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Randeep Kumar
Department of Chemistry, College of Basic Sciences and Humanities, G.B. Pant University of Agriculture & Technology, Pantnagar, U.S. Nagar, Uttarakhand, India

Ravendra Kumar
Department of Chemistry, College of Basic Sciences and Humanities, G.B. Pant University of Agriculture & Technology, Pantnagar, U.S. Nagar, Uttarakhand, India

Om Prakash
Department of Chemistry, College of Basic Sciences and Humanities, G.B. Pant University of Agriculture & Technology, Pantnagar, U.S. Nagar, Uttarakhand, India

RM Srivastava
Department of Entomology, College of Agriculture, G.B. Pant University of Agriculture & Technology, Pantnagar, U.S. Nagar, Uttarakhand, India

AK Pant
Department of Chemistry, College of Basic Sciences and Humanities, G.B. Pant University of Agriculture & Technology, Pantnagar, U.S. Nagar, Uttarakhand, India

Correspondence
Ravendra Kumar
Department of Chemistry, College of Basic Sciences and Humanities, G.B. Pant University of Agriculture & Technology, Pantnagar, U.S. Nagar, Uttarakhand, India

Chemical composition, *in vitro* antioxidant, anti-inflammatory and antifeedant properties in the essential oil of Asian marsh weed *Limnophila indica* L. (Druce)

Randeep Kumar, Ravendra Kumar, Om Prakash, RM Srivastava and AK Pant

Abstract
Limnophila indica L. (Druce) is being estimated for the essential oil composition, *in-vitro* antioxidant, anti-inflammatory and insect antifeeding activity of this plant was evaluated. The GC-MS analysis led to identification of thirty-five compounds comprising of 90.9% of total oil composition. The major compound of the oil identified was *epi*-cyclocolorenone (27.7%). The oil was screened for antioxidant activity using reducing power assay, metal chelating of Fe^{+2} assay, NO and DPPH radical scavenging assay with IC_{50} 21.19±0.06, 22.77±0.11, 15.74±0.06 and 14.52±0.05 µg/ml respectively. *In-vitro* anti-inflammatory activity assessment of the essential oil results in its activity with IC_{50} 19.28±0.06 µg/ml. And also showed potent insect antifeeding action in a sequentially dose dependent manner against *Spilosoma obliqua*.

Keywords: GC-MS analysis, epi-cyclocolorenone, *In-vitro* anti-inflammatory, antioxidant

Introduction
Plantaginaceae an important family in the angiospermic flowering plants includes 120 genera comprising of 7,055 species including *Limnophila indica* L. (Druce). The word Limnophila derived of a latin word meaning pond loving and popularly known by the name Ambulia (Asian marshweed) [1]. The genus *Limnophila* comprises the short heighted herbaceous plants [2] which are basically semi-aquatic plants inhabiting marshy areas and are widespread in tropical and subtropical regions of the world including Asian countries, West Africa, Southern Iraq, Korea, Southern Japanand Northern Australia [3]. The species *Limnophila indica* L. (Druce) has been widely exploited in the field of traditional medicine and found to possess medicinal values such as antiseptic, anti-dysentry, anti-dyspesia, anti-filariasis, carminative, anti-shigella, antacid, antimicrobial, hepatoprotective and cytotoxic agent [4].
Present research is to investigate the complete identification of the compounds of essential oil through GC-MS, *in-vitro* antioxidant, anti-inflammatory and the insect antifeeding activity for the first time to be reported on the essential oil of the plant.

Materials and Methods
Collection of Plant material
The plant material was collected from tarai region of Uttarakhand, India in the month of December 2017. The plant was identified and the herbarium voucher number GBPUH-980 was submitted to G. B. Pant University Herbarium, Department of Biological Sciences, C.B.S.H., G.B.P.U.A.T., Pantnagar, Uttarakhand, India.

Isolation of essential oil
Hydro-distillation method was used for isolation of essential oils from aerial plant part of *L. indica*. The fresh aerial plant part was cut into small size pieces, hydrodistilled for 8 hours. Then, the essential oil was extracted with hexane and desiccated with the help of anhydrous Na_2SO_4.

GC-MS Analysis
The essential oil was analyzed on GCMS-QP2010 Ultra DB-5 column (30m×0.25mm and film thickness 0.25µm). The column temperature was programmed for 60-210ºC at the rate of 3ºC/min and then again upto 280^{0}C at the rate of 8ºC/min and then hold upto 11min.

Helium gas at the rate of 1.21 ml/min was used as the carrier gas at the injector temperature at 210°C. MS were recorded under EI condition (70 ev) with injection volume of 0.1 µL with split mode of 1:100. 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 [5].

Evaluation of antioxidant activity

Nitric oxide (NO) radical scavenging activity

The NO radical scavenging activity was screened following the developed protocol generally being practiced with slight modifications [6]. Briefly the reaction mixture consisting of essential oil (5-25 µL) and 0.5mL of 10mM sodiumnitroprusside, incubated at 25^{0}C for 180min. Griess reagent was added in the reaction mixture and absorbance was taken at 546 nm. Ascorbic acid was used as the standard antioxidant. The % nitric radical scavenging activity was calculated as per the formula:

$100 \times (V_t / V_c - 1)$

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

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

Standard protocol for the free radical scavenging activity of the essential oil was followed **[7]**. Various concentrations essential oil (5-25 µL) were mixed with 5mL of 0.004% DPPH and kept in dark for half an hour for incubation and absorbance was taken at 517nm. BHT (Butylated Hydroxyl Toluene) was used as standard antioxidant. The % inhibition of DPPH free radical was calculated by using the formula:

$100 \times (V_t / V_c - 1)$

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

Metal chelating activity

The metal chelating activity of Fe^{2+} of essential oil was screened by the method adopted by Kumar *et al*., 2012 **[7]**. Reaction mixture consisting of 0.1 ml (2mM) $FeCl_2.4H_2O$, 0.2 ml (5mM) ferrozine and methanol was added to makeup final volume upto 5 ml with various concentrations of essential oil (5-25 µL) and was incubated for half an hour. The absorbance was taken at 562nm. Citric acid was used as the standard antioxidant. % Metal chelating activity was evaluated using the formula:

$100 \times (V_t / V_c - 1)$

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

Reducing power activity

The reducing power activity of essential oil was done as per the developed protocol explained by Parki *et al*., 2017 **[8]**. Various concentrations of essential oil (5-25 µL) were added to 2.5 ml of phosphate buffer (200 mM, pH= 6.6), 2.5 ml of 1% $K_3Fe(CN)_6$ and kept for 20 min incubation at 50±1^{0}C and then added to it is 2.5mlof trichloroacetic acid followed by centrifugation at 650 RPM for 10 min. 1 ml of supernatent obtained was mixed with 5 ml distilled water and 1 ml of ferric chloride (0.1%). Absorbance was recorded at 700nm. Catechin was used as the standard antioxidant. The % reducing power of the essential oil was calculated using the formula:

$100 \times (V_t / V_c - 1)$

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

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*., 2012 [9]. The reaction mixture consisting of essential oil (5-25 µL), 100 ppm (200µL) fresh albumin protein, 2.8 ml of freshly prepared phosphate buffered saline (PBS) of pH 6.4 and make up the final volume to 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 was used as standard. The percent inhibition was calculated by the formula:

% Inhibition $=100 \times (V_t / V_c - 1)$

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

Evaluation of antifeeding activity

Test insect

The Bihar hairy caterpillar, *Spilosoma obliqua* belonging to the family Erebidae of the order Lepidoptera is a key pest of pulse crop in India. The damage is mostly due to the third and its onward instars. It is a polyphagous pest and infests the plant by defoliating the leaves causing serious damage to several plant species belonging to several families [10].

Collection of larvae and maintenance

Larvae of the insect were collected from the soyabean (*Glycine max*) field at Crop Research Center, G.B.P.U.A.T., Pantnagar, Uttarakhand, India during the July month. The insects were reared in the laboratory at 27^{0}C temperature and 75-80%relative humidity in a jar covered with muslin cloth. The larvae were specially feed on fresh leaves of Soyabean on daily basis. The full grown fourth instar larvae already kept for 24 hours starvation were used to investigate the antifeeding activity.

Experimental procedure

The experiment was carried out in PGRL (Post Graduate Research Laboratory), Department of Entomology, G.B. Pant University of Agriculture and Technology, Pantnagar as per the developed protocol **[11]**. The experiment was assessed in petri plates with moisture papers at bottom to maintain proper humid condition and to keep the treated leaves fresh. Leaves of known area of 25 cm^2 was taken, dipped in various concentrations of essential oil for 1 min, air dried and then transferred to the petri plates for feeding the insects. 24 hours starved fourth instar test insect larvae were released as one insect per petri plate. Readings were taken after 12 hours interval at 12, 24, 36, 48 hours. Graph paper method was used to measure the leaf area consumed by the insects. And then calculating % antifeeding activity of essential oil 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 are conducted in triplicates and the data expressed as mean ± standard deviation. Data illustrated in the graph were subjected to ANOVA ($p<0.01$) for *in-vitro* antioxidant and anti-inflammatory 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. Regression line method was used to calculate $IC_{50,}$ RP50and IB_{50}.

Result and Discussion

Chemical composition

The yield of essential oil *Limnophila indica* L. (Druce) of aerial part was 0.90%. Using GC-MS analysis technique thirty-five compounds were identified contributing to 90.9% of total oil composition. Chemical composition of the oil was mainly dominated by the *epi*-cyclocolorenone (27.7%), α-gurjunene (9.7%), 5-hydroxy-cis-calamenene (7.0%), β-caryophyllene (7.0%), α-pinene (5.6%), limonene (5.3%), myrcene (3.5%), δ-cadinene (3.3%), cadin-4-en-10-ol (3.2%), viridiflorol (2.8%), shyobunol (2.7%), nerolidol (2.5%), 9-*epi*-E-caryophyllene (1.9%), himbaccol (1.2%), endo-1-bourbonanol (1.1%) and γ-gurjunene (1.1%). The other minor constituents contributing <1.0% were also identified. The detailed list of compounds has been presented in (Table 1).

Antioxidant activity

Nitric oxide radical scavenging activity

The scavenging effect of different concentrations of essential oil (5-25 µL) of *L. indica* was screened and analyzed, resulting in significantly different ($p<0.01$) inhibition effect with a wide range of inhibition ranging from 50.90±0.00% to 80.91±0.22% at various concentrations conferring its antioxidant property that is substantial but slightly lower compared to standard antioxidant ascorbic acid with respective IC_{50} values 15.74±0.06 µg/ml and 7.82±0.15 µg/ml as illustrated in Table 2.

DPPH radical scavenging activity

The radical scavenging activity of the essential oil of *L. indica* resulted its antioxidant activity in a sequential dose dependent manner. At all concentrations (5-25 µL) the oil exhibited profound inhibition effect on DPPH radical formed which was analyzed to be significantly different ($p<0.01$) in the range of 27.16±0.25% to 72.83±0.05% having its potent antioxidant activity validated with its IC_{50} value compared to that of the standard antioxidant BHT (Table 2.).

Metal chelating activity of Fe^{+2}

Increased disruption of complex formation of Fe^{+2} by ferrozine with increasing concentration reports the metal chelating activity at all concentrations (5-25 µL) of essential oil of *L. indica* in a concentration dependent manner. Percent metal chelation at various concentrations ranging from 29.90±0.09% to 52.41±0.12% was reported indicating its potent antioxidant activity. IC_{50} value of standard antioxidant citric acid 22.77±0.11 µg/ml and that of oil 11.13±0.17 µg/ml signifies the metal chelation property (Table 2.).

Reducing power activity

The reducing power activity of *L. indica* essential oil was found to be active at all selected dose levels (5-25 µL). The percent reduction ranging from 89.42±0.05% to 40.14±0.13% clearly exhibits the significant property. RP_{50} value of the oil and standard antioxidant catechin reports found to be 21.19±0.06 µg/ml and 18.12±0.01 µg/ml respectively indicates the oil as a potential antioxidant of same degree of effect as that of the standard. (Table 2)

The antioxidant activity of *L. indica* essential oil in present study are in aggrement of the previous reports of essential oil containing α-pinene [12], cyclocolorenone [13], limonene [14], β-caryophyllene [15], 5-hydroxy-cis-calamenene [16], α-gurjunene [17].

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

The essential oil 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 20.16±0.79% to 58.45±0.26% (Table 3), statistical analysis reported to be significantly different ($p<0.01$). The results were also validated by IB_{50} values 19.28±0.06 µg/ml and 10.15±0.10 µg/ml of oil and standard anti-inflammatory agent diclofenac sodium respectively. Compounds like cyclocolorenone [18], limonene [19], α-pinene [20] and β-caryophyllene [21] has been reported to possess anti-inflammatory activity. These compounds are also reported in the chemical composition of *L. indica* essential oil.

Evaluation of insect antifeeding activity

In present study the antifeeding activity of essential oil of *L. indica* was investigated against *S. obliqua*, and results exhibited the significant antifeeding activity in the oil in a sequentially dose dependent manner. At lowest concentration of 5 ppm it was 16.78, 7.02, 0, 0% at the consecutive time intervals of 12, 24, 36, 48 hours respectively. However, at 10 ppm it was found to be 43.28, 35.92, 4.50, 2.18 % respectively. At 15 ppm it was 95.18, 73.16, 44.88, 39.13%. At 20 ppm it was 100, 100, 98.80, 96.54% while at 25 ppm 100% antifeeding activity was observed in the same time frame. (Table 4). The present research confirms the antifeedant activity of the essential oil of *L. indica* exhibited significant antifeeding activity at the dose levels of 20 and 25 ppm. The results are in total agreement of the previous report revealing the antifeeding activity of α-pinene, β-caryophyllene, limonene [22,23], calamenene [24], cyclocolorenone insect repellent activity [25], compounds which have also being identified in the present investigation.

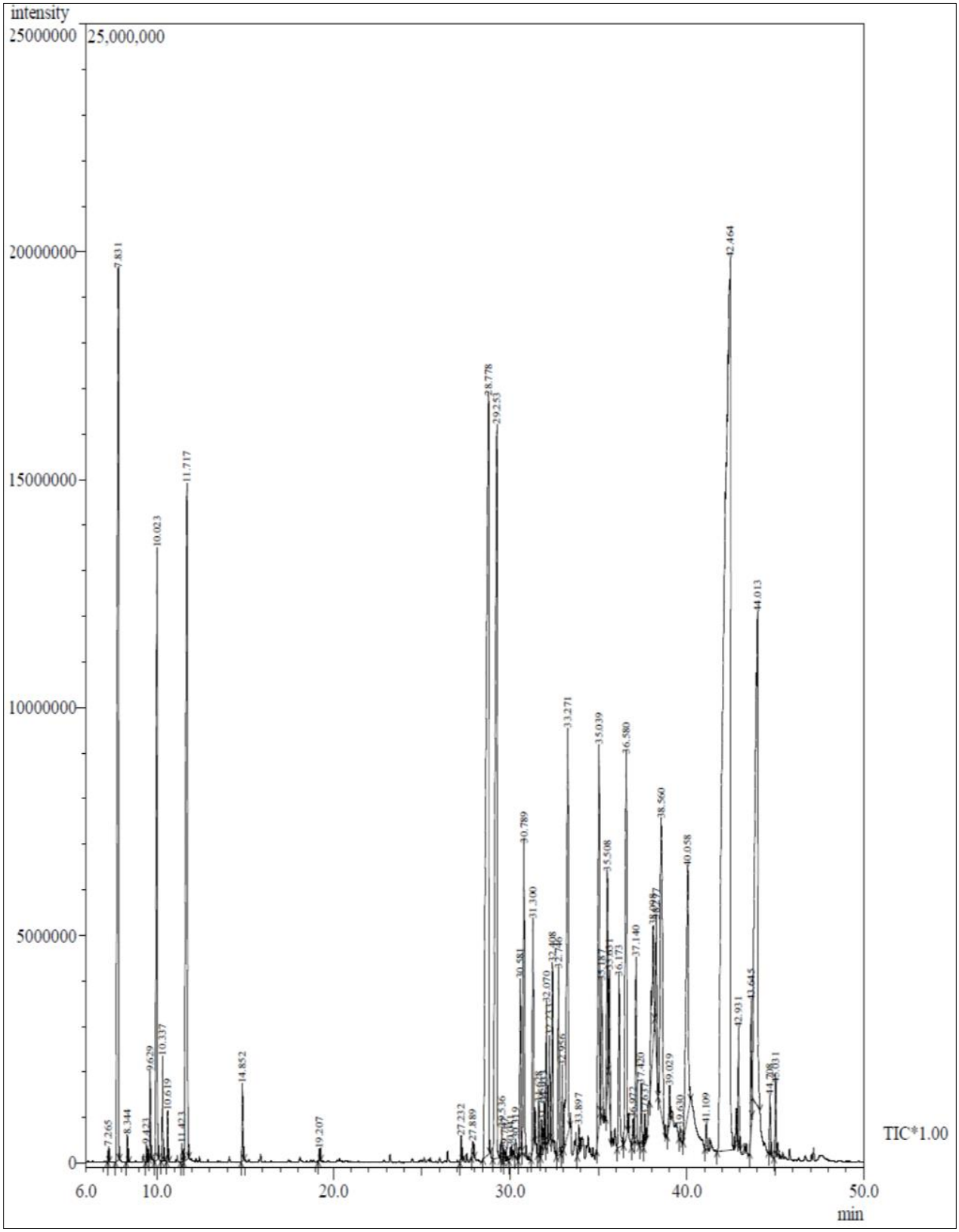

Fig 1: Gas chromatogram of essential oil aerial plant part of *Limnophila indica* L. (Druce)

Table 1: Chemical composition (%) of essential oil of aerial plant part of *Limnophila indica* L. (Druce).

RI_{exp}	RI_{lit}	Compounds name	Peak Area %
939	939	α-pinene	5.6
950	953	camphene	t
978	980	β-pinene	t
991	992	myrcene	3.5
1007	1005	α-phellandrene	0.2
1018	1018	α-terpinene	0.4
1025	1026	p-cymene	t
1030	1033	limonene	5.3

1082	1080	linalool	0.3
1186	1192	α-terpineol	t
1349	1352	α-cubebene	0.1
-	1375	β-elemene	t
1392	1390	β-cubebene	0.3
1406	1409	α-gurjunene	9.7
1416	1415	*cis*-α-bergamotene	t
1433	1432	β-copaene	0.1
-	1452	α-humulen	0.9
1464	1464	9-epi-caryophyllene	1.9
1466	1467	muurola-4(14)-5-diene<cis>	0.1
1476	1473	γ-gurjunene	1.1
1492	1485	B-selinene	0.2
1494	1467	β-caryophyllene	7.0
1504	1508	α-E,E-farnesene	1.0
1512	1512	γ-cadinene	0.4
1518	1524	δ-cadinene	3.3
1523	1515	1-endo-bourbonanol	1.1
1530	1546	himbaccol	1.2
1531	1534	nerolidol	2.5
1538	1537	α-cadinene	0.9
1594	1590	viridiflorol	2.8
1631	1626	epicubenol	0.4
1659	1648	cadin-4-en-10-ol	3.2
1689	1687	shyobunol	2.7
1715	1713	5-hydroxy-cis-calamenene	7.0
1771	1774	*epi*-cyclocolorenone	27.7
		Total	90.9
Monoterpenoid hydrocarbon		15.0 %	
Oxygenated hydrocarbon		0.3 %	
Sesquiterpenoid hydrocarbon		26.1 %	
Oxygenated sesuiterpenoid		49.5 %	
Total		90.9 %	

Notes: t- traces (0.1%), RI_{exp}- Experimental retention indices, RI_{lit}- Literature retention indices.

Table 2: Antioxidant activity in terms of IC_{50} & RP_{50} values for essential oil of *Limnophila indica*.

Sample and standards	NO radical scavenging activity	DPPH radical scavenging activity	Metal chelating activity of Fe^{+2}	Reducing power activity
**LIEO	15.74±0.06	14.28±0.06	22.77±0.11	21.19±0.06
*Ascorbic acid	7.82±0.15	-	-	-
*BHT	-	9.28±0.13	-	-
*Citric acid	-	-	11.13±0.17	-
*Catechin	-	-	-	18.12±0.01

Notes: IC_{50} of various antioxidant activity of essential oil of aerial plant part of *Limnophila indica* (LIEO) *versus* their respective standard antioxidant. Data obtained of percent inhibition are statistically analyzed to be significantly different (p<0.01) and IC_{50} values calculated with regression line method.

Table 3: *In-vitro* anti-inflammatory activity in terms of IB_{50} values for essential oil of *Limnophila indica*.

Sample and standards	IB_{50} Value
**LIEO	19.28±0.06
*Diclofenac sodium	10.15±0.10

Notes: %*In-vitro* anti-inflammatory activity of essential oil of aerial plant part of *Limnophila indica* (LIEO) *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 4: % Antifeeding activity of essential oil of *Limnophila indica*.

Doses (ppm)	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	11.55±2.95	16.78	20.03±3.13	7.02	25.00±0.00	0	25.00±0.00	0
10	6.41±1.91	43.28	8.87±2.49	44.42	22.84±1.96	4.50	19.59±0.00	2.18
15	0.40±1.30	95.18	3.57±1.39	73.16	9.51±1.33	44.88	8.95±0.00	39.13
20	0.00±0.44	100	0.00±0.88	100	0.15±1.07	98.80	0.44±1.66	96.54
25	0.00±0.25	100	0.00±1.08	100	0.00±0.86	100	0.00±1.61	100
Control	16.21±2.79		23.06±2.02		25.00±0.00		25.00±0.00	

Notes: % Antifeeding values calculated on comparison with control. Data are statistically analyzed (p <0.05) to be significantly different.

Conclusion

As per the results of the present research it is evident that the essential oil of *Limnophila indica* L. (Druce) is a potent antioxidant and anti-inflammatory agent indicating its potentiality in the field of food, pharmaceutical and cosmetic industry. The plant essential oil also possesses a substantial insect antifeeding activity predicting its possible use as a botanical pesticide acting as a substitute to synthetic pesticides and can be an important component of sustainable agriculture.

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