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Phytochemical Profiling, Antibacterial Potential and GC-MS Analysis of Methanol Leaf Extract of *Enicostemma axillare*

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A B S T R A C T

Aim of the study is to identify and characterize the bioactive compounds from methanol leaf extract of *Enicostemma axillare* responsible for the bactericidal activity. The *in vitro* antibacterial activity was determined by disc diffusion method against *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterococcus faecalis* and *Bacillus subtilis*. Gas Chromatography-Mass Spectrometry (GC-MS) studies were carried out to identify the presence of bioactive compounds. Antibacterial studies reveal a dose-dependent zone of inhibition. A maximum zone of inhibition was observed with reference to *Klebsiella pneumoniae* (12mm) followed by *Escherichia coli* (10mm). Observation of GC-MS results shows the presence of antibacterial compounds glycine, N- methyl ester, octadecatrienoic acid, 1-heptatriacetanol, and tetramethyl-2-hexadecen-1-ol were identified. It is concluded that the methanolic leaf extract from *Enicostemma axillare* possesses the potential compounds responsible for the antibacterial activity.

Introduction

Enicostemma axillare is a perennial herb found throughout India and common in coastal areas. It is commonly known as Vellarugu in Tamil, Chotachirayata in Hindi, Mamejavo in Gujarati and Nagajivha in Bengal. The plant possesses anti-inflammatory (Sadique *et al.*, 1987), hypoglycemic (Murali *et al.*, 2002) and anti-cancer (Jyoti *et al.*, 2003), antimalarial, antipyretic (Varier, 2001) properties. Further crude extract from these plants was used in folk medicine to treat diabetes mellitus, rheumatism, abdominal ulcers, hernia, swelling, itching and insect poisoning (Kirtikar and Basu, 1999). It is also used traditionally for blood purifier and to treat snake bites (Garg, 2000-2001). Infectious diseases are the leading cause of death worldwide (Gannon, 2000). The re-emergence of deadly infectious diseases, and the increasing prevalence of antimicrobial resistant strains presents a formidable threat to public health and welfare.

Recently, significant evidence has emerged which indicates that markedly different microbial pathogens use common strategies to cause infection and disease (Wilson *et al.*, 2002). The indiscriminate use of various antibiotics increases their resistance to microorganisms, giving rise to multidrug resistant strains, which has become a global

concern (Shariff, 2001). This paves the way for the search and identification of a new antibacterial compound. Isolation of antibacterial compounds from the medicinal plants seems to be a potential alternative to the control of multidrug resistant strains. The medicinal plants possess a wide variety of chemical substances which could be explored for pharmaceutical applications (Periyasami and Kaliyaperumal, 2016; Malakar and Choudhury, 2015). This study showed the presence of novel compounds with antibacterial activity in the methanol leaf extract from *Enicostemma axillare*.

Materials and Methods

Collection and identification of *Enicostemma axillare*

The whole plants of *Enicostemma axillare* (Fig. 1) were collected from Pudukkottai district of Tamil Nadu, India. The plants were authenticated by Captain Srinivasa Murthi, Multi Drug Research Center for Siddha and Ayurveda, Chennai, 600029 (Voucher specimen No: 00299).

The leaves were dried under shade, then ground into a uniform powder using a mechanical blender and stored in an airtight bottle at room temperature.

Chemicals and reagents

Methanol, Dimethyl sulfoxide (DMSO), Muller Hinton agar and other chemicals used for the phytochemical studies and disk diffusion methods were brought from Himedia Laboratory, Mumbai, India.

Extract preparation

Fifty grams of the shade dried and powdered material (leaves) were packed and extracted from methanol using a Soxhlet apparatus for 12 h at 45°C. The sample was concentrated using rotary evaporator and stored at 4 ^{0}C for further studies.

Preliminary phytochemical analysis

The crude plant extract was subjected to qualitative phytochemical tests to identify various classes of bioactive chemical constituents present in the leaves using standard procedures (Harborne, 1973; Edeoga and Gomina, 2000). Visible color change or precipitate formation was taken into consideration in the presence (+) or absence (–) of particular active constituents.

Collection and maintenance of bacterial strains

The pure cultures such as *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterococcus faecalis* and *Bacillus subtilis* were collected from King Institute of Preventive medicine, Guindy, Chennai. The organisms were sub-cultured and maintained in the laboratory

In vitro antibacterial activity by disc diffusion method

Antibacterial activities of the leaf extract were determined using a disc diffusion method (Kirby-Bauer, 1966). Freshly prepared Mueller-Hinton Agar plates were seeded with the bacterial inoculum (10^{-5}) to obtain lawn culture. Sterile discs (~6mm diameter) impregnated with concentrations of methanol leaf extracts (10, 15, 20, 25 mg/ml) were placed on the inoculated Mueller Hinton agar.

Amikacin (30 µg/disc) was used as standard antibiotics. The plates were then incubated at 37ºC for 18 h, the zone of inhibition determined by measuring the diameter of the inhibition zone (mm) using a transparent scale.

GC-MS spectral analysis of *Enicostemma axillare* methanolic leaf extract

The components of test sample were evaporated in the injection port of the GC equipment which consists of GC-MS (Jeol GC Mate). The GC column was equipped with a capillary column (30mm x 0.25mm), composed of 100% Dimethyl poly siloxane). For Mass Spectra detection, an electron ionization system with a ionizing energy of 70 eV was used. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 1.4 ml/min and an injection volume of 2µl was employed (split ratio of 10:1) Injector temperature 250°C, Ion source temperature 280°C. The oven temperature was programmed from 110°C (isothermal for 2 min.), with an increase of 10°C/min to 200°C, then 5 °C/min to 280°C, ending with a 9min isothermal at 280°C. Mass spectrum was taken at 70 eV a scan interval. The chromatogram and mass spectra were evaluated using the software embedded in the GC-MS system.

Identification of phyto compounds

Interpretation of the mass spectrum was conducted using the database of National Institute Standard and Technology (NIST) (NIST, 2010). The compound bioactivity prediction is based on Dr. Duke's Phytochemical and Ethnobotanical Databases (Duke, 2002). The relative percentage amount of each photo-component was calculated by comparing its average peak area to the total area.

Results and Discussion

Medicinal plants are the richest source of secondary metabolites with various biological activities. In the present study phytochemicals from the methanolic leaf extract of *Enicostemma axillare* revealed the presence of alkaloid, coumarin, flavonoid, tannin, phenols, cardiac glycoside, anthocyanin, carbohydrates, quinine and terpenoid. Among these, the strong presence of phytocompounds was alkaloid and flavonoid, quinones, coumarins were moderate presence and saponin, steroids, leucoanthocyanin were absent in this plant methanol leaf extract (Table 1). The presence of different phytoconstituents in the whole plant extract from *Enicostemma axillare* has been reported earlier by Leelaprakash and Mohan Das (2012). Leaves of medicinal palnts possess antibacterial activity can be supplemented against moderately sensitive antibiotics (Kamath *et al.,* 2017).

Phenolic compounds present in the *Enicostemma axillare* may contribute to its antibacterial activity and thus the plant is used in herbal treatment. A phenol has been found to be used in the preparation of some antimicrobial and antioxidant compounds (Brewer, 2011). There was a close correlation between the antibacterial activity and the amount of polyphenols, flavonoids and flavonols found in the plant. Total phenolic and total flavonoid contents were directly related to the antioxidant and antibacterial potential of the plant extracts (Bagdassarian *et al.,* 2013). Alkaloids are considered as the largest group of secondary plant metabolites having nitrogenous bases which also exhibit a range of pharmaceutical activity such as antimicrobial property (Hadi and Bremner, 2001).

The analysis of the results in table 2 shows that the crude extracts from the tested plant show maximum activity against *E. coli* and *Klebsiella pneumoniae*. The zone of inhibition observed with reference to drug-resistant *E. coli* is higher than the standard antibiotic amikacin with the concentration of 30µg/ml. The antibacterial activity was more pronounced on gram-negative bacteria

(*Klebsiella pneumoniae* and *E. coli*) than the gram positive bacteria (*Bacillus subtilis*).

The results of the present studies are in agreement with the above findings as the methanolic leaf extract shows a strong presence of alkaloids, flavonoids along with terpenoids, quinine, coumarins, tannin, cardiac glycosides, anthocyanin. These phytocompounds mainly targeting the cell wall of bacteria (Cowan, 1999) inactivates microbial enzymes, cell wall protein (Jones *et al.*, 1994) and also cause loss of cellular function (Kazmi *et al.*, 1994). Hence, the methanolic extract shows maximum antibacterial activity against *E. coli* (8-10mm) at 10, 15, 20 and 25 mg/ml concentration of plant extracts.

Table.1 Phytoconstituents of methanol leaf extract of *Enicostemma axillare*

Phytoconstituents	**Methanol Extract**
Alkaloids	+++
Flavonoids	++
Tannin	+
Cardiac glycosides	+
Phenol	+
Steroids	_
Terpenoids	+
Anthocyanin	+
Leuco anthocyanin	_
Quinone	++
Saponin	_
Protein	+
Carbohydrate	+
Phenol	+

Table.2 Major Components of the methanol leaf extract of *Enicostemma axillare*

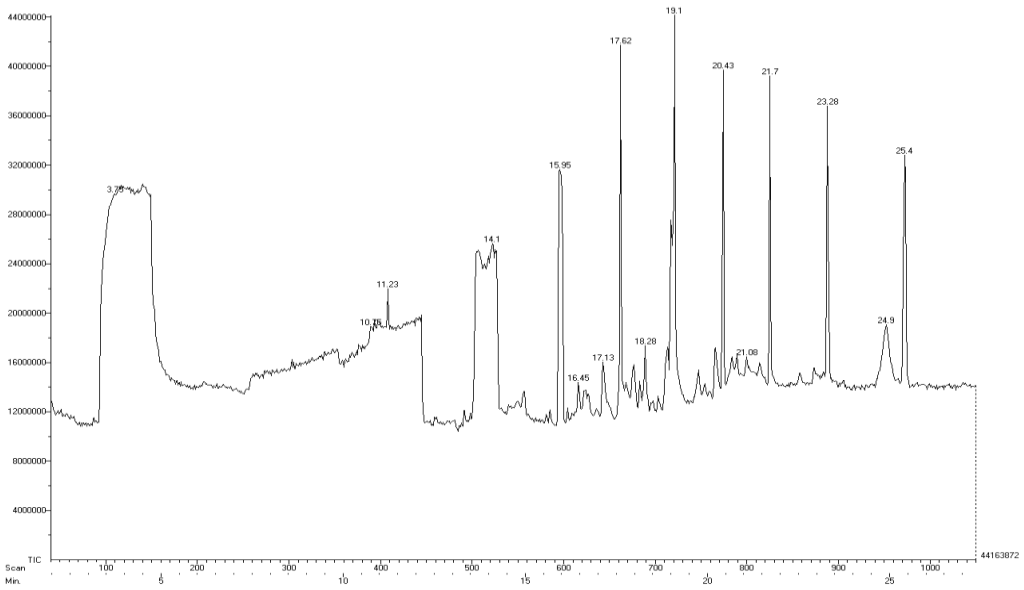
S.No	**Name of the compounds**	**RT**	**Area %**	**M.W**	**M.F**
1	Propanoic acid -2(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl)	19.12	6.4	208.21	$C_{11}H_{12}O_4$
2	Glycine,N-[(3a,5a,7a12a-Oxo-3,7,12-Tris [(trimethylsily) cholan-24-y] methyl ester	20.45	7.72	185.10	$C_5H_6F_3NO_3$
3	Acetamide,N-methyl-N-[4[4-fluoro-1-heahydropyridyl]2-butynyl]-	3.95	4.32	179.22	$C_{13}H_9N$
4	Benzolic acid, 3-methyl-2-trimethylsilyloxy-trimethylsilyl ester	11.25	6.7	135.18	C_7H_5NS
5	9,12,15-Octadecatrienoic acid, 2-trimethylsilyl0oxy] -1-[(trimethylsilyl) Oxy] ethyl ester	16.05	11.3	312.00	$C_{20}H_{40}O_2$
6	Cyclopropanebutanoic acid	17.17	8.71	268.00	$C_{17}H_{32}O_2$
7	1-Heptatriacotanol	20.17	7.5	536.00	$C_{37}H_{76}O$
8	3,7,11,15-Tetramethyl-2- hexadecen-1-ol (Phytol)	10.75	9.85	296.00	$C_{20}H_{40}O$

Table.3 Antibacterial efficacy of *Enicostemma axillare* methanol leaf extract against different Microorganisms

Microorganisms	**Diameters of zones of Inhibition (in mm)**				**Amikacin**
	10µg (A)	15 µg (B)	20 µg (C)	25 µg (D)	30µg/ml (E)
Escherichia coli	8	9	9	10	8
Enterococcus faecalis	7	8	9	9	14
Klebsiella pneumonia	7	8	9	12	15
Proteus mirabilis	6	7	8	9	15
Bacillus subtilis	7	7	7	8	15

Fig.1 *Enicostemma axillare*

Fig.2 GC-MS spectral analysis of *Enicostemma axillare* methanolic leaf extract



The result pertaining to GC-MS analysis leads to the identification of the number of compounds from the GC fractions of the methanolic leaf extract from *Enicostemma axillare* and these compounds were identified with mass spectrometry attached to GC. The results presented in table 3 and figure 2 reveals the presence of the major components Propanoic acid -2(3acetoxy4, 4, 14 trimethylandrost8en17yl,),Glycine,N[(3a,5a,7 a12 aoxo3,7,12tris[(trimethylsily)cholan-24-y] methylester, Acetamide,N-methy lN[4 [4 fluoro1heahydropyridyl]2-butynyl]-, Benzolicacid, 3-methyl-2-trimethylsilyloxy-trimethylsilylester, 9,12,15-Octa decatrienoic acid, 2-trimethylsilyloxy]-1-[(trimethylsilyl) oxy] ethyl ester, Cyclopropane butanoic acid,1-Heptatriacotanol and 3,7,11,15-Tetramethyl-2- hexadecen-1-ol of which many of the identified compounds seems to possess many biological activities.

GC-MS analysis of methanolic leaf extracts shows the presence of 8 compounds, of which Glycine, N, (3a,5a,7a,12a)24oxo3,7,12tris ((trimethylsily)) cholan-24yl) methyl ester, 9,12,15- Octadecatrienoicacid, 2 trimethylsilyl0oxy] -1-[(trimethylsilyl) Oxy] ethyl ester, 1-Heptatriacotanol and 3,7,11,15-tetramethylhexadec-2-en-1-ol were responsible for the antibacterial activity. The previous studies are in support of the above findings (Adeneye and Agbaje, 2008; Senthilkumar and Kamaraj, 2010; Kumaradevan *et al.,* 2015; Anand and Gokulakrishnan, 2012). In addition, compounds with anti-diabetic (Propanoic acid -2 (3-acetoxy-4, 4, 14-trimethylandrost-8-en-17-yl) and anti-cancer activity (Benzolic acid, 3-methyl-2-trimethylsilyloxy-trimethylsilyl ester) were also observed.

It is evident from the results that the presence of a major antimicrobial compound phytol was responsible for the antibacterial activity. The phytol is the compound which inactivates the protein and enzymes present in the microorganisms. In addition, the compound has no remarkable toxicity and possess high stability (Alqasoumi *et al.,* 2012; Khasawneh *et al.,* 2011; Dagla *et al.,* 2012). Thus, it is evident from the present findings that phytol along with other phytoconstituents plays a major role in the inhibition of the growth of drug resistant *E. coli.*

In conclusion biologically active molecules phytol from the leaves of *Enicostemma axillare,* acts against the multi-drug resistant bacterial strains such as *E. coli* and *Klebsiella pneumoniae.* Isolation and purification of the compounds will paves the way for the identification of novel compound with potential microbial activity.

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