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FLUORESCENCE, FT-IR AND GC-MS DETERMINATION OF BIOACTIVE CONSTITUENTS OF LEAF EXTRACT OF *CLITORIA TERNATEA*

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ABSTRACT

Medicinal plants play a key role in human health care from ancient period. India is blessed with several herbal plants for the treatment of major and minor health problems without studying any phytochemical in detail. Plants are a rich source of bioactive constituents with interesting biological activities and are considered to be less toxic and free from side effects compared to synthetic drugs. *Clitoria ternatea* a valuable medicinal plant used in Ayurveda and Siddha medicine for the treatment of various diseases. The present study was to analyze the bioactive compounds from ethanolic extract of leaf of *Clitoria ternatea* L. by FT-IR and GC-MS techniques. The FT-IR analysis identified the functional groups such as primary alcohols, phenolics, alkane, alkynes and ketones. GC-MS revealed the presence of various bioactive compounds like -(2-ethylhexyl) phthalate, (2S,5S)-2,5-dimethyl-2-phenyl-1,3-dioxolane-4-one, 5,10-dihexyl-5,10-dihydroindolo (3,2-b) indole-2,7 dicarbaldehyde, 9,12-octadecadienoic acid (Z,Z), 2-hydroxy-1-(hydroxyl methyl) ethyl ester (CAS), neophytadiene and nonacosane (CAS). Phytochemical analysis showed the presence of various bioactive compounds which are known to exhibit medicinal activities.

KEYWORDS: *Clitoria ternatea*, **phytochemicals, fourier transform infrared spectroscopy, GC-MS**

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INTRODUCTION

Millions of people in the third world still use herbal medicines because they believe in them and regard them as their own system of medicine[1,2]. Many higher plants are known to be the main source of drug therapy in traditional medicine[3]. It occupies an important place in the health care systems of developing countries. The World Health Organization estimates that 80% of the people living in developing countries are almost completely dependent on the traditional medicine as therapeutic remedies for their primary health care needs. In India traditional communities like tribal and rural populations are frequently using the crude extracts of local plants for medicinal and other purposes because it is cheaper, more accessible and blends readily into the people's socio-cultural life than orthodox medicine[4,5]. Plants consist of number of biologically active chemical compounds which are formed during the plants normal metabolic processes. These chemicals are often referred to as "secondary metabolites". In addition to these substances, plants contain other chemical compounds; those can act as agents to prevent considerable side effects of the main active substances or to assist in the assimilation of the main substances. Plants have an ability to synthesize aromatic substances, mainly secondary metabolites of which 12,000 have been isolated, the number estimated to be less than 10% of the total. Secondary metabolites are sought after because they are known to exhibit numerous biological activities that promote health effects[6]. The WHO encouraged developing countries to use herbal medicine which they have been traditionally using for centuries[7]. They have identified 3000 plants from the forests of India and other tropical countries which can be used as medicine. A proper scientific evaluation and screening of plants by pharmacological tests followed by chemical investigations are necessary[8,9]. The main objective of this study was to investigate the phytochemical composition present in the ethanolic extract of leaf of *Clitoria ternatea* L. *Clitoria ternatea* Linn. (family: fabaceae) is a perennial twisting herbaceous plant distributed in India, China, Bangladesh, South and Central America, East and West Indies. It is commonly called "shankpushpi". In traditional Ayurvedic medicine, it could serve as therapeutic agents for various diseases like urinogenital disorder, bronchitis, purgative, diuretic, anthelmintic, rheumatism[10,11,12,13], antipyretic, anti-inflammatory, analgesic[14], anticancer activity[15], antihelmintic[16] and neurological disorders[17]. Recent studies showed that it has hypoglycemic and anti-hyperlipidemic activities[18,19].

MATERIALS AND METHODS

Collection and authentication of plant material

Fresh leaf of *Clitoria ternatea* L. (*C.ternatea*) was collected from SKM herbal research centre, Erode, Tamil Nadu, India. The plant was identified and authenticated by dr. V.R.Mohan, Associate professor, Department of Botany, V.O.Chidambaram college, Tuticorin. A voucher specimen (No. VOCB 2453) was deposited in ethnopharmacology unit, research Department of Botany, V.O.Chidambaram college, Tuticorin, Tamil Nadu.

Fluorescence analysis

Fluorescence analysis acts as a pharmacognostic parameter for identification and standardization of a particular drug from its adulterants[20]. Freshly collected leaf of *C.ternatea* was washed with distilled water and dried under shade for two weeks. The shade dried leaves was coarsely powdered separately. The powdered material was kept in airtight containers until use. Powdered drug of different parts of plant give different fluorescences under ultraviolet radiation. Each fluorescence characteristic of the treated sample was observed under ordinary light and then under UV light of both long and short wave lengths. Therefore florescence evaluation is used for identification of plant and powdered drug[21]. The fluorescence analysis of dried powdered of leaf of *C.ternatea* was carried out by treating with different chemicals. If the substances themselves are not fluorescent, they may often be converted into fluorescent derivatives or decomposition products by applying different reagents. A small quantity (1 gm) of dried and finely powdered leaf of *C.ternatea* was treated with freshly prepared acids, alkaline solutions and different solvents. The drug powder was treated with acids *viz*., 1N HCl, Conc. HCl; 50% H_2SO_4; Conc. H_2SO_4; 50% HNO_3; Conc. HNO_3; picric acid and acetic acid. The drug powder was treated with alkaline solutions *viz*., aqueous NaOH; alcoholic NaOH. The drug powder was treated with different solvents *viz*., acetone, benzene, chloroform, petroleum ether, methanol, ethanol etc. They were subjected to fluorescence analysis in daylight and in short UV- light (254 nm) and long UV- light (365 nm).

Preliminary phytochemical screening

About 500 gm of dried coarse powdered sample was weighed and subjected to 1250 ml of successive solvent extraction. The extraction was carried out with the following solvents in increasing order of polarity *viz.,* petroleum ether, chloroform, methanol, ethanol followed by water in a soxhlet extractor for 24 hrs. The extracts were filtered through Whatmann no.41 filter paper separately and the extracts were concentrated in vacuum at 60ºC using a rotary evaporator. To evaporate the remaining solvent, the extracts were kept in an oven at a temperature of 40-50ºC for 8 hrs. Later the extracts were used for qualitative identification of various phytochemical constituents as per the standard procedures[22,23].

FT-IR spectroscopy study

Fourier transform infrared spectroscopy (FT-IR) analysis was performed using Perkin Elmer spectrophotometer system, which was used to detect the characteristic peaks and their functional groups. For FT-IR spectrophotometer analysis, the extract was centrifuged at 3000 rpm for 10 min and filtered through Whatmann no. 1 filter paper by using high pressure vacuum pump. The sample was diluted to 1:10 with the same solvent. The extract was scanned in the wavelength ranging from 300-1100 nm using Perkin Elmer spectrophotometer and the characteristic peaks were detected. The peak values of the FT-IR was recorded. Each and every analysis was repeated twice for the spectrum confirmation.

GC-MS analysis

Gas chromatography-mass spectrometry (GC-MS) plays a key role in the analysis of unknown component of plant origin. The chemical composition of ethanolic extract of leaf of *C.ternatea* was subjected to GC-MS analysis. GC-MS analysis of the extract was performed using a GC clarus 500 Perkin-Elmer system comprising a AOC–20i autosampler and gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following condition: equipped with a column elite-1, fused silica capillary column (30 m x 0.25 mm id x 1µm df, composed of 100% dimethyl poly siloxane), operating in electron impact mode at 70 ev; helium gas (99.999%) was used as carrier gas at a constant flow rate of 1 ml/min and an injection volume of 2 µl was employed (split ratio of 10:1). The injector temperature is set at 250°C and ion-source temperature is 280°C. The oven temperature was programmed from 110°C (isothermal for 2 min), with an increase for 10°C/min, to 200°C/ min, then 5°C/ min to 280°C/ min, ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70 ev; a scan interval of 0.5 seconds and fragments from 45 to 450 Da. Total GC running time was 36 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Software adopted to handle mass spectra and chromatograms was a turbo mass ver. 5.0. Interpretation on mass spectrum of GC-MS was conducted using the database of national institute of standard and technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained[24].

RESULTS

Fluorescence analysis

The behaviour of the drug powder with different reagents will be helpful in characterization of the crude drug. After treating with different chemical reagents, the fluorescent behavior of the powdered leaf material of *C.ternatea* was observed in day light and under UV light at 254 nm and the observation was presented in table 1. Among the various chemical treatments, the powdered leaf of *C.ternatea* showed the characteristic fluorescent green colour when treated with 1N alcoholic NaOH, 50% H_2SO_4, 50% HNO_3, HNO_3+NH_3 and acetone under short UV light.

Table 1
UV and visible fluorescence analysis of leaf powder of *C.ternatea* with different chemicals

S.No.	Experiments	Visible/ day light	UV – light	
			254 nm (short wave length)	365 nm (long wavelength)
1.	Powder as such	Pale brown	Light green	Dark blue
2.	Powder +1N NaOH(aqueous)	Light green	Yellowish green	Dark blue
3.	Powder +1N NaOH(alcohol)	Brownish yellow	Fluorescent green	Dark brown
4.	Powder +1N HCl	Pale brown	Light green	Dark blue
5.	Powder +Conc. H_2SO_4	Light green	Yellowish green	Dark blue
6.	Powder +50% H_2SO_4	Brownish yellow	Fluorescent green	Black
7.	Powder +Conc.HNO_3	Reddish orange	Yellowish green	Dark brown
8.	Powder +Conc.HCl	Light green	Yellowish green	Dark blue
9.	Powder +50%HNO_3	Reddish brown	Fluorescent green	Dark blue
10.	Powder +40%NaOH+ 10% lead acetate	Yellowish orange	Light green	Black
11.	Powder +acetic acid	Light green	Yellowish green	Dark blue
12.	Powder +ferric chloride	Yellowish green	Light green	Black
13.	Powder +HNO_3 +NH_3	Light green	Fluorescent green	Dark blue
14.	Powder +NH_3	Light green	Yellowish green	Dark brown
15.	Powder +benzene	Yellowish green	Light green	Dark blue
16.	Powder +petroleum ether	Light green	Yellowish green	Black
17.	Powder +acetone	Greenish yellow	Fluorescent green	Dark blue
18.	Powder +chloroform	Brownish green	Yellowish green	Black
19.	Powder +methanol	Brown	Light green	Dark blue
20.	Powder +ethanol	Pale yellow	Yellowish green	Dark blue

Preliminary phytochemical screening

The distribution of different phytochemical constituents in petroleum ether, chloroform, methanol, ethanol and water extracts of powder of leaf of *C.ternatea* was evaluated qualitatively and summarized in table 2.

Table 2
Qualitative phytochemical screening of different extracts of leaf of *C.ternatea*

Phytochemicals	Petroleum ether	Chloroform	Methanol	Ethanol	Water
Alkaloids	-	-	+	+	+
Anthraquinone	-	-	+	+	-
Catechin	-	-	+	+	+
Coumarin	-	-	+	+	+
Flavonoids	-	-	+	+	+
Glycosides	-	-	+	+	+
Phenols	-	-	+	+	+
Proteins and free amino acids	-	-	+	+	+

Quinones	+	+	-	-	+
Saponins	-	-	+	+	+
Steroids	-	-	+	+	+
Sugars	+	-	+	+	+
Tannins	-	-	+	+	+
Terpenoids	+	+	+	-	+
Xanthoprotiens	-	+	+	+	+
Fixed oil	-	+	+	-	-

note: + = present, - = absent

From the table 2, it was evident that a wide range of active compounds like alkaloids, flavonoids, glycosides, phenols, tannins, steroids, saponins, anthraquinone, catechin, coumarin, quinine, sugar, terpenoids and xanthoproteins were present in the methanol and ethanol extract of leaf of *C.ternatea*. Whereas in other solvents like petroleum ether and chloroform, compounds like alkaloids, catechin, saponins, steroids, terpenoids and other biologically active compounds were absent in the test sample.

FT-IR spectroscopy study

FT-IR spectroscopy was used to detect the characteristic peaks and their functional groups of plant extracts. The crude extract of leaf of *C.ternatea* was passed to FT-IR analysis. The functional groups of the components were separated based on its peak ratio. The FT-IR spectrum chromatogram profile of ethanolic extract of leaf of *C.ternatea* was illustrated in the figure 1. The characteristic absorption peak was shown in table 3 and FT-IR peak values and functional groups were depicted in table 4.

Figure 1
FT-IR chromatogram of ethanolic extract of *C.ternatea* leaf

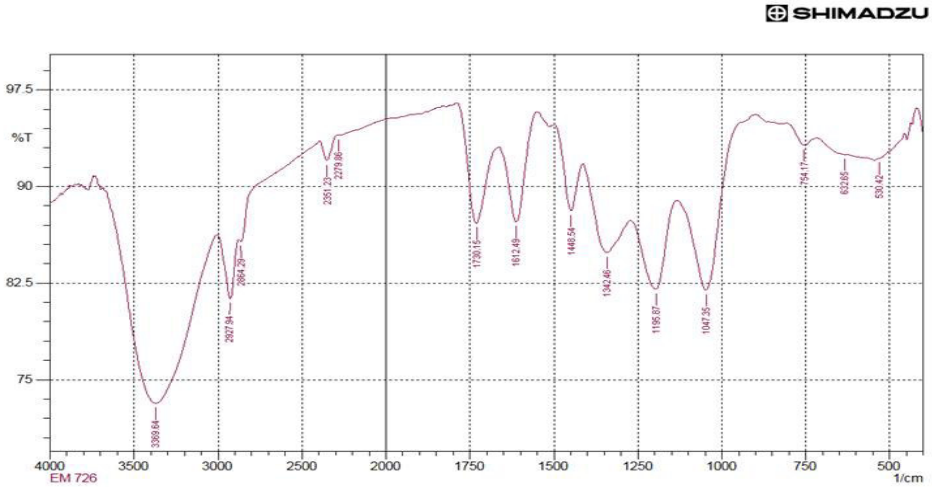


Table 3
Characteristic absorption peak of ethanolic extract of *C.ternatea* leaf

	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	530.42	92.169	0.049	532.35	457.13	2.394	0.074
2	632.65	92.468	0.107	717.52	626.87	2.879	0.073
3	754.17	93.206	1.021	806.25	719.45	2.397	0.196
4	1047.35	81.948	9.387	1132.21	898.83	11.649	3.596
5	1195.87	82.021	6.187	1271.09	1134.14	9.619	2.11
6	1342.46	84.86	4.668	1413.82	1273.02	8.547	1.764
7	1448.54	88.134	4.817	1498.69	1415.75	3.406	0.879
8	1612.49	87.244	7.03	1662.64	1550.77	4.48	1.696
9	1730.15	87.136	7.733	1788.01	1664.57	4.96	2.053
10	2279.86	93.961	0.076	2291.43	2052.26	5.954	0.085
11	2351.23	91.997	1.73	2393.66	2293.36	3.203	0.399
12	2864.29	85.714	0.37	2875.86	2395.59	19.729	0.034
13	2927.94	81.286	4.753	3007.02	2877.79	9.843	1.426
14	3369.64	73.168	14.935	3670.54	3008.95	66.14	29.174

Table 4
FT-IR peak values and functional groups of ethanolic extract of leaf of *C.ternatea*

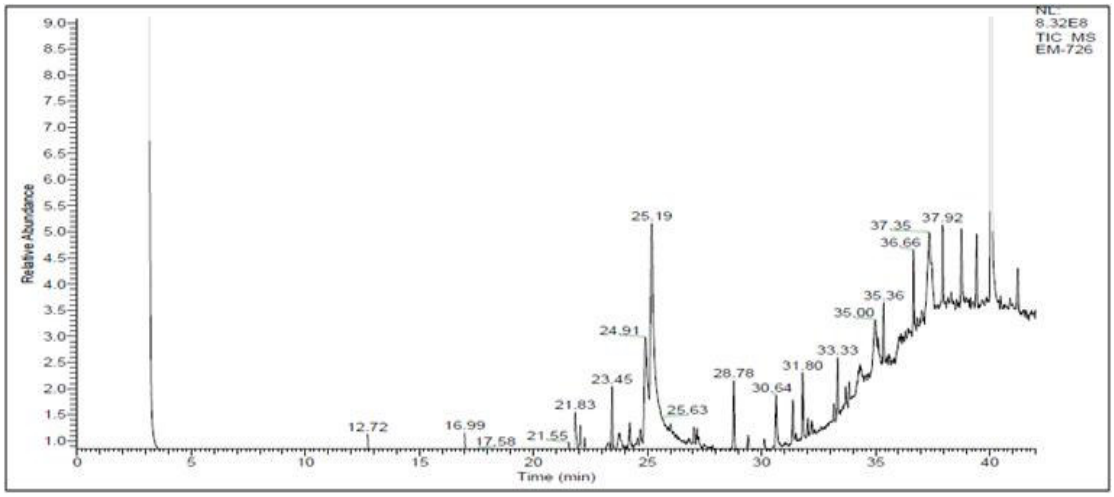
Bond/ stretching	Frequency (cm^{-1})	Functional groups
O-H stretch	3369.64	Alcohols and phenolics
C-H stretch	2927.94 & 2864.29	Alkanes
C-O stretch	1342.46, 1195.87 & 1047.35	Primary alcohols/ phenolics
C-H deformation	1448.54 & 632.65	Alkynes
C≡C stretch	2279.86	Alkynes
C=O stretch	1730.15 & 1612.49	Ketones

The FT-IT spectrum exhibited strong absorption bands at IR (kbr)υ cm^{-1} [bt]. From the table 4, alcohols and phenolics (O-H stretching) showed peak at 3369.64cm^{-1}, alkanes (C-H stretching) showed peaks at 2927.94 and 2864.29 cm^{-1}, alkynes (C-H deformation and C≡C stretching) were noticed in leaf of *C.ternatea*.

GC-MS analysis

Different phytochemical compounds of the ethanolic extract of leaf of *C.ternatea* was examined by using GC-MS. The chromatogram of the extract was revealed in figure 2 and summarized in table 5.

Figure 2
GC-MS chromotogram of ethanolic extract of *C.ternatea* leaf



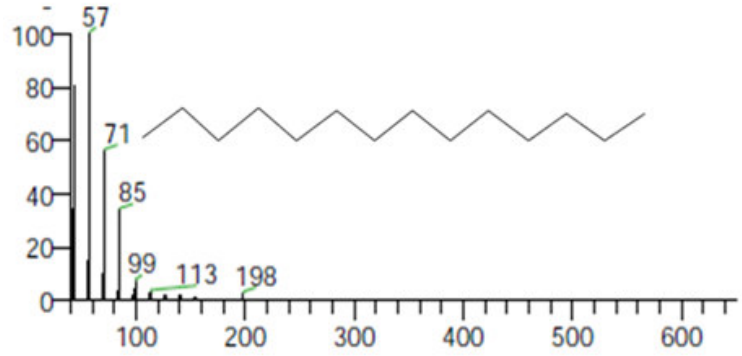
GC-MS chromatogram of *C.ternatea* leaf extract showed 14 peaks indicated the presence of 14 phytochemical constituents (Figure 2). Table 5 revealed that the percentage of major bioactive compounds such as di-(2-ethylhexyl) phthalate (53.06%), (2S,5S)-2,5-dimethyl-2-phenyl-1,3-dioxolane-4-one (14.39%), 5,10-dihexyl-5,10-dihydroindolo (3,2-b) indole-2,7 dicarbaldehyde (4.59%), 9,12-octadecadienoic acid (Z,Z), 2-hydroxy-1-(hydroxyl methyl) ethyl ester (CAS) (3.01%), neophytadiene (1.94%) and nonacosane (CAS) (1.64%) were found as the major compounds in the ethanolic extract of leaf of *C.ternatea.*

Table 5
Bioactive compounds present in ethanolic extract of *C.ternatea* leaf

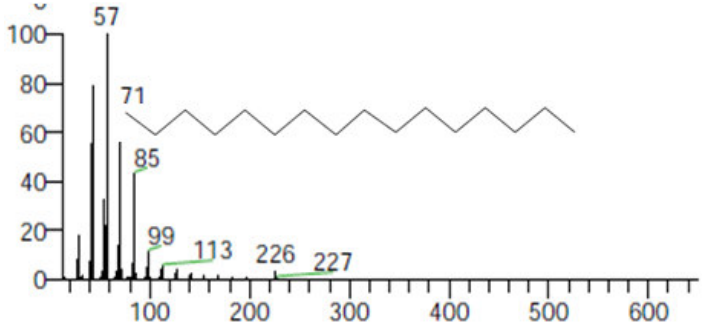
S. No.	Compounds	Retention time (min)	Peak area %	Molecular Formula	Probability
1.	Tetradecane (CAS)	12.74	0.71	$C_{14}H_{30}$	38.89
2.	Hexadecane	16.99	0.55	$C_{16}H_{34}$	41.85
3.	À-methoxycarbonyl-2-vinylstilbene	21.85	1.28	$C_{18}H_{16}O_2$	76.34
4.	Neophytadiene	23.45	1.94	$C_{20}H_{38}$	25.17
5.	(2S,5S)-2,5-dimethyl-2-phenyl-1,3-dioxolane-4-one	25.19	14.39	$C_{11}H_{12}O_3$	34.76
6.	Hexadecanoic acid, ethyl ester (CAS)	28.78	1.64	$C_{18}H_{36}O_2$	77.76
7.	5-(ethoxycarbonyl)-6-methyl-4-isopropyl-3,4-dihydropyrimidin-2(1H)-one	30.64	1.36	$C_{11}H_{18}N_2O_3$	51.78
8.	Phytol	31.80	1.10	$C_{20}H_{40}O$	59.77
9.	7,8,17,18-tetrahydro-35-methoxy-1,3,21,23-tetramethyl-16H,31H-5,9,15,19-dimethano-10,14-methano-26,30-nitrilo-6H,25H-dibenzo(b,s)(1,21,4,8,14,18) dioxatetraazacyclooctacosine	33.33	0.67	$C_{38}H_{41}N_5O_5$	60.41
10.	9,12-octadecadienoic acid (Z,Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester (CAS)	34.98	3.01	$C_{21}H_{38}O_4$	41.93
11.	Nonacosane (CAS)	35.36	1.64	$C_{29}H_{60}$	10.75
12.	Pentacosane	36.66	1.06	$C_{25}H_{52}$	22.60
13.	5,10-dihexyl-5,10-dihydroindolo(3,2-b)indole-2,7-dicarbaldehyde	37.35	4.59	$C_{28}H_{34}N_2O_2$	39.27
14.	Di-(2-ethylhexyl) phthalate	40.08	53.06	$C_{24}H_{38}O_4$	39.66

GC-MS mass spectra detected some bioactive compounds from ethanolic extract of *C.ternatea* leaf. Mass matching of the spectrums of prominent compounds present in the experimental sample was done with the spectrum of standard compounds of NIST library as shown in figure 3.

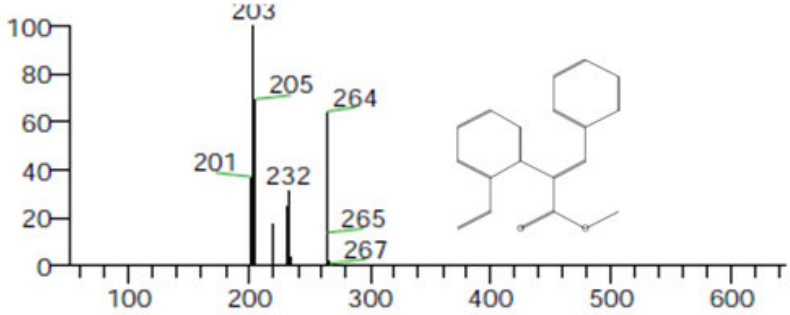
Figure 3
GC-MS mass spectra of a few compounds detected from ethanolic extract of *C.ternatea* leaf



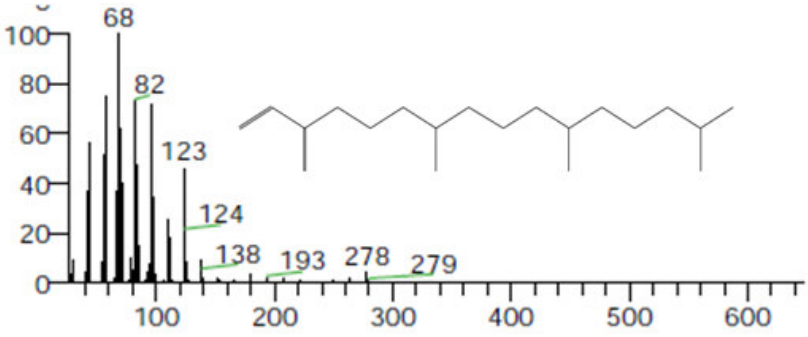
A) mass spectra of tetradecane ($C_{14}H_{30}$)



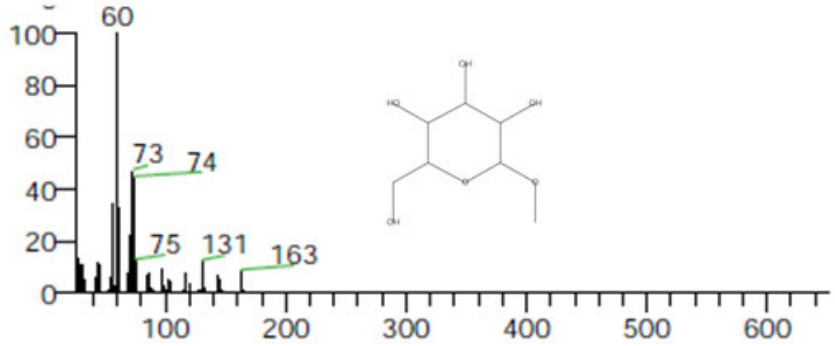
B) mass spectra of hexadecane ($C_{16}H_{34}$)



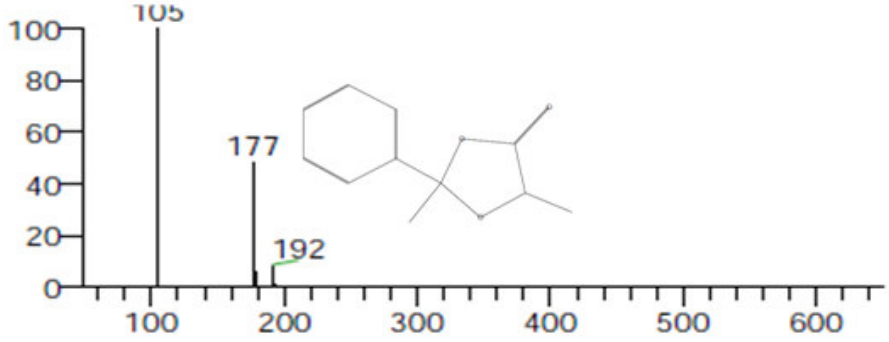
C) mass spectra of α-methoxycarbonyl-2-vinylstilbene ($C_{18}H_{16}O_2$)



D) mass spectra of neophytadiene ($C_{20}H_{38}$)



E) Mass spectra of α-D-glucopyranoside, methyl (CAS) $C_{17}H_{14}O_6$)



F) mass spectra of (2S,5S)-2,5-dimethyl-2-phenyl-1,3-dioxolane-4-one ($C_{11}H_{12}O_3$)

DISCUSSIONS

Fluorescence analysis

Fluorescence analysis is effectively sensitive which can be used to characterize the crude drugs[25]. The fluorescent colour is specific for each compound. Plant material gives different coloration when treated with various chemicals. The crude drug when viewed under UV light showed different fluorescence at different wavelengths. This is due to the presence of different phytochemical constituents in the drug[26,27]. Flavonones which are light yellow in aqueous condition, under UV light, turns to bright yellow under alkaline conditions. Phytosterols, when treated with 50% H_2SO_4 shows green fluorescence under UV light. Coumaric acid appears yellowish green in alkaline condition under short UV radiation. In the same way terpenoids, especially sapogenins, exhibits yellow green fluorescence under short UV light[28]. Quinine, aconitin, berberin and emetin show specific colours of fluorescence (aconitin - light blue; berberin - light yellow; emetin - orange). Fixed oils and fats show the least fluorescence[29]. In the present study, the fluorescent analysis clearly showed that the powdered leaf of *C.ternatea* exhibited clear fluorescence behaviours at different radiations. The major bioactive compounds present in the crude drugs were found to be coumarins, flavonoids, tannins, alkaloids, steroids and saponins.

Preliminary phytochemical screening

Crude extracts and medicines manufactured on the principles of natural compounds even by pharmaceutical companies, may lead to large scale exposure of humans to natural products. The first step towards this goal is phytochemical analysis of plant extracts from traditional preparations used in popular medicine[30]. In the present study, occurrence of wide range of active phytochemical compounds such as alkaloids, anthraquinone, catechins, coumarins, flavonoids, glycosides, phenols, quinones, saponins, steroids, sugars, tannins and xanthoproteins were seen in methonal and ethanol extracts when compared to other solvents indicated that these compounds were not disturbed during the preparation and storage in the former solvents. Ethanol is one of the good solvents in plant extractions which include low toxicity, inability to cause the extract to complex or dissociate easy evaporation at low heat, preservative action and stable. Hence the ethanol extract of leaf of *C.ternatea* was used for further investigation which includes identification of pharmacologically active phytochemical compounds.

FT-IR *spectroscopy study*

FT-IR is proved to be a reliable and sensitive method used for identification of different bonds. Secondary metabolites functional groups were separated based on its peak values in the region of infrared radiation[31]. The spectra showed substantial overlap of each absorption spectrum of various components, each band represents an overall overlap of some characteristic absorption peaks of functional groups in the test sample. In the current study, the FT-IR analysis of ethanolic extract of leaf of *C.ternatea* showed different peaks. It confirmed the presence of different functional groups such as alcohols, phenolics, primary alcohols, alkanes, alkynes and ketones which might be a perfect product for any type of pharmaceutical applications. The above infrared functional group characteristics were cited in literature[32,33].

GC-MS analysis

GC-MS analysis is used to determine whether the plant species contains any individual compound or group of compounds and also used to understanding the nature of active principles in medicinal plants. The heights of the peak indicate the relative concentrations of the components present in the extract. The spectrum profile of GC-MS confirmed the presence of major components with their retention time. On comparison of the mass spectra of the constituent with the NIST library, the phytochemicals were characterized and identified. In the present study, among the identified phytochemicals in the investigated sample, hexadecanoic acid, ethyl ester (CAS) has antioxidant property and hypocholesterolemic activity. 9, 12-octadecadienoic acid (Z,Z)- has hypocholesterolemic, hepatoprotective and cancer preventive activity. Using Dr.Duke's phytochemical and ethnobotanical database (online), the biological activity of the identified phytocomponents were ascertained.

CONCLUSION

Medicinal plants are used for screening of the secondary metabolites in research institutes which proves to be helpful in pharmaceutical companies for manufacturing of new drugs for the treatment of various diseases and disorders. From this study, it can be concluded that the leaf of *C.ternatea* contains different phytochemicals, which might be a perfect product for anti-hyperglycemic and anti-hyperlipidemic activities.

CONFLICT OF INTEREST

Conflict of interest declared none.

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