Gas chromatography-mass spectroscopy profiling of leaf extracts of *Stachytarpheta urticifolia* (Salisb) based on antioxidant activities

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Abstract
The Stachytarpheta urticifolia is one of the widely used herbs as folk medicine for treating various disorders by many tribal communities. Present study was to explore the antioxidant potential of S. urticifolia leaf that was extracted with solvents hexane, ethyl acetate and methanol. An antioxidant activity was studied by using previous published and established methods using ABTS, hydrogen peroxide and nitric oxide. Extracts possessing highest potential antioxidant activities were further profiled using gas chromatography and mass spectrophotometer instrumentation. S. utricifolia leaves have shown significant antioxidant potential with methanol extract having inhibition concentration values IC_{50} = 75.1 7± 0.4 µg/ml and IC_{50} = 110.05 ± 7.1 µg /ml for the ABTS and hydrogen peroxide tests respectively whereas as IC_{50} value was 79.01±2.02 µg/ml for the nitric oxide test.

The outcome affirms the role of S. urticifolia as a good source of antioxidant agents. Methanolic extract of S. urticifolia leaves was further subjected for Gas Chromatography-Mass Spectroscopic (GC–MS) analysis. In the GC-MS chromatogram, 15 compounds were obtained with the highest percentage of peak area of 44.15 and later identified as 0,0- Diethyl (1-formyl pentyl selenophosphate) with a retention time of 23.805 sec.

Keywords: *Stachytarpheta urticifolia*, antioxidant activity, ABTS, hydrogen peroxide, nitric oxide, gas chromatography-mass spectroscopy.

Introduction

The herbal plants are used as a remedy for various disorders and infections from ages by tribes staying in interior or remote areas. Even today after much advancement in medicine, many people are choosing traditional medicine using herbs because of lower side effects. Medicinal plants are having a mixture of bioactive metabolites produced for their primary or secondary metabolism which can be used to induce notable metabolic activities against syndromes and diseases. Secondary metabolites are gaining thrust and extensive research is being carried out to explore the bioactive metabolites for activities like antioxidant as one of the primary interests along with other activities like antibacterial, anti-inflammatory, anti-cancer, analgesic etc.[1,6,14]

Antioxidant metabolites are compounds which can reduce or prevent reactive oxygen species (ROS) generated in the body during various metabolisms. Detoxification disproportion in ROS generated leading to oxidative push that causes cellular damage due to free radicals[4,9,19]. Toxic effects of free radicals are well established in metabolic disorders like cancer, diabetes, arthritis, neurodegenerative etc.[16] Antioxidants are compounds with an ability to neutralize the free radicals produced in the body and thereby help to reduce their negative effects[23]. Protective role of antioxidants on human body is unbiased across the studies and many analytical methods have been developed for determination of antioxidant activities[10].

Secondary metabolites with antioxidant activities are identified from numerous therapeutic and aromatic plants as a key source as preventing and protecting compounds and are used in foods, cosmetics and pharmaceutics industries[2]. The *S. urticifolia* is a multi-branched woody base herb that grows upto1.5m tall and is generally found in the sub tropical areas of Eastern Ghats. The name Stachytarpheta word is derived from Greek words “stachys” and “tarphys” means spike inflorescence which is a common character to many species in this genus.

The *S. urticifolia* is used in ethnomedicine by folk medicine practitioners to treat sore skin wounds as topical application using macerated leaves and roots. Though *S. urticifolia* plant is being used as a folk medicine for many years, only very few scientific reports are available to evaluate phytochemical constituents and their efficacy. The aim of the current study was to evaluate the antioxidant potential of *S. urticifolia* leaves extracted with hexane, ethyl acetate and methanol and to study the antioxidant activity with ABTS, hydrogen peroxide and nitric oxide scavenging assays. Chemical compounds present in the antioxidant rich extracts were analysed using gas chromatography coupled to MS detector.

Material and Methods

S. urticifolia leaves were collected from Damaku rai village located in the Araku valley of Alluri Sitaramaraju district, in the State of Andhra Pradesh, India. Plant was authenticated by Dr. P. Venkaiah, Professor of the Department of Botany, Andhra University and deposited in herbarium with assigned reference number 28083. The fresh leaves of *S. urticifolia*

were air dried at room temperature under shade approximately for 2 weeks. Later, dried leaves were powdered and stored in a glass bottle in the dark for further analyses.

Preparation of Crude Extracts: Dried leaves of *S. urticifolia* with known weight (50 gm) were extracted using Soxhlet extraction apparatus (Labline Pvt. Ltd., India) in each 250 ml of solvents hexane, ethyl acetate and methanol and crude extracts were further concentrated using rotavapor apparatus (Aditya Scientific Ltd., India). Concentrated crude extracts were weighed and stored in airtight dark bottles and were kept in a refrigerator at 4 °C until use. GC-MS analysis was carried out using 5gm dried leaves powder defatted in 50ml of hexane followed by sonication in ultrasonic bath (130 KHz) for 45 min. After sonication, samples were taken out from the bath and centrifuged. Supernatant was collected, filtered through Whatmann filter paper, dried using rotavapor apparatus, weighed and reconstituted in hexane prior to GC-MS analysis.

Determination of Antioxidant Activity: The solvent extracted leaf samples were analysed for antioxidant potential with earlier reported methods using ABTS, hydrogen peroxide and nitric oxide methods.

2,2-Azino-bis(3-ethylbenzothiazoline-6-sulphonic) Acid (ABTS) Radical Scavenging Activity: The radical scavenging activity of ABTS radical was determined with slight modifications as per the method published by Re et al[17]. Briefly, the stock solution of ABTS was prepared by taking ABTS aqueous solution (7 mM) in 2.45 mM aqueous solution of potassium persulfate and the solution was kept in dark for 12-16 hours at room temperature. The stock solution of ABTS was reacted with methanol and the absorbance at 734 nm was recorded using spectrophotometer. Fifteen micro litters of test samples (leaf extract) were taken and added to 185 µL of diluted solution ABTS and incubated for 10 min. The antioxidant activity results were expressed as IC_{50} (µg/ml) and the percentage of inhibition was calculated by the following equation:

$$\text{Percent ABTS Scavengind effect} = \frac{(A0 - A1)}{A0} \times 100$$

where A0 is absorbance of the control and A1 is absorbance of the sample.

Hydrogen Peroxide Scavenging Activity: Hydrogen peroxide radical scavenging activity by the leaves extracts was studied by method published earlier[18]. Series of concentrations of solvent extracts were taken and added with 40 mM H_2O_2 solution (0.6 mL) in phosphate buffer (pH 7.4). Samples were incubated for 10 minutes, then absorbance was measured at 230 nm against phosphate buffer as negative control. Positive control was prepared by taking 0.1–1 mg/ml L-ascorbic acid in distilled water. The % of H_2O_2 scavenging activity was calculated by using the following equation:

Scavenging activity (%) = (Absorbance of control) – (Absorbance of sample) / (Absorbance of control) ×100

Nitric Oxide Scavenging Activity: Leaf extract with nitric oxide scavenging activity was done by using method developed by Garratt[11]. Briefly, leaf extracts were prepared by taking 1ml of extract (10–100 µg/ml) and added with 0.5 ml of 10 mM sodium nitroprusside (in phosphate buffer saline pH 7.4). Incubate at 25 °C for 3 hr, add equal volumes of freshly prepared Griess reagent (1% of sulphanilamide in 5% phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride solution prepared in distilled water). Absorbances were recorded at 546 nm. Blank sample was prepared without sodium nitroprusside and at the same time, the control was prepared by taking gallic acid and ascorbic acid (10–100 µg/ ml) in phosphate buffer. The % of NO radical scavenging activity was calculated from the formula:

NO radical scavenging activity (%) = (Absorbance of control- Absorbance of sample/ Absorbance of control) × 10

Gas Chromatography -Mass Spectroscopy: The GC-MS analysis of the leaves extract was performed on a GC-MS-QP2020 system (Shimadzu, Kyoto, Japan) with SLB-5ms column (Merck KgaA) (30m length × 0.25mm diameter × and 0.25 µm thickness) "AOC-20i" system auto-injector. System was maintained at a temperature of 50°C initially and increased up to 350°C with gradual increase rate of 3 °C/ min and holding time of 5 min. 1.0 µl of sample was injected to the column at temperature 280 °C and helium was used as carrier with linear velocity of 30 cm/s and inlet pressure at 26.7 KPa, EI source temperature at 220°C and interface temperature at 250°C MS spectra were obtained with mass range and event time of 0.2 Sec. using full scan mode.

Chemical compounds present in the samples were expressed in the GC chromatogram as relative quantity based on the percent of peak area and compounds were identified by using the software FFNSC 4.0 (Shimadzu) and W11N17.

Based on the MS similarity match and LRI filter, compounds are identified. Linear retention indices (LRI) were calculated by using a reference of saturated alkanes mixture C7-C40 (49452-U, Merck) and by using a software GCMS solution ver. 4.50 (Shimadzu). Data files were collected and processed.

Statistical Analysis: All the experiments were done in triplicate and results are expressed as average with standard deviation and all the data were analysed by using SPSS Statistics for Windows, version 21 (IBM Corp., USA). Analysis of variation (ANOVA) was performed for the comparison of means between groups and differences were considered significantly when the 'p' value is less than 0.05.

Results

Antioxidant activity: ABTS radical scavenging activity by leaves extracts of *S.urticifolia* was evaluated and results with IC 50 values are presented in table 1.The antioxidant activities is based on the redox properties of the extracts as reducing agents by breaking free radical chain and donating hydrogen particle or avoiding formation of peroxide[13]. The therapeutic *S. urticifolia* leaf extracts are rich in antioxidants and the percentage of scavenging activities using solvents was presented in figure 1.

ABTS Radical Scavenging Activity: The radical of ABTS was formed in the steady form in potassium persulphate before the absorbance. Antioxidant ability of the leaf extracts was observed by decolorizing the reaction mixture when added. The methanol extract of *S. urticifolia* leaves extract showed highest percent inhibition with 75.17 ± 0.4 µg/ml and percent inhibition of remaining two solvent extracts hexane and ethyl acetate 88.05±1.6 µg/ ml, 109.8±4.8 µg/ml respectively.

Hydrogen peroxide scavenging assay: The *S. urticifolia* leaf extracts capability to scavenge hydrogen peroxide was investigated against standard ascorbic acid and the results are presented in figure 2. The methanol leaves extracts of *S. urticifolia* exhibited the highest hydrogen peroxide scavenging capacity, with an inhibitory concentration value of ascorbic acid as a standard (IC_{50} = 110.05 ± 7.1 µg/ml), ethyl acetate (IC_{50} = 145.78±5.8 µg /ml) and hexane (IC_{50} = 165.56±14.1 µg /ml) leaves extracts of *S, urticifolia* (Table 2).

Nitric oxide scavenging assay: *S. utricifolia* leaf extract with the nitric oxide scavenging activity was determined as described by Boora et al[5] and the results were expressed as the percentage of scavenging activity shown in figure 3. All the leaf extracts possessed dose-dependent nitric oxide radicle scavenging activity. The methanol leaf extract was found to be more efficient with IC_{50} values of 79.01 ± 2.02 µg/ml and ascorbic acid with IC_{50} values was 16.5 ± 2.12 µg/ml, respectively. The hexane and ethyl acetate leaf extracts of *S. urticifolia* were having IC_{50} values of 104.98±4.2 µg/mL and 114.68±5.7 µg/ml respectively (Table 3).

Table 1
ABTS radicle scavenging activity of *S. urticifolia* hexane, ethyl acetate and methanol leaves extracts.

Conc. of *S. urticifolia* leaves Extracts	ABTS Radicle Scavenging Activity (µg/ml)		
	Hexane	Ethyl acetate	Methanol
20	7.38± 1.02	10.4±0.02	12.2±1.2
40	12.8±1.2	14.6±1.09	20.8±2.2
60	28.6±1.02	30.4±1.12	40.8±2.09
80	38.4±1.00	39.8±1.09	40.4±2.12
100	42.6±2.00	40.6±1.12	54.6±2.18
150	52.4±1.24	62.4±1.08	68.4±2.10
200	60.4±1.80	72.6±1.62	76.8±2.26
IC_{50}	109.8±4.8	88.05±1.6	75.17± 0.4

The results were expressed as Mean ±SD (n=3)

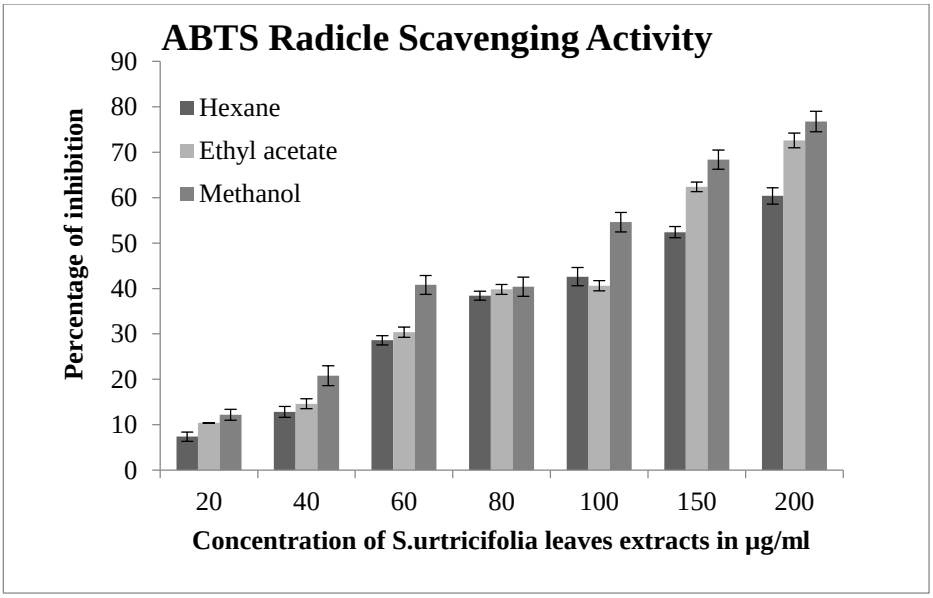

Figure 1: ABTS radical scavenging activity of *S. urticifolia* extracts of leaves.

Gas Chromatography-Mass Spectroscopy Analyses: The GC-MS data of the n-hexane fraction of *S. urticifolia* methanol extract showed the presence of ketones, steroids, esters, lipids and alkenes (Figure 4, Table 4). A total of 15 compounds were positively detected and the highest peak area with percentage of 44.15 similar to 0,0- Diethyl (1-formyl pentyl selenophosphate) with a retention time of 23.805 was the most abundant one, followed by the second highest percentage of peak area of 31.82 naming Carbetapentane with a retention time of 3.228 with similarity in percentage with range from 89 to 98%.

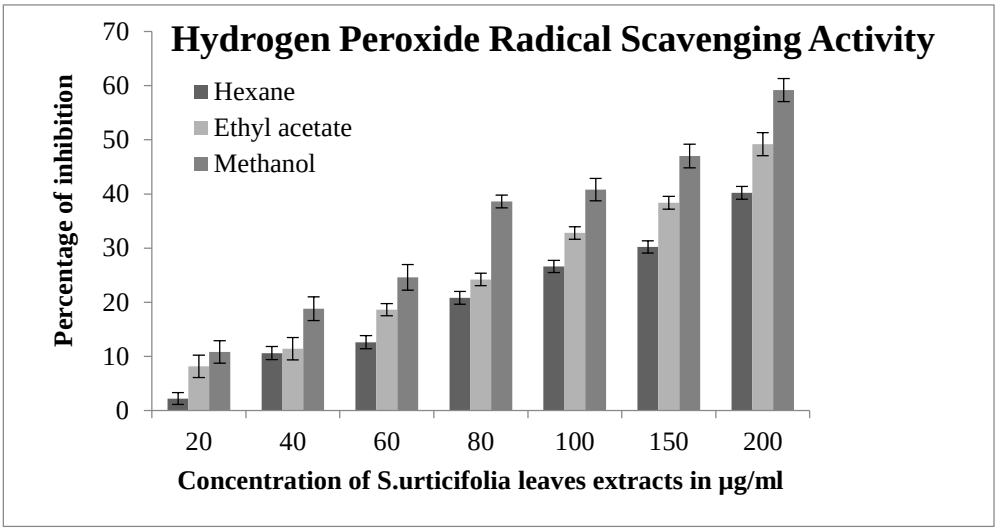

Figure 2: Hydrogen Peroxide Radical scavenging assay of *S. urticifolia* leaves extracts.

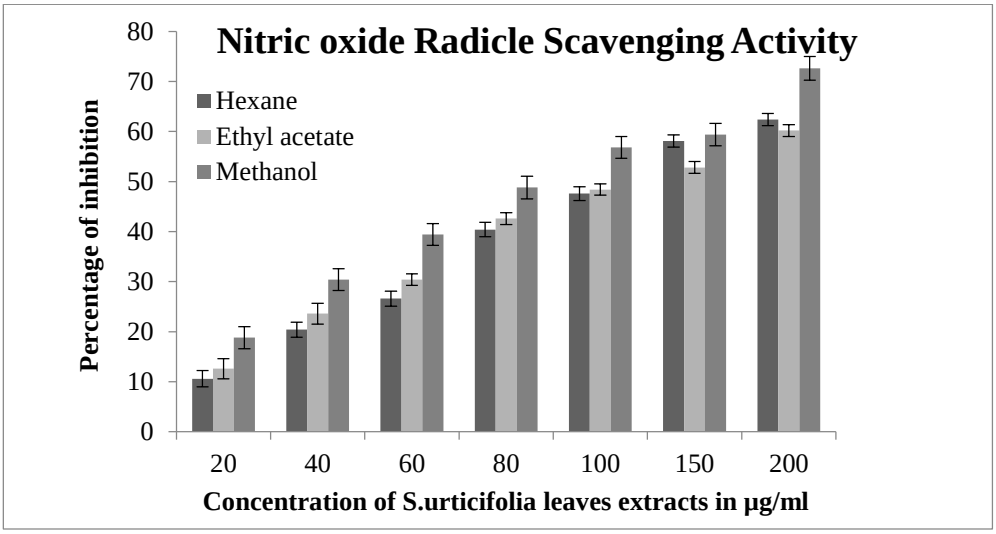

Figure 3: Nitric oxide radical scavenging activity of *S.urticifolia* leaves extracts

Table 2
Hydrogen peroxide radicle scavenging activity of *S. urticifolia* of hexane, ethyl acetate and methanol leaves extracts

Conc. of *S.urticifolia* leaves Extracts	Hydrogen Peroxide Radicle Scavenging Activity (µg/ml)		
	Hexane	**Ethyl acetate**	**Methanol**
20	2.18± 1.10	8.16±2.06	10.8±2.08
40	10.6±1.19	11.4±2.06	18.8±2.18
60	12.6±1.22	18.6±1.12	24.6±2.36
80	20.8±1.19	24.2±1.16	38.6±1.18
100	26.6±1.12	32.8±1.14	40.8±2.06
150	30.2±1.12	38.4±1.18	47.0±2.19
200	40.2±1.18	49.2±2.12	59.2±2.14
IC_{50}	165.56±14.1	145.78±5.8	110.05 ± 7.1

The results were expressed as Mean ±SD (n=3)

Table 3
Nitric oxide radicle scavenging activity of *S.urticifolia* of hexane, ethyl acetate and methanol leaves extracts

Conc. of *S.urticifolia* leaves Extracts	Nitric oxide Radicle Scavenging Activity (µg/ml)		
	Hexane	Ethyl acetate	Methanol
20	10.6± 1.62	12.6±2.02	18.8±2.22
40	20.4±1.52	23.6±2.08	30.4±2.18
60	26.6±1.48	30.4±1.18	39.4±2.18
80	40.4±1.42	42.6±1.18	48.8±2.28
100	47.6±1.38	48.4±1.12	56.8±2.18
150	58.1±1.22	52.8±1.18	59.4±2.24
200	62.4±1.20	60.2±1.18	72.6±2.36
IC_{50}	104.98±4.2	114.68±5.7	79.01±2.02

The results were expressed as Mean ±SD (n=3)

Table 4
Total ionic chromatogram (GC-MS) of *S. urticifolia* methanolic leaf extract obtained with temperature of EI 70 eV using a capillary column of DB×LB with the gas as the carrier.

S.N.	Retention Time	Name of the compound	Molecular formula	Molecular weight	Peak area %
1	3.228	Carbetapentane	$C_{20}H_{31}NO_3$	333	31.82
2	4.238	2-Propenoicacid, 2-methyl propyl ester	$C_7H_{12}O_2$	128	0.33
3	8.272	Bornyl acetate	$C_{12}H_{20}O_2$	196	0.53
4	10.273	Methanone (1-hydroxy cyclohexyl) Phenyl-	$C_{13}H_{16}O_2$	204	0.39
5	10.65	1(3H)-Isobenzofuranone,3-ethoxy-	$C_{10}H_{10}O_3$	178	0.05
6	12.042	4-Hydroxy-6-(4-methoxyphenyl)-2-pyranone	$C_{12}H_{10}O_4$	214.34	3.44
7	13.633	1-(Benzyl-3-(4-nitophenyl)pyrrolo (2,1-a) Phthalazine	$C_{24}H_{15}N_3O_3$	228.37	1.56
8	15.082	Pyridine-3-Carboxamide,2,4,6-trichloro-N-(4-methoxyphenyl)-5-nitro	$C_{13}H_8Cl_3$	256.42	2.49
9	16.503	3-n-Pentadecyl-2,4-dinitrophenol	$C_{21}H_{24}N_2O_5$	298.5	1.07
10	16.699	Dibenzoxazabicycloundecane	$C_{20}H_{23}NO_4$	296.49	4.28
11	17.097	Ergost-25-ene-3,6-diane,5,12 dihydroxy(5a,12a)	$C_{28}H_{44}O_4$	444	4.41
12	17.677	Lup-20(2a)-en-3-ol,acetate(3a)	$C_{32}H_{52}O_2$	468	0.23
13	18.423	E-ethyl(2)-3-(4-Acetylphenylthio) Cinnamate	$C_{19}H_{18}O_3S$	326	0.3
14	23.805	0,0-Diethyl(1-formyl pentyl selenophosphate	$Ci_0H_2iO_4PSe$	316	44.15
15	26.974	Ethyl-1-benzyl-4,6-dibromo-8-mehyl-2-oxoindoline-3-Carboxylate	$C_{l9}H_{17}Br_2NO_3$	465	4.94

Discussion

Studies were done on other *Stachytarpheta* sps like *S. jamaicensis, S. cayennensis, S. Angustifolia, S. gesnerioides, S.indica* etc. Our studies on *S. urticifolia* on antioxidant activities are similar with the other species reported from *Stachytarpheta* genus[15].

The three solvent leaf extracts of *S. urticifolia* possess antioxidant activity with significant variability between solvent extracts and the methods used: the methanol extract has the highest antioxidant properties with values of IC_{50} = 98.3 ±0.4 µg/ml and IC_{50} = 735 ± 49.5 mg/ml by ABTS and hydrogen peroxide assays respectively and for nitric oxide assay, the value was 81 ± 1. µg/ml. The ethyl acetate extract which contained the highest level of phenolic compounds and flavonoids yielded the smallest IC_{50} values in agreement with previous studies[10,20-22]. ABTS and hydrogen peroxide have antioxidant activity higher than the one described[3].

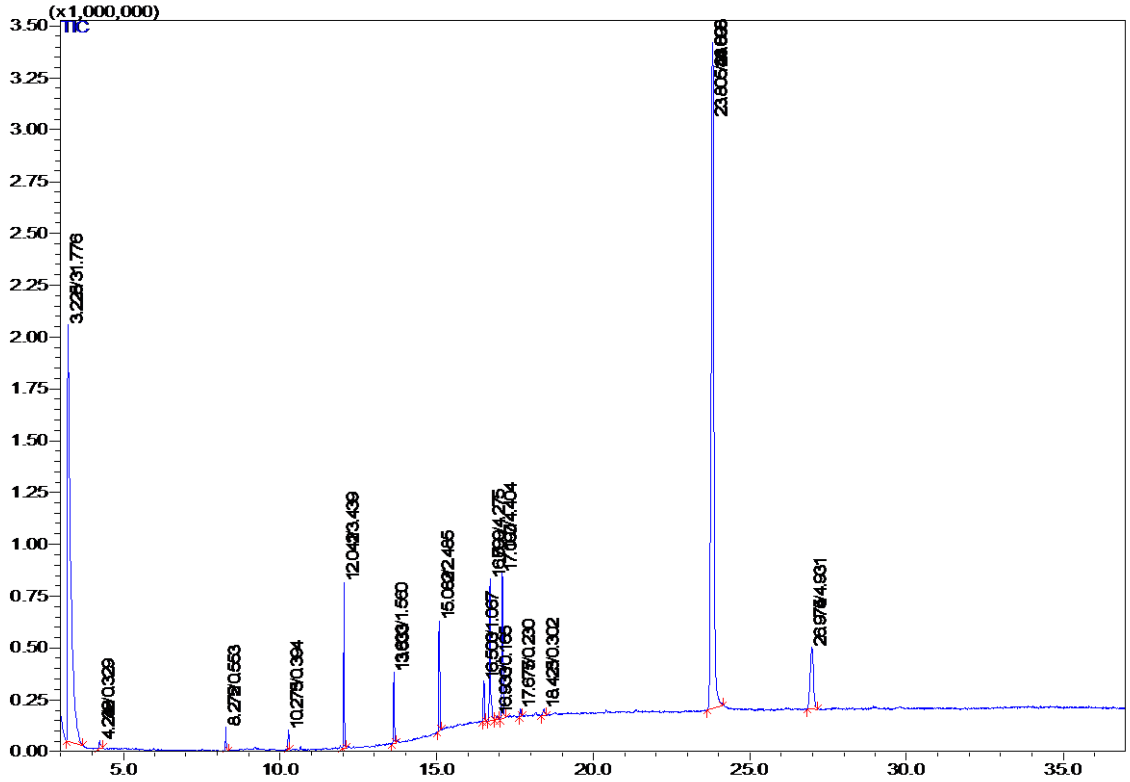

Figure 4: GC-MS chromatogram of S. *urtricifolia* methanolic leaf extract

In addition, literature available has proved that *S. urticifolia* leaf extracts contain antioxidant compounds that help to prevent various disorders and diseases[7,8]. Similar study was conducted by Yuvaraj et al[24] on *S. indica* using GC-MS instrumentation revealing around 27 compounds in which about 10 compounds have known bioactivities that can correlate with the medicinal activity of the plant.

Conclusion

The antioxidant activities of hexane, ethyl acetate and methanol leaves extract of *S. urticifolia* extracted in solvents hexane, ethyl acetate and methanol were investigated by ABTS, hydrogen peroxide and nitric oxide. The antioxidant profile results indicate that the methanolic leaf extract shows the highest activity compared to hexane and ethyl acetate. The methanol extract results showed 15 compounds belonging to different chemical classes. The results achieved are very promising.

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