Pharmacogenetic properties of *Mentha spicata* L. leaves and isolation of L-Carvone from its leaves

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ABSTRACT

The present study aimed to explore the phytochemical constituents, antioxidant properties, and characterization of M. spicata L. leaves, with a focus on isolating and identifying L-Carvone. Phytochemical screening was performed using the condensation method, while extracts were prepared with solvents of increasing polarity. Qualitative analysis of phytochemicals was carried out on different solvent extracts, whereas quantitative estimation and antioxidant assays were performed on the hydroethanolic (50:50) extract. GC-MS and FT-IR analyses were used for characterization, and Soxhlet extraction followed by column chromatography enabled isolation of compounds. TLC and HPLC confirmed the presence of L-Carvone. Results indicated that the hydroethanolic extract was rich in steroids and exhibited strong antioxidant activity, with GC-MS, FT-IR, and chromatographic methods confirming L-Carvone in the isolated fraction.

Keywords: Analytical standardization, isolation, L-Carvone, phytochemistry, spearmint

INTRODUCTION

Spearmint (*Mentha spicata* L.), a member of the Lamiaceae family rich in essential oils (Amel *et al*., 2022), is widely cultivated and valued for its antifungal, antimicrobial, insecticidal, antioxidant, and therapeutic properties (Kumar *et al*., 2023), including treatment of fever, bronchitis, gastritis, headaches, and nausea (Choudhury *et al*., 2006). With restrictions on synthetic antioxidants due to health concerns (Gomez, 2003; Mitra *et al*., 2009), natural sources like mint are important antioxidants. Often the fresh leaves of *M. spicata* L., are used as condiments (Hasan Jone *et al*., 2022). Peppermint (*Mentha* × *piperita*), a hybrid of *M. spicata* and *M. aquatica*, also shows potential in cardiovascular disorders (Nagarajan and Doss, 2023). The main constituent of spearmint oil is L-Carvone, along with limonene (Snoussi *et al*., 2015), responsible for its aroma and strong antimicrobial activity (Scherer *et al*., 2013). Since spearmint oil contains at least 28 organic compounds (Diaz-Maroto *et al*., 2003), rationalizing its traditional use requires focused studies. This work was aimed to isolate L-Carvone using Soxhlet extraction followed by column chromatography, with TLC, FTIR, and HPLC to confirm its presence. Our microscale method reduced solvent and silica gel use and employed FTIR and HPLC as confirmatory techniques.

MATERIALS AND METHODS

All analytical-grade chemicals and reagents were procured from HiMedia Laboratories Pvt. Ltd., India. Leaves of spearmint were collected from farms in Coimbatore, authenticated by the Botanical Survey of India, Southern Regional Centre

(BSI/SRC/5/23/2021/Tech/137). Fresh leaves were washed, shade-dried (5–10 days), powdered, and extracted with solvents of increasing polarity (petroleum ether, acetone, chloroform, ethanol, hydroethanol 50:50, and water) using cold maceration (10 g/100 mL, 72 h). Filtrates were concentrated, lyophilized, and stored for analysis.

Qualitative and quantitative analysis

Hydroethanolic (50:50) extracts of spearmint leaves were concentrated at 45 °C and stored at –20 °C. Screening confirmed carbohydrates, proteins, alkaloids, flavonoids, glycosides, terpenoids, phenols, tannins, saponins, steroids, and fats. Quantitative assays estimated carbohydrates, proteins, phenols, flavonoids, steroids, and tannins (Sugumar *et al*., 2019).

Antioxidant analysis

Antioxidant potential was evaluated by DPPH dot-blot, DPPH, nitric oxide, and hydroxyl radical scavenging assays. Enzymic assays included catalase, SOD, and glutathione peroxidase, while non-enzymic antioxidants measured were ascorbic acid (Omaye *et al*., 1979), tocopherol, and reduced glutathione. Total antioxidant capacity was assessed by reducing power, phosphomolybdenum, and FRAP (Jethinlalkhosh *et al*., 2016) determinations.

Characterization

Phytocomponents were analyzed by GC-MS (Shimadzu QP2010, Elite-1 fused silica column, 100% dimethyl polysiloxane) and FTIR (Shimadzu spectroscope, 400–4000 cm^{-1}, 16 cm^{-1} resolution) (Nagarajan and Victor Arokia, 2024).

Soxhlet extraction

Mint leaf powder (10 g) was extracted in a 250 mL Soxhlet using ethanol (99.9%), which gave higher yield than hydroethanol. Extraction with 100 mL ethanol at 78 °C for 1.5 h produced a crude extract, which was filtered (Whatman No.1), concentrated on a hot water bath, weighed, and stored in amber vials at –8 °C (Alelign *et al*., 2020).

Column, TLC, and HPLC

L-Carvone isolation was carried out by Elmastas (2006) method with modifications. Crude extract (2 g) was fractionated on silica gel (50 g) using solvent mixture (benzene:toluene:ethyl acetate:ethanol, 3.5:2.5:2.5:1.5), which yielded L-Carvone fractions. TLC (silica gel 60F254, Merck) separation using toluene:ethyl acetate:ethanol (4.5:3.5:2) fractionated terpenoid which was vizualized using vanillin–sulfuric acid (Mar *et al*., 2020). HPLC was performed on a Shimadzu LC-2030 Plus with UV detection (272 nm). Injected 20 µL sample in acetonitrile:water (45:55, v/v) at a speed of 1.0 mL/min, and at 40 °C, for 20 minutes, which confirmed L-Carvone peaks.

RESULTS AND DISCUSSION

Qualitative and Quantitative phytochemical analysis

Phytochemical screening revealed the highest metabolite content in hydroethanolic extract, followed by chloroform, benzene, acetone, petroleum ether, ethanol, and water, (Table 1) consistent with Paikara & Pandey (2018). Quantitative analysis of the hydroethanolic extract (Figure 1) showed carbohydrates (34.60 ± 0.79 mg/g) and proteins (20.67 ± 0.53 mg/g), comparable to Scherer *et al*. (2013). Phenols (40.13 ± 2.5 mg/g) exceeded aqueous extract values reported by Kanatt *et al*. (2007), supporting their usefulness in cardiovascular, cancer, metabolic, and aging-related disorders (Yuan *et al*., 2011). Flavonoids (54.50 ± 0.7 mg/g) were significantly of higher values than those reported with aqueous solution (Kanatt *et al*., 2007). The other antimicrobial, anticancer, and anti-inflammatory properties were as reported earlier (Panche *et al*., 2016). Steroids (80.56 ± 0.40 mg/g) and tannins (54.03 ± 0.35 mg/g) were also abundant of which, the latter is known for antioxidant, cardioprotective, and anticancer effects (Labieniec *et al*., 2003).

Antioxidant activity

Antioxidant evaluation confirmed strong free radical scavenging ability. As shown in the Table 2, in the DPPH assay, the extract showed IC_{50} = 221.34 µg/ml, close to ascorbic acid (255.75 µg/ml) and ethanolic

extract reported by Mata *et al*. (2007), IC_{50} = 140.97 µg/ml). NO scavenging activity (IC50 = 288.18 µg/ml) slightly outperformed the standard (314.47 µg/ml), while hydroxyl radical scavenging (IC_{50} = 316.46 µg/ml) activities was stronger than aqueous extract values reported by Kanatt *et al*. (2007), IC_{50} = 498.3 µg/ml), likely due to phenolic hydrogen-donating ability. Dot-blot assay (200–1000 µg/ml) further confirmed free radical scavenging through visible yellow spot formation against a purple background (Figure 2).

Enzymic and non-enzymatic antioxidants

Enzymatic antioxidants were also prominent, with catalase (1.948 ± 0.02 µmol H_2O_2 /min/mg protein), SOD (1.308 ± 0.05 U/mg), and glutathione peroxidase (2.36 ± 0.24 U/mg) activities (Figure 3), in line with the SOD activity (aqueous extracted) reported by Kanatt *et al*. (2007). These enzymes provide anti-inflammatory and anticancer-preventive benefits. Non-enzymic antioxidants (Figure 4) included ascorbic acid (23.06 ± 0.02 mg/g), α-tocopherol (31.63 ± 0.12 mg/g), and glutathione (18 ± 0.6 mg/g), reinforcing the strong antioxidant capacity of the hydroethanolic extract (Klimczak *et al*., 2007).

Total antioxidant assay

Phenolics and flavonoids largely contributed to the antioxidant capacity of spearmint. Reducing power assay showed 59.75% inhibition at 500 µg/ml, comparable to ascorbic acid (62.25%) with RC_{50} values of 351.62 and 336.47 µg/ml (Table 3). Phosphomolybdenum assay gave IC_{50} of 254.20 µg/ml (extract) vs. 246.18 µg/ml (standard), while FRAP assay showed IC_{50} values of 261.64 µg/ml (sample) and 257.47 µg/ml (standard), as reported by Mandana *et al*. (2011), who had observed high antioxidant activity (71.00 ± 2.65%) *via* Supercritical Carbon Dioxide ($SC\text{-}CO_2$) extraction.

GC-MS and FTIR analysis of spearmint leaves

GC-MS identified 37 compounds (Figure 5), with major constituents β-sitosterol (46.05%), L-carvone [2-Cyclohexen-1-one, 2-methyl-5-(1-methylethenyl)-(R)] (17.28%), phytol (7.07%), and ergost-5-en-3-ol (4.88%) at retention times 22.285, 9.053, 17.385, and 20.974 min, respectively. L-Carvone, the bioactive signature compound, was selected for isolation. FTIR spectra (Figure 6) confirmed functional groups including phenols, aldehydes/ketones, carboxylic acids, ethers, alkanes, aliphatic amines, alkenes, and aromatics as showed in Table 4.

L-Carvone extraction

Soxhlet extraction followed by column chromatography (benzene to ethanol; ethyl acetate: toluene:ethanol, 5.5:3:1.5 v/v) (Figure 7) yielded 50 mg of L-carvone as a pale-yellow oil. Fractions 3–12 showed a pale-green TLC spot (Rf = 0.42), consistent with the reported Rf value of carvone (0.44) (Stecher, 1968), confirming its identity (Figure 8). The compound was further verified by FTIR.

FTIR analysis of L-Carvone

FTIR spectra of the hydroethanolic extract (Figure 9) confirmed the compound as L-carvone, matching the standard (NIST, 2018). In Table 5 and Figure 9, peak values for phenols (3618.46 cm^{-1}), aromatics (1435.04 cm^{-1}), aldehydes (2731.20 cm^{-1}), benzene derivatives (894.97 cm^{-1}), and halogens (570.93 cm^{-1}) were consistent with Truzzi *et al*. (2022).

High Performance Liquid Chromatography

Standard L-carvone of 20–100 µg/mL showed linearity (R^2 = 0.9927) with retention times of 14.819–15.079 minutes as shown in Table 6. The standard and isolated compound results of HPLC Figure 10 & 11 showed a similar retention time of 14.994 and 14.041 minute respectively. Clear, sharp peaks confirmed purity and successful extraction using benzene:ethyl acetate:toluene:ethanol (3.5:2.5:2.5:1.5), validating identity and suitability for bioassays.

CONCLUSION

Spearmint leaves are rich in L-carvone, isolated and characterized using simple, effective techniques. Such refined methods meet the growing demand for bioactive compounds as food supplements. Despite modern medicine, traditional remedies remain vital, especially for underprivileged populations. Hence, documenting and standardizing phytoconstituents like L-carvone is crucial, alongside innovations in extraction, analysis, and clinical evaluations.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 1: Preliminary phytochemical analysis of *M. spicata* L. leaves.

Phytochemical analysis test name	Aqueous	Ethanol	Hydro Ethanol	Petroleum Ether	Chloroform	Acetone	Benzene
Carbohydrate							
Benedict's	+	+	-	+	-	+	-
Fehling's	+	++	+	+++	++	+	+++
Molisch's	-	+	+	+	+	-	+
Protein							
Biuret's	+	-	-	-	+	+	-
Ninhydrin	++	+	+	-	-	++	-
Alkaloids							
Dragendorff's	-	+	+	++	+	-	++
Wagner's	++	-	+	++	+	-	++
Mayer's	-	-	+	-	-	-	+
Flavonoids							
Alkaline reagent	+	-	+	+	+	-	+
Zinc HCl	+	+	++	+	++	+	++
Glycosides							
Borntrager's	+	+	+++	-	+	-	++
Keller- Killani	-	-	+	+	+	++	++
Legal's	++	-	+++	++	+++	+++	++
Terpenoids							
Salkowski	+	+++	++	-	+	++	++
Libermann-Burchard's	+	++	+++	+	+	++	+
Phenols							
Ferric chloride	-	++	++	-	+	+	-
Lead acetate	+	++	+++	+	+	+	+
Tannins							
Ferric chloride	+	+	+	-	+	+	+
Lead acetate	-	++	+	+	-	+	+
Saponin							
Foam	+	++	++	++	+++	+	+
Steroids							
Salkowsi's	+	+++	++	-	+	++	++
Libermann-Burchard	+	++	+++	+	+	+	+
Fats							
Cupric hydroxide	+	+++	++	+	++	+	++

+++ indicates **excess,** ++ indicates **strong,** + indicates **weak** concentrations, and
- indicates **absence** of phytochemicals in different extracts of leaves of spearmint.

Table 2: Free radical scavenging activity of spearmint sample.

Concentration (µg/mL)	DPPH (%)		Nitric Oxide Free Radical Scavenging (%)		Hydroxyl Radical Scavenging (%)	
	Ascorbic acid	*M. spicata* L.	Ascorbic acid	*M. spicata* L.	Ascorbic acid	*M. spicata* L.
100	61.63 ± 1.12	58.75 ± 2.04	42.86 ± 0.98	38.57 ± 1.62	42.00 ± 1.05	40.00 ± 1.75
200	75.25 ± 1.35	62.50 ± 2.30	48.57 ± 1.12	44.29 ± 1.85	50.00 ± 1.20	45.00 ± 1.90
300	81.13 ± 1.42	67.50 ± 2.55	57.14 ± 1.25	50.00 ± 2.05	55.00 ± 1.28	51.00 ± 2.10
400	85.13 ± 1.50	73.75 ± 2.80	64.29 ± 1.35	60.00 ± 2.20	62.00 ± 1.35	59.00 ± 2.15
500	89.25 ± 1.62	78.75 ± 2.95	77.14 ± 1.48	71.43 ± 2.40	74.00 ± 1.45	70.00 ± 2.25

Table 3: Total antioxidant activity of spearmint sample.

Concentration (µg/mL)	Reducing Power Assay (%)		Phosphomolybdenum Assay (%)		FRAP Assay (%)	
	Ascorbic acid	*M. spicata* L.	Ascorbic acid	*M. spicata* L.	Ascorbic acid	*M. spicata* L.
100	38.63 ± 0.75	33.75 ± 1.25	58.41 ± 1.12	53.74 ± 2.05	43.00 ± 0.86	41.37 ± 1.60
200	44.25 ± 0.88	42.50 ± 1.35	64.02 ± 1.22	63.41 ± 2.15	57.28 ± 1.05	55.89 ± 1.85
300	50.13 ± 0.95	48.50 ± 1.45	70.41 ± 1.35	68.74 ± 2.30	62.08 ± 1.10	61.18 ± 1.95
400	57.13 ± 1.05	54.75 ± 1.55	77.05 ± 1.45	74.85 ± 2.45	69.44 ± 1.20	69.00 ± 2.05
500	62.25 ± 1.15	59.75 ± 1.65	82.27 ± 1.55	79.08 ± 2.55	89.26 ± 1.35	87.71 ± 2.20

Table 4: FTIR frequency values and functional groups of ethanolic extract of spearmint leaves

S.No	Frequency (cm^{-1})	Reference frequency (cm^{-1})	Functional group	Compounds	Intensity
1.	3726.47	3749.62	O-H	Phenol	Strong
2.	2978.09	2975	C-H	Alkenes	Medium
3.	2360.87	2400-3200	N-H	Ammonium ions	Multiple broad peaks
4.	2337.72	2400-3200	N-H	Ammonium ions	Multiple broad peaks
5.	1728.22	1725	C=O	Aldehyde/Ketone	Influenced by conjugation
6.	1604.77	1550-1610	C=O	COOH derivates	Medium
7.	1381.03	1380	N-O	Nitro compounds	Weaker
8.	1249.87	1220-1260	C-O	Ether	Strong
9.	1149.57	1100-1200	C-X	Fluoroalkanes	Two strong broad bands
10.	1072.42	1020-1220	C-N	Aliphatic amines	Often overlapped
11.	956.69	956.69	C-H	Alkenes	Strong
12.	810.10	800-860	C-H	Aromatic	Strong
13.	671.23	670-700	C-H	Alkenes	Strong
14.	547.78	540-760	C-X	Chloroalkanes	Weak to medium
15.	509.21	500-600	C-X	Bromoalkanes	Medium to strong

cm^{-1} can be abbreviated as centimetre power minus 1.

Table 5: FTIR frequency values and functional groups of isolated terpene compound L-Carvone.

S.No.	Frequency (cm^{-1})	Reference Frequency (cm^{-1})	Functional Group	Compound	Intensity
1.	3618.46	3610	O-H	Phenol	Medium
2.	2924.09	2925	C-H	Alkyl	Medium to strong
3.	2731.20	2720	C-H	Aldehyde derivates	Medium
4.	1674.21	1675	C=C	Alkenes	Medium
5.	1435.04	1450	C=C	Aromatic	Weak to strong
6.	1242.16	1220- 1260	C-O	Aromatic ethers	Medium
7.	964.41	965	C-H	Alkene	Strong
8.	894.97	860- 900	C-H	Aromatic benzene	Strong

Table 6: Data for the calibration curve of L-Carvone quantification using HPLC.

S No	Injection Volume (ul)	Concentration (ug/ml)	Area Under the Curve (AUC)
Stock Standard (1000ug/ml)			
1	20	20	2041372
2	20	40	4332665
3	20	60	5939478
4	20	80	7571862
5	20	100	9059949
***M. spicata* L. Extract Sample**			
1	L-Carvone Extract	60	5027556.81

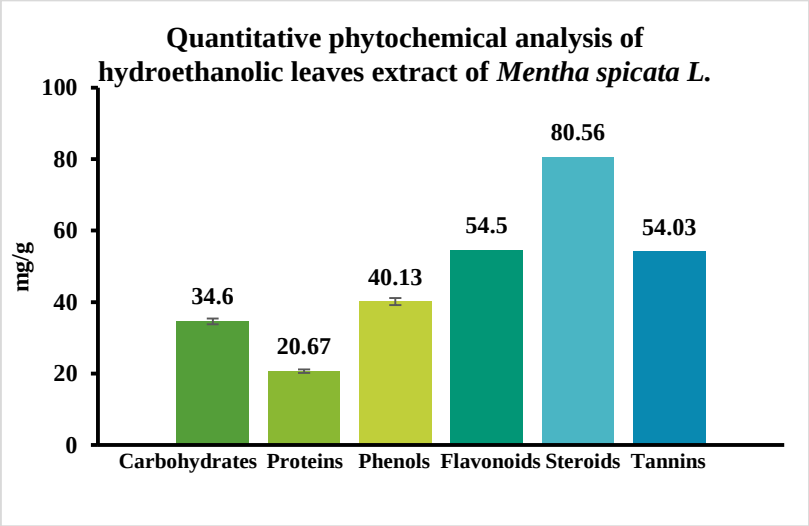


Figure 1. Quantitative phytochemical analysis of hydroethanolic leaves extract of spearmint.

200µg 400µg 600µg 800µg 1000µg

Figure 2. DPPH-dot-blot of spearmint sample.

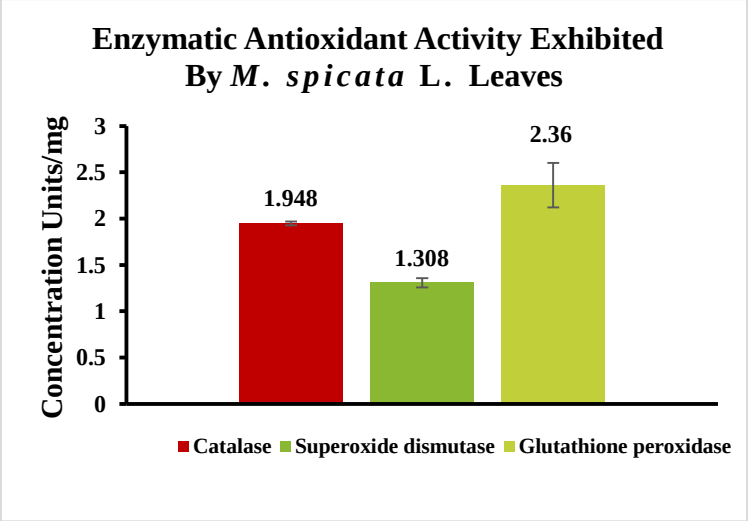


Figure 3. Enzymatic antioxidant activity of spearmint sample.

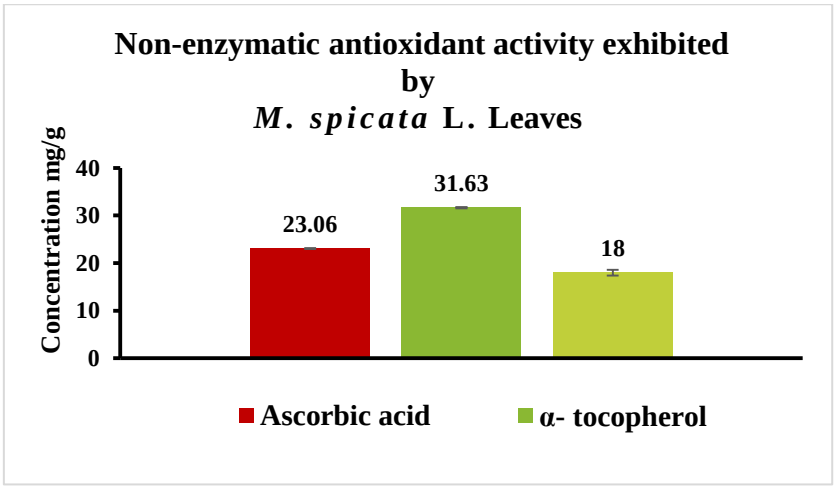


Figure 4. Non-enzymatic antioxidant activity of spearmint.

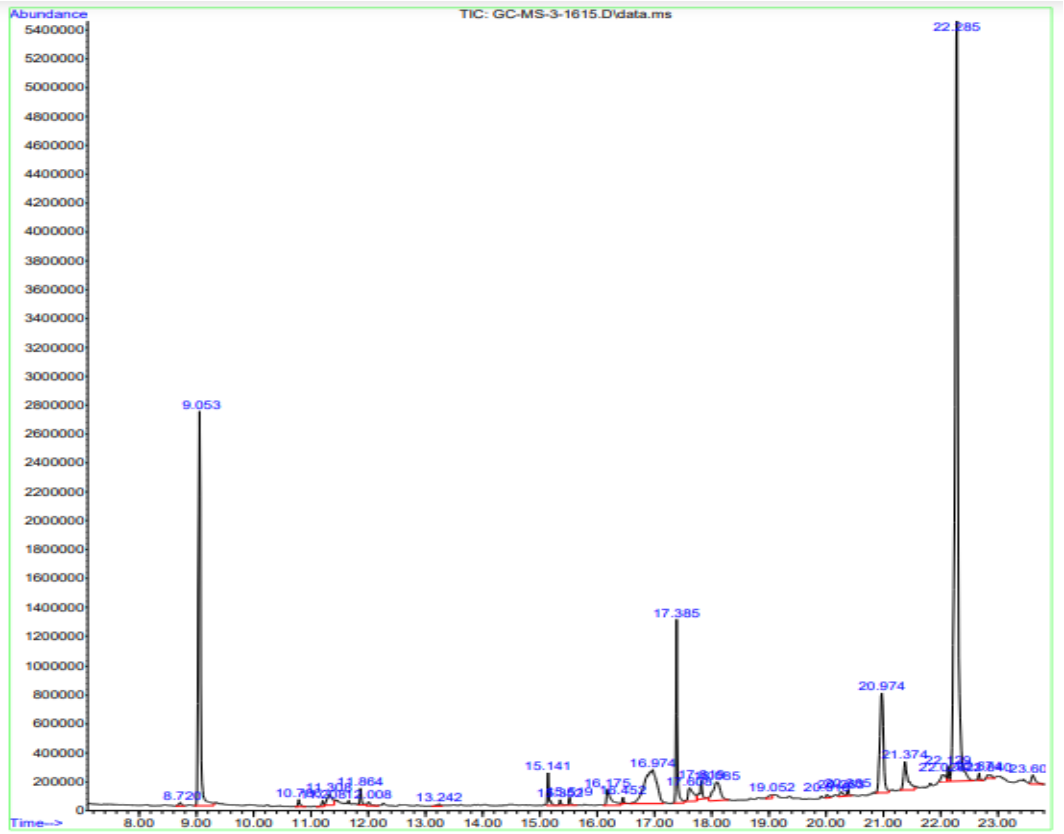


Figure 5. The result of spearmint GCMS chromatogram.

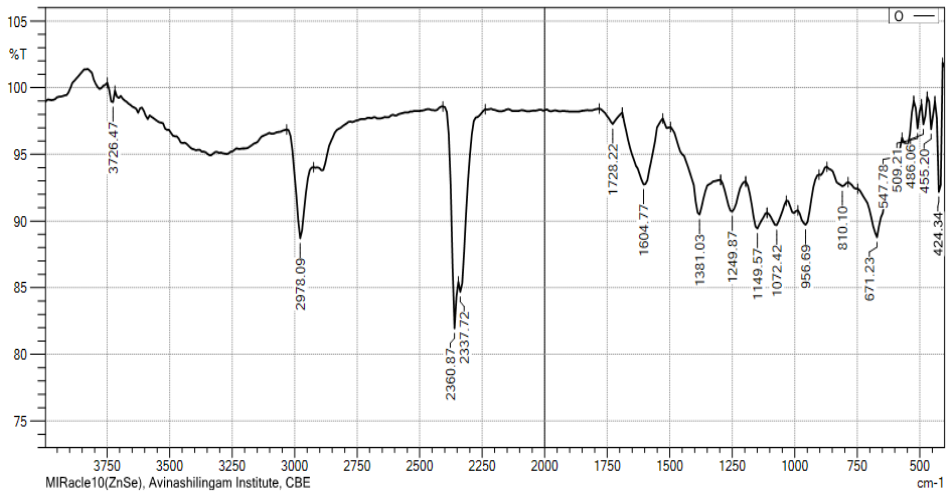


Figure 6. FTIR- spectrum wave numbers of ethanolic extract of spearmint leaves.

Figure 7. Column chromatography setup for isolating terpene compound L-Carvone by using increasing polarity of the eluent by beginning with pure benzene and ending with ethanol.

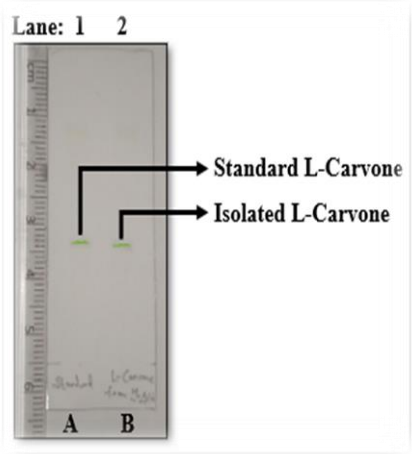


Figure 8. TLC separation of L-Carvone: - A and B represent Standard L-Carvone and isolated L-Carvone respectively. Green spot (Lane - 2) is that of isolated L-Carvone and the Rf value (0.42) of it matches with Rf value (0.44) reported by Stecher 1968. The solvent system used is benzene:toluene:ethyl acetate:ethanol (3.5:2.5:2.5:1.5, v/v).

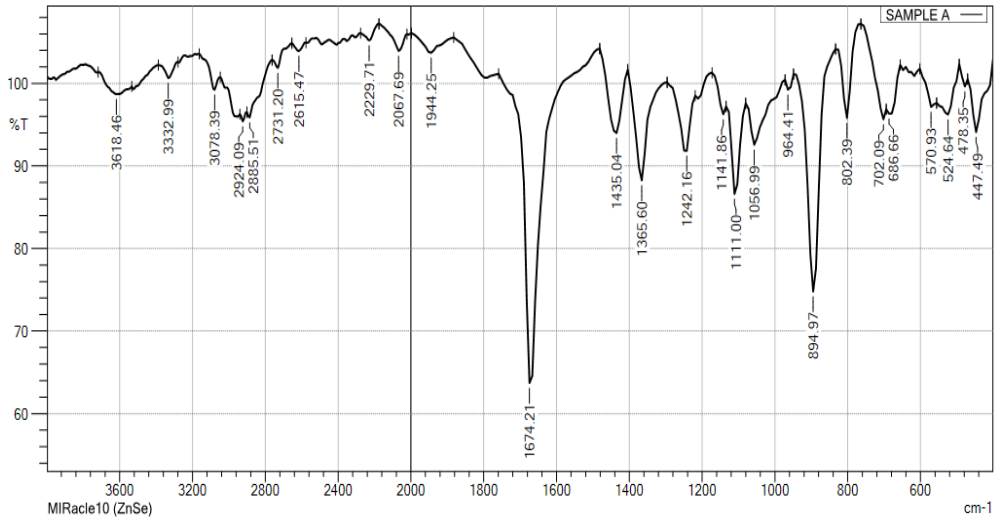
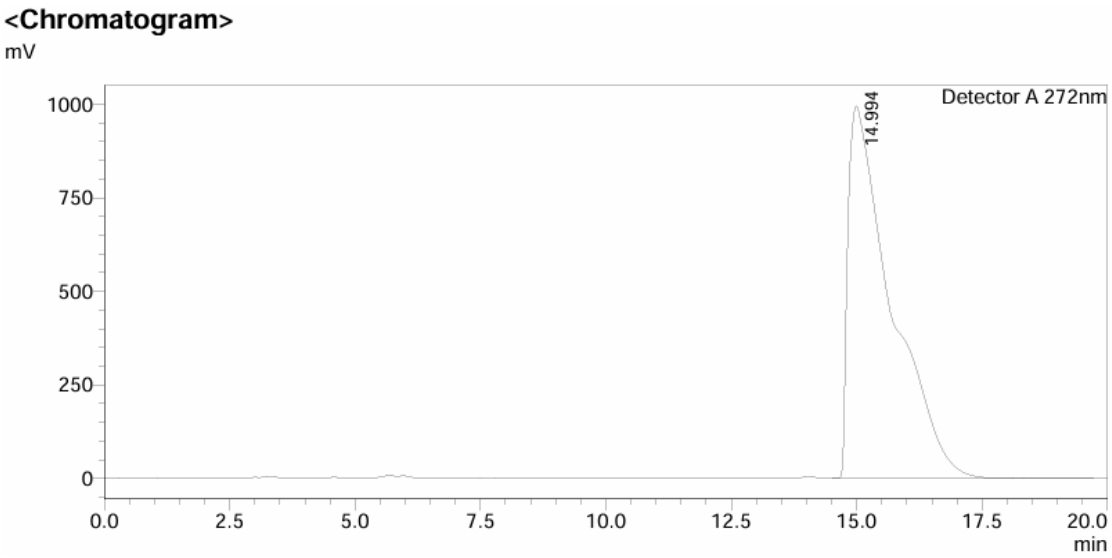


Figure 9. FTIR- spectrum of isolated terpenoid compound L-Carvone.

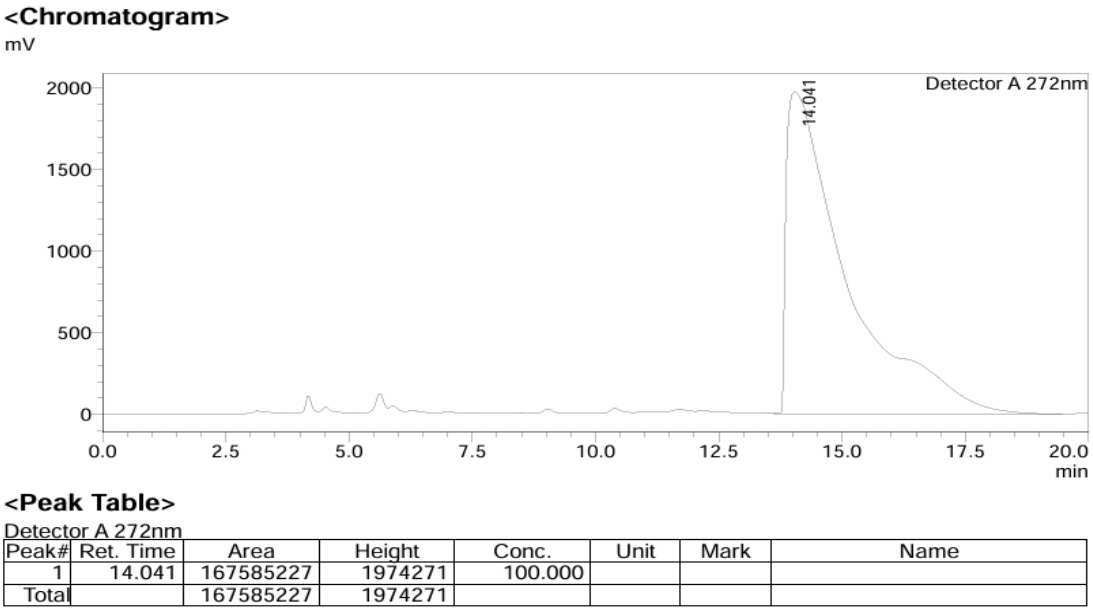


<Peak Table>

Detector A 272nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	14.994	59394787	994068	100.000			
Total		59394787	994068				

Figure 10. HPLC chromatogram of L-Carvone standard (100 µg/mL): A distinct peak appeared at 14.994 minutes at 272 nm, confirming the pure L-Carvone standard. The peak area and height were 59,394,787 mVs and 994,068 mV, respectively, validating the standard's retention and peak characteristics.



<Peak Table>

Detector A 272nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	14.041	167585227	1974271	100.000			
Total		167585227	1974271				

Figure 11. HPLC chromatogram of isolated L-Carvone sample (200 µg/mL). A distinct peak was observed at a retention time of 14.041 minutes at 272 nm, confirming the elution of L-Carvone. The peak area and height were 167,585,227 mVs and 1,974,271 mV, respectively, validating the similar retention and peak characteristics of the standard compound.