International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2019; 7(1): 1691-1696

Received: 10-11-2018
Accepted: 13-12-2018

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Phytochemical analysis, *in-vitro* antioxidant, anti-inflammatory and insect antifeeding activity of methanolic extract of *Limnophila indica* (L.) Druce

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Abstract
Present research reports the chemical composition, total phenolics, antioxidant and insect antifeeding activity of the methanolic extract of the *Limnophila indica* (L.) Druce. GC-MS analysis of the extract led to identification of fifty-two components constituting 74.3% of total extract composition mainly dominated by β-monolinolein (12.9%) and linoelaidic acid (11.6%). The extract possesses significant amount of total phenolics, flavonoids and ortho-dihydric phenols. Antioxidant activity assessment via NO radical scavenging, DPPH radical scavenging, metal chelating of Fe^{+2}, reducing power activity reveals the plant possesses potential antioxidant property with respective IC_{50} values 42.48±1.87, 31.98±0.59, 59.02±0.73, 53.28±1.21 µg/ml. *In-vitro* anti-inflammatory activity screening of the extract revealed the extract acting as potentially active anti-inflammatory agent with IB_{50} value 43.98±2.66. Insect antifeeding activity monitored through no choice leaf dip method against *Spilosoma obliqua* resulting dose and time dependent antifeeding activity.

Keywords: *Limnophila indica*, β-monolinolein, Antioxidant, Antifeeding, *Spilosoma obliqua*

Introduction
Limnophila indica (L.) Druce (Plantaginaceae) commonly known as Asian Marshweed is a perennial aquatic herb may be of submerged, emerged or amphibious nature. The plant is widely distributed throughout South-East Asia, tropical and subtropical regions of Africa, Australlia, North America and India having its broad spectrum use in traditional system of medicine [1, 2]. Extracts of the plant *L. indica* revealed to be rich in alkaloids, flavonoids and triterpenes constituents responsible for significant biological and pharmacological activities like anti-dysentry, antibiotics, carminative, anti-dyspesia, hepatoprotective, cytotoxic, antiseptic, anti-elephantiasis, anti-diarrheal, antacid, anti-shigella, antibacterial and antifungal [2-7].

Present study reports the systematic identification of chemical composition, quantitatively total phenolics estimation, *in-vitro* antioxidant, anti-inflammatory and insect antifeeding activity of the methanolic extracts of the aerial plant part of *L. indica*.

Materials and Methods
Collection of Plant material
The collection of the plant material was done in the month of December 2017 from Tarai region of Uttarakhand, India. The plant identification was done by Dr. D.S. Rawat, Assistant Professor (Plant Taxonomist), Department of Biological Sciences, C.B.S.H., G.B.P.U. A. & T., Pantnagar, Uttarakhand, India. The plant specimen was submitted to G. B. Pant University Herbarium, Department of Biological Sciences, CBSH, Pantnagar and provided with the herbarium voucher number GBPUH-980.

Preparation of extract
The aerial plant part of *L. indica* was extracted in methanol using cold percolation method. The extract obtained was recorded to be 102 gm from aerial plant part extract of 1.25 kg.

The extract obtained was filtered and concentrated using rotary evaporator stored at 4 ºC for further analysis and biological activity estimation.

GC-MS Analysis

The plant extract was analyzed on GCMS-QP2010 Ultra Rtx-5MS column (30m×0.25mm and film thickness 0.25µm). The column temperature was programmed for 50-210ºC at the rate of 3ºC/min and then again upto 280^{0}C at the rate of 8ºC/min. Helium gas at the rate of 1.21 ml/min column flow and 69.0 kPa pressure was used as the carrier gas at the injector temperature at 260 ºC. MS were recorded under EI condition (70 ev) with injection volume of 0.1 µL with split mode of 1:120. Identification of the constituents of the essential oil done by comparing their mass spectra fragmentation pattern and their retention indices with that of MS library (NIST14.lib, FFNSC2.lib, WILEY8.LIB) and comparing the spectra with literature data [8].

Phytochemical assay

Total phenolic assay

The total phenol content estimation of the extract was done using Folin-Ciocalteu reagent [9] with minor adjustments. Reaction mixture prepared by mixing 0.5 ml of the extract, 1ml Folin -Ciocalteu reagent, 1 ml (7%) Na_2CO_3, 5 ml distilled water and allowed to stand for 30 min. Absorbance was recorded at 765 nm. Total phenol content expressed as gallic acid equivalent (GAE) in mg/g.

Total flavonoids assay

Method developed by Choi *et al*. [10] was adopted for total flavonoids assay. 1ml of plant extract mixed with 1.25 ml of distilled water, 75µl (5%) $NaNO_3$. After incubation for 5 min 150µl (10%) $AlCl_3$, 500 µl of 1M NaOH and 275 µl were added and mixed absorbance measured at 510 nm. Total flvonoids content expressed as catechin equivalent (CNE) in mg/g.

Ortho-dihydric phenol content estimation

1 ml of extract mixed with 1ml (0.5N) HCl, 1ml Arnow's reagent, 2ml (1N) NaOH and 4.5 ml of distilled water. Absorbance measured at 515 nm. Total ortho-dihydric phenol content was expressed with catechol equivalent (CLE) in mg/g [11].

Evaluation of antioxidant activity

Nitric oxide (NO) radical scavenging activity

Standard protocol for the free radical scavenging activity of the plant extract was followed [12]. The reaction mixture containing the plant extract (50-250 µg/ml) added with 0.5mL of 10Mm sodiumnitroprusside in phosphate buffered saline and kept for incubation at 25 ºC for 180mins. Thereafter added the freshly prepared Griess reagent (equimolar mixture of 1% sulphanilamide in 2.5% phosphoric acid + 0.1% naphthylethylene diamine dihydrochloride in 2.5% phosphoric acid) and absorbance was taken at 546 nm. Ascorbic acid as the standard antioxidant was used. The % NO radical inhibition was calculated with the formula:

$$IC\% = \frac{(A_0 - A_t)}{A_0} \times 100$$

Where, $A_{0=}$ Absorbance of control, A_t= Absorbance of sample, IC = Inhibitory concentration.

DPPH (2,2-diphenyl-2-picrylhydrazyl) free radical scavenging activity

The DPPH radical scavenging activity was screened following the developed protocol generally being practiced with slight modifications [13]. Various concentrations of plant extract (50-250 µg/ml) added to 5mL of 0.004% methanol solution of DPPH and kept in dark for half an hour for incubation and absorbance was taken at 517nm. BHT (Butylated Hydroxyl Toluene) as standard antioxidant was used. The % DPPH radical inhibition was calculated by using the formula:

$$IC\% = \frac{(A_0 - A_t)}{A_0} \times 100$$

Where, A_0 = Absorbance of Control, A_t = Absorbance of sample, IC = Inhibitory concentration

Metal chelating activity

The metal chelating activity of Fe^{2+} of plant extract was as per the developed protocol explained by Kumar *et al*. [14]. Reaction mixture consisting of 0.1 ml (2mM) $FeCl_2.4H_2O$, 0.2 ml (5mM) ferrozine and methanol making final volume upto 5 ml with various concentrations of plant extract (50-250 µg/ml) and kept for incubation for half an hour. The absorbance was taken at 562nm. Citric acid as the standard antioxidant was used. % Fe^{2+}chelation inhibition was evaluated using the formula:

$$IC\% = \frac{(A_0 - A_t)}{A_0} \times 100$$

Where, $A_{0=}$ Absorbance of control, A_t= Absorbance of sample, IC = Inhibitory concentration.

Reducing power activity

The reducing power activity of plant extract was screened by the method adopted by Parki *et al*. [13]. Various concentrations of plant extract (50-250 µg/ml) were mixed with solution containing 2.5 ml of phosphate buffer (200 mM, pH= 6.6) and 2.5 ml of 1% $K_3Fe(CN)_6$ and kept for incubation for 20 min at 50±1^{0}C and then 2.5ml of trichloroacetic acid was added to it and centrifuged for 10 min at 650 RPM. 1 ml of supernatent along with 5 ml distilled wateradded to 1 ml of ferric chloride (0.1%) was used to record absorbance at 700nm. Catechin as the standard antioxidant was used. The % reduction was calculated using the formula:

$$\%\ \text{Reducing Power} = \frac{(A_0 - A_t)}{A_0} \times 100$$

Where, $A_{0=}$ Absorbance of control, A_t= Absorbance of sample

Evaluation of *In-vitro* anti-inflammatory activity

In-vitro anti-inflammatory activity was screened as per the developed protocol along with minor adjustments in the protocol being practiced by Kar *et al*., [15]. The reaction mixture consisting of plant extract (50-250 µg/ml), 100 ppm (200µL) fresh albumin protein, 2.8 ml of freshly prepared phosphate buffered saline (PBS) of pH 6.4. The final volume made upto 5ml. The solution was kept in incubation at 37^{0}C for 15min and then at 70^{0}C for 5min. After cooling the

absorbance was measured at 660nm. Diclofenac sodium of various concentrations were used as standard. The percent inhibition was calculated by the formula:

% Inhibition =100 × (V_t / V_c- 1)

Where, V_t = absorbance of sample, V_c = absorbance of control

Evaluation of antifeeding activity

Test insect

The Bihar hairy caterpillar, *Spilosoma obliqua* (Family: Erebidae, Order: Lepidoptera) is an important pest of pulse crop in northern India. The damage causing stage is mainly the third and its onward instars [16, 17]. The pest is mainly polyphagous in nature damaging the plant by defoliation of the leaves [18].

Collection of larvae and maintenance

Insect larvae were collected from the soyabean (*Glycine max*) field of the Crop Research Center, G.B.P.U.A. & T., Pantnagar, Uttarakhand, India during the July month. Rearing performed in the laboratory conditions (Temperature: 27^{0}C and Relative humidity: 75-80%) in a glass jar covered with muslin cloth. Feeding was done with fresh leaves of Soyabean on daily basis. The fourth instar larvae kept for 24 hours starvation and used to investigate the antifeeding activity.

Experimental procedure

The antifeeding property of the plant extract was assessed as per the developed standard protocol [19]**.** The experiment was conducted in petri plates with bottom spread with moisture papers ensuring proper humid condition and keeping the treated leaves fresh. 25 cm^{2}of known area leaves dipped in various concentrations of plant extract (5-25%), air dried and transferred to the petri plates for feeding. The fourth instar larvae starved for 24 hours were released as one insect per petri plate. Readings were taken at 12, 24, 36 and 48 hours after the release of the larvae. And then calculation of the % antifeeding activity of the plant extract was done as per the formula:

$$\text{Percent antifeeding: } \frac{\text{Leaf area consumed in control} - \text{leaf area consumed in treatment}}{\text{Leaf area consumed in control} + \text{leaf area consumed in treatment}} \times 100$$

Statistical analysis

All the experiments were conducted in triplicates and the data expressed as mean±standard deviation. Data obtained of all the experiments were subjected to ANOVA (p<0.01) for *in-vitro* antioxidant activity while ANOVA (p<0.05) for insect antifeeding activity with two factor analysis with replication via. SPSS software. Data analyzed were found to be significantly different at the respective level of significance. IC_{50} and RP_{50}were calculated by regression line method.

Result and discussion

Chemical composition

The chemical constituent investigation of the methanolic extract of *L. indica* via GC-MS analysis results in identification of fifty-two compounds comprising a total of 74.3% of total extract composition as tabulated in Table 1. As per the abundance the major compounds identified were β-monolinolein (12.9%), linoelaidic acid (11.6%), 2-methyl-Z,Z-3,13-octadecadienol (5.6%), 3,5-dihydroxy-6-methyl-2,3-dihydro-4h-pyran-4-one (5.2%), cubitene (4.1%), n-hexandecanoic acid (3.8%), cyclooctyl acetate (3.0%), hydroxylmethyl furfural (2.5%), curcumenone (1.7%), capric acid (1.6%), stearic acid (1.4%), furaneol (1.4%), furfuryl alcohol (1.3%), 9-epi-β-cyclocolorenone (1.2%) and 2-octylcyclopropen-1-heptanol (1.2%), besides other compounds having individual concentration less than 1.0%.

Total phenols, flavonoids and ortho-dihydric phenol content

The total phenolic content (TPC) of methanolic extract of *L. indica* (LIME) in terms of mg/gm of Gallic Acid Equivalent (mg GAE/gm)) and the results are displayed in Table 2. The total flavonoids content (TFC) of LIME was determined in terms of mg of Catechin Equivalent per gm (mg CNE/gm). The ortho-dihydric phenol content (ODP) of LIME was determined in terms of mg of Catechol Equivalent per gm (mg CLE/gm).

Antioxidant activity

The antioxidant activities of the methanolic extract of *L. indica* were assessed with NO (Nitric oxide) radical scavenging, DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging, metal chelating of Fe^{+2}, reducing power activity. The plant exhibited to inhibit the radical formation, metal chelation of Fe^{+2} and reducing power of Fe^{+3} to Fe^{+2} in a sequentially concentration dependent way at all concentrations (50-250 µg/ml). As the percent inhibition was found to increase with increasing concentrations in case of radical scavenging and metal chelating activity while percent reducing decreases with increasing concentrations in reducing power activity and statistically analyzed to significantly different (p<0.01) at all the tested concentrations.

Later on, the IC_{50} (Inhibitory concentration) values of tested activity confirms the plant exhibiting a substantial but significant antioxidant property having IC_{50} values of the extract that is 42.48±1.87, 31.98±0.59, 59.02±0.73, 53.28±1.21 µg/ml while that of standard antioxidant 7.82±0.15, 9.28±0.09, 11.13±0.17, 18.12±0.01 µg/ml respectively for ascorbic acid, BHT (Butylated hydroxyl toluene), citric acid and catechin in case of NO radical scavenging, DPPH radical scavenging, metal chelating of Fe^{+2}, reducing power activity respectively as presented in Table 3. Significant antioxidant properties of the extract might be possibly due to the significant amount of phenols, flavonoids and ortho-dihydric phenols [20]. The methanolic extract of *L. aromatica* was also found to possess significant antioxidant properties [21]. Since, the methanolic extract of *L. indica* consists of complex mixture of numerous components. The major and minor components may also be responsible for the antioxidant properties. Overall the order in which plant possessing the activity is DPPH radical scavenging > NO radical scavenging > reducing power > metal chelating as lower the IC_{50} value greater is the antioxidant activity.

***In-vitro* anti-inflammatory activity**

The methanolic extract of the plant *Limnophila indica* exhibited the potential to inhibit protein denaturation at all tested concentrations of the essential oil (5-25 µL) having percent inhibition from 51.07±0.73% to 88.14±0.26% (Table 4), statistical analysis reported to be significantly different (p<0.01). The results were also validated by IB_{50} values

43.98±2.66 µg/ml and 10.15±0.10 µg/ml of the extract and standard anti-inflammatory agent diclofenac sodium respectively.

Antifeeding activity

The antifeeding activity screening of the methanolic extract *L. indica* against *Spilosoma obliqua* (Bihar hairy caterpillar) was assessed using no choice/ non preferential leaf dip method. The higher antifeeding index indicates decreased rate of feeding by the insect. The result suggests the extract possessing significant capability to inhibit the feeding activity of the test insect at all the tested concentrations and at all the time intervals and the results analyzed to be significant ($p<0.05$) for all the replications. The antifeeding activity in the extract was found to be highest at 25 ppm and sequentially decreases upto lowest antifeeding activity at 5 ppm in concentration dependent manner. And also the antifeeding activity is found to highest after 12 hours interval after the treatment application and decreases with the subsequent time intervals as represented in Table 5. Crude extracts of various plant species constituting active compounds like sesquiterpenes, diterpenoids, triterpenes, lactones, quinolene, phenolics, fatty acids, saponins, alkaloids, exhibited antifeeding, insecticidal and growth inhibitory properties [22]. Therefore, the antifeeding activity of methanolic extract of *L. indica* could be due to the major or minor constituents of the extract.

Table 1: Chemical composition of the methanolic extract of *L. indica.*

Sl. No.	Compounds	RI*	% Peak Area
1	diacetone alcohol	834	0.6
2	2-hydroxycyclopent-2-en-1-one	883	0.6
3	anulene	888	0.3
4	furfuryl alcohol	-	1.3
5	furaneol	1022	1.4
6	2,2-dimethyl-3-vinyl-bicyclo[2.2.1]heptane	1027	0.3
7	isocitronellol	1074	0.1
8	5-methylfuran-2-carbaldehyde	-	0.9
9	2,4-dihydroxy-2,5-dimethyl-3(2h)-furanone	1173	0.3
10	hydroxy methyl furfural	1225	2.5
11	3,5-dihydroxy-6-methyl-2,3-dihydro-4h-pyran-4-one	1269	5.2
12	cyclooctyl acetate	1287	3.0
13	4-hydroxy-3-methoxystyrene	1293	0.4
14	4a(2h)-naphthalenol, octahydro-4,8a-dimethyl-,(4.alpha.,4a.alpha.,8a.beta.)-	1386	0.4
15	cis-1,3-dideuterio-1,3-cyclohexandiamine	-	0.5
16	2,3-dihydro-benzofuran	-	0.4
17	4,5-dimethyl-1,2,3,6,7,8,8a,8b-octahydrobiphenylene	1443	0.7
18	3-cyclopentylpropionic acid, 2-dimethylaminoethyl ester	1474	0.2
19	2,6,10,10-tetramethylbicyclo[7.2.0]undeca-2,6-diene	1499	0.2
20	tetradec-7-Z-enal	1593	0.5
21	viridiflorol	1594	0.2
22	capric acid	-	1.6
23	benzene, 1,2,4-trimethoxy-5-(1-propenyl)-,	-	0.9
24	ethylene glycol, monosalicylate	1623	0.7
25	3-buten-2-one, 4-(2-hydroxy-2,6,6-trimethylcyclohexyl)-	1669	0.7
26	phthalic acid	-	t
27	*cis*-5-hydroxy-calamenene	1715	0.3
28	cyclocolorenone	1757	0.8
29	phytone	1841	0.2
30	neocurdione	1870	0.8
31	curdione	1870	0.1
32	cubitene	1882	4.1
33	curcumenone	-	1.7
34	9-*epi*-β-Caryophyllene	-	1.2
35	n-hexadecanoic acid	1977	3.8
36	n-eicosane	2000	0.1
37	9-methyl-10,12-hexadecadien-1-ol acetate	2029	0.7
38	phytol	2045	0.3
39	2-octylcyclopropene-1-heptanol	2056	1.2
40	9,12-octadecenoic acid, methyl ester	2093	0.5
41	coronarine	-	0.6
42	linoelaidic acid	-	11.6
43	2-methyl-Z,Z-3,13-octadecadienol	2104	5.6
44	stearic acid	2165	1.4
45	linolenic acid	2191	0.3
46	ethyl linoleate	2193	0.8
47	n-docosane	2200	0.1
48	palmitic acid-β-monoglyceride	2498	0.4
49	β-monolinolein	2713	12.9
50	squalene	2914	0.1
51	n-hentriacontane	3100	0.2
52	3-α-mannobiose	3560	0.4

Table 2: Biochemical assay of the methanolic extract of *L. indica*.

Phytochemical assay	LIME
Total phenolic content	115.04±3.04 mg/gm of GAE
Total flavonoids content	25.90±2.09 mg/gm of CNE
Ortho dihydric phenol content	41.76±3.86 mg/gm of CLE

Notes: LIME- methanolic extract of *L. indica*, GAE- gallic acid equivalent, CNE- Catechin equivalent, CLE- Catechol equivalent.

Table 3: Antioxidant activities of the methanolic extract of *L. indica* expressed in terms of their IC_{50} values.

Sample and standards	NO radical scavenging activity	DPPH radical scavenging activity	Metal chelating activity of Fe^{+2}	Reducing power activity
**LIME	42.48±1.87	31.98±0.59	59.02±0.73	53.28±1.21
*Ascorbic acid	7.82±0.15	-	-	-
*BHT	-	9.28±0.09	-	-
*Citric acid	-	-	11.13±0.17	-
*Catechin	-	-	-	18.12±0.01

Notes: LIME- Methanolic extract of *L. indica*, BHT- Butylated hydroxyl toluene, NO- Nitric oxide, DPPH- 2,2-diphenyl-1-picrylhydrazyl. **sample, *standard antioxidant. All the IC_{50} values expressed as mean±standard deviation taken in triplicates and analyzed to be significantly different ($p<0.01$).

Table 4: *In-vitro* anti-inflammatory activity in terms of IB_{50} values for methanolic extract of *L. indica*.

Sample and standards	IB_{50} Value
**LIME	43.98±2.66
*Diclofenac sodium	10.15±0.10

Notes: % *In-vitro* anti-inflammatory activity of methanolic extract of aerial plant part of *Limnophila indica* (LIME) *versus* the standard anti-inflammatory agent (Diclofenac sodium). IB_{50}values plotted as mean±standard deviation with percent inhibition at various concentrations are significantly different ($p<0.01$).

Table 5: Insect antifeeding activity assessment of methanolic extract of *L. indica*.

Doses (%)	After 12 hours		After 24 hours		After 36 hours		After 48 hours	
	Leaf area consumed	% Antifeeding activity	Leaf area consumed	%Antifeeding activity	Leaf area consumed	% Antifeeding activity	Leaf area consumed	%Antifeeding activity
5	14.96±2.95	2.44	23.19±3.13	1.36	25.00±0.00	0	25.00±0.00	0
10	11.99±1.91	13.42	18.27±2.49	13.18	23.29±1.96	3.54	25.00±0.00	0
15	04.90±1.30	52.42	9.20±1.39	44.29	21.46±1.33	7.60	25.00±0.00	0
20	00.58±0.44	92.87	4.54±0.88	67.99	09.52±1.07	44.82	20.28±1.66	10.40
25	00.19±0.25	97.52	1.27±1.08	89.88	07.66±0.86	53.06	18.32±1.61	15.41
Control	15.71±2.79	-	23.83±2.02	-	25.00±0.00	-	25.00±0.00	-

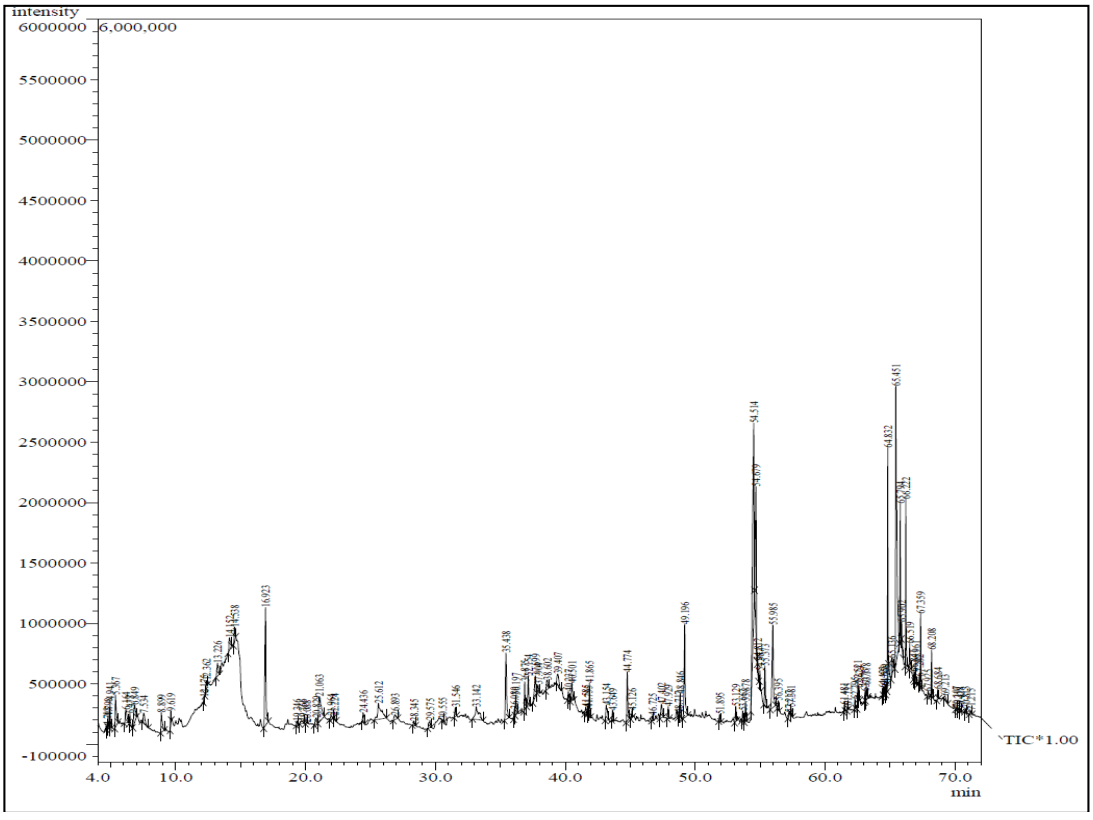

Fig 1: Gas chromatogram of methanolic extract of *L. indica*.

Conclusion

Present research result confirms a potential antioxidant activity in the methanolic extract of the plant *L. indica* supporting their use in traditional medicine and also indicating the plant to supplement as an important component in food, cosmetic and pharmaceutical industry. In addition, investigation of insect antifeeding activity against *S. obliqua* results the plant as a potent antifeeding agent indicating its insecticidal values. The result confirms its potential to control the pest population in pulse crops and also an opportunity to develop botanical insecticide to promote sustainable agriculture.

Acknowledgement

Author hereby acknowledges Dr. D.S. Rawat, Dept. of Biological Sciences, College of Basic Sciences and Humanities, G.B.P.U.A. & T., Pantnagar in identifying the plant specimen and providing the herbarium number. The author also thanks Dr. R.M. Srivastava, Dept. of Entomology, College of Agriculture, G.B.P.U.A. & T., Pantnagar for guiding in insect antifeeding activity. Thanks to Advanced Instrumentation Research Facility, J.N.U., New Delhi for facilitating GC-MS analysis of the plant sample.

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