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Molecular Profiling And Antioxidant Potential Of Citrus Limon (L.) Burm.F Fruits

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ABSTRACT

Citrus limonL. Burm F. was subjected to preliminary screening for phytochemicals, followed by molecular characterization using GC-MS and antioxidant analysis,using aqueous, petroleum ether, ethyl acetate, chloroform and ethanol extract of fruits. Qualitative analysis showed the presence of phenols, flavonoids and triterpenoids was present in ethanol, ethylacetate and aqueous extract with maximumtotal phenolic content of of 40.1 ± 0.7 mg/100g, flavonoid content analysis result showed 65.8 ± 0.8 mg /100g, and total terpenoidof 44.7 ± 0.16 mg/100g in the ethanol extract of fruits. The antioxidant potential of ethanolic fruit extract evaluated through DPPH assay exhibited an IC_{50} value of 85.83 % strong antioxidant activity when compared to other solvents. While SARS assay of the ethanol extract had IC_{50} values of 84.38 %. The chloroform fruit extract of plant showed very low antioxidant activity. The GC-MS analysis ofethanolic fruit extract revealed the presence of 9 compounds which were eluted at various interval of time. The chemical compounds Urs-12-en-3-ol, acetate (3-beta) showed the highest sharp peak of 57.97 % at a retention time (RT) of 19.759 minutes, indicating the presence of the compound Urs-12-en-3-ol, acetate(3.beta). The smallest peak with the retention time (Rt) of 13.178 minute had the corresponding compound identified as 9-Undecenal, 2,10 –dimethyl.

Key words: Citrus limon,phytochemical analysis, antioxidant, DPPH, SARSA, GC-MS analysis.

Introduction

C. limonis one of the most widely produced commercial fruit crops in the planet. It is one of the world's largest plant species, with 40 different kinds found all over the world. Citrus limon fruit has the highest level of eriocitrin in comparison to other Citrus sps. as well as important quantities of phenolic acids (ferulic acid or synaptic acid) which are localized mainly in the juice. Finally the most known is ascorbic acid, commonly known as vitamin C, which is highlights as a powerful antioxidant molecule and an effective free radical scavenger [1]. These fruits contain antioxidative,

antitumor, and antibacterial compounds such as phenolic, flavonoids, vitamins, and essential oils. C. limoncontains numerous significant natural chemical compounds such as citric acid, ascorbic acid, minerals, and flavonoids. Although its health benefits have long been associated to its vitamin C content, it has recently been shown that flavonoids also play a role in this regard. Flavonoids, have a variety of biological roles, including antioxidative, anti-inflammatory, antiallergic, antiviral, antiproliferative, antimutagenic, and anticarcinogenic properties.Overall, lemon fruits, which are high in flavonoids, are an essential part of a healthy diet, especially in the prevention of diseases such as obesity, diabetes, blood cholesterol lowering, cardio vascular disease, and some types of cancer. Because its phenolic molecule can inhibit cellular oxidative processes in the central nervous system, C. limon essential oil may play a modulatory role in the treatment of neurodegenerative disorders.There are many studies used the extract of citrus fruits like lemon, orange and grape because they have significant antimicrobial activity [2].

Materials and Methods:

3.1.1 Collection of Plant Material

Fruits of Citrus limon collected from plants growing at Kudunkulam of Tirunelveli district of Tamil Nadu in India was used for this investigation. Fresh fruits were washed and dried in shade. After drying, the plant material was macerated using mixer grinder. Then the powder was stored in air tight containers and kept in refrigerator for future use.

3.1.2 Preparation of plant extracts

The dried fruits of Citrus limon were extracted using the procedure of [3].10 grams of plant powder and 250ml of solvents like ethyl acetate, chloroform methanol and ethanol separated by successive solvent method in a soxhlet extractor for 8 hours and temperature not exceeding the boiling point of the solvent. The extracts were filtered using whatman (No 1) filter paper and then concentrated in vacuum at 40 degree Celsius using rotary evaporator. The residues obtained were stored in a freezer until further experiments.

3.1.4 Qualitative phytochemical test :

The extracts of each solvent was used to analyze the presence of different phytochemical constituents using standard procedures.

3.1.5 Quantitative Analysis

3.1.5.1 Determination of flavonoids:

Total flavonoid content was determined by aluminium chloride method using catechin as a standard[4]. 1ml of test sample and 4 ml of water was added to a volumetric flask. After 5 min 0.3 ml of 5 % sodium nitrite, 0.3 ml of 10% aluminium chloride was added. After 6 min incubation at room temperature, 2 ml of 1 M sodium hydroxide was added to the reaction mixture. Immediately the final volume was made up to 10 ml with distilled water. The absorbance of the reaction mixture was measured at 510 nm against a blank spectrophotometrically.

3.1.5.2 Determination of Total Phenols

The total phenolics content in different solvent extracts was determined with theFolin- Ciocalteu's reagent using the procedure [4]of different concentrations of the 1 ml of the extract were mixed with 0.4 ml of reagent . After 5 min 4 ml of 7% sodium carbonate solution was added. The final volume of the tubes were made upto 10 ml with distilled water and allowed to stand for 90 min at room temperature. Absorbance of sample was measured against the blank at 750 nm using a spectrophotometer.

3.1.5.3 Determination of Terpenoids

Total terpenoid content was determined by the method of [5].And the absorbance was read at 538 nm using UV/visible spectrophotometer.

3.2 Determination of antioxidant activity of the extracts

3.2.1 Superoxide anion radical scavenging (SARS) assay

The assay for superoxide anion radical scavenging activity was supported by riboflavin-light-NBT system [6]. 1 ml of extracts was taken at different concentrations (20, 40, 60, 80 and 100 µg/ml) and mixed with 0.1 ml of riboflavin solution (20 µg), 0.2 ml of EDTA solution (12 mM), 0.2 ml of methanol and 0. 1 ml of nitro-blue tetrazoliumn(0.5 mM) were mixed in test tube and reaction mixture was diluted up to 3 ml with phosphate buffer (50 mM). After 20 min of incubation at room temperature, the absorbance was measured at 560 nm. Ascorbic acid was used as standard. The scavenging ability of the plant extract was determined using the following equation:

Scavenging effect (%) = [(control OD − sample OD) / (control OD)] × 100

3.2.2 DPPH(2,2 Diphenyl-1-picryl-hydrazyl-hydrate) scavenging assay

DPPH assay was done following the procedure of [6].An aliquot of 0.5 ml of sample solution in methanol was mixed with 2.5 ml of 0.5 mM methanolic solution of DPPH. The mixture was shaken vigorously and incubated for 30 minutes in the dark at room temperature. The absorbance was measured at 517 nm using UV spectrophotometer. Ascorbic acid was used as a positive control. DPPH free radical scavenging ability (%) was calculated by using the following formula.

$$\% \text{ of inhibition} = \frac{\text{OD of control} - \text{OD of sample}}{\text{OD of control}} \text{ X } 100$$

3.3 GC-MS Analysis

GC-MS analysis of theethanolextract of Citrus limonwas performed the following procedure [7], using a Perkin-Elmer GC Clarus 500 system comprising an AOC-201 auto sample and a gas chromatograph interfaced to a mass spectrometer (GC-MS) equipped with a Elite 5MS (5% biphenyl /95% dimethyl poly siloxance) fused a capillary column (30 X 0.25mm 1D X 0.25mm df). For GS-MS detection an electron ionization system was operated in electron impact mode with ionization energy of 70ev. Helium gas (99.999%) was used as a carrier gas at a constant flow rate of 1ml/min, and an injection volume of 2ml was employed. The injector temperature was maintained at 200^0C, the ion source temperature was 200^0C, the oven temperature was programmed from 110^0 C, (isothermal for 2min), with an increase of 10^0C/min to 200^0C, then 5^0C/min to 280^0C, ending with a 9 min isothermal at 280^0C, mass spectra were taken at 70ev, a scan interval of 0.5S and fragments from 45 to 450 Da. The solvent delay was 0 to 2 min, and the total GC/Ms running time was 36min. the relative percentage amount of each component was calculated by comparing its average peak area to the total areas. The mass detector used in this analysis was Turbo Mass Gold Perkin Elmer and the software adopted to handle mass spectra and chromatograms was a Turbo Mass Ver-5.2.The compounds were identified using online NIST library.

4. RESULTS

4.1 Phytochemical screening of Citrus Limon

To screen for the existence of therapeutically significant bioactive chemicals, phytochemical analysis was performed on Citrus limon fruits. The dried fruits of trees grown at Tirunelveli district were screened. The samples were extracted using solvents such as aqueous, petroleum ether, ethyl acetate, chloroform, and ethanol in a series of successive steps. Steroids, triterpenoids, reducing sugars, phenolic groups, proteins, alkaloids, flavonoids, catechins, tannins, anthroquinonesaponins, aminoacids, and sugars were among the secondary metabolites tested. Maximum phytochemicals was detected in the ethanol extract of fruits (Table1).Steroids, triterpenoids, reducing sugars, phenolic groups, proteins, alkaloids, flavonoids, tannins, anthroquinones, saponins, aminoacids, and

sugars were detected in the ethanol extract however catechins were not.Quantitative analysis of ethanol, aqueous, and ethylacetate fruit extractsshowed the presence of phenols, flavonoids and triterpenoids.The total phenolic content of the fruitsin the ethanol, aqueous and ethylacetate extractwas 40.1 ± 0.7, 23.6 ± 0.53, 20.1 ± 0.61 mg/100g, while flavonoid content was calculated to 65.8 ± 0.8, 35.7 ± 0.61, 19.88 ± mg/100gm and total terpenoid present in the sample came to 44.7 ± 0.16, 43.2 ± 0.53, 21.8 ± 0.61 mg/100gm respectively.

Table 1 Phytochemical characterization of Citrus limon fruit extract

S. No	Phytochemical Test	Different Solvent extract				
		Aqueous	Petroleum Ether	Ethyl Acetate	Chloroform Extract	Ethanol Extract
1	Steroids	+	-	+	+	+
2	Triterpenoids	+	+	+	-	+
3	Reducing Sugar	+	-	-	-	+
4	Phenolic Group	+	-	-	-	+
5	Protein	+	-	-	-	+
6	Alkaloids	+	+	-	-	+
7	Flavonoids	+	-	+	+	+
8	Catechin	-	-	-	-	-
9	Tannins	+	-	+	-	+
10	Anthroquinones	-	-	-	-	+
11	Saponins	-	-	+	-	+
12	Aminoacids	+	-	-	-	+
13	Sugars	+	+	-	-	+

4.2 Antioxidant Activity of Citrus limon

Antioxidant potential of C.limon extracts (ethanol, aqueous, ethylacetate, chloroform, petroleum ether) was estimated through the DPPH radical scavenging activity and SARSA potential. In DPPH and SARS assays the ethanol fruit extract exhibited strong antioxidant activity. The inhibition percentage trend among the extracts was ethanol ≥ aqueous ≥ ethyl acetate ≥petroleum ether ≥ chloroform . In the DPPH assay, IC_{50} value of ethanol was 85.83% followed by aqueous 98.61%, ethyl acetate extract had 102.24%, petroleum ether 122.24% and chloroform extract had 130.2% (Table 2).

Table 2 Antioxidant potential of C. limon fruit by DPPH assay

Concentration	Ethanol	Aqueous	Ethyl acetate	Chloroform	Petroleum ether	Ascorbic Acid
20 µg /ml	4.4	6.1	1.7	4.1	3.1	20.2
40 µg /ml	20.3	16.8	2.2	17.9	7.7	37.7
60 µg /ml	32.8	29.6	12.7	22.6	17.8	55.7
80 µg /ml	46.6	41.5	43.2	34.6	31.9	70.7
100 µg /ml	60.7	50.7	48.9	38.4	40.9	91.6
IC 50	85.83	98.61	102.24	130.2	122.24	53.05
	Percentage of Inhibition (%)					

In SARS assay IC_{50} value of ethanol was calculated to 84.38% followed by ethylacetate 90.09%, petroleum ether 95.46%, aqueous 99.40% and chloroform extract had 128.53 %(Table 3). Statistical analysis supported the findings by revealing highly significant values in the antioxidant activity of plants. The P value for the DPPH assay was 0.2392, whereas the P value for the superoxide anion radical scavenging assay was 0.3277 at 5% significance.

Concentration	Ethanol	Aqueous	Ethyl acetate	Chloroform	Petroleum ether	Ascorbic Acid
20 µg /ml	5.4	5.8	8.6	1.6	7.9	21.7
40 µg /ml	12.3	12.8	16.8	6.7	18.1	41.4
60 µg /ml	26.7	35.0	28.9	16.9	28.0	62.2
80 µg /ml	47.4	38.8	39.5	24.8	41.9	75.2
100 µg /ml	67.9	50.3	55.5	38.9	52.1	83
IC 50	84.38	99.40	90.09	128.53	95.46	48.30
	Percentage of Inhibition (%)					

Table 3Antioxidant potential of C. limon fruit by Superoxide anion radical scavenging assay

4.3 GC – MS Analysis

GC-MS was used to examine the components of Citrus limon ethanol fruit extracts. Thechromatogram is presented in (Fig 1, Table 4). Nine compounds were found in an ethanol fruit extract of Citrus limon grown at Tirunelveli district. The ethanolic extractdisplayed distinct peaks at varied retention times in the GC-MS profile. The chemical Urs-12-en-3-ol, acetate (3.beta) showed

the highest sharp peak of 57.97 % with a retention time (RT) of 19.759 minutes. Beta-Amyrin, a compound, at peak of 21.8 % at retention time of 18.038 minutes. The peak with a retention time (RT) of 17.594 minutes was identified as corresponding to 2H-3, 9a-Methano-1-benzoxepin, Octahydro-2, 2, 5a, 9-tetramethuyl – [3R-(3.alpha.5a.alpha., 9.alpha., 9a.alpha)]. Squalene, a compound, with a peak of 2.79%at retention time of 19.390 minutes. The compound silicic acid, diethyl bis(trimethylsilyl) ester was identified to haveretention time (RT) of 16.516 minutes. The compound 1,4-Bis(trimethyl silyl) benzene was identified by the retention time (Rt) of 18.426 minutes. The pyrene, hexadeca hydro compound has a peak of 2.08 at a retention duration of 14.965 minutes. The presence of 5-Hexenoic acid, 5-methul, was recognized by the retention time (RT) of 10.531 minutes. The peak with the retention time (RT) of 13.178 minutes had a compound identified as 9-Undecenal, 2, 10-dimethy.

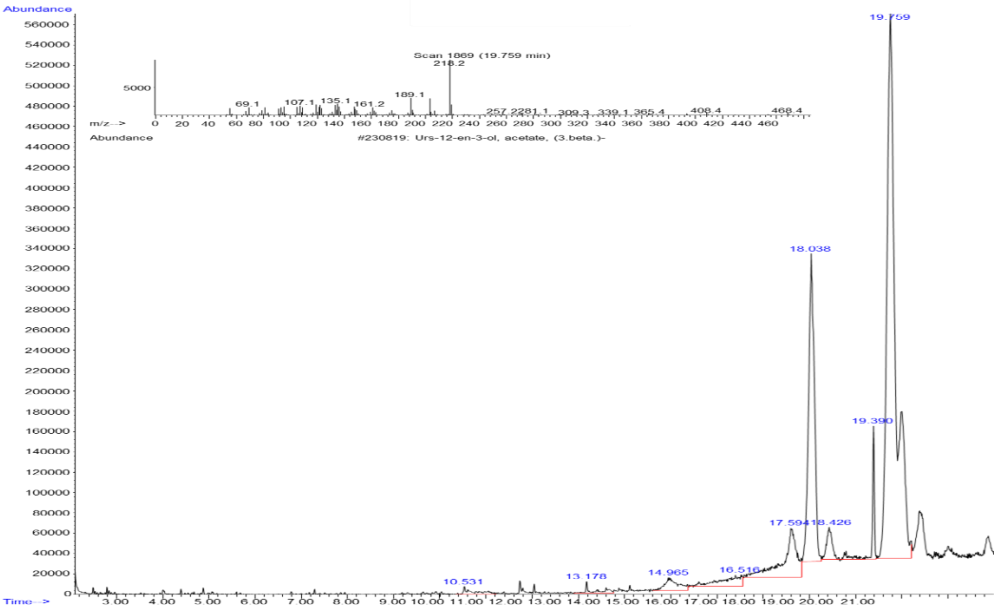

Figure 1: The GC-MS chromatogram of Citrus limonethanolic fruit extract

5. DISCUSSION

Phytochemicals Analysis

Lemon fruit is rich in natural chemical components such as phenolic compounds (flavonoids) and other nutrients and non-nutrients (Vitamins, Minerals, Dietary fibre, Essential oils and Carotenoids). Because of their natural antioxidant qualities, the contents of Vitamin C and flavonoids have been linked to health-promoting actions and attributes. Overall, lemon fruits, which are high in flavonoids, are an essential part of a healthy diet, especially in the prevention of diseases such as obesity, diabetes, blood cholesterol lowering, cardio vascular disease, and some types of cancer. Because its

phenolic molecule can inhibit cellular oxidative processes in the central nervous system, C limon essential oil may play a modulatory role in the treatment of neurodegenerative disorders.

The presence of limonoids in Citrus fruits, which could use against various clinically identified bacterial strains [8]. Lemon juice as an expected antibacterial specialist against diarrhoea causing microorganism [9].Our studies have demonstrated the occurrence of significant amount of secondary metabolites such as tannin, steroids, reducing sugar, proteins and high content of carbohydrates in the different extracts analysed and in the ethanolic extract expressed the presence of maximum number of metabolites analysed.Ethanol has the ability to attract glycosides [10], polyacetylenes, sterols [11], polyphenols, tannins, flavonols, terpenoids, and alkaloids [12]. The choice of ethanol as the extraction solvent was considered to provide many advantages over other organic solvents, which is relatively safer (less toxic).While the chloroform extract of fruits showed less number ofphytochemicals.Thephytochemicals detected are well known to contain non-nutritive plant chemicals that possess varying degrees of disease- preventive antimicrobial and antioxidant molecules. The current study has shown valuable newer sources possessing antibacterial activity and hormonal stimulation [13].

Total phenols, flavonoids, and terpenoids compounds which confer antioxidantof C. limonaqueous ethyl acetate and ethanol fruit extract was evaluated in this study. The total phenolic content of the fruitsin the ethanol, aqueous and ethylacetate extract showed 40.1 ± 0.7, 23.6 ± 0.53, 20.1 ± 0.61 mg/100g, while flavonoid content was calculated to 65.8 ± 0.8, 35.7 ± 0.61, 19.88 ± mg/100gm and total terpenoid present in the sample came to 44.7 ± 0.16, 43.2 ± 0.53, 21.8 ± 0.61 mg/100gm respectively.Antioxidants can be defined as bioactive compounds that inhibit or delay the oxidation of molecules. The antioxidant effect is predominantly because of their redox properties [14]. Polyphenol content might depend on different factors like genotypic contrasts, geographic and climatic conditions, season of gather [15]. The antioxidant property of the fruit might also be from the presence of vitamins, anthocyanins, phenolics, and tannins [16].

Antioxidant assays carried out in the ethanolic fruit extract showed high inhibition activity of DPPHand the ethyl acetate extract had the lowest values.The inhibitory activity of of the ethanol extract could be correlated to the presence of higher levels of phenols, flavonoids and terpenoids in it [17,18].The number of hydroxyl groups in a phenolic molecule affects its capacity as an antioxidant. Phenolic has a tendency to donate hydrogen atoms or electrons from its hydroxyl groups to free radicals [19]. As for flavonoids, the number and location of the aromatic hydroxyl groups in their structure affect their antioxidant capacity [20].

GC-MS analysis of C. limon

Gas chromatography is used in a wide range of applications. However, the separation and analysis of multi-component mixtures such as essential oils, hydrocarbons, and solvents is performed using gas chromatography. In recent years, GC-MS investigations have become more common in the research of medicinal plants, since this approach has proven to be a useful tool for determining non-polar components such as volatile essential oils, fatty acids, lipids, and alkaloids [21].

In the present investigation the GC-MS profile of ethanol fruit extract of C. limonshowed nine compounds distributed at 57.97 Peaks at retention time of 19.759 minutes Among them, (1) Urs-12-en-3-ol, acetate, (2) (3.beta.), beta.-Amyrin, (3) Squalene, and (4) 2H-3,9a-Methano-1-benzoxepin, octahydro-2,2,5a,9-tetramethyl-,[3R-(3.alpha.,5a.alpha.,9.alpha.,9a.alpha are major compounds.

Urs-12-en-3-ol, acetate, (3.beta.) is known to have antimicrobial, antioxidant, anticancer and cytotoxic potential and has been reported in Orthosiphon spp.[22], Ficusvariegata[23], Hypochaerisradicata[24]. Beta-Amyrin: the essential oil of resin of Protiumheptaphyllum showed broad range of antibacterial activity, beta-Amyrin isolated from the stem bark of Alstoniaboonei showed anti-inflammatory activity[25]. The pharmacological effects beta amyrin isolated from Protiumheptaphyllum[26] also adds that this compound hasmany biological activities like, antimicrobial, anti-inflammatory [27], anticonvulsant, analgesic, antihyperglycemic, antidepressive, antipancreatitic, gastroprotective, hepatoprotective, anticholytic, and hypolipidemic effects [28].

Squalene (2.38%) present in fixed oil from Sudanese Ziziphusspina Christi Fruits Pulp [29].Camellia oleifera exhibited potential for antibacterial and antioxidant activity and the main chemical constituent of this plant is squalene, which along with other phytochemicals contribute above said activities [30]. Essential oil of Spinaciaoleracea leaves has antimicrobial property shown due to presence of squalene (0.233%) present in it [31].

6. CONCLUSION

In conclusion, the present investigation has indicated that the extracts from C. limoncontain high enough levels of phenols andflavonoid compounds, which exhibit powerful antioxidant properties, expressed by its capacity to scavenge DPPH and SARS radicals. This preliminary work could be a promising lead for the development of new drugs for the prevention and treatment of oxidative stress related diseases or as food additives.

7. ACKNOWLEDGMENT

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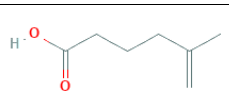
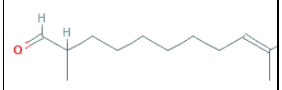
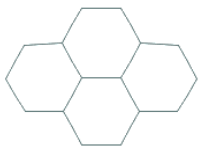
Peak No.	Compound Name	Retention Time (Min.)	Molecular Weight (G/mol.)	Molecular Formula	Structure
1	5-Hexenoic acid, 5-methyl-	10.531	128.17	$C_7H_{12}O_2$	
2	9-Undecenal, 2,10-dimethy	13.178	196.33	$C_{13}H_{24}O$	
3	Pyrene, hexadecahydro	14.965	218.38	$C_{16}H_{26}$	

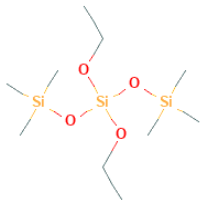
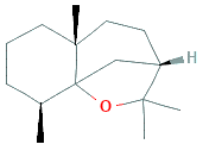
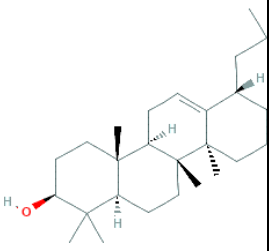
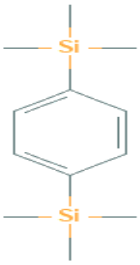
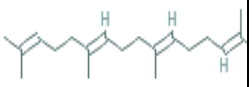
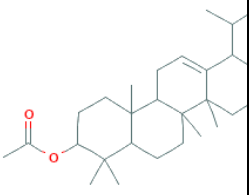
4	Silicic acid, diethyl bis(trimethylsilyl) ester	16.516	296.52	$C_{10}H_{28}O_4Si_3$	
5	2H-3,9a-Methano-1-benzoxepin, octahydro-2,2,5a,9-tetramethyl-, [3R-(3.alpha.,5a.alpha.,9.alpha.,9a.alpha	17.594	222.37	$C_{15}H_{26}O$	
6	beta.-Amyrin	18.038	426.7	$C_{30}H_{50}O$	
7	1,4-Bis(trimethylsilyl)benzene	18.426	222.47	$C_{12}H_{22}Si_2$	
8	Squalene	19.390	410.7	$C_{30}H_{50}$	
9	Urs-12-en-3-ol, acetate, (3.beta.)	19.759	468.8	$C_{32}H_{52}O_2$	

Table 4. The phytochemical components in ethanol fruits extracts of Citrus limon from Tirunelveli District were identified using gas chromatography-mass spectrometry