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

GC-MS analysis of the hexane extract of *Limnophila indica* (L.) Druce, its total phenolics, *in-vitro* antioxidant, anti-inflammatory and antifeeding activity against *Spilosoma obliqua*

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

Abstract
The present study was carried out for analysis of hexane extract of *Limnophila indica* and evaluated for total phenolics, *in-vitro* antioxidant, anti-inflammatory and antifeeding activity. The n-hexane extract was obtained by Soxhlet apparatus and analysed by GC-MS. GC-MS analysis leads to identification of thirty-eight compounds in extract comprising of 83.9% of total extract composition, containing aristolone (40.3%) as the major compound. Phytochemical analysis resulted in potentially significant total phenolics, total flavonoids and total ortho-dihydric phenol content. Antioxidant activity of extract assessment using NO, DPPH radical scavenging, metal chelating and reducing power activity resulted significant antioxidant activity presumably due to qualitative and quantitative difference of their antioxidative components. Also concentration dependent inhibition of protein (albumin) denaturation exhibited in assessment of *in-vitro* anti-inflammatory activity in the extract. Percent antifeeding analysis on *Spilosoma obliqua* results into higher range of inhibition of feeding action in a dose dependent manner and more prominent at higher concentrations (20 and 25 ppm).

Keywords: *Limnophila indica*, antioxidant, anti-inflammatory, antifeeding, *Spilosoma obliqua*

1. Introduction
Spilosoma obliqua Walk. (Bihar hairy caterpillar) (Lepidoptera: Erebidae) is a potent pest of pulse crops causing serious damage to leguminous crops in northern and central part of India [1,2]. Pulses accounts for the second most important crops after cereals and a rich source of protein. The insect larvae mainly Infests green gram and black gram and accounts for 30% of the total yield losses in moong and urd bean [3]. The oxidative damage caused due to reactive oxygen species and free radicals produced of normal metabolic processes induces severe damage to lipid, protein, nucleic acids and in severe cases causes coronary diseases. The health promoting effects due to antioxidant derived of plant origin aroused of the protective effects of antioxidants against reactive oxygen species and the free radicals [4]. Similarly, the inflammation in body triggered due to entry of foreign agents inside the body and may be due to the defective immune responses in the body. Various steroidal and nonsteroidal drugs having side-effects have been administered to counteract triggered immune responses [5]. The potential of the medicinal plants to serve as an antioxidant, anti-inflammatory and antifeedant agents may be due to the presence of secondary metabolites such as phenols, flavonoids, alkaloids, terpenoids, saponins, gums, glycosides, polyphenols, tannins and coumarins in their chemical compositions and also synergistical effects of major and minor constituents [6-8].
Limnophila indica (L.) Druce (Plantaginaceae) is an aquatic, perennial herb found as submerged or emergent stem plant. Naturally it is inhabitant of fresh water reservoirs and marshy land. The submerged stems are smooth havingfeathery leaves while the emergent stems remain covered of flat shiny hairs. Flowers may be of pink, white, blue or purple to lavender coloured, stalkless, axillary or solitary, sessile or pedicellate and borne in the leaf axis. The fruit is generally capsulated containing around 150 seeds. The plant is known for its medicinal uses in traditional system of medicine such as antiseptic, anti-dysentry, anti-dyspesia, anti-filariasis, carminative, anti-shigella, antacid, antimicrobial, hepatoprotective and cytotoxic agent [9]. Present research is to investigate the systemic identification of chemical composition of the hexane extract of L. *indica*, its total phenolics, *in-vitro* antioxidant, anti-anti-inflammatory activity and insect antifeeding activity.

2. Materials and Methods

2.1. 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 by Dr. D.S. Rawat, Assistant Professor (Plant Taxonomist), Department of Biological Sciences, College of Basic Sciences and Humanities, G.B.P.U. A. & T., Pantnagar, Uttarakhand, India. The herbarium voucher number GBPUH-980 was also provided by him and was submitted to G. B. Pant University Herbarium, Department of Biological Sciences, CBSH, Pantnagar.

2.2. Preparation of extract

The aerial plant part of *L. indica* was chopped, shade dried, powdered and extracted in hexane using Soxhlet apparatus. The extract obtained was filtered and concentrated using rotary evaporator. After drying the yield of the extract was recorded to be 3.0% and stored at 4^0C for further analysis and biological activity estimation.

2.3. 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 280^0C 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 260ºC. 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 [10].

2.4. Phytochemical assay

2.4.1. Total phenolic assay

The total phenol content estimation of the extract was done using Folin-Ciocalteu reagent (FCR) [11] 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.

2.4.2. Total flavonoids assay

Method developed by Choi *et al*., 2006 [12] was adopted for total flavonoids assay. In this method 1ml of plant extract was mixed with 1.25 ml of distilled water and 75µl (5%) $NaNO_3$ and incubated for 5 min. 150µl (10%) $AlCl_3$, 500 µl of 1M NaOH and 275 µl were added and mixed after incubation. Absorbance was then measured at 510 nm. Total flavonoid content was expressed as catechin equivalent (CNE) in mg/g.

2.4.3. 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 [13].

2.5. Evaluation of antioxidant activity

2.5.1. NO radical scavenging activity

The NO radical scavenging activity was screened following the developed protocol generally being practiced with slight modifications [14]. Briefly the reaction mixture consisting of plant extracts (50-250 µg/ml) mixed with 0.5mL of 10mM sodium nitroprusside in phosphate buffered saline and incubated at 25^0C for 180 min. Griess reagent was added in the reaction mixture and absorbance was taken at 546 nm. Ascorbic acid was used as the standard antioxidant. The% NO radical scavenging activity was calculated as per the formula:

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

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

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

Standard protocol for the free radical scavenging activity of the plant extracts was followed [15]. Various concentrations plant extracts (50-250 µg/ml) were mixed with 5mL of 0.004% methanol solution of DPPH. The reaction mixture was kept in dark for half an hour for incubation and absorbance was taken at 517nm. BHT was used as standard antioxidant. The% inhibition of DPPH free radical was calculated by using the formula:

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

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

2.5.3. Metal chelating activity

The metal chelating activity of Fe^{2+} of plant extracts was screened by the method adopted by Kumar *et al*., 2012 [15]. Reaction mixture consisting of 0.1 ml (2mM) $FeCl_2.4H_2O$, 0.2 ml (5mM) ferrozine and methanol was added and made up the final volume upto 5 ml with various concentrations of plant extracts (50-250 µg/ml) and was incubated for half an hour. The absorbance was taken at 562nm. Citric acid was used as the standard antioxidant. Percent metal chelating activity was evaluated using the formula:

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

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

2.5.4. Reducing power activity

The reducing power activity of plant extracts was done as per the developed protocol by Parki *et al*., 2017 [16]. Various concentrations of plant extracts (50-250 µg/ml) 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 ± 1^0C and the added 2.5ml of trichloroacetic acid, and centrifuged at 650 RPM for 10 min. 1 ml of supernatant, 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:

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

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

2.6. Evaluation of In-vitro anti-inflammatory activity

In-vitro anti-inflammatory activity was screened as per the developed protocol by Kar *et al*., 2012 [17]. The reaction mixture consisting of plant extracts (50-250 µg/ml), 100 ppm (200µL) fresh albumin protein, 2.8 ml of freshly prepared phosphate buffered saline (PBS) of pH 6.4 and made up the final volume to 5ml and then incubated at 37^0C for 15min and then at 70^0C for 5min. After cooling the absorbance was

measured at 660nm. Diclofenac sodium was 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

2.7. Evaluation of antifeeding activity

2.7.1. Test insect

The Bihar hairy caterpillar, *Spilosoma obliqua* is a key pest of pulse crop in India. The damage is mostly due to the third and its onward instars [1, 2]. The pest is a polyphagous, infests by defoliation causing serious damage to several pulse crops [18].

2.7.2. Collection of larvae and maintenance

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

2.7.3. Experimental procedure

The experiment was carried out as per the developed protocol [19]. 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 were taken and dipped in various concentrations of plant extracts (5-25%) for 1 min. These were then air dried and transferred to the petri plates for feeding the insects. The 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 percent antifeeding activity of essential oil as per the formula:

Percent antifeeding:

2.8. Statistical analysis

All the experiments were 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 $IC_{50,}$ $RP_{50}$and IB_{50}.

3. Results

3.1. Chemical composition

The combination of GC-MS analysis of hexane extract of *L. indica* reveals the presence of thirty-seven compounds together accounts for 83.9% of the total extract composition. The results revealed the hexane extract to be mainly dominated by aristolone (40.3%), 5-hydroxycalamenene (12.2%), hexadecanoic acid (3.3%), (Z)-7-hexadecenal (2.7%), δ-cadinene (2.6%), neophytadiene (2.6%), α-cadinol (2.0%), ledol (2.3%), hexadecanoic acid methyl ester (1.9%), hexadecanoic acid trimethylsilyl ester (1.7%), stigmasta-5,22-dien-3-ol (1.2%), linoleic (1.1%), nerolidol (1.1%) and phytol (1.0%), besides other components having individual concentration less than 1.0%. Complete identification of the extract composition is tabulated in Table 1.

3.2. Quantitative phytochemical analysis

Table 1: Chemical composition of hexane extract of *L. indica*.

Retention Indices	Compounds	% Peak Area
		LIHE
1117	2,4-dimethyl-1-decene	0.2
1121	2,5,6-trimethyldecane	0.2
-	tetradecane	0.2
1469	δ-cadinene	2.6
1530	Ledol	2.3
1547	α-calacorene	0.1
1564	Nerolidol	1.1
1574	Aristolone	40.3
1580	α-cadinol	2.0
-	Shyobunol	0.4
1623	ethylene glycolmonosalicylate	0.5
1757	5-hydroxycalamenene	12.2
-	neophytadiene	2.6
1808	(Z)-7-hexadecenal	2.7
1878	hexadecanoic acid methyl ester	1.9
1968	hexadecanoic acid	3.3
-	β-eudesmene	0.9
-	2-methyl-decane	0.4
1987	Hexadecanoic acid trimethylsilyl ester	1.7
-	eremophilone	0.2
2045	Phytol	1.0
2077	octadecanoic acid methyl ester	0.1
2093	11,14-octadecadienoic acid methyl ester	0.5
2167	octadecanoic acid	0.2
2183	Linoleic	1.1
2186	octadecanoicacidtrimethylsilyl ester	0.6
-	linoelaidicacidtrimethylsilyl ester	0.2
2210	α-linolenic acid	0.5
-	Nonadecane	0.2
-	Docosane	0.3
-	stigmasta-5,22-dien-3-ol	1.2
2797	Stigmasterol	0.3
2789	β-sitosterol	0.2
2722	stigmasta-4,22-dien-3-one	0.3
-	stigmast-4-en-3-one	0.4
2804	Octacosane	0.7
3149	vitamin E	0.3
Total		83.9

Notes: LIHE- Hexane extract of *L. indica.*

3.2.1. Total phenol content (TPC)

TPC estimation using FCR resulted in significant amount of total phenols in the extract as expressed in terms of mg/gm of Gallic Acid Equivalent (mg GAE/gm)) and the results are displayed in Table 2. Hexane extract (104.79±0.57 mg/g GAE) comprises of significantly higher phenol content.

3.2.2. Total flavonoid content (TFC)

Total flavonoid content of the extract Were determined by Catechin calibration curve and expressed in terms of mg of Catechin Equivalent per gm (mg CNE/gm) (Table 2). Potentially higher flavonoid content reported in hexane extract (19.55±0.82 mg/g CNE).

3.2.3. Total Ortho-dihydric phenol content

Catechol calibration curve drawn to estimate total Ortho-dihydric phenol content in the extract and expressed in terms of mg of Catechol Equivalent per gm (mg CLE/gm). (Table 2). The result reports the hexane extract (35.06±0.61 mg/g CLE) to possess higher Ortho-dihydric phenol content.

Table 2: Phytochemical assay of the hexane extract of *L. indica*.

Phytochemical assay	LIHE
Total phenolic content (TPC)	104.79±0.57 mg/g GAE
Total flavonoids content (TFC)	19.55±0.82 mg/g CNE
Ortho dihydric phenol content (ODP)	35.06±0.61 mg/g CLE

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

3.3. Antioxidant activity

3.3.1. NO radical scavenging activity

NO radical scavenging effect of the extract at various dose levels of 50-250 µg/ml was found to be significant and substantial when compared to standard antioxidant ascorbic acid (Table 3.). IC_{50} (52.44±3.38µg/ml) value represents the potentiality in the extract to act as antioxidant agent against standard ascorbic acid (IC_{50}7.82±0.15).

3.3.2. DPPH radical scavenging activity

The radical scavenging ability of the extract was observed to vary in a dose dependent manner at all concentrations (50-250 µg/ml) and also statistical analysis reveals the significant antioxidant activity in the extract when compared to standard antioxidant (p<0.01) and the results observed to be significantly different at all concentrations represented in the form IC_{50} value of 40.96±1.18µg/ml.

3.3.3. Metal chelating activity of Fe^{+3}

From the quantitative analysis of the metal chelating activity of Fe^{+3}, the result reveals the extract to possess potent antioxidant property in hexane extract (IC_{50} 69.15±1.13 µg/ml). The extract exhibit remarkable activity in concentration dependent manner and significantly different chelating activity compared to standard antioxidant citric acid (p<0.01) at all tested concentrations (50-250 µg/ml).

3.3.4. Reducing power activity Fe^{+3} to Fe^{+2}

At all the concentrations of 50-250 µg/ml, the extract displayed significant reducing power Fe^{+3} to Fe^{+2} but substantial compared to standard antioxidant catechin (p<0.01). The analysis reveals extract to possess significant activity in hexane extract indicated by IC_{50}58.48±0.45 µg/ml (Table 3).

Table 3: Antioxidant activity of hexane extract of *L. indica* represented in terms of IC_{50} values.

Sample and standards	NO radical scavenging activity	DPPH radical scavenging activity	Metal chelating activity of Fe^{+2}	Reducing power activity
**LIHE	52.44±3.38	40.96±1.18	69.15±1.13	58.48±0.45
*Ascorbic acid	7.82±0.15	-	-	-
*BHT	-	9.28±0.09	-	-
*Citric acid	-	-	11.13±0.17	-
*Catechin	-	-	-	18.12±0.01

Notes: **samples, *standard antioxidant, LIHE- Hexane extract of *L. indica*, BHT- Butylated Hydroxyl Toluene.

3.4. In-vitro anti-inflammatory activity

Graded concentrations (50-250 µg/ml) of the plant extract was assessed for inhibition of protein denaturation as a measure of anti-inflammatory activity. And the results were found to be satisfactory and significant at all concentrations and even substantial with that of standard diclofenac sodium (p<0.01) (Table 4).

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

Sample and standards	IB_{50} Value
**LIHE	54.52±2.13
*Diclofenac sodium	10.15±0.10

Notes: **samples, *standard antioxidant, LIHE- Hexane extract of *L. indica.*

3.5. Insect antifeeding activity

Antifeeding property of hexane extract of *L. indica* was assessed by measuring the efficacy of plant extract to inhibit feeding habit of the fourth instar larvae of *S. obliqua*. As per the results obtained (Table 5.), it was revealed that like other nutraceutical activity performed the hexane extract was found to be more efficient antifeedant than that of the control as the percent antifeeding activity was found to be vary from 94.8-100% in the extract. Hexane extract was found to inhibit feeding rate by 100% at higher concentrations of 20% and 25% at all-time intervals of 12, 24, 36 and 48 hours after treatment. Although significant antifeeding action was observed at lower concentrations also and the results are statistically analyzed to be significantly different (p<0.05) at all the concentrations (5-25%) and at all-time intervals in sequentially dose and time dependent manner.

Table 5: Insect antifeeding activity of hexane extract of *L. indica* represented in percentage.

Doses (%)	After 12 hours		After 24 hours		After 36 hours		After 48 hours	
	% Antifeeding activity		% Antifeeding activity		% Antifeeding activity		% Antifeeding activity	
	% Feeding	LIHE	% Feeding	LIHE	% Feeding	LIHE	% Feeding	LIHE
5	1.64	94.80	14.6	73.29	61.12	22.94	100.00	0.00
10	1.40	95.54	5.9±	88.16	10.58	80.41	50.88	32.55
15	0.00	100.00	3.22	93.41	6.42	87.63	28.54	55.58
20	0.00	100.00	0.00	100.00	0.00	100.00	0.00	100.00
25	0.00	100.00	0.00	100.00	0.00	100.00	0.00	100.00
Control	62.84		94.78		97.53		100.00	

Notes: LIHE- Hexane extract of *L. indica*.

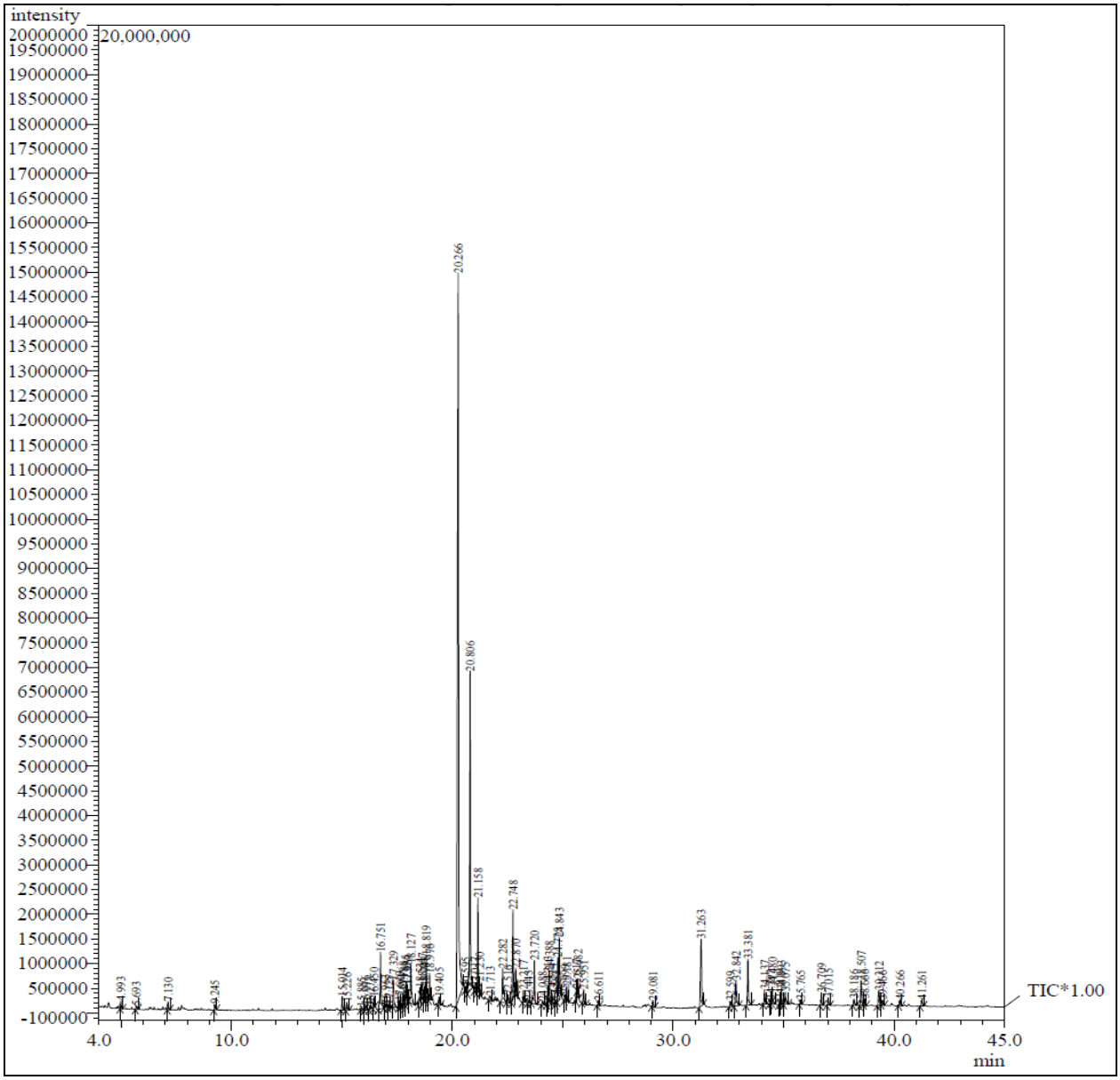

Fig 1: Gas chromatogram of hexane extract of *L. indica*.

4. Discussion

The present study regarding phytochemical quantification reports the substantial amount of TPC, TFC and ODP in hexane extract, significantly higher phenol and flavonoid content in hexane extract. The result supports the previous report suggesting higher phenolics and flavonoids content in hexane extract **[20].** Overall the results report the extract to be potentially efficient antioxidant having activity in the order of DPPH radical scavenging > NO radical scavenging > reducing power > metal chelating. Thus, can be inferred that the various biological activities like *in-vitro* antioxidant and anti-inflammatory activity is the cumulative result of TPC, TFC and ODP content. The results are in consistent with previous research reports showing close relationship between total phenolics and biological activities [21]. Antifeeding screening of the extract resulted very efficient antifeedancy rate at all the tested concentrations and at all the time intervals after the treatment in hexane extract. In recent years there is an upsurge in the field of insect pest management through botanical pesticides [22]. Many researchers have reported antifeeding activity of neem based botanicals against *Spilosoma obliqua* [23, 24]. The results obtained are in support of previous other researches reporting antifeeding properties in the plant extracts [25] containing aristolone [26], stigmasterol [27], phytol [28], cadinene [29], 5-hydroxycalamenene [30], hexadecanoic acid [31], compounds that are also reported in the chemical constituents the plant extracts of L. *indica*. Also, antifeeding activity in the extract may be due to the significant amount of the phenolic constituents in the extract [32].

5. Conclusion

The results obtained confirmthe plant extracts of *L. indica* to be a potent antioxidant, anti-inflammatory agent and also a significant antifeedant agent. The results signify that the plant can be potentially used to form various medicine formulations for health related issues like oxidative stress or inflammation in the body. Antifeeding action of the plant signifies its potentiality in the field of organic/sustainable agriculture to reduce the reliance on the chemical pesticides and to promote botanical pesticides to reclaim various health hazards occurring in nature due to chemical pesticides.

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