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Original Article

SEPARATION AND IDENTIFICATION OF PHYTOCHEMICALS FROM *LILIUM POLYPHYLLUM* D. DON (*KSHIRKAKOLI*), AN INGREDIENT OF *ASHTAVARGA*

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Abstract: The primary goal of present study was qualitative analysis and mass detection of Lilium polyphyllum D.Don (Kshirkakoli) with antiaging ethno-botanical records in northern India. The dried root samples were extracted with 9:1 methanol/water (MeOH/H_2O) solution. Sepbox 2D-2000 a multicolumn 2D-HPLC was used to separate the root extract with C 18 analytical column and mobile phase consisting acetonitrile (ACN)/MeOH/H_2O for each gradient resulted in 103 fraction. The method provides rapid and reproducible results. Screening was carried out by UV detector data. Among them 3 fractions were selected for gas chromatography/mass spectrometry (GC/MS). Total 26 compounds with different similarity index (SI) were identified through GC/ MS analysis. Highest similarity index (89%) of R-(-)-Cyclohexylethylamine and lowest similarity index (71%) of Macdougallin was observed.

Key word: *Lilium polyphyllum D.Don*, Sepbox-2000

INTRODUCTION

Growing awareness of harmful side effects of modern medicine has led to interest in medicinal plants all over the world [1]. *Ashtavarga* include eight medicinal planta viz., *Jeevak* (*Malaxis acuminata* D.Don), *Rishbhak* (*Malaxis muscifera* (Lindley) Kuntze), *Meda* (*Polygonatum verticillatum* (L.) All.), *Mahameda* (*Polygonatum cirrhifolium* (Wall.) Royle), *Kakoli* (*Roscoea procera* Wall.), *Kshirkakoli* (*Lilium polyphyllum* D.Don), *Riddhi (Habenaria edgeworthii* H.f.*)*, *Vriddhi (Habenaria intermedia* D.Don). All of these plants have their natural habitats in Himalaya particularly the North-West Himalaya in Jammu & Kashmir, Uttarakhand and Himachal Pradesh between elevations of 1500-4000 m. Their natural habitats are specific in ecological environment and hence these occur only in small pockets. *Ashtavarga* is important ingredient of various *Ayurvedic* formulations such as *Chyavanprasha* [2]. It is cooling, tasty, nutritious tonic, aphrodisiac, nourishes body and increases *kapha*. They are beneficial in seminal weakness, increases fat in the body, heals bone fracture and cures *vata*, *pitta*, and *rakta doshas*, abnormal thirst, burning sensation in the body, consumption, fever and diabetic condition. It is one of the excellent combinations of herbal drugs which restores health immediately, strengthens immunity system and rectifies defects in anabolism or body growth processes and works as antioxidant in the body. That is why *Aswani Kumars* invented it for curing the frail, emaciated sick body of *Chayavan Rishi* who regained youthful condition as it is documented [3]. *Lilium polyphyllum D.Don* (*Kshirkakoli*) is generally found in open slopes, grassy lands in forests. It is rare and occurs between 1800-3800 m altitudes. Its leaves are scaly. The plant

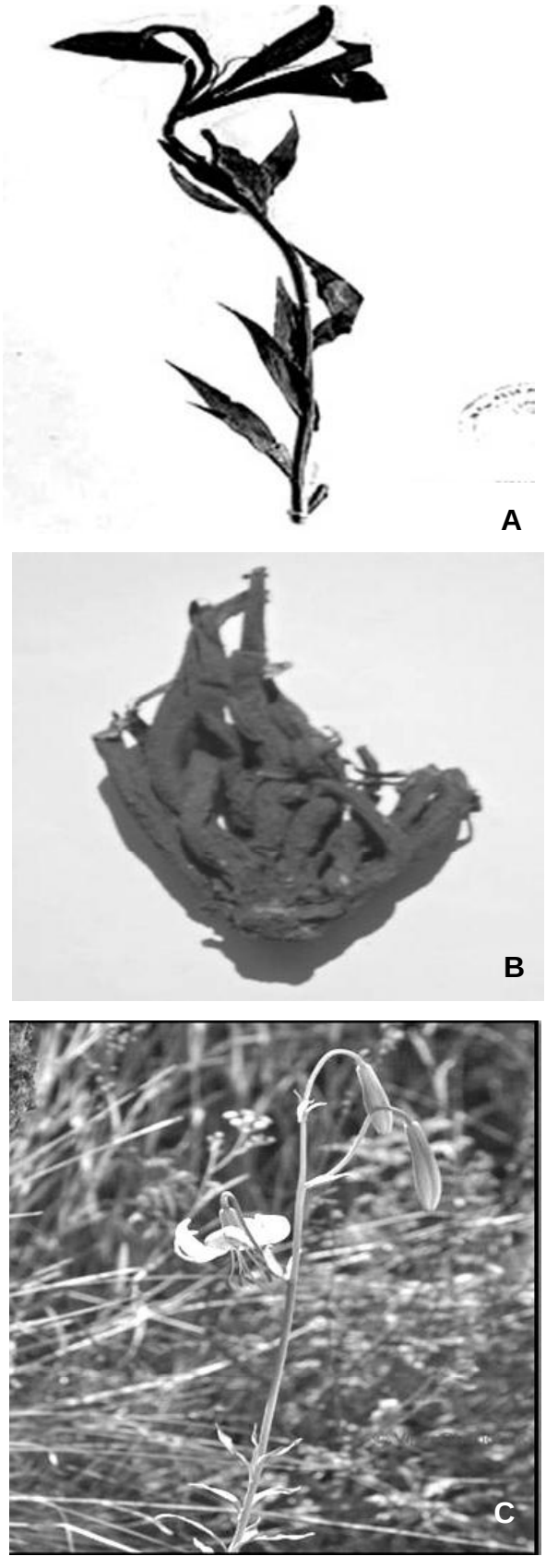

Fig. 1: Dry root of *Lilium polyphyllum* D.Don (*Kshirkakoli*) with its morphology: A, B, C, Photo from herbarium, roots (economic part), morphology (Fresh plant), respectively.

originates from bulbs. Stems are 30-100 cm high, erect (Fig. 1) [4]. It is Sweet in taste, cold in potency, pacifies *vata* and *pitta*. It is cooling and spermopiotic. It has soothing, astringent and anti-inflammatory properties. The bulbs are sweet, bitter, refrigerant, galactagogue, expectorant, aphrodisiac, diuretic, antipyretic and tonic. It is beneficial in fever, burning sensation and phthisis. Also they are useful in agalatia, cough, bronchitis, vitiated conditions of *pitta*, seminal weakness, strangury, hyperdipsia, intermittent fevers, hematemesis, rheumatalgia and general disability [3]. The *Kshirkakoli* is an endangered species and it is very important to know the active ingredients present in this plant because of its high medicinal value. So far, no detailed study of these active ingredients (phytochemicals) has been done. Therefore, the present investigation was conducted to characterize the molecular mass of these compounds.

MATERIALS AND METHODS

Plant material: The roots of *Kshirkakoli* were collected from forest region of Himalaya at the altitudes ranging from 1800-3800 m with the help of tribal people. The detail taxonomy of this plant was worked out from literature. (Fig 1).

Extraction and isolation: The roots were cut into small pieces, air-dried at room temperature in a dust-free environment and ground with laboratory grinder at 35°C. After determination of solubility, methanol and water were selected as solvent for extraction. The powdered roots were dissolved in 90% MeOH as per the ratio of 100mg/1.5ml solvent. The solution was filtered through 0.2 micron filter paper to ensure that no particles were present in solution and placed in 100 ml round- bottomed flask in rotary evaporator (Ingos, Czech Republic) for evaporation of solvents, the rpm was not more than 90. An optimum value was 60rpm. The remaining moisture was removed by lyophilizing (Operon, Korea) the extract at low temperature [1].

(a) Sample preparation and injection column packing: For column preparation, 2 gm of fine extract powder with 4 gm pure silica (from Sepiatec, Germany) was mixed through rotary evaporator. Fine powder of sample was used for injection column packing. Free-flowing dry mixture transferred carefully into column with one end sealed, remaining column was filled up with silica, with knocking

Table 1: Sepbox and multimode reader data of various fractions. *Abbreviations:* UV, ultraviolet; mAU, milliampaier unit; min, minute; Abs, absorbance; λ max, absorption maxima; nm, nano meter.

Sepbox report							Multimode reader data	
Fraction no.	Start [min]	Stop [min]	Area UV [mAU]	Water (%)	Methanol (%)	Acetonitrile (%)	Abs	λ max [nm]
73	4.15	5.03	3.111	78	-	22	3.7991	200
92	4.05	7.43	15.579	62	-	38	3.8641	510
93	7.45	7.73	1.385	62	-	38	3.9538	550
94	7.75	9.53	6.535	62	-	38	3.9920	580
95	9.55	10.30	1.593	62	-	38	3.9278	500
96	10.33	11.53	2.361	62	-	38	3.9107	440
97	11.55	11.92	0.247	62	-	38	3.9699	200
98	11.93	12.67	0.439	62	-	38	3.9295	690
99	12.68	16.07	0.696	62	-	38	3.7740	700
100	16.08	16.43	0.038	62	-	38	3.8500	290
101	21.48	21.85	0.036	62	-	38	3.9863	660
102	66.62	70.00	0.344	-	50	50	3.8922	410
103	70.02	71.63	0.149	-	50	50	3.9710	200

Table 2: List of phytochemicals isolated and identified from *Lilium polyphyllum* D. Don (*Kshirkakoli*). Abbreviations*:* SI-similarity index; g/mol - Gram per moles; RT - retention time; min - minute, MW- molecular weight.

S.N.	Fraction No.	Name of the Compound	SI %	Chemical Formula	MW g/mol	Chemical Nature	RT (min)
1	73	1-(5-Bicyclo[2.2.1]heptyl) ethylamine	80	$C_9H_{17}N$	139.23	Chiral amine	25.95
2	73	R-(-)-Cyclohexylethylamine	89	$C_8H_{17}N$	127.23	Chiral amine	26.63
3	73	(S)-(+)-1-Cyclohexylethylamine	82	$C_8H_{17}N$	127.23	Chiral amine	27.84
4	73	8α-Acetoxyelemol	81	$C_{17}H_{28}O_3$	280.40	Oxygenated sesquiterpene	42.59
5	73	Pregan-20-one, 2-hydroxy-5,6-epoxy-15-methyl	78	$C_{22}H_{34}O_3$	346.50	Steroid	42.87
6	73	Tris(dimethylamino) methane	77	$C_7H_{19}N_3$	145.25	Cycloaminoalkane	43.39
7	73	1-(3,5-Dimethyl-1-adamantanoyl) emicarbazide	73	$C_{14}H_{23}N_3O_2$	265.35	Adamantane derivative	43.93
8	73	alpha-Methyl-1-adamantanemethylamine	73	$C_{12}H_{21}N$	179.30	Adamantane derivative	44.34
9	92	Estran-3-one, 17-(acetyloxy)-2-methyl, (2 alpha, 5 alpha, 17 beta)	75	$C_{21}H_{32}O_3$	332.47	Steroid	37.02
10	92	1,3-Adamantanediacetamide	74	$C_{14}H_{22}N_2O_2$	250.33	Adamantane derivative	38.05
11	92	Eudesmol	76	$C_{15}H_{26}O$	222.37	Oxygenated sesquiterpene	39.20
12	92	Palustrol	78	$C_{15}H_{26}O$	222.37	Sesquiterpene	39.38
13	92	Columbin	75	$C_{20}H_{22}O_6$	358.38	Diterpenoidfuranolactone	40.00
14	92	1-(3,5-Dimethyl-1-adamantanoyl) semicarbazide	75	$C_{14}H_{23}N_3O_2$	265.35	Adamantane derivative	42.84
15	92	Macdougallin	71	$C_{28}H_{48}O_2$	416.67	Sterol	56.16
16	94	7-Oxabicyclo[4.1.0]heptane, 1-methyl-4-(2-methyloxiranyl)	77	$C_{10}H_{16}O_2$	168.23	Oxygenated monoterpene	37.22
17	94	Veridiflorol	79	$C_{15}H_{26}O$	222.37	Sesquiterpenic alcohol	38.33
18	94	(1S,2E,4S,5R,7E,11E)-Cembra-2,7,11-trien-4,5-diol	73	$C_{20}H_{34}O_2$	306.48	Cembranoid	38.87
19	94	cis-Z-.alpha.-Bisabolene epoxide	84	$C_{15}H_{24}O$	220.35	Oxygenated Sesquiterpene	38.97
20	94	Kessane	80	$C_{15}H_{26}O$	222.37	Sesquiterpene	39.33
21	94	Thujopsan-2-alpha-ol	80	$C_{15}H_{26}O$	222.37	Oxygenated sesquiterpenes	39.42
22	94	Longipinanol	83	$C_{15}H_{26}O$	222.37	Oxygenated sesquiterpene	39.68
23	94	Columbin	76	$C_{20}H_{22}O_6$	358.38	Diterpenoidfuranolactone	42.01
24	94	Docosahexaenoic acid, 1,2,3-propanetriyl ester	72	$C_{69}H_{98}O_6$	1023.51	Fattyacyl ester	49.19
25	94	Caryophyllene<14-hydroxy-9-epi-(e)>	74	$C_{15}H_{24}O$	220.35	Oxygenated sesquiterpenes	51.02
26	94	1-Heptatriacotanol	81	$C_{37}H_{76}O$	536.99	Alcoholic compound	55.03

carefully against column to allow that material to settle. The column was then packed and connected to valve 4, to which injection column was connected with the Sepbox 2D-2000.

(b) Separation of phytochemicals: The Sepbox concept is based on combination of HPLC and solid-phase extraction (SPE). The sample introduced via injection loop and separated at separation column 1 using a low pressure gradient. Water, methanol and acetonitrile were used as a mobile phase in gradient run. The polarity of the eluent from separation column 1 was increased by the addition of the water to such an extent that the fraction eluted from separation column 1 was adsorbed onto the 15 non-polar trap columns. The trapped fraction was then passed through column 2 where the final separation was to be completed. Different components were

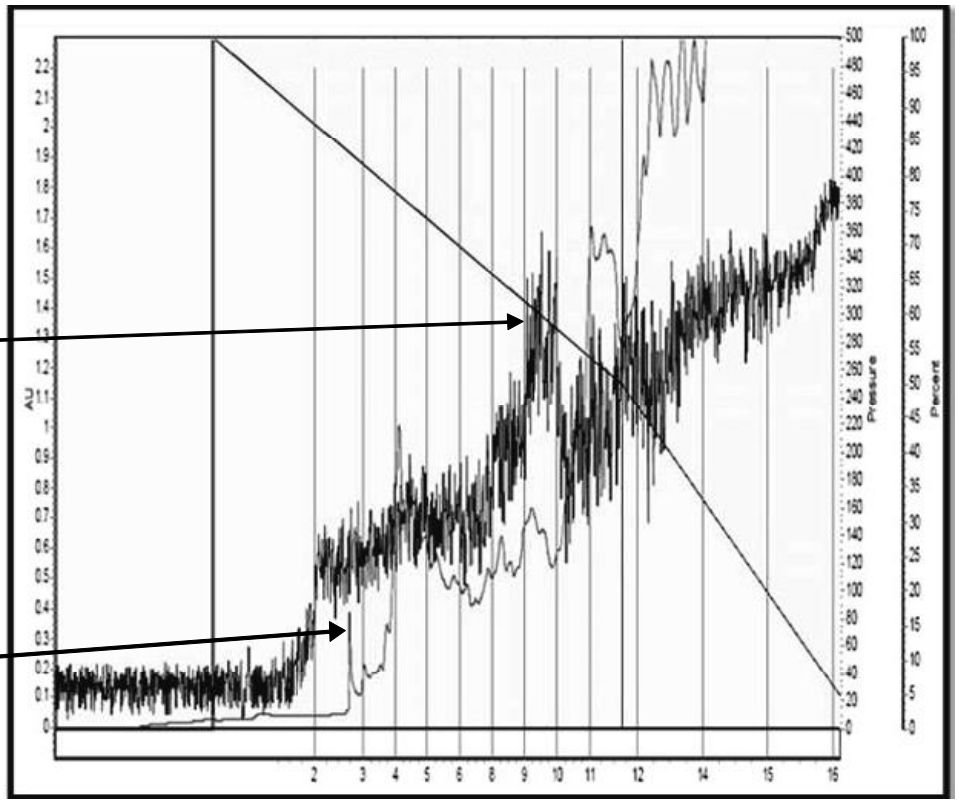

Fig. 2: The main separation chromatogram of *Lilium polyphyllum* D.Don (*Kshirkakoli*).

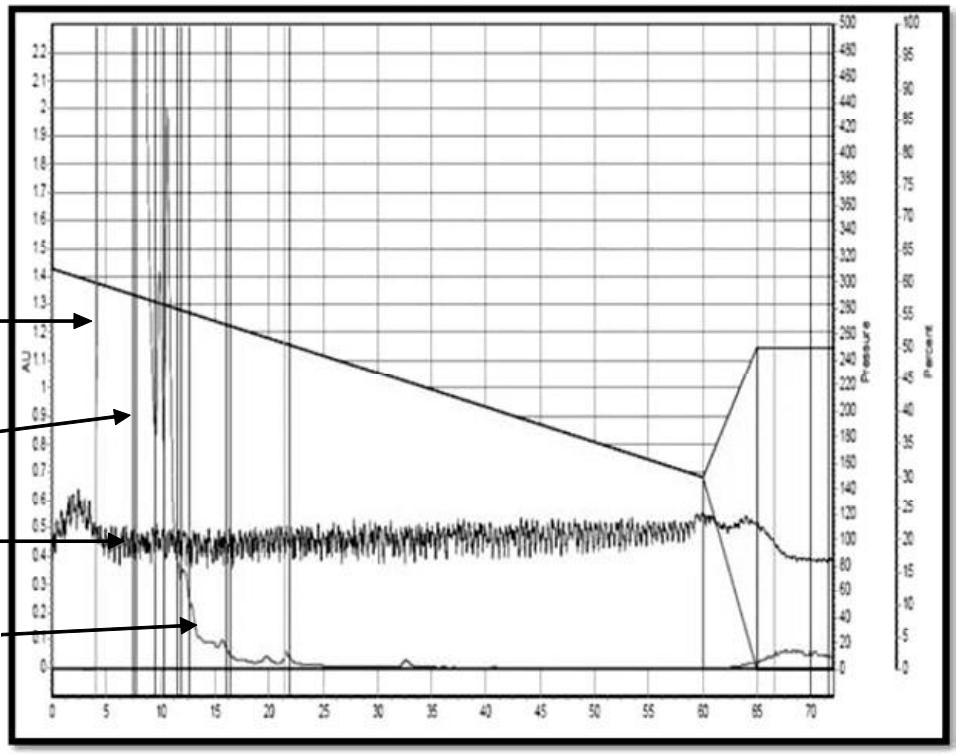

Fig. 3: Second separation chromatogrm of methanolic extract of root powder of *Kshirkakoli* in Column 12; step 10.

eluted and fractions were collected in vials using fraction collector. The method was programmed by method edit software with fraction parameter as described in earlier literature [1, 5, 6, 7].

Screening of fractions: Fractions were plated in 96 vial plate for spectral maxima analysis with 200-700 nm wavelength, using skanIt software in multimode reader for screening. Fractions of different colour, different absorption maxima were screened for GC/MS analysis. Also, the fractions with higher volume and higher UV area were selected for GC/MS analysis [1].

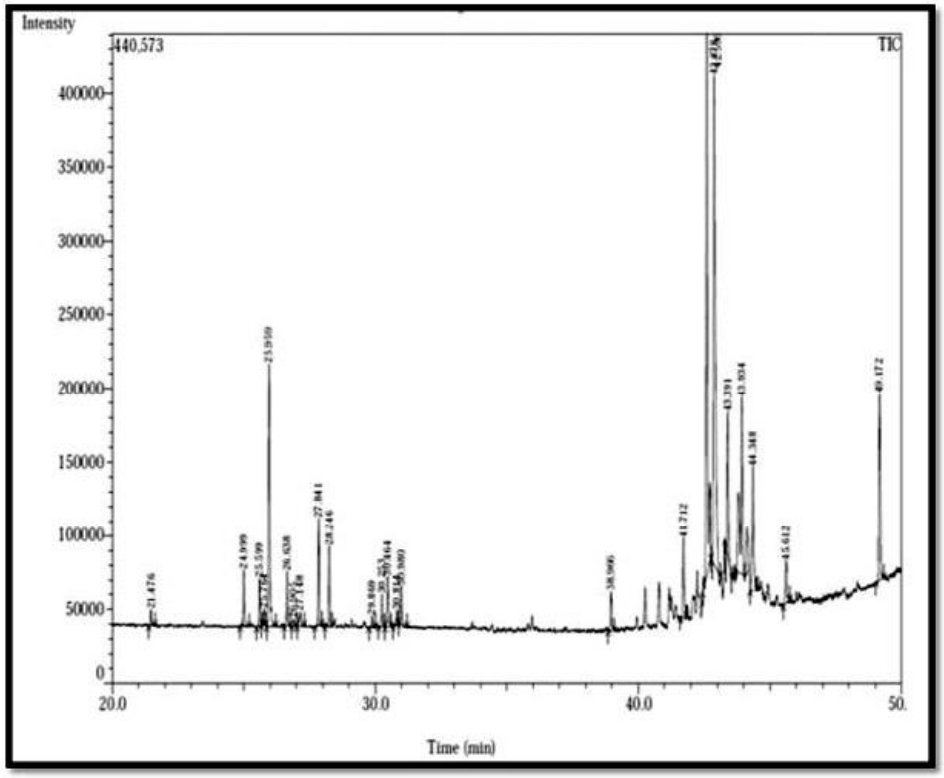

Fig. 4: GC chromatogram of fraction no. 73.

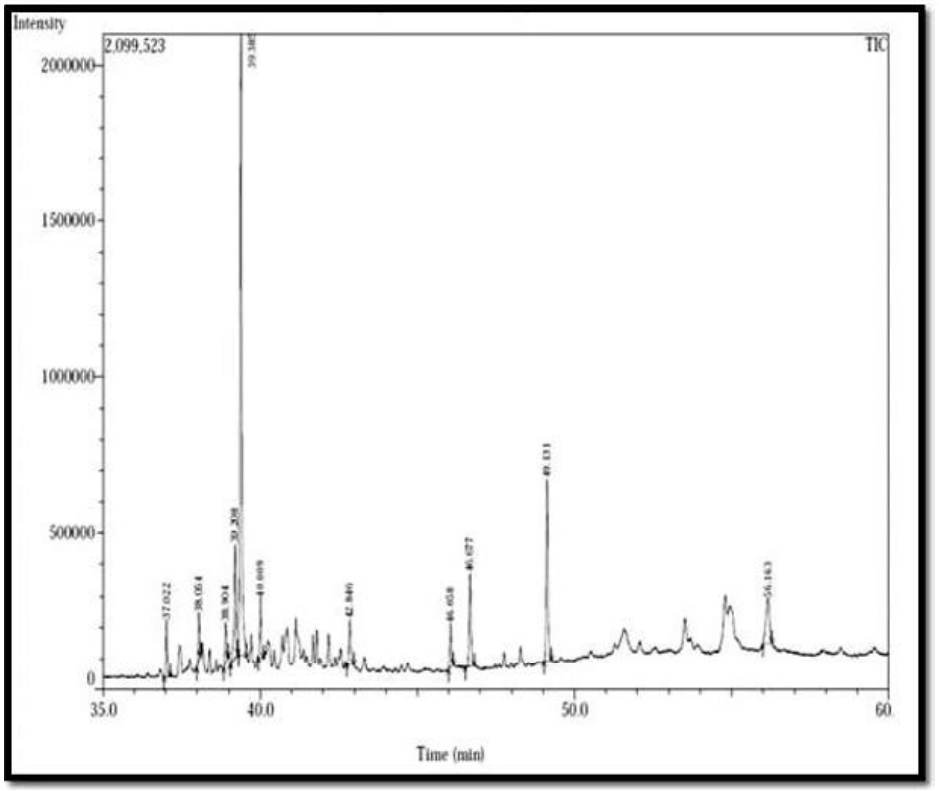

Fig. 5: GC chromatogram of fraction no. 92**.**

Identification of phytochemicals by GC/MS analysis:

For identifica-tion of 3 fractions, GC/MS (Shimadzu GC-2010 Plus, column- Rtx-5 MS, 30 m × 0.25 mm) was used. Fractions were lyophilized in order to concentrate, and then dissolved in very low amount solvent in which they were eluted. After setup of GC/ MS parameter, 2 µl of sample was injected to GC and spectra observed. The data were analyzed after completion of sample run; the desired peak was subjected to MS libraries (Mass range- m/z 1.5- 1090) for identification [8].

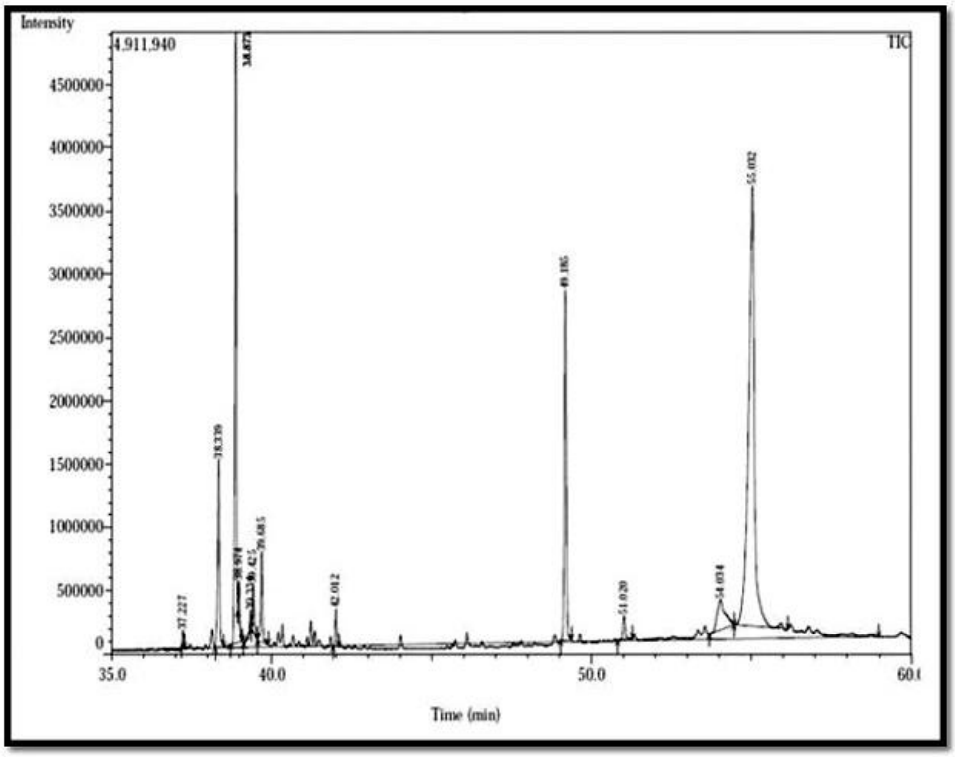

Fig. 6: GC chromatogram of fraction no. 94.

RESULTS

Separation of phytochemicals and screening of fractions: Dried roots of *L. polyphyllum* were extracted to yield a 90% MeOH extract, which was injected to Sepbox 2D-2000 for sepatation of phytochemicals**.** Through Sepbox 2D-2000, 103 fractions were collected from the root extract of *Lilium polyphyllum* D.Don (*Kshirkakoli*) within 24 hour. Sepbox was proved standard technology for the separation of compounds from plant root extract because it allowed processing sample automatically and would make up to 30 times faster than by using a conventional process. After completion of main separation (Fig. 2), according to trap column, 10 columns were selected for the second separation. Detail results (not whole) of second separation of methanolic extract of root powder of *Kshirkakoli* in step 10, column 12 from Sepbox chromatogram (Fig. 3) and Multimode reader data are given (Table 1). Out of 103 fractions, only 3 fractions (i.e. fraction no. 73, 92 and 94) were selected based on area under UV, absorption maxima (Table 1) and also as per volume and color of fraction. In case of multimode reader, all selected fractions have absorbance in the range of 3.68 to 3.99.

Identification of phytochemicals by GC/MS analysis: Selected 3 fractions (73, 92 and 94) separated by Sepbox were subjected for (GC-MS) analysis. Total twenty four peaks were observed by GC analysis of fraction no.73 (Fig 4). While eleven peaks from fraction no.92 (Fig 5) and twelve peaks from fraction no. 94 (Fig 6) were observed by GC analysis. However, only sharp peaks were taken into consideration for identification of compounds using MS. A total of twenty six phytochemical were identified from three selected fractions i.e. fraction no.73, 92 and 94 of *Lilium polyphyllum* D. Don (*Kshirkakoli*) by (GC-MS) with different similarity index. Highest similarity index was 89 % of R-(-)-Cyclohexylethylamine and lowest was 71 % of Macdougallin (Table 2).

DISCUSSION

All of the above compounds, listed in table 2, contain medicinal and therapeutic values. 7-oxabicyclo [4.1.0]heptane, 1-methyl-4-(2-methyloxiranyl) contains anti-microbial and anxiolytic activity. (S)-(+)-1-Cyclohexylethylamine contains anti-cancer activity, stimulates the central nervous system, accelerates the respiratory movements [9].

Longipinanol contains anti-microbial activity [10]. Medicinal properties of Pregan-20-one, 2-hydroxy-5,6-epoxy-15-methyl are not clearly known but other compounds of this chemical nature found neuroactive steroids.

The notable medicinal properties of columbin are anti-diabetic, anti-periodic, anti-spasmodic, anti-inflammatory, anti-arthritic, anti-oxidant, anti-allergic, anti-stress, anti-leprotic, anti-malarial, hepatoprotective, immunomodulatory and anti-neoplastic activities [11]. Palustrol contains anti-fungal, anti-bacterial and anti-convulsant activities. Eudesmol inhibits potassium– ATPase activity, used for treatment of urinary and colon diseases, possess antimutagenic activity, also has potentiating effect on succinylcholine-induced neuromuscular blockade. It also modifies neuronal functions [12, 13]. Veridiflorol contains anti-inflammatory, antispasmodic and diuretic, antibacterial, anti-tumor, anti-spasmodic, anti-listerial activity [14,15]. Macdougallin has anti-inflammatory activity, cytotoxic activity against several human cancer cell lines [16]. cis-Z-.alpha.-Bisabolene epoxide acts as a pheromone [17]. Kessane induces sleeping time in rodents and also contains anti-microbial activity. Medicinal properties of 1-(5-bicyclo[2.2.1]heptyl)-ethylamine are not clearly known but other compounds of this chemical nature found antimicrobial agents, as well as algae inhibitors. They are especially useful as hard surface disinfectants and as additives to oil well drilling muds, injection brines and industrial waters where microbial control is desired.

Also, chiral amine derivatives are useful for binding to histamine H_3 receptor sites and for providing therapeutic agents for histamine H_3 mediated diseases. Generally, Tris (dimethylamino) methane is not intended for diagnostic or therapeutic use. It is used for research only. Medicinal properties of 1-(3,5-Dimethyl-1-adamantanoyl) semicarbazide are not clearly known but other compounds of this chemical nature found to contain anti *influenza virus* activity. 1-Adamantanemethylamine, alpha,-methyl is a stronger inhibitor of *Sindbis virus* reproduction. Its hydrochloride formation at concentrations of 10-25 mg/ml depresses the RNA-dependent RNA polymerase induction in a culture of cells infected with *Influenza virus* (*fowl plague virus*). Furthermore, its hydrochloride formation inhibits the in vitro proliferative response of human peripheral blood lymphocytes to mitogenic and antigenic stimulation [18,19].

Thujopsan-2-alpha-ol contains anti-microbial activity and other related compound to it, contains anti-inflammatory, healing, and antiseptic activities. Thus most of the compouds identified by (GC-MS) are having antioxidant, anti-microbial activity and other therapeutic values. To the best of our knowledge, there is no chemical investigation for the chemical profile of *Lilium polyphyllum* although it is used one of components of *Ashtavarga* in *Ayurveda* since ancient. Identified compounds may be further validated by authentic standards.

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