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Comparative GC-MS profiling of wild *Calamintha incana* leaf essential oils: influence of geographical origin on chemical composition

Abstract

Herbal medicine is widely practiced in Palestine, largely due to the region's diverse topography, which gives rise to varied climatic conditions and fosters rich biodiversity. *Calamintha incana (C. incana)* is among the most widely utilized medicinal plants in herbal teas, traditionally esteemed for its therapeutic effects and distinctive aroma. The essential oils extracted from wild *C. incana* are rich in volatile and semi-volatile secondary metabolites, whose composition can vary with geographic origin, environmental conditions, and harvest time. The current study aims to investigate these metabolites in the leaves of wild *C. incana* using steam distillation (SD) and gas chromatography–mass spectrometry (GC-MS). It is hypothesized that the bioactive compounds present in wild populations may possess therapeutic potential. Air-dried leaves were collected from ten different locations across Palestine. Essential oils were extracted via SD, yielding an average of approximately 0.4% (*wt/wt*). The oils were subsequently analyzed using GC-MS in electron impact (EI) mode. Seventeen components were separated with high resolution and identified by comparison with the NIST mass spectral library and Kovats retention indices (KIs) of authentic standards. The analysis revealed pulegone, p-menthan-3-one, and caryophyllene oxide as the major constituents. Pulegone was the dominant component in all samples, with concentrations ranging from 28.64% to 58.97%.

Keywords: *Calamintha incana*, essential oils, secondary metabolites, GC-MS, folk medicine, Palestinian flora

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Abbreviations: GC-MS, gas chromatography–mass spectrometry; EI, electron impact mode; *C. incana, Calamintha incana; wt/wt*, weight-by-weight; SD, steam distillation; KI, Kovats retention index; NIST, national institute of standards and technology; Da, dalton; TIC, total ion chromatogram; RT, retention time

Introduction

Palestine, with its diverse topographical features and varying climatic conditions, is home to a rich biodiversity, including numerous plant species with recognized medicinal properties.[1] Over 700 of these plants are traditionally used in Palestinian folk medicine, but scientific documentation of their bioactive compounds and therapeutic potentials remains scarce.[2,3] One such plant, *Calamintha incana* (*C. incana*), belonging to the mint family Lamiaceae, is widely found in the region.[4] In Palestinian folk medicine, *C. incana* is valued for both its therapeutic and culinary uses. The leaves are often boiled to prepare a tea-like infusion known for its pleasant aroma. Traditionally, the plant has been used to alleviate digestive problems, respiratory conditions, elevated blood pressure, and abdominal discomfort. However, its use is generally not advised for children or pregnant women. While *C. incana* has long been a part of Palestinian herbal medicine, particularly for treating various ailments, there is a notable lack of research on its chemical composition and pharmacological properties. This gap in the scientific literature limits a comprehensive understanding of the plant's potential. Photo 1 displays an image of wild *C. incana*.

Photo 1 Image of wild *C. incana.*

Essential oils, which are aromatic liquids extracted from plant materials, have been a focal point in natural product research due to their diverse therapeutic properties.[1] These oils are composed of volatile compounds that play a significant role in plant defense mechanisms and may also contribute to their medicinal efficacy. In particular, essential oils from plants in the *Calamintha* genus are known to contain various bioactive compounds such as terpenoids and phenolic compounds.[3–10] These compounds have been associated with multiple pharmacological activities, including antioxidant, antimicrobial, and antifungal effects, which are increasingly relevant in light of the growing concerns over antibiotic resistance and the rising incidence of diseases linked to oxidative stress.[5–9] For instance, essential oils of *Calamintha* species have shown significant antimicrobial activity against a range of bacterial and fungal pathogens, which makes them a potential natural source for alternative therapeutic agents.[7,8]

Despite the promising pharmacological activity of *Calamintha* species, there is limited research on the specific chemical profile of *C. incana's* essential oils, especially in the context of Palestinian plants.[2] Existing studies on related *Calamintha* species, primarily from Southern Europe and Turkey, have reported varying essential oil compositions influenced by factors such as geographic origin, environmental conditions, and harvesting time.[4] Common compounds found in *Calamintha* essential oils include pulegone, menthone, carvone, limonene, and 1,8-cineole, among others.[4–7] However, these studies have often focused on species other than *C. incana*, leaving a gap in our knowledge regarding the composition and bioactivity of this particular species found in Palestine.

The scarcity of research on the pharmacological properties, essential oil composition of *C. incana* in Palestine highlights the need for further investigation into this plant. This study aims to address these gaps by analyzing the volatile and semi-volatile secondary metabolites of *C. incana* using steam distillation (SD) and gas chromatography-mass spectrometry (GC-MS).

Materials and methods

Material and instruments

GC-grade n-hexane and anhydrous sodium sulfate were procured from Sigma-Aldrich Inc. (USA). The Kovats retention index (KI) reagent, containing a standard mixture of even-numbered alkanes ranging from C10 to C40, was obtained from Fluka, Switzerland. All reference standards and other chemicals used in this study were generously provided by the Central Public Health Laboratory, Ministry of Health, Ramallah, Palestine. The instruments and tools utilized in this study included a simple distillation setup (Clevenger apparatus), an analytical balance (Sartorius, accuracy ±0.0001g, Germany), a rotary evaporator (Steroglass-strike202, Italy), Whatman No. 1 filter papers, separatory funnels, glass funnels, graduated cylinders, micropipettes, Erlenmeyer flasks, amber glass bottles (300 mL and 25 mL), and 2 mL amber GC vials. All equipment was kindly provided by the Central Public Health Laboratory, Ministry of Health, Ramallah, Palestine. The essential oils were analyzed using a PerkinElmer Clarus 600 Gas Chromatograph coupled with a mass spectrometer (USA). The GC-MS system operated under electron impact ionization (EI) at 70 eV. A PerkinElmer autosampler was employed with 2 mL vials for sample injection. The gas chromatograph was equipped with a DB-5 MS fused silica capillary column (Restek, USA), composed of 5% diphenyl and 95% dimethyl polysiloxane, measuring 28 m × 0.25 mm with a film thickness of 0.25 µm.

Collection and extraction of the essential oils from wild *C. incana*

The leaves of wild *C. incana* were collected from ten distinct locations across Palestine, including Kifor Niema (Ramallah), Bait Seera (Ramallah), Bait Rema (Ramallah), Kalandia (Jerusalem), Jericho, Nablus, Anabta (Tulkarm), Ya'bad (Jenin), Kifor Thelth (Qalqilya), and Hebron.. The collected leaves were air-dried at room temperature for approximately one week in a dark environment, then stored in sealed paper bags away from light. The identification and authentication of *C. incana* were carried out by Dr. Khalid Sawalha, Professor of Botany in the Biology Department, and Faculty of Science & Technology at Al-Quds University. The essential oils from *C. incana* leaves were extracted through steam distillation using a Clevenger-type apparatus for duration of three hours. The resulting distillate was extracted twice with 100 mL of hexane. The essential oil was then recovered by evaporating the hexane using a rotary evaporator. The hexane layers were pooled and dried using anhydrous sodium sulfate (the average oil yield was about 0.4% *wt/wt*). Subsequently, 500 µL of the hexane extract was diluted to a final volume of 1 mL with hexane, and 1 µL of this diluted solution was analyzed by GC-MS using an optimized condition.

Chromatographic GC-MS analysis

A PerkinElmer EI-GC-MS system was utilized for analysis. Helium was used as the carrier gas at a constant flow rate of 1 mL/min. The injector temperature was maintained at 240°C, with the ion source set at 250°C and the interface at 260°C. A split ratio of 1:20 was applied throughout the analysis. The temperature program for the column started at 60°C (held for 2 minutes), then increased to 100°C at a rate of 3°C/min, followed by a rise to 280°C at 15°C/min, where it was held for an additional 5 minutes. A solvent cut time of 5 minutes was set to remove the solvent peak and ensure consistent response. The mass spectrometer scanned a mass range from 50 to 500 Da with a scan interval of 0.2 seconds. Compound identification was primarily based on comparing their MS spectra with those in the NIST mass spectral library. Additionally, the Kovats Retention Index (KI) was calculated to further confirm the identity of the compounds.

Results and discussion

Separation and identification of essential oil components

The essential oils of *C. incana* were analyzed using EI-GC-MS, and the components were identified by comparing their spectra with the NIST library and by calculating KI. Seventeen major components were successfully identified and separated at high resolution. The name, molecular formula, retention time, and KI values are provided in Table 1. The reference KI values for each compound were obtained from the NIST library. The identified peaks of the *C. incana* components from different locations are shown in the GC-MS Total Ion Chromatogram (TIC) in Figure 1 (a-d).

All essential oil samples were analyzed in triplicate *($n = 3$)* using GC-MS. The relative standard deviation (RSD%) was calculated for both retention times (RT) and peak areas, as presented in Table 2 and Table 3, respectively. The RSD% values for retention times demonstrated excellent consistency, while those for peak areas remained within acceptable limits, even for components present at low concentration levels. The GC-MS analysis indicated that *C. incana* essential oil is predominantly composed of monoterpenoids and oxygenated monoterpenoids, with minor contributions from sesquiterpenes. Among the identified compounds, pulegone-an oxygenated monoterpenoid-was the most abundant, with an average concentration exceeding 50%.

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Table 1 Identified compounds, molecular formulas, retention times, and KI values

#	Component	Formula	t_R (min.)	Calculated KI	Reference KI
1	1S-α-Pinene	$C_{10}H_{16}$	7.08	953	948
2	4(10)-Thujene	$C_{10}H_{16}$	8.42	1019	975
3	Limonene	$C_{10}H_{16}$	9.97	1081	1020
4	4-Iodo-2,6-dioxa-adamantane	$C_8H_{11}IO_2$	13.73	1197	1234
5	Isomenthone	$C_{10}H_{18}O$	13.97	1203	1166
6	p-Menthan-3-one	$C_{10}H_{18}O$	14.24	1210	1151
7	Isopulegone	$C_{10}H_{16}O$	14.59	1219	1179
8	p-Menthan-3-ol	$C_{10}H_{20}O$	14.92	1227	1164
9	cis-1,3,trans-1,4-(±)- Menthol	$C_{10}H_{20}O$	14.99	1228	1164
10	2-Isopropyl-2,5-dimethylcyclohexanone	$C_{11}H_{20}O$	15.8	1247	1221
11	Pulegone	$C_{10}H_{16}O$	16.4	1260	1216
12	Piperitenone	$C_{10}H_{14}O$	19.29	1317	1315
13	m-Xylene-2,5-diol	$C_8H_{10}O_2$	19.85	1327	1348
14	β-Caryophyllene	$C_{15}H_{24}$	21.19	1456	1424
15	β-Cubebene	$C_{15}H_{24}$	22.77	1348	1384
16	Caryophylene oxide	$C_{15}H_{24}O$	25.33	1561	1575

Table 2 Relative Standard Deviation (RSD %) of Retention Times (RT) for Each Peak *(n = 3)*

Component	Average	SD	RSD%
1S-α-Pinene	7.083	0.006	0.078
4(10)-Thujene	8.429	0.016	0.195
Limonene	9.976	0.012	0.119
4-Iodo-2,6-dioxa-adamantane	13.741	0.016	0.114
Isomenthone	13.977	0.011	0.079
p-Menthan-3-one	14.245	0.012	0.082
Isopulegone	14.595	0.017	0.117
p-Menthan-3-ol	14.928	0.012	0.08
cis-1,3, trans-1,4- (±)-Menthol	14.999	0.012	0.081
2-Isopropyl-2,5-dimethylcyclohexanone	15.806	0.011	0.068
Pulegone	16.408	0.01	0.063
Piperitenone	19.295	0.011	0.059
m-Xylene-2,5-diol	19.852	0.004	0.02
β-Caryophyllene	21.193	0.006	0.029
β-Cubebene	22.776	0.005	0.024
Caryophylene oxide	25.337	0.008	0.032

Table 3 Relative Standard Deviation (RSD %) of Peak Area for Each Peak *(n = 3)*

Component	Average	SD	RSD%
1S-α-Pinene	27202.75	680.25	2.501
4(10)-Thujene	31725.5	1530.2	4.823
Limonene	112318.8	1194.25	1.063
4-Iodo-2,6-dioxa-adamantane	397029.1	4271.8	1.076
Isomenthone	19605.3	481.2	2.454
p-Menthan-3-one	1046703	22117.85	2.113
Isopulegone	109953.6	555.25	0.505
p-Menthan-3-ol	161640.3	2097.8	1.298
cis-1,3,trans-1,4-(±)-Menthol	790007.3	1674.45	0.212
2-Isopropyl-2,5-dimethylcyclohe-xanone	147771.4	5928.85	4.012
Pulegone	6633522	89918.75	1.356
Piperitenone	13262.8	606.6	4.574
m-Xylene-2,5-diol	129692.7	7154.45	5.516
β-Caryophyllene	52351.2	748	1.429
β-Cubebene	15085.07	1030.75	6.83
Caryophylene oxide	305325.2	3383.3	1.108

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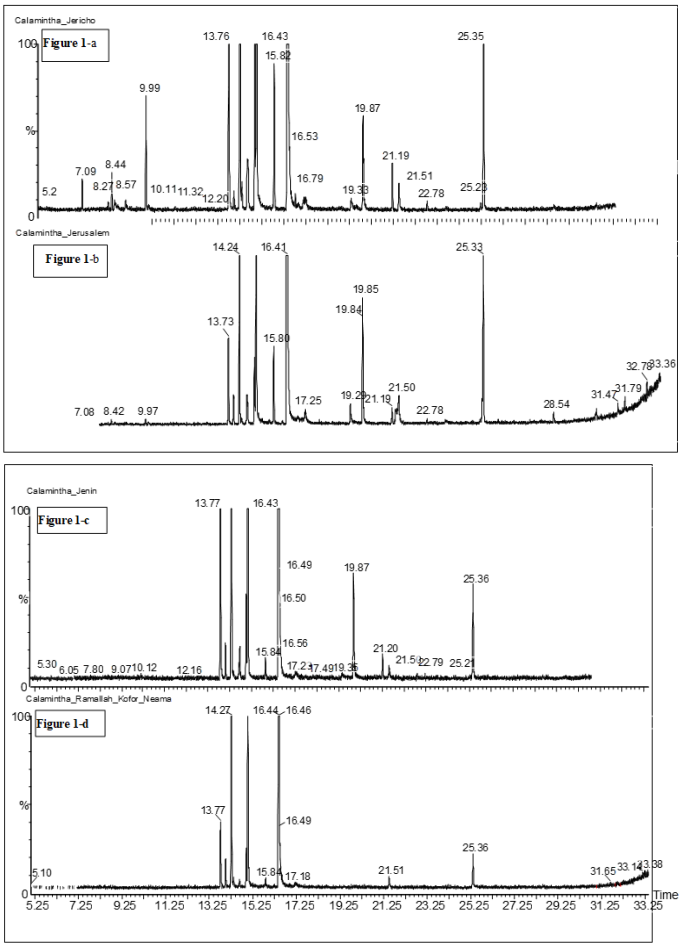

Figure 1 (a-d) GC-MS chromatogram (TIC) of *C. incana* from different locations.

Figure 2 illustrates the chemical structures of the major compounds identified in the essential oil of *C. incana*. These include key monoterpenoids and oxygenated monoterpenoids such as pulegone, menthol, isomenthone, and limonene, which together constitute the majority of the oil's composition. The structural diversity of these compounds contributes significantly to the biological activity and characteristic aroma profile of *C. incana*. Pulegone, in particular, was found to be the most abundant constituent, with a structure typical of oxygenated monoterpenes, known for their potent biological properties.

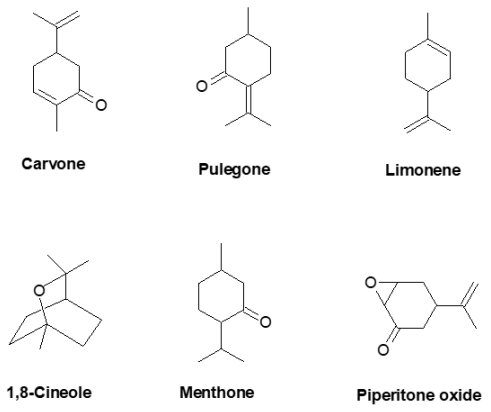

Figure 2 Chemical structures of the major compounds in *C. incana.*

Figure 3 presents a histogram of wild *C. incana* samples collected from all locations. The results highlight specific indigenous volatile compounds that varied in concentration across different regions, allowing each location to be distinguished based on the relative abundance of these components.

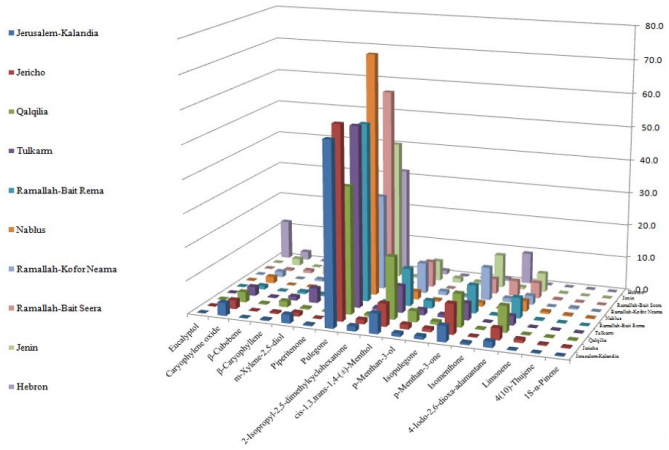

Figure 3 Distribution of *C. incana* Constituents across all locations.

Pulegone, the primary constituent of *C. incana*, is a monoterpene ketone commonly found in the leaves and flowering tops of various species within the mint family (*Lamiaceae*). It is colorless oil characterized by a strong, sharp mint-like aroma. The concentration of pulegone in essential oils can vary significantly depending on multiple factors, including the plant's origin, annual climatic conditions, harvest time, plant maturity, fertilization practices, geographic location, and time of planting. Figure 4 highlights the major components identified, with particular emphasis on pulegone, p-menthan-3-one, cis-1,3, trans-1,4-(±)-menthol, and caryophyllene oxide. However, it was clear that specific indigenous volatiles were present that differentiated each location. For instance, the Hebron sample contained only a limited number of components compared to the others. Additionally, Eucalyptol was identified as the second most abundant component (11.8%), following Pulegone (32.3%), a pattern not observed in samples from other locations.

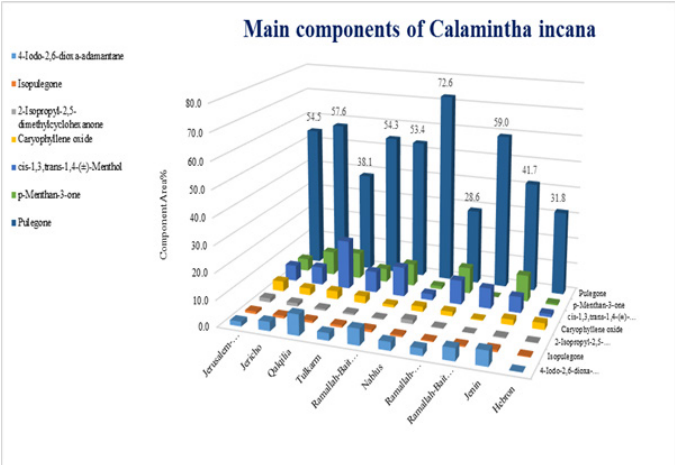

Figure 4 Predominant constituents of *C. incana* across all sampling sites.

Conclusion

This study reports, for the first time, the identification of seventeen volatile and semi-volatile constituents in the leaves of wild *C. incana* collected from various regions in Palestine. Pulegone was

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consistently identified as the major component, comprising over 50% of the essential oil content across samples. In addition to pulegone, several minor compounds were detected, including monoterpenes, oxygenated monoterpenes, and sesquiterpenes such as 1S-α-pinene, limonene, isomenthone, and caryophyllene oxide. The predominance of pulegone suggests it may be the primary bioactive constituent responsible for the plant's pharmacological effects. These findings contribute valuable insights into the chemical composition and potential medicinal value of *C. incana*.

Acknowledgments

None.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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None.

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