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Research Article

THE ANTIBACTERIAL ACTIVITY AND PHYTOCHEMICALS OF THE LEAVES OF *STYLOSANTHES FRUTICOSA*

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Abstract

The antibacterial activity and phytochemicals of the leaves of *Stylosanthes fruticosa* were evaluated against three Gram-positive bacteria viz. *Bacillus cereus, Staphylococcus faecalis, Staphylococcus aureus* and five Gram-negative bacteria viz. *Klebsiella pneumonia Pseudomonas aeruginosa, Escherichia coli, Salmonella typhimurium, and Proteus vulgaris*. Both polar and nonpolar extracts viz. acetone, chloroform, ethanol, and aqueous extracts were prepared and studied for antibacterial activity using disc diffusion method. The majority of the significant antibacterial activity was observed in the ethanol extracts. The other solvent extracts showed satisfactory results. In general, gram-negative bacteria are more resistant to antibiotics than gram-positive bacteria. The resistance is due to the differences in their cell wall composition. In gram-negative bacteria the outer membrane acts as a great barrier to many environmental substances including antibiotics. Presence of thick murine layer in the cell wall prevents the entry of the inhibitors. But our results revealed a controversy report that gram-positive bacteria are more susceptible to the crude extracts than gram-negative bacteria. The results which are obtained with acetone, ethanol and chloroform extract of leaf exhibited significant antibacterial activity, a property that supports traditional use of the plant inthe treatment of some diseases as broad spectrum antibacterial agents.

Keywords: Antibacterial activity, *Stylosanthes fruticosa*, phytochemicals, medicinal plant activity.

1. Introduction

Nature has been a source of medicinal agents since times immemorial. The importance of herbs in the management of human ailments cannot be overemphasized. It is clear that the plant kingdom harbors an inexhaustible source of active ingredients invaluable in the management of many intractable diseases. Furthermore, the active components of herbal remedies have the advantage of being combined with many other substances that appear to be inactive. However, these complementary components give the plant as a whole a safety and efficiency much superior to that of its isolated and pure active components[1].

Antibiotic resistance has become a global concern[2]. There has been an increasing incidence of multiple resistances in human pathogenic microorganisms in recent years, largely due to indiscriminate use of commercial antimicrobial drugs commonly employed in thetreatment of infectious diseases. This has forced scientist to search for new substances from various sources like the medicinal plants. Search for new antibacterial agents should be continued by screening many plant families. Recent work revealed the potential of several herbs as sources of drugs [3]. The screening of plant extracts and plant products for antimicrobial activity has shown that higher plants represent a potential source of novel antibiotic prototypes[4].

Numerous studies have identified compounds within herbal plants that are effective antibiotics[5]. Traditional healing systems around the world that utilize herbal remedies are animportant source for

the discovery of new antibiotics[6]; some traditional remedieshave already produced compounds that are effective against antibiotic-resistant strains of bacteria [7]. The results of this indicate the need for further research into traditional health systems[8]. It also facilitates pharmacological studies leading to synthesis of a more potent drug with reduced toxicity [9].The need of the hour is to screen a number of medicinal plants for promising biological activity.

In the present work twelve different medicinal plants each belonging to different families was evaluated for their antibacterial properties.

***1.2 Morphological Description*:** *Stylosanthes fruticosa* (Family:*Fabacea*) commonly known as *Wild Lucerne*. Copiously branching woody herb, ascending shrub or under shrub, reaching 50 cm in height. Branches densely clothed with short yellowish pubescence. Leaflets oblanceolate narrowed to both ends, long mucronate at the apex, 9 to 18 mm long, prominently nerved, and both surfaces nearly glabrous, Flowers in dense oblong terminal heads. Pod with two articulations, about 6 mm long, both faces and remains of style densely. Beaks 1.5 to 3 mm long and the plant have evenly pubescent stems. It is a perennial which may behave as an annual in the subtropics.

Distributions: Native to the South Sahelian and North Sudanian ecozones from Senegal to Rep. of Sudan (Kordofan) and to East and South Africa. Found in the Sudan, Nigeria, Kenya, Uganda, Tanzania, Zambia, Mozambique, Zimbabwe, South Africa and south India.[10,11,12]

Stylosanthes fruticosa is much sought after by all kinds of livestock and is grazed heavily by stock in the Sudan and Tanzania[13]. This stylo is suitable for the rehabilitation of fallow land[14] The present investigation deals with extraction of essential biological active compounds. This study will help to design the new drugs for many incurable drugs.

2. Materials and Methods

2.1 Collection of plant Material: The leaves of *Stylosanthes fruticosa* were collected from the Bharadhidasan university herbarium, Trichirappalli, Tamil Nadu, India. They were identified and authenticated by the Bharadhidasan university herbarium, Trichirappalli, Tamil Nadu, India.

2.2 Preparation of Plant Extract: The healthy disease free plant samples of *Stylosanthes fruticosa* (Retz.) Alston were collected in and around of Bharathidasan University, Tiruchirappalli. The samples were washed in running tap water for 10 minutes to remove soil particles and adhered debris. Then the samples were washed thoroughly with sterile distilled water. The leaves were then cut and dried in shade at room temperature for a period of one week and ground into powder. The leaf powder was dissolved in various solvents by crude extraction method. The complete extraction was carried out for one week with the following solvent in the increasing order of polarity. The solvents taken for this study are ethanol, acetone, chloroform, water.

2.3 Phytochemical analysis: The three different solvents prepared were taken and preliminary phytochemical analysis is done by using the standard procedure of Brindha *et al*[15] to identify the presence of some secondary metabolites.

2.4 Determination of antibacterial activity:

2.4.1 Disc diffusion method: Preliminary screening of the extracts was carried out by disc diffusion method[3]. Briefly, freshly grown liquid culture of the test pathogens were seeded over the nutrient agar plates with a sterile swab. Sterile filter paper discs of eight mm diameter were soaked with 40 µl of 50 mg/ml the extracts and air dried to evaporate the solvent and the discs were applied over the seeded MHA plates at equidistance. The plates were incubated at 37°C for 18 - 24 h. After the incubation period, the plates were observed for a clearance zone around the discs which indicates a positive antibacterial activity of the respective extracts. The clearance zones formed around each disc were measured. Each experiment was carried out in triplicates. The mean ± SD of the inhibition zone was taken for evaluating the antibacterial activity of the extracts.

3. Results and Discussion

3.1. Antibacterial Activity (Disc Diffusion Method): The antibacterial activity of the ethanol, acetone and chloroform extracts *Stylosanthes fruticosa* evaluated by disc diffusion method against eight different pathogenic bacteria. The results were observed in terms of inhibition zone around each

disc caused by diffusion of antibacterial substances from the plant extract impregnated disc into the surrounding medium. Among the three extracts tested, the ethanolic extract exhibited high degree of inhibition followed by chloroform and acetone. The inhibition zones formed by standard antibiotic disc (chloramphenicol 30 mcg/disc) and those filter paper discs injected with ethanol, acetone and chloroform (negative controls) are also listed. The diameter of inhibition zones for each of the samples were compared with standard antibiotics (Table-I).

3.1a. Ethanolic leaf Extract: The ethanolic leaf extract showed significant antibacterial activity against the test bacteria. The zones of inhibition were higher in *Klebsiella pneumoniae* (1.53 mm). Moderate activity was associated with *Pseudomonas aeruginosa* (0.93 mm) and *Proteus vulgaris* (0.85 mm). There was no inhibition associated with *Escherichia coli* and *Salmonella typhimurium.* Ethanol extract of *Indfigofera subulata* exhibiting highest potency in *Staphylococcus aureus*[16]

Table:I Antibacterial activity of *Stylosathes fruticosa*against various bacteria (Disc diffusion method)

Organism	Diameter of inhibition zone in mm (Mean*)				
	Acetone extract (30 µg/disc)	**Chloroform extract (30 µg/disc)**	**Ethanol extract (30 µg/disc)**	**Aqueous extract (30 µg/disc)**	**Standard# antibiotic (Chloramphenicol) (30 mcg/disc**
Gram-positive bacteria:					
Bacillus cereus	–	0.36 ± 0.20	–	–	1.0 ± 0.01
Staphylococcus faecalis	–	–	–	–	1.2 ± 0.21
Staphylococcus auerus	–	–	–	–	1.1 ± 0.02
Bacillus cereus	–	0.36 ± 0.20	–	–	1.0 ± 0.01
Gram-negative bacteria:					
Proteus vulgaris	–	–	0.85 ± 0.55	–	1.4 ± 0.15
Salmonella typhimurium	–	0.2 ± 0.24	–	–	1.2 ± 0.05
Escherichia coli	1.3 ± 0.20	0.4 ± 0.1	–	–	1.3 ± 0.15
Klebsiella pneumoniae	0.63± 0.25	–	1.53 ± 0.15	–	1.0 ± 0.10
Pseudomonas aeruginosa	0.9 ± 0.01	–	0.93 ± 0.11	–	1.3 ± 0.10

* : Mean of triplicate
± : Standard Deviation
: Chloramphenicol
– : Absence of measurable inhibitory action

3.1b. Acetone leaf extract: The acetone extract showed higher zones of inhibition in *Escherichia coli* (1.3 mm) and *Pseudomonas aeruginosa* (0.9 mm). Low degree of inhibition was observed in *Klebsiella pneumoniae* (0.63 mm). There was no inhibition associated with *Salmonella typhimurium, Proteus vulgaris* and*Bacillus cereus.* The acetone extract of *Gymnema Silvestre* showed higher zone of inhibition in the pathogenic bacteria [17].

3.1c. Chloroform leaf extract: The chloroform extract showed low degree of inhibition was observed in *Escherichia coli* (0.4 cm), *Bacillus cereus* (0.3 cm) and *Salmonella typhimurium*(0.2 cm). There was no inhibition associated with *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Proteus vulgaris*. The chloroform extract of this plant *G. kollimalayanum* expressed significant antibacterial activity (Table-I).

3.1d. Aqueous leaf extract: There are no activities found. The majority of the significant antibacterial activity was observed in the ethanol extracts. The other solvent extracts showed

satisfactory results. In general, gram-negative bacteria were more resistant to antibiotics than gram-positive bacteria [18]. The resistance is due to the differences in their cell wall composition. In gram-negative bacteria the outer membrane acts as a great barrier to many environmental substances including antibiotics [19]. Presence of thick murine layer in the cell wall prevents the entry of the entry of the inhibitors [20]. But our results revealed a controversy report that gram-positive bacteria were more susceptible to the crude extracts than gram-negative bacteria.

3.2. Biochemical Analysis

3.2a. Phytochemical Analysis: The preliminary phytochemical analysis which was performed by Brindha *et al*[15]. Method shows the presence of the following secondary metabolites in three different solvents.

Table:II

S. No.	Phytocompounds	Acetone extract	Chloroform extract	Ethanol extract
1.	Steroids	–	–	+
2.	Triterpenoids	+	+	–
3.	Sugar	–	+	+
4.	Alkaloids	+	–	+
5.	Phenols	+	–	+
6.	Catechin	–	+	–
7.	Flavanoids	–	–	+
8.	Saponins	+	–	–
9.	Tannins	+	–	–
10.	Amino acids	+	–	–
11.	Reducing Sugar	–	+	–
12.	Anthroquinone	–	–	–

Note: '+' Represents as Present; '–' Represents as absent

This method gives an initial idea about the presence or absence of secondary metabolites. From this result, further studies using advanced the techniques like UV-Vis, FTIR and GC-MS can be performed to identify the prime compounds and their therapeutical activities.

3.2b. UV-Vis Spectroscopy: The plant sample extracts of two different solvents (acetone, ethanol) was taken for this study and the data obtained is as follows:

Table:III

S. No	Acetone		Ethanol	
	Nanometer	Absorption value	Nanometer	Absorption value
1.	405	1.820	264	1.920
2.	501	0.621	272	1.976
3.	605	0.574	276	1.9860
4.	661	1.009	405	0.591

The qualitative UV-Vis spectrum profile of *Stylosanthes fruticosa* (Retz.) Alston, acetone extract was selected from 390 to 1100 nm due to sharpness of peaks and proper baseline. The profile showed the peaks at 390 to 1100 nm and the profile showed the peaks 405, 534, 605 and 661 nm with absorption 1.8206, 0.6211, 0.5741, 1.0093 respectively. The UV-Vis spectrum of *S. fruticosa*, the ethanol extract was taken at the wavelength of 240 to 1100 nm. The profile showed the peaks at 605 and 661 nm with the absorption of 0.5741 and 1.0093 respectively (Table 3; Fig. 1-2).

Fig:I UV-Visible spectrum of Acetone extract of *Stylosanthes fruticosa*

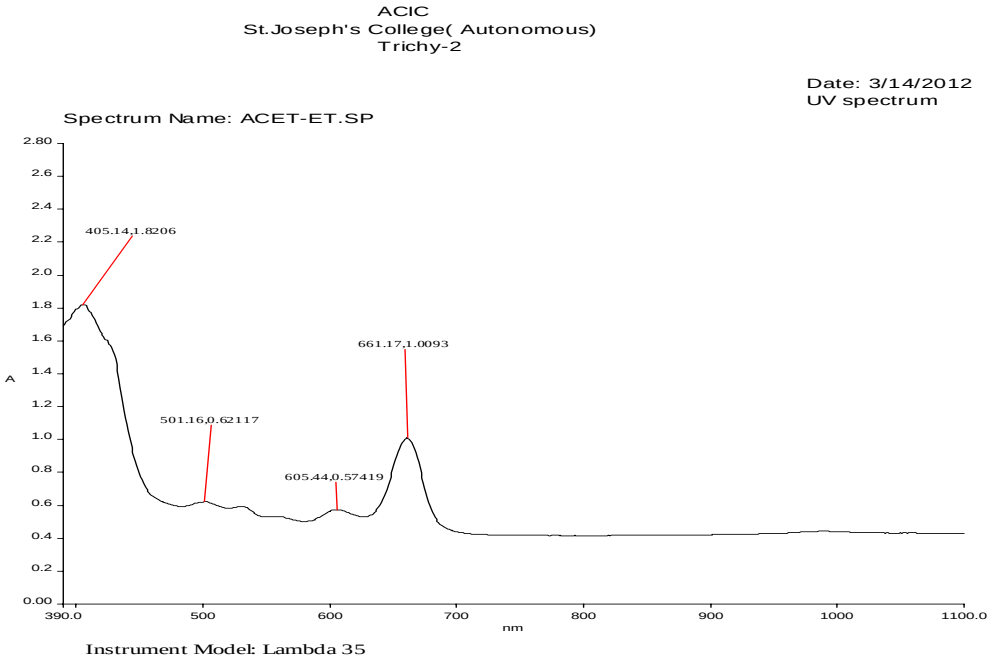
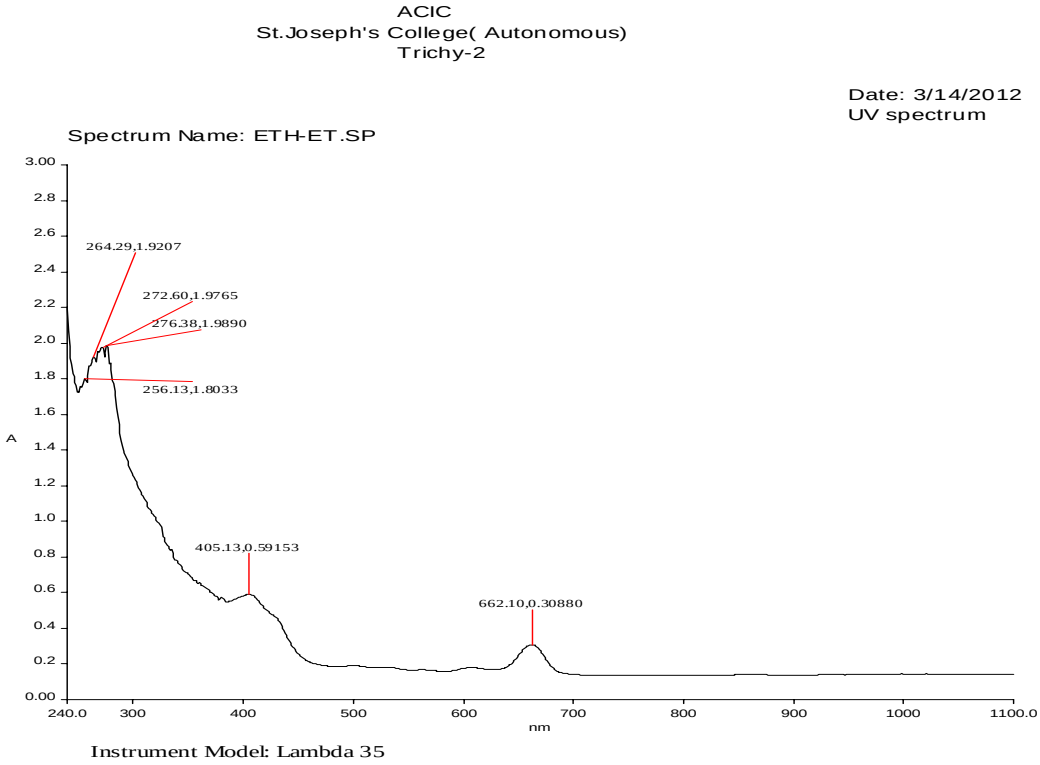


Fig:IIUV-Visible spectrum of Ethanol extract of *Stylosanthes fruticosa*



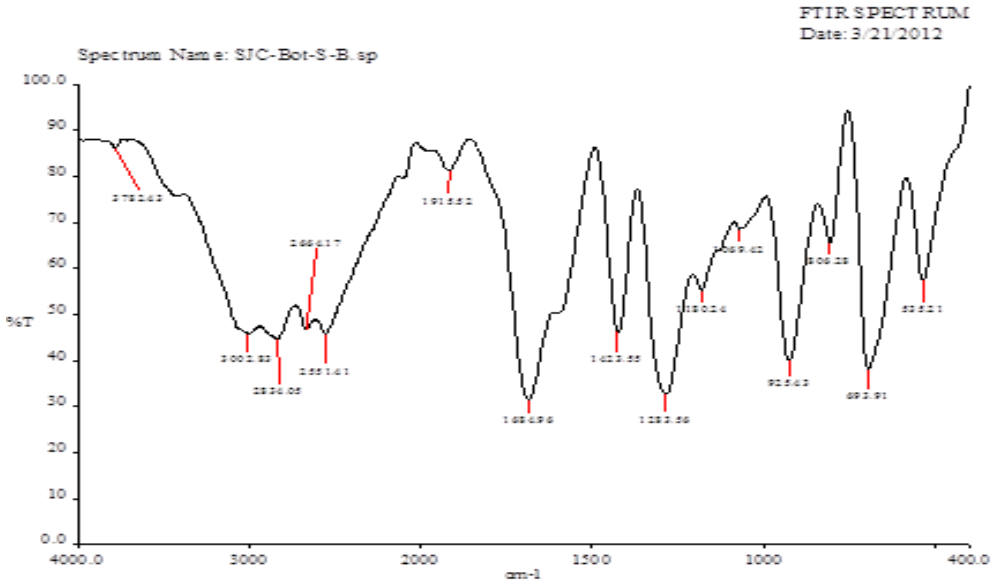
3. 2c. FTIR Spectroscopy: Performing the next advanced phytochemical analysis technique of FTIR the presence of various functional groups of different compounds was found. The solvent had its respective functional group like amines, cycloalkanes, carboxylic acids, amino acid, halogen compounds, ethers, non-conjugated diense *etc.* (Table-4; Fig. 3).

Table:IV The peak values of ethanolic extract is as follows:

S. No.	Peak Value	Functional Group
1.	3782.43	Phenol
2.	3002.83	Alkene
3.	2834.05	Alkane
4.	2664.17	Ester
5.	2551.41	Para Benzene
6.	1915.52	Alkene
7.	1684.96	Alkene
8.	1423.55	Nitrogen
9.	1283.56	Secondary Alcohol
10.	1180.24	Nitro compounds

The FTIR spectrum was identify the functional group of the active components based on peak value in the infrared radiation. The FTIR peak values and functional groups were listed above the table. The FTIR spectrum was illustrated in the graphical representation. The FTIR spectrum confirmed the presence of phenol, alkene, alkane, secondary alcohol, para benzene and nitrogen. Hence the crude extracts subjected to UV-vis and FTIR analysis used for the identification of chemical constituents present *S. fruticosa.* In addition, UV-vis and FTIR spectroscopy is proved to be reliable and sensitive method.

Fig:III FTIR analysis of Ethanolic Extracts of *Stylosanthes fruticosa*

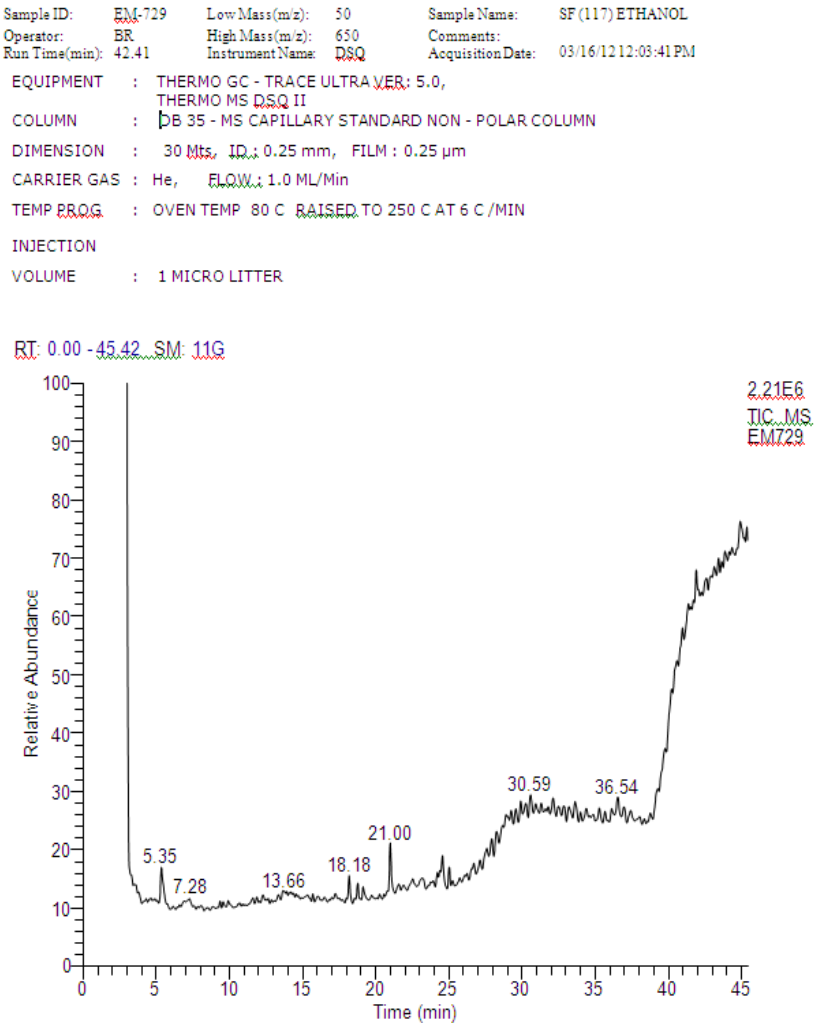


3. 2d. GC-MS analysis: The plant sample on subjecting to GC-MS provides the result of different peaks determining the presence of twelve different compounds. The molecular weight of these compounds is also known. By interpreting these compounds, it is found that this plant possess various therapeutical uses. The numbers at various peaks are the retention time in minutes. The mass spectrometer analyses the compounds eluted at different times to identify the nature and structure of the compounds. The large compound fragments into small compounds giving rise to appearance of peaks at different m/2 ratios. The mass spectra are fingerprint of that compound which can be identified from the data library (Table 5& 6; Fig. 4 to 16).

Table-V Compounds identified the *Stylosanthes fruticosa* extract

S. No.	RT	Name of Compound	Molecular formula	MW	Peak Area %
1.	5.35	Phenol, 2-(phenylethyl)	$C_{14}H_{14}O$	198	5.11
2.	5.35	Undecane, 2-methyl-(CAS)	$C_{12}H_26$	170	5.11
3.	5.35	Propanic acid 2-hydroxy-butyl ester (CAS)	$C_7H_{14}O_3$	146	5.11
4.	18.18	Trans-4-(3-carbethoxy-3-butenyl)-2-propytetrahydrofuran	$C_{14}H_{24}O_4$	256	3.18
5.	21.00	Dodecanoic acid, Methy-ester (CAS)	$C_{13}H_{26}O_2$	214	6.58
6.	21.00	Nonanoic acid Methyl ester (CAS)	$C_{10}H_{20}O_2$	172	6.58
7.	24.56	Trans-5-Hexyl-1,4-dioxane-2-carboxylic acid	$C_{11}H_{20}O_4$	216	9.26
8.	27.92	Propanoic acid 2-methyl-2-Methylpropyl ester (CAS)	$C_8H_{16}O_2$	144	2.79
9.	29.90	Ethyl 2,4,5-Trifluoro-a-oxo-3-(trifluoromethyl) benzenepropanate	$C_{12}H_8F_6O_3$	314	2.34
10.	30.56	Azidodiphenyl-borane	$C_{12}H_{10}BN_3$	207	3.68
11.	32.88	Hazaleamide monoeptixide	$C_{18}H_{29}NO_2$	291	2.55
9.	36.97	Ethyl 2,4,5-Trifluoro-a-oxo-3-(trifluoromethyl) benzenepropanate	$C_{12}H_8F_6O_3$	314	2.34

Fig:IV Gas chromatography and mass spectroscopy (GC-MS) analysis of ethanolic sample of *Stylosanthes fruticosa* (Retz.) Alston



Conclusion

The medicinal plant *Stylosanthes fruticosa* (Retz.) Alston. belong to the family Fabaceae was tested for the study of antibacterial and phytochemical studies. The antibacterial activity is being detected by disc diffusion assay, by using organic solvent such as acetone, chloroform, and ethanol while tested against three gram-positive and five gram-negative bacteria. The result shows that the ethanol, acetone, chloroform and aqueous extracts of leaves of *Stylosanthes fruticosa* possess measurable antibacterial activities against all the bacterial strains tested, while aqueous extracts of the plant did not produce any measurable antibacterial activity. The effect of various extracts of leaves on the sensitivity of eight selected bacteria by disc plate method has been shown in Table-1. The gram-negative bacteria have more inhibition zones than gram-positive bacteria. One gram-positive and five gram-negative bacteria showed activity either in one or two extracts. *Klebsiella pneumaniae* and *Pseudomonas aeruginosa* showed activity in acetone and ethanol extracts, whereas *Escherichia coli* showed activity in acetone and chloroform extracts. *Salmonella typhimurium*showed activity in chloroform extract only whereas in gram-positive bacteria *Bacillus cereus* showed activity in chloroform extract only whereas *Proteus vulgaris* showed activity in ethanolic extract only. The study has the potency of the plant crude extract on the tested microorganisms which is indicates the medicinal value of the plant extract.

The phytochemical screening chemical constituents of the plants studied showed that the leaves were rich in sugar, amino acids and secondary metabolites like steroids, alkaloids, flavonoids, saponins, tannin *etc*. (Table-2). They were known to show medicinal activity as well as exhibiting physiological activity[19]. The presence of saponin in *Stylosanthes fruticosa* in the present study is in corroborate with the opinion of Gill [21] who noted that saponin is one of the active constituents. Also, the presence of saponin contradicts the observation of Taylor-smit [22] who reported that saponin was absent in this taxon.

Further phytochemical investigation where done using UV-Vis and FTIR to find out the peacks obtained by the two extracts. The peaks identified by the extracts show the presence of functional groups (Tables 3 and 4). GC-MS analysis was done using the organic solvent ethanol and it shows the presence of twelve different chemical compounds present in the plant sample. The sample was extracted with ethanol because of the effect of antibacterial activity in this solvent. GC-MS analysis also provides the spectrum for the ethanolic extract.

The different chemical compounds identified in GC-MS analysis are as follows: Phenol, 2-(phenylethyl), Undecane, 2-methyl-(CAS), Propanic acid 2-hydroxy-butyl ester (CAS), Trans-4-(3-carbethoxy-3-butenyl)-2-propytetra-hydrofuran, Dodecanoic acid, Methy-ester (CAS), Nonanoic acid Methyl ester (CAS), Trans-5-Hexyl-1,4-dioxane-2-carboxylic acid, Propanoic acid 2-methyl-2-Methylpropyl ester (CAS), Ethyl 2,4,5-Trifluoro-a-oxo-3-(trifluoromethyl) benzenepropanate, Azidodiphenyl-borane, Hazaleamide monoeptixide, and (SE)-3-Acetoxy-6,10-methyl (1,59 andecadien-2-one)

The chemical compound, molecular formula, molecular weight, percentage of peak area of the compounds is reported in Table-6. The chemical nature of the compound, structure and their therapeutic uses. From the above studies, it is concluded that this plant has certain bioactive principles and medicinal uses. The chemical compounds present in this plant from the characteristics nature of its medicinal uses. The plants studied here can be seen as a potential source of useful drugs. Further studies are going on these plants in order to isolate, identify, characterize and elucidate the structure of the bioactive compounds. The antimicrobial activities of these plants for the treatments of the diseases as claimed by traditional healers are also being investigated.

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