
| | ii) Benedict's test | ++ |
| | iii)Camnelisation | ++ |
| | iv)Selwinoff's test | ++ |
| | v)Fehling's test | ++ |
| Proteins and amino acids | i)Millons test | + |
| | ii)Ninhydrin test | + |
| Saponins | i)Froth test | + |
| | ii)Hemolysis test | + |
| Flavonoids | i)Shinoda test | ++ |
| | ii)Zinc hydrochloride reducing test | ++ |
| | iii)Alkaline reagent test | ++ |
| Vitamin-C | DNPH | - |
| Fats and fixed oils | i)Stain test | - |
| | ii)Saponification test | - |

Key: **++** = high concentration; **+**= low concentration; **-** = absents

**Fig.1a: The solvent system selected for the TLC of EAME was Toluene: Etheylacetate: Diethylamine (7:2:1). The Rf values of the 5 distinct spots were 2.03, 1.61, 1.36, 1.26, 1.12.**

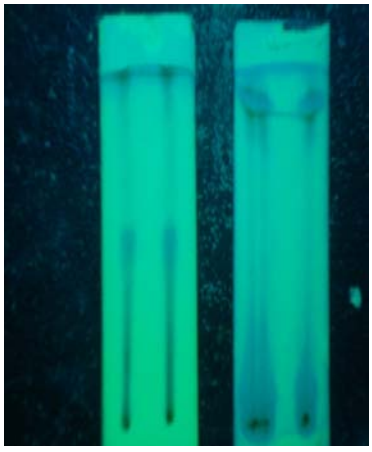

**Fig. 1b: The solvent system selected for the TLC of EAME was $CHCL_3$: Glacial acetic acid: Methanol: $H_2O$ (64:32:12:8). The Rf values of the 2 distinct spots were 0.49, 1.07**

**Fig. 1c: The solvent system selected for the TLC of EAME was $CHCL_3$: Methanol (9:1). The Rf values of the 4 distinct spots were 5.71, 4.62, 2.68, 2.31.**

**Fig. 1d: The solvent system selected for the TLC of EAME was Ethyl acetate: Methanol: Water (100: 13.5: 10). The Rf values of the 3 distinct spots were 1.91, 1.59, 1.45**

**Fig.1e: The solvent system selected for the TLC of EAME was Toluene: CHCL3: Ethanol (4:4:1). The Rf values of the 2 distinct spots were 3.26, 1.87**

**Fig. 1: TLC plat of the EAME**

**Table 3: Rf Value of EAME major constituent in various solvent systems**

| Solvent Systems | Number of spots visible by UV/open light | Rf Value |
|---|---|---|
| Toluene : Etheylacetate: Diethylamine (7:2:1) | 5 | 2.03 |
| | | 1.61 |
| | | 1.36 |
| | | 1.26 |
| | | 1.12 |
| Toluene: Ethyl acetate: Glacial acetic acid (12.5 :7.5 :0.5) | No spots | - |
| $CHCL_3$: Glacial acetic acid: Methanol: $H_2O$ | 2 | 0.49 |
| ( 64:32:12:8) | | 1.07 |
| $CHCL_3$: Methanol (9:1) | 4 | 5.71 |
| | | 4.62 |
| | | 2.68 |
| | | 2.31 |
| Ethyl acetate: Butanol: Formic acid :Water (3:2:0.5:2) - | No spots | - |
| Ethyl acetate: Formic acid: Glacial acetic acid:H2O (100:11:11:26) - | No spots | - |
| Ethyl acetate: Ethanol: Acetic acid : H2O (8:1:1:8) | No spots | - |
| Ethyl acetate: Methanol: Water (100: 13.5: 10) | 3 | 1.91 |
| | | 1.59 |
| | | 1.45 |
| Toluene : CHCL3: Ethanol (4:4:1) | 2 | 3.26 |
| | | 1.87 |

**Antimicrobial activity**

Antimicrobial activity on Gram positive, Gram negative and some fungal strains have been performed by Disc diffusion method and

the results are presented in Table 4, Table 5, Figure 2 and Figure 3. Ciprofloxacin and Fluconazole were used as standard antibiotics. In antibacterial study, EAME showed significant inhibitory activity against all tested pathogenic bacterial strains. *Micrococcus luteus*, *Vibrio cholera* are more sensitive as EAME showed 14mm of inhibitory zone. EAME displayed remarkable antifungal activity against *Aspergillus niger, Aspergillus fumigates, Microsporum gypseum, Candida albicans and Monascus ruber* which is close to the Fluconazole inhibitory activity.

**Table 4: Antibacterial activity of EAME by Disc Diffusion method**

| S. No. | Microorganisms | Zone of inhibition (mm) Mean±SD | |
|---|---|---|---|
| | | EAME (100µg/disc) | Ciprofloxacin (5µg/Disc) |
| 1. | *Corynebacterium* (NCIM - 2640) | 11±1.0 | 21±0.5 |
| 2. | *Bacillus subtilis* (NCIM - 2063) | 13±2.4 | 19±0.1 |
| 3. | *Rhodospirillum* (NCIM - 5128) | 12±1.2 | 21±0.2 |
| 4. | *Salmonella paratyphi* (NCIM - 2501) | 11±1.0 | 28±0.4 |
| 5. | *Klebsiella pneumoniae* (NCIM - 2707) | 11±1.5 | 24±0.2 |
| 6. | *Micrococcus luteus* (NCIM - 2169) | 14±1.4 | 25±0.6 |
| 7. | *Staphylococcus albus (*NCIM - 2178), | 11±2.3 | 19±0.1 |
| 8. | *Vibrio cholerae* (MTCC - 1738) | 14±1.2 | 25±0.2 |
| 9. | *Escherichia coli* (NCIM - 2065) | 12±0.5 | 23±0.1 |
| 10. | *Pseudomonas aeruginosa* (NCIM - 2200) | 12±0.9 | 16±0.3 |

Key: n=3

**Table 5: Antifungal activity of EAME by Disc Diffusion method**

| S. No | Microorganisms | Zone of inhibition (mm) Mean±SD | |
|---|---|---|---|
| | | EAME (200µg/disc) | Fluconazole (20µg/disc) |
| 1. | *Aspergillus niger* (MTCC – 1344) | 11±0.2 | 10±0.1 |
| 2. | *Aspergillus fumigatus* (MTCC - 1811) | 10±1.1 | 09±0.5 |
| 3. | *Microsporum gypseum* (MTCC - 2819) | 09±0.8 | 10±0.1 |
| 4. | *Candida albicans* (NCIM - 3100) | 12±0.2 | 15±0.2 |
| 5. | *Monascus ruber* (MTCC - 2326) | 10±1.8 | 11±0.4 |

Key: n=3

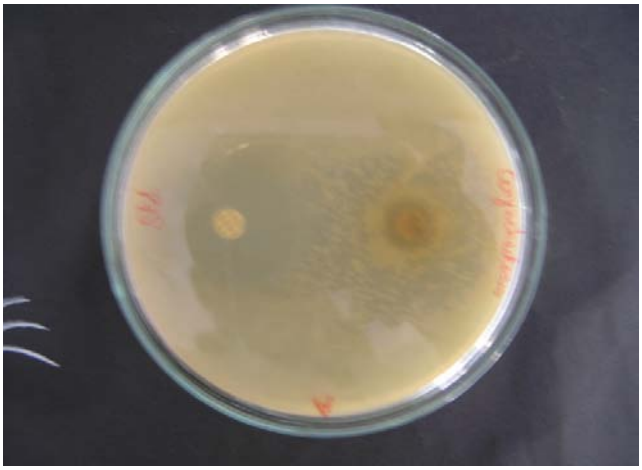

**Fig 2a:** ***Corynebacterium***

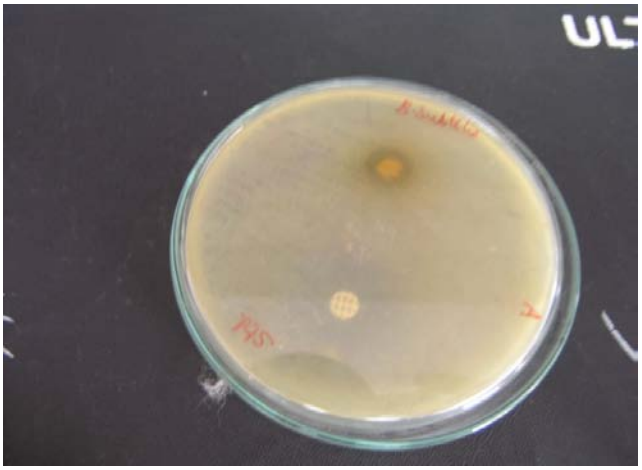

**Fig 2b:** ***Bacillus subtilis***

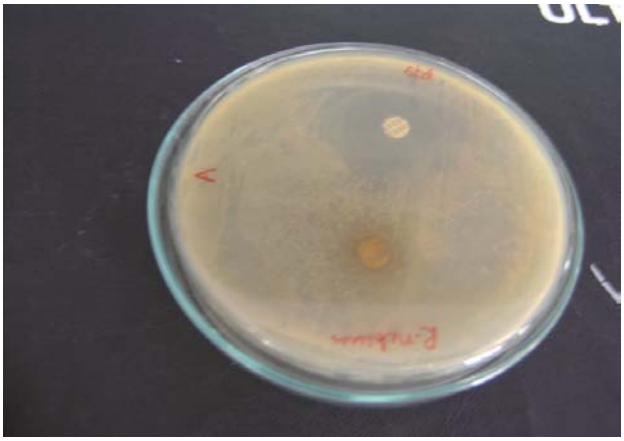

**Fig 2c:** ***Rhodospirillum***

**Fig 1d:** ***Salmonella paratyphi***

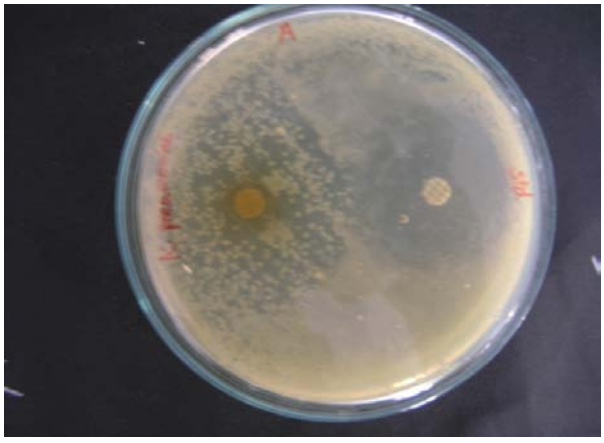

**Fig 2e:** ***Klebsiella pneumoniae***

**Fig 2F: Micrococcus luteus**

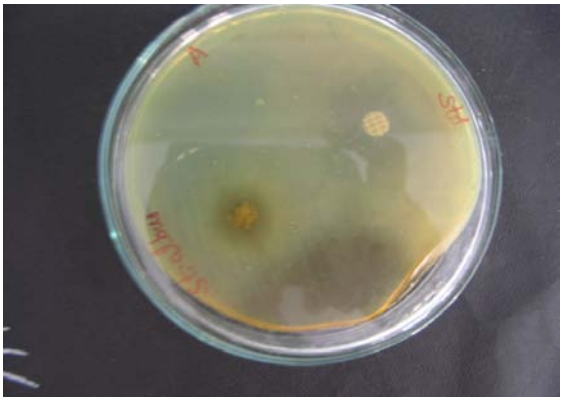

**Fig 2g:** ***Staphylococcus albus***

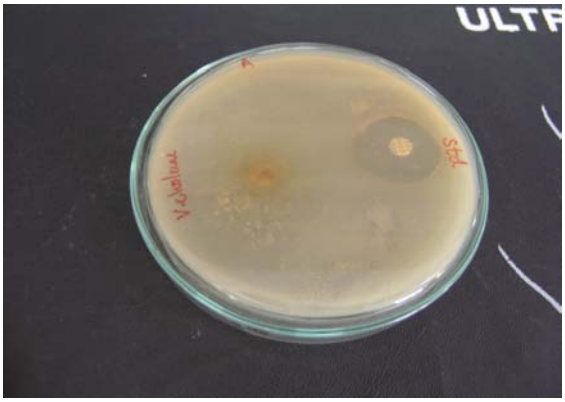

**Fig 2h:** ***Vibrio cholerae***

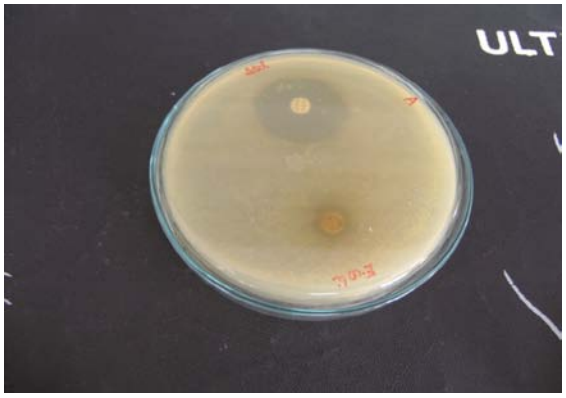

**Fig 2i:** ***Escherichia coli***

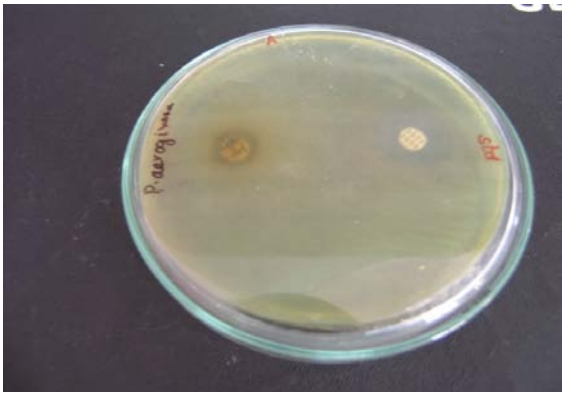

**Fig 2j:** ***Pseudomonas aeruginosa***

**Fig. 2: Antibacterial activity of EAME**

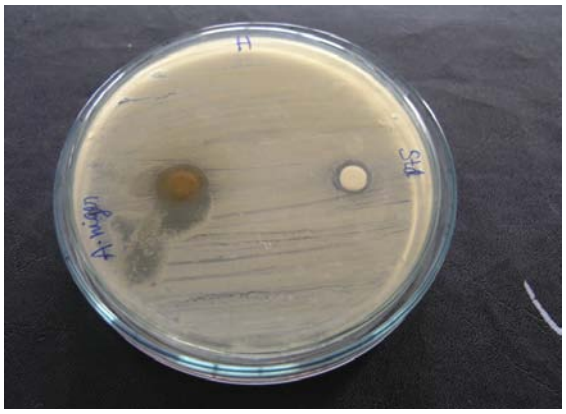

**Fig 2a:** ***Aspergillus niger***

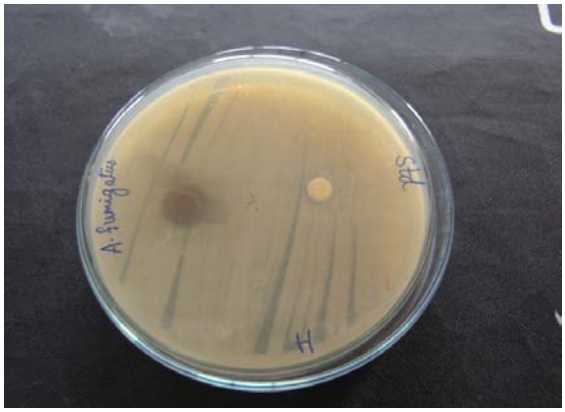

**Fig 3b:** ***Aspergillus fumigates***

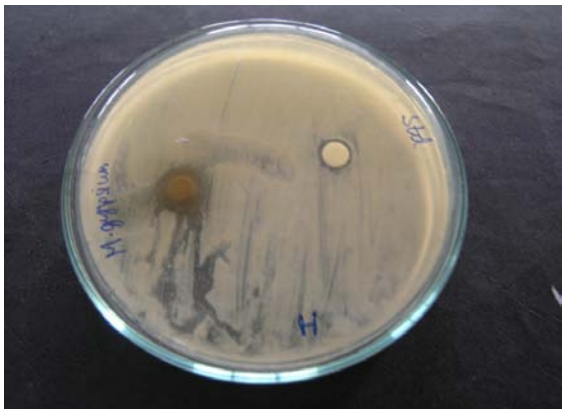

**Fig 3c:** ***Microsporum gypseum***

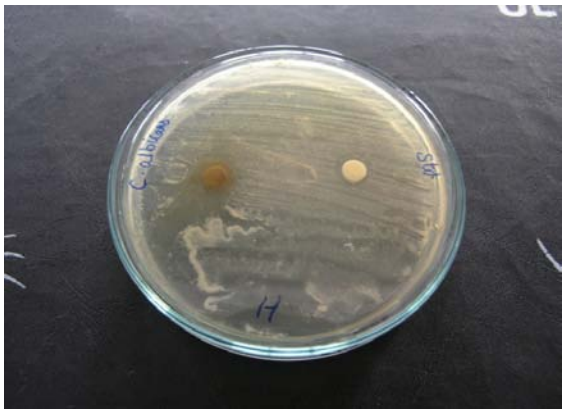

**Fig 3d:** ***Candida albicans***

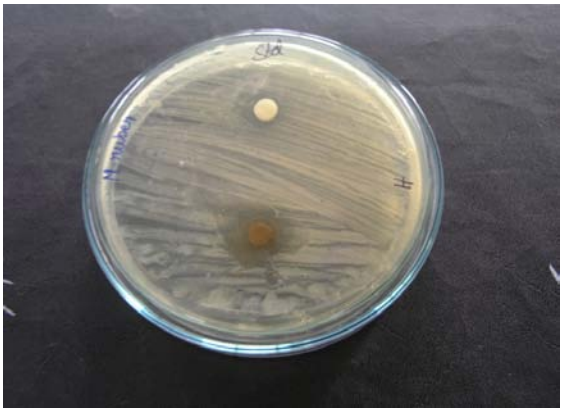

**Fig 3e:** ***Monascus ruber***

**Fig. 3: Antifungal activity of EAME**

## DISCUSSION

Antibiotics provide the main basis for the therapy of microbial infections. However, the high genetic variability of microorganisms enables them to rapidly evade the action of antibiotics by developing antibiotic resistance. Thus, there has been a continuing search for new and more potent antibiotics [20]. In the present investigation, the methanol extract of whole plant of *Enicostemma axillare* was evaluated for its antibacterial, antifungal potential against important pathogenic microorganisms.

EAME showed strong antimicrobial activity, which certainly indicates that methanolic extracts contain higher concentration of active antimicrobial agents. These may include Alkaloids, Glycosides, Tannins, Carbohydrates, Proteins and amino acids, Saponins and Flavonoids. Previous studies had also demonstrated that the whole plant of *Enicostemma axillare* is very rich in all the above phytochemicals which are all found in other solvent extracts such as aqueous and chloroform [21]. Among the different solvent extracts of *Enicostemma axillare,* 85% methanolic extract contain higher concentration of the phytoconstituents like flavonoids, tannins etc. and this is taken for further pharmacological evaluation [22]. The phytochemical analysis and TLC report revealed that the active principle responsible for the antimicrobial activity is a phytochemicals. The TLC analysis under the solvent system Toluene: Etheylacetate: Diethylamine (7:2:1), $CHCL_3$: Glacial acetic acid: Methanol: $H_2O$ (64:32:12:8), $CHCL_3$: Methanol (9:1), Ethyl acetate: Methanol: Water (100: 13.5: 10) and Toluene: CHCL3: Ethanol (4:4:1) shows 5, 2, 4, 3, 2 distinct spots. These observations will stimulate further research in the field of phytochemistry and also in the clinical application of phytochemical constituents of *Enicostemma axillare.*

## CONCLUSION

In our research we concluded that the obtained results are considered significant for further study to isolate the compounds responsible for the antimicrobial activity and suggesting the possibility of finding potent antimicrobial agents from *Enicostemma axillare* extracts.

## ACKNOWLEDGEMENT

The authors thank the principal, KMCH College of pharmacy, Coimbatore, India, for providing the necessary facilities to carry out the work.

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