J. Mountain Res. P-ISSN: 0974-3030, E-ISSN: 2582-5011 DOI: https://doi.org/10.51220/jmr.v21-i1.9
Vol. 21(1), (2026) 71-80

Antioxidant Potential and Chemical Characterization Of The Essential Oil Of *Spilanthes Calva* DC From Lower Himalayan Region Of Uttarakhand

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Received:05.04.2026; Revised: 24.5.2026; Accepted: 29.5.2026

Abstract: *Spilanthes calva* DC (Asteraceae), also known as "toothache plant" or "paracress", is an aromatic plant found in India whose flowers are still used in folk medicine and are rich in essential oil. Present study investigates the first ever chemical composition profile of essential oil from *S. calva* flowers. The essential oil was extracted by using hydro-distillation method. *in vitro* Antioxidant potential of essential oil using seven different methods was also evaluated. Out of all methods, in six methods viz., ABTS radical scavenging activity, Cuprac Assay, Hydroxyl free radical scavenging assay, NO scavenging assay, FRAP Assay and Super oxide Anion radical scavenging Assay, *S. calva* essential oil shows better antioxidant activity than respective standard. IC_{50} value for each antioxidant assay was calculated by using Graph Pad Prism 6 software. The essential oil was analysed by gas chromatography-mass spectrometry (GC-MS). Total 31 compounds were identified from flowers essential oil of *Spilanthes calva* DC accounting for 97.55% of total composition. The essential oil yield obtained was 0.32%. p-Menth-1-ene (31.54%), myrcene (12.44%), cis-α-ocimene (11.25%) and (E)-caryophyllene (11.15%) are dominating constituents present in essential oil.

Key words: *Spilanthes calva* DC • Essential oil • Antioxidant Potential • Chemical Characterization • p-Menth-1-ene.

Introduction

Spilanthes calva DC belongs to *Spilanthes* genus of Asteraceae family, which is widely distributed in tropical and subtropical regions of the world (Tiwari *et al.* 2011). It is native to South America (Begum *et al.* 2008) and widely distributed in Bangladesh, India, Brazil, China, South Asia, West Indies, New Caledonia, Thailand, tropical Africa and Malaysia (Nasrin and Hossain 2015). It is an annual/ short-lived herb with golden yellow flowers and usually grows in sandstones, riversides, dry soil and waste places (Tiwari *et al.* 2011, Begum *et al.* 2008, Nasrin and Hossain 2015). It is commonly known as marhatitiga (Bangla) (Ramproshad *et al.* 2019), toothache plant (Dube *et al.* 2013) and paracress plant (English). In Bangladesh, people use fresh leaves of the plant in their salads, despite the fact that this herb is not traditionally cultivated for culinary purposes. However, nowadays, people are increasingly experimenting with it in search of new flavours (Ramproshad *et al.* 2019). There are over 55 species of genus Spilanthes which have been reported from different areas and region around the world. *S. acmella*, *S. alba*, *S. calva*, *S. americana*, *S. paniculata*, *S. ciliata*, *S. mauritiana*, *S. oleracea*, *S. radicans*, *S. filicaulis*, *S. ocymifolia*, *S. uliginosa* are some of those species (Tiwari *et al*. 2011, Devi *et al.* 2016). This plant is renowned for its unique characteristics and diverse range of ailments, including gum troubles, inflammation, pain, scabies, toothache, rheumatism, throat complaints, dry cough, scurvy, for treating various parasitic diseases, for liver disorder, tuberculosis and paralysis of tongue both in traditional and contemporary contexts. Its distinctive name, "toothache plant," alludes to one of its most notable features: the numbing sensation it produces

when its leaves or flowers are chewed, which can alleviate toothache and other oral discomforts (Paulraj *et al.* 2013, Paulraj *et al.* 2014, Yasuda *et al.* 1980). *S. calva* is a rich source of bioactive compounds; the most active biomolecule is spilanthol (Yasuda *et al.* 1980) which has significant local anaesthetic, anti-inflammatory,
and analgesic properties.

To the best of our knowledge, there has been no previous report on the chemical compositions, antioxidant and antimicrobial activity analysis of essential oil of *S. calva* from foothills of North India. Therefore, the aim of this study is to evaluate phytochemical screening and antioxidant potential of essential oil of flower of *Spilanthes calva* DC from foothills of Kumaon region of Uttarakhand, India.

Material and Methods

Collection of plant material and Identification of plant species

Fresh 630 g flowers of *Spilanthes calva* DC were collected during April 2023 from wild from Nainital district of Kumaon region of Uttarakhand (latitude 29°21'55.5" N and longitude 79° 32'36.2" E) at an elevation of about 1365 meter from sea level. Plant sample was identified from RARI, Ranikhet (Uttarakhand) and a sample voucher (specimen no. 7, accession no. 21230) submitted at RARI Ranikhet, Herbarium (Uttarakhand, India) of Central Council for Research in Ayurvedic Sciences (Ministry of AYUSH, Government of India).

Essential oil Extraction Method

Fresh flowers (630 grams) of *Spilanthes calva* DC were firstly cleaned and then hydro-distilled for 4 h using Clevenger apparatus. The oil samples were collected and dried over anhydrous sodium sulphate. Before GC-FID/GC-MS analysis, the oil samples were kept in sealed vials in a refrigerator without any additional treatment.

GC and GC-MS Interpretation

The essential oil samples (0.2 µL, undiluted) were subjected to gas chromatographic analysis using a Shimadzu QP-2010 Ultra GC system. Separation was achieved on an Rxi-5 SIL MS capillary column measuring 30 m × 0.25 mm with a film thickness of 0.25 µm. Helium was employed as the carrier gas at a constant flow rate of 1.21 mL/min, maintaining an average linear velocity of 39.9 cm/s under a pressure of 69.0 kPa. The oven temperature program was initiated at 50°C with a holding period of 2 min, then increased to 210°C at a rate of 3°C/min and held for another 2 min, followed by a rise to 280°C at 8°C/min with a final hold of 9 min. The injector and flame ionization detector (FID) temperatures were both maintained at 260°C, while the split ratio was adjusted to 1:40.

GC–MS characterization of the essential oil was performed on a Shimadzu QP-2010 Ultra GC/MS instrument equipped with the same Rxi-5 SIL MS column (30 m × 0.25 mm i.d., film thickness 0.25 µm). The oven temperature was programmed from 50°C to 280°C at a heating rate of 3°C/min. Helium served as the carrier gas with a constant flow rate of 1.21 mL/min. A 0.1 µL aliquot of the sample diluted in acetone was injected at an injector temperature of 260°C using a split ratio of 1:40. Mass spectra were recorded under electron ionization mode at 70 eV across a scan range of 45–650 amu. The ion source and interface temperatures were maintained at 230°C and 280°C, respectively.

Quantitative estimation of the constituents was carried out by the external standard calibration method. The relative composition of the volatile compounds was determined from normalized FID peak areas without applying correction factors. The floral essential oils of *Spilanthes calva* DC were analysed through both GC and GC/MS techniques. Identification of individual components was accomplished by comparing their retention indices, calculated relative to a homologous series of n-alkanes (C9–C33) on an Rtx-5MS

fused silica capillary column, together with spectral matching using NIST (NIH version 2.1) and WILEY (7th edition) mass spectral libraries, as well as previously published MS and RI reference data. (Adams *et al.* 2006, Adams 2007, Babushak *et al.* 2011, Goodner 2008).

Evaluation of Antioxidant activity

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

Different concentrations (0–5%) of the test compound (5 µL each) were added to 0.1 mL of 0.1 mM DPPH solution in a 96-well plate. The reactions were performed in triplicate. Duplicate blank wells were prepared containing 0.2 mL of DMSO/methanol and 5 µL of the respective compound concentrations. The plate was incubated in the dark for 30 minutes. After incubation, the decrease in absorbance was measured at 495 nm using a microplate reader (iMark, Bio-Rad). A reaction mixture containing 20 µL of deionized water served as the control. The percentage of DPPH radical scavenging activity was calculated relative to the control using the equation below, and the IC_{50} values were determined using GraphPad Prism 6 software (Abdulrasheed and Busari 2020, Imam *et al.* 2011).

DPPH Scavenging activity = $((Abs_{Control} - Abs_{Sample})/Abs_{Control}) \times 100$

Where, $Abs_{Control}$ and Abs_{Sample} represent the absorbance value of control sample and test sample respectively.

ABTS radical scavenging

ABTS radicals were generated by reacting 7 mM ABTS solution with 2.4 mM ammonium persulfate (APS), followed by a 100-fold dilution to obtain the ABTS radical working solution. Different concentrations (0–2.5%) of the test samples (10 µL each) were added to 200 µL of the ABTS radical reagent in a 96-well plate, alongside a standard (ascorbic acid, 5 mg/mL). The mixture was incubated at room temperature for 10 minutes in the dark place. After incubation, the decrease in absorbance was recorded at 750 nm using a microplate reader. (iMark, Bio-Rad). Results were expressed relative to the negative control, and IC_{50} values were determined using GraphPad Prism 6 software (Cao and Prior 1998, Gupta *et al.* 2009, Kambayashi *et al.* 2009).

Cuprac assay

Different concentrations of the test compound (0–2.5%) were prepared, and 10 µL of each sample was added to 200 µL of the reagent mixture in a 96-well plate. The reactions were performed in triplicate. Duplicate blank wells were prepared containing 200 µL of methanol and 10 µL of the test compound at the same concentrations (0–2.5%) or standard (ascorbic acid, 0–50 µg/mL). The plate was incubated in the dark for 30 minutes at room temperature. After incubation, the absorbance was measured at 490 nm using a microplate reader (iMark, Bio-Rad). A reaction mixture containing 20 µL of deionized water served as the control. The radical scavenging activity was expressed as the percentage of inhibition relative to the control, and the IC_{50} values were determined using GraphPad Prism 6 software (Apak *et al.* 2007, Rubio *et al.* 2016).

Hydroxyl free radical scavenging assay

A total of 66 µL of reagent mixture (containing 10 µL EDTA [0.5 M], 24.14 mg deoxyribose, 88 µL $FeCl_3$ (10 mg/mL), 28 µL H_2O_2 (6%), and distilled water up to 33 mL) was added to each well of a 96-well plate. Subsequently, 10 µL of the test sample (0–5%), 10 µL of ascorbic acid and 24 µL of phosphate buffer (50 mM, pH 7.4) were added sequentially. The plate was preserved at 37°C temperature for one hour. Gallic acid (0–50 µg/mL) was used as the standard. Following incubation, 50 µL of 10% trichloroacetic acid (TCA) and 50 µL of 1% thio-barbituric acid (TBA) were added to each well, resulting in the formation of a pink chromogen. The absorbance was then measured at 540 nm using a microplate reader (iMark, Bio-Rad). IC_{50} values were determined using GraphPad Prism 6 software (Hazra *et al.* 2008, Rahman *et al.* 2015).

NO scavenging assay

A total reaction volume was formulated by mixing 50 µL of 10 mM sodium nitroprusside (Fisher Scientific, Cat. No. 27864) with 40 µL of distilled water and 10 µL of either the experimental sample, reference standard [gallic acid (SRL, Cat. No. 13142)], or blank solution. This solution mixture was allowed to stand for 15 min at room temperature under light to facilitate nitric oxide generation. Following incubation, Each reaction was treated with 100 µL of Griess reagent and control well, and the plate was further incubated at room temperature for 5–10 minutes to allow chromophore formation and stabilization. The absorbance was measured at 540 nm and 660 nm using a microplate reader (iMark, Bio-Rad). IC_{50} values were determined using GraphPad Prism 6 software (Rao *et al.* 2013).

FRAP assay

Different concentrations of the test compound (0–2.5%) and the standard (ascorbic acid; SRL, Cat. No. 23006; 0–50 µg/mL) were prepared. In each well, 10 µL of test sample or standard was combined with 40 µL of 0.2 M sodium phosphate buffer of pH value 6.6 and 50 µL of 1% potassium ferricyanide [$K_3Fe(CN)_6$] (SRL, Cat. No. 15766). After thorough vortexing, the reaction system was incubated at 50°C for 20 minutes. After incubation, 50 µL of 10% trichloroacetic acid (SRL, Cat. No. 92390) was mixed to it, followed by 50 µL of deionized water and 50 µL of 0.1% $FeCl_3$ (Fisher Scientific, Cat. No. 23585). The absorbance of the developed coloured solution was recorded at 750 nm against a blank using a microplate reader (iMark, Bio-Rad). The IC_{50} values were determined using GraphPad Prism 6 software (Rao *et al.* 2013).

Super oxide anion radical scavenging assay

Different concentrations of the test material were mixed with riboflavin solution and incubated under light at room temperature for 30 minutes. After incubation, the reaction mixture was combined thoroughly, and the absorbance was measured at 560 nm using an ELISA plate reader (iMark, Bio-Rad, USA). The IC_{50} values were calculated using GraphPad Prism 6 software (Noda *et al.* 1997). Some of the content of all above sections is based on findings from my previous published work (Karakoti *et al.* 2022, Karakoti *et al.* 2024).

Results and Discussion

In vitro antioxidant activity

The antioxidant activity of the essential oils was measured in terms of seven parameters, in all of which the flower oil of *Spilanthes calva* DC (sample SPC) showed good antioxidant activity in a dose dependent manner. Smaller the IC_{50} value, greater the antioxidant potential. **Table 1** represents IC_{50} of SPC sample in six different methods determining the antioxidant potential. The Graphical representation of antioxidant potential of standard and sample SPC is shown in **Fig 1**.

Table 1. Antioxidant activity of flower essential oil of *Spilanthes calva* DC (sample SPC)

Sample/ Standard	Antioxidant activity in terms of IC_{50} (µg/ml)						
	DPPH radical scavenging activity	ABTS radical scavenging activity	Cuprac Assay	Hydroxyl free radical scavenging assay	NO scavenging assay	FRAP Assay	Super oxide Anion radical scavenging Assay
SPC	2.55	0.5378	0.06292	3.035	1.92	0.3201	1.323
Ascorbic acid*	1.773	0.8237	-	-	-	4.258	-
Trolox*	-	-	1.38	-	-	-	-
Gallic acid*	-	-	-	14.71	12.28	-	-
Quercetin*	-	-	-	-	-	-	28.97

IC_{50} = Half maximal inhibitory concentration; SPC= flower essential oil of *S. calva*; * = Standard antioxidant.

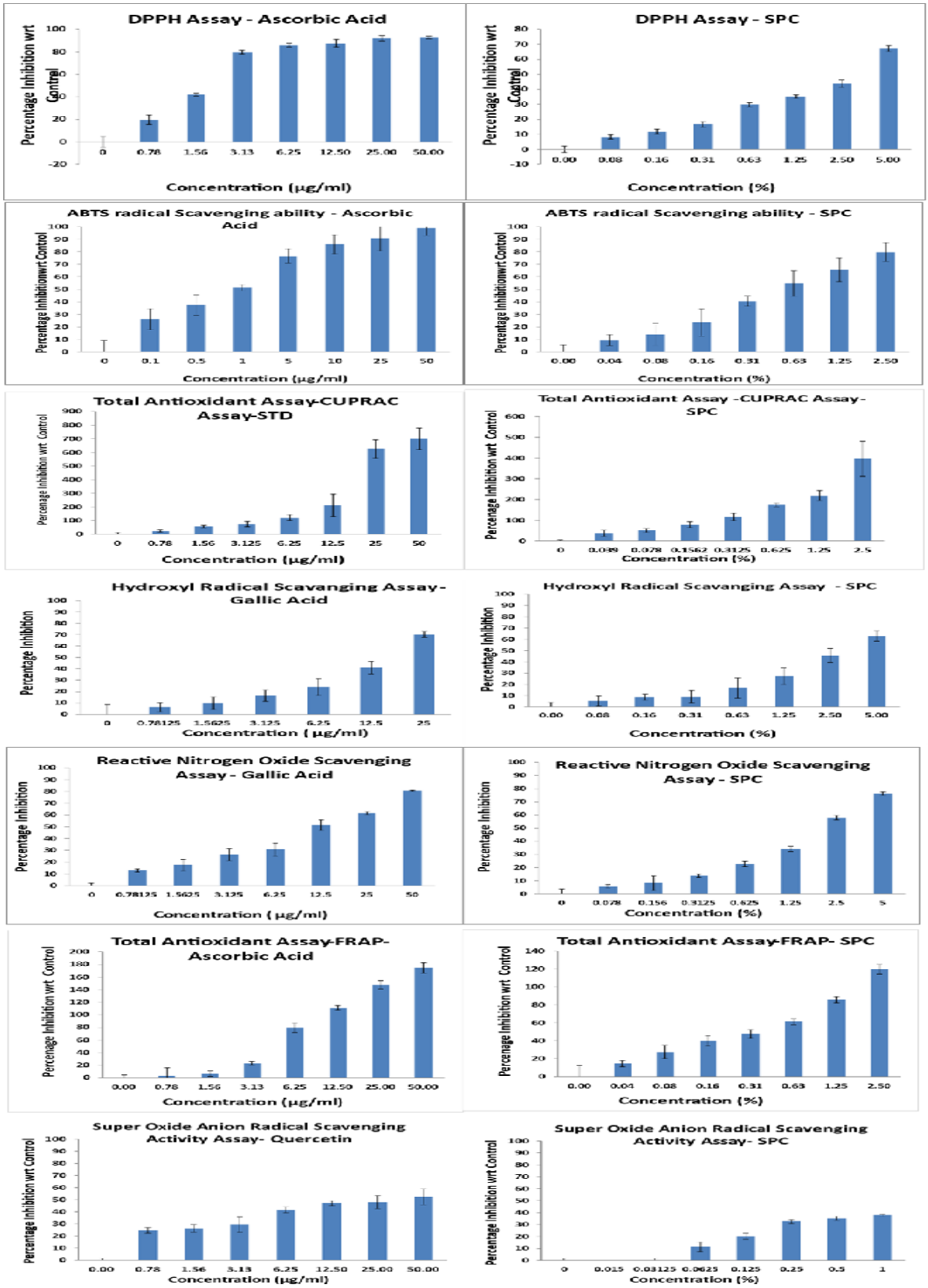

Fig 1. Graphs representing antioxidant potential of standard and sample SPC

Out of seven methods, in six methods viz., ABTS radical scavenging activity, Cuprac Assay, Hydroxyl free radical scavenging assay, NO scavenging assay, FRAP Assay and Super oxide Anion radical scavenging Assay, *S. calva* essential oil shows better antioxidant activity than respective standard, while only for DPPH radical scavenging assay, IC_{50} value of oil sample is greater than standard. Although there is no data available on antioxidant activity of *S. calva* flower essential oil, however antioxidant activity from Bangladesh (Ramproshad *et al.* 2019, Ali *et al.* 2011) and India (Badami *et al.* 2007, Devi *et al.* 2017, Pali and Bhatnagar 2021) has been reported earlier for plant and various part extract.

Chemical Characterization

The essential oil yield from flowers of 630 g was 2.0 ml (0.32 % v/w). **Table 2** summarizes the identified oil constituents. In the essential oil of *Spilanthes calva* DC flowers, 31 compounds were characterized, accounting for 97.55% of the total content. Gas Chromatogram of *Spilanthes calva* flower essential oil is shown in **Fig 2**.

Table 2. Chemical characterization of *Spilanthes calva* DC flower essential oil

S. No.	Compound	Retention Time	RI Obs	RILit	% Composition
1.	α-Thujene	6.4	927	925	0.03
2.	α-Pinene	6.6	933	939	0.43
3.	Sabinene	8.1	972	968	5.39
4.	β-Pinene	8.2	978	964	2.06
5.	β-Myrcene	8.8	990	988	12.44
6.	p-Mentha-1(7),8-diene	9.2	1004	1002	0.52
7.	α-Phellandrene	9.3	1007	1006	0.04
8.	α-Terpinene	9.8	1018	1019	0.06
9.	p-Menth-1-ene	10.5	1022	1030	31.54
10.	cis-α-Ocimene	10.8	1032	1038	11.25
11.	E-β-Ocimene	11.1	1046	1043	0.23
12.	γ-Terpinene	11.5	1058	1052	0.10
13.	α-Terpinolene	12.7	1086	1087	0.02
14.	Linalool	13.5	1095	1098	0.05
15.	(E)-4,8-Dimethyl nona-1,3,7-triene	14.0	1113	1118	0.03
16.	(-)-4-Terpineol	17.0	1167	1174	0.37
17.	β-Elemene	26.1	1390	1395	0.81
18.	β-Maaliene	26.5	1415	1420	0.09
19.	(E)-Caryophyllene	27.483	1424	1427	11.15
20.	β-Copaene	27.8	1433	1435	0.06
21.	trans-α-Bergamotene	27.9	1434	1438	0.09
22.	α-Humulene	28.8	1454	1454	0.96
23.	Germacrene D	30.1	1480	1482	9.61
24.	Bicyclogermacrene	30.5	1497	1494	2.70
25.	(E, E)-α-Farnesene	31.0	1504	1506	1.34
26.	δ-Cadinene	31.4	1518	1522	0.14
27.	β-Sesqui phellandrene	31.8	1529	1537	4.62
28.	(E)-Nerolidol	33.3	1561	1564	0.39
29.	Carotol	34.8	1601	1600	0.69
30.	T Muurolol	36.9	1645	1640	0.24
31.	Khusinol	38.0	1677	1675	0.10
	Total Composition		97.55		

	Essential Oil Yield w/v (%)	0.32
S. No.	Class	6.6
1	Monoterpene hydrocarbon	8.1
2	Oxygenated monoterpenes	8.2
3	Sesquiterpene hydrocarbon	8.8
4	Oxygenated sesquiterpenes	9.2

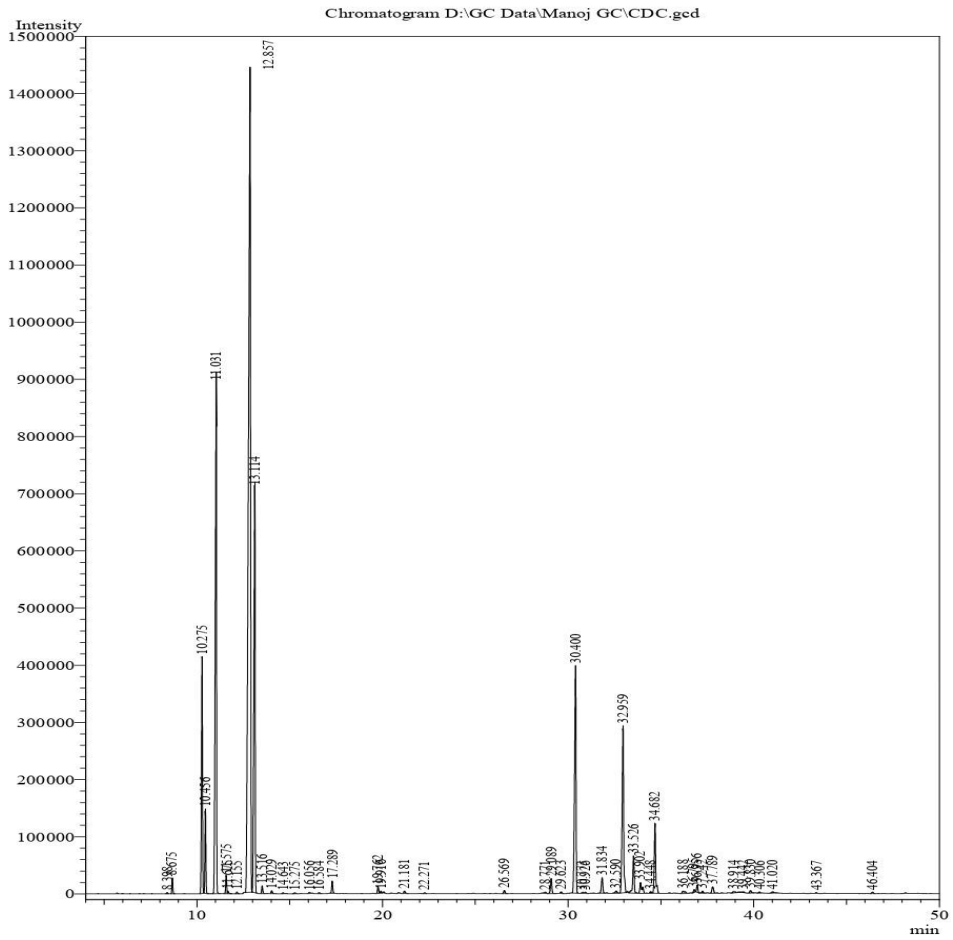

Fig 2. Gas Chromatogram of *Spilanthes calva* flower essential oil

The monoterpene hydrocarbons (64.12%) are dominant class of compounds followed by sesquiterpene hydrocarbons (31.57%) and oxygenated sesquiterpenes (1.42%), while some other class of compounds viz., alcohols, acids, diterpenes, diterpenoids are also found in trace amount. The essential oil of flowers was dominated by monoterpene hydrocarbons with p-menth-1-ene (31.54%), myrcene (12.44%) and cis-α-ocimene (11.25%) (E)-caryophyllene (11.15%) as major constituents. The other constituents present in essential oil are germacrene D (9.61%), sabinene (5.39%), β-sesqui phellandrene (4.62%), bicyclogermacrene (2.70%), β-pinene (2.06%) and 14-hydroxy-4,5-dihydro-caryophyllene (1.09%). Previous study from South India (Jirovetz et al., 2005) reports (E)-2-hexenol (25.7%), 2-tridecanone (13.1%), hexanol (11.0%), (Z)-3-hexanol (5.1%), germacrene D (11.1%) and β-caryophyllene (10.8%) as dominating components in fresh plant essential oil of *S. calva*. Another report from Bangladesh (Begum et al 2008) reported caryophyllene oxide (24.14%), caryophyllene (22.19%), limonene (21.79%), myrcene

(9.02%), sabinene (8.15%), β-cis-ocimene (7.52%) and β-pinene (7.17%) as major constituents in inflorescence essential oil, while in our study major constituents are entirely different from both the findings. 40 new compounds (α-thujene, α-pinene, p-mentha-1(7),8-diene, α-phellandrene, α-terpinene, p-menthene… **table 2**) have been identified with reference to inflorescence essential oil from Bangladesh[2]. This may be due to the change in climatic, geographical conditions (Dobhal *et al.* 2024, Moghaddam *et al.* 2023, Santos *et al*. 2023).

Conclusion

The current study, first ever reported chemical characterisation of flower essential oil of *Spilanthes calva* DC along with its *in vitro* antioxidant potential by seven different methods from India. GC/GC-MS analysis, indicates p-menth-1-ene (31.54%), myrcene (12.44%), cis-α-ocimene (11.25%) as major compounds in essential oil of flower. Among seven out of six methods viz. ABTS, Hydroxyl free radical scavenging, Cuprac, NO scavenging, FRAP, and Super oxide anion radical scavenging assay *S. calva* flower essential oil show better antioxidant activity than respective standard, which means essential oil of S. *calva* flower was an excellent antioxidant. However *in vivo* studies have to be done for better understanding. These findings clearly indicate the useful insights regarding *S. calva*.

Acknowledgements

We acknowledge the Council of Scientific and Industrial Research (CSIR), Government of India, through the award of a Senior Research Fellowship (SRF) to Manoj Karakoti (Ref no. May 2018-121661), which provide financial assistance to this work and Dr Deepshikha Arya for plant identification.

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