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Chemo-geographical variations in volatile profiles of *Mentha longifolia* (L.) populations from Iran

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Abstract

Mint (*Mentha*) has been used in traditional medicine for centuries and is a key species in the Lamiaceae family, with seven varieties found in the Iranian flora. Despite its extensive use in conventional medicine, limited research has focused on the detailed chemical profiling of this species in specific ecological contexts. This study investigates the essential oil composition of *M. longifolia* ecotypes naturally growing in the northwest region of Iran, highlighting the species' chemical diversity and its potential applications in pharmaceutical and agricultural industries. Using high-performance liquid chromatography (HPLC), 20 compounds were identified, accounting for 92.82 % to 100 % of the total oil composition. The main compounds were polegune (7.18-52.23 %), menthone (10.18-32.54 %) and piperitenone oxide (0.77-16.01 %). Additionally, oleanolic acid and ursolic acid, two isomeric triterpenes with recognized therapeutic potential, were quantified in the ecotype samples, with concentrations ranging from 0.17-8.07 mg/g and 0.24-2.94 mg/g, respectively. At a 50 % similarity, the essential oil properties were classified into two sub-clusters: Cluster I, which mainly consisted of six ecotypes and Cluster II, which included two. Cluster analysis revealed two subgroups of ecotypes based on their essential oil profiles, suggesting environmental factors may influence the chemical composition. The findings underscore the significant variation in bioactive compounds among *M. longifolia* ecotypes and provide valuable insights for selecting and cultivating chemotypes with enhanced medicinal or aromatic properties. This study contributes to the growing body of knowledge on *M. longifolia* and supports its broader application in natural product development and sustainable agriculture.

Keywords

Azerbaijan provinces; mint; oleanolic acid; phytochemical; polegune

Introduction

The Lamiaceae (Labiatae) family is significant for its medicinal properties, attributed to its high content of flavonoids, phenolic acids and essential oils. This family is also famous for its aromatic species and includes more than 7,200 species and 240 genera (1). The genus Mentha includes around 25-30 species, with most growing in wet areas such as rivers and are found throughout the worlds' temperate regions. They are extensively distributed across North America, Central and Southern Europe, South Africa, Southwest Asia and Australia (2). *Mentha longifolia* L., commonly called horsemint, is a wild perennial herb that can reach a height of up to 2 meters. Several species

of Mentha have demonstrated a range of biological activities, including antibacterial and antifungal effects (3). This herb is also highly effective in treating irritable bowel syndrome. *M. longifolia* L. syrup has been recognized as a safe and effective remedy for inducing and regulating menstrual bleeding in women with menstrual disorders (4). Various species of Mentha have been used in traditional medicine to treat bronchitis, flatulence, anorexia and liver ailments due to their anti-inflammatory, carminative, antiemetic, diaphoretic, antispasmodic and anticatarrhal properties (5, 6). Mint plants are abundant in phenolic compounds and other substances known for their antioxidant properties. Various flavonoids and phenolic compounds have been identified within these plants (7).

Oleanolic acid and ursolic acid are common constituents found in plants. These two triterpenes can exist as aglycones of saponins or as free acids. Studies indicate the widespread presence of these triterpenes within the Lamiaceae family (1). Oleanolic acid and ursolic acid share similar pharmacological effects due to the similarity in their chemical structures. Previous studies provide extensive and significant evidence of their anti-inflammatory properties, hepatoprotective, antitumor, anti-HIV, antimicrobial, antifungal, gastroprotective (14), hypoglycemic and antihyperlipidemic effects (8-15). Due to their non-toxic properties, oleanolic and ursolic acids have been used in cosmetics and various health products (9). Unfortunately, there is limited information regarding oleanolic and ursolic acids in *M. longifolia* L. With the advancement of modern chromatographic systems, rapid and efficient methods are now available for standardizing herbal drugs based on their natural compounds. Terpenoids are the most common and structurally diverse natural products found in many plants, with over 20000 known compounds. They are significant due to their extensive industrial applications, including flavouring agents, perfumes and insecticides (8). Oleanolic and ursolic acids are particularly noteworthy among the bioactive compounds identified in the Lamiaceae family. Oleanolic acid and ursolic acid are isomers that are challenging to separate because of their similar chemical structures. In recent years, various methods have been developed for the quantitative analysis of oleanolic acid and ursolic acid in different plants, with high-performance liquid chromatography (HPLC) being one of the most widely used techniques, gas chromatography (GC), cyclodextrin-modified micellar electrokinetic chromatography (CD-MEKC), micellar electrokinetic capillary chromatography (MECC) and nonaqueous capillary electrophoresis (NACE) (16-18). A critical review of the literature regarding Iran Mint reveals that they have been the subject of several studies. The essential oil composition was studied and have shown that the essential oils of *M. longifolia* could also enhance the antimicrobial activity (19, 20)

However, To the best of our knowledge, no comprehensive studies have analyzed the essential oil composition or quantified oleanolic acid and ursolic acid levels in *M. longifolia* ecotypes from Northwest Iran. This study aims to fill this research gap by investigating the phytochemical composition of wild *M. longifolia* from different regions of Iran. Furthermore, it seeks to quantify oleanolic acid and ursolic acid concentrations using HPLC, providing new insights into this speciess' chemical diversity and medicinal potential. By building on prior research, this study advances the understanding of *M. longifolia* and its potential applications in pharmaceutical and natural product development. The findings of this study provide critical insights into the phytochemical variability of essential oils in Mentha species, driven by genetic and environmental factors. These results contribute to strategically selecting and breeding high-value Mentha cultivars with optimized essential oil profiles for targeted therapeutic and pharmaceutical applications. Moreover, the identified bioactive compounds can be a foundation for developing standardized, natural products with enhanced efficacy and market potential. It will also contribute to future studies for breeding purposes and determining the degree of relatedness among the wild genotypes.

Materials and Methods

First, with the help of reliable sources and the information of local people, the habitats of *M. longifolia* were identified in the northwest and west of Iran. The aerial parts (leaves and flowering branches) of *M. longifolia* were collected from eight regions in the Azerbaijan provinces of Iran in September 2017 (Table 1). The herbarium of Tabriz University in Iran identified the plants. The collected samples were then dried and ground into small particles. The natural distribution of *M. longifolia* in the Azerbaijan provinces is depicted in Fig. 1. The dried powder of the aerial parts was then stored in plastic bags and kept frozen until the testing date. In total, eight ecotypes were collected and labelled from A_1 to A_8.

Identifying the essential oil and phytochemical compounds in M. longifolia ecotypes

The essential oil preparation **:** 100 g of dried *M. longifolia* aerial part powder was subjected to hydro-distillation for 3 hr using a Clevenger apparatus to extract the essential oil. The essential oil was separated, dried over anhydrous sodium sulfate and stored in a dark container at 4 °C until

Table 1. *M. longifolia* ecotypes are classified according to their geographic origin and environmental conditions, including altitude, temperature and humidity

Sample	Location	Height (m)	Slope	Latitude	Longitude	Temp (°C)	RH (%)
A_1	West Azerbaijan-Khoy	964	NE	38 51 23	45 12 45	12.62	58.5
A_2	West Azerbaijan- Oroumieh	1465	SW	37 17 41	45 07 49	11.9	61.16
A_3	West Azerbaijan-Sardasht	1283	Flat	36 8 24	45 30 59	14.57	50.16
A_4	East Azerbaijan-Maragheh	1496	SW	37 25 22	46 14 44	15	50.33
A_5	East Azerbaijan-Oskoo	2083	W	37 49 34	46 16 06	12.47	52.16
A_6	East Azerbaijan-Bostan abad	1911	NE	37 43 48	46 54 44	7.6	61.48
A_7	West Azerbaijan-Salmas	1452	N	38 14 24	44 49 38	11.6	58.08
A_8	East Azerbaijan-Azershahr	2123	NE	37 43 25	46 11 53	15.2	52.28

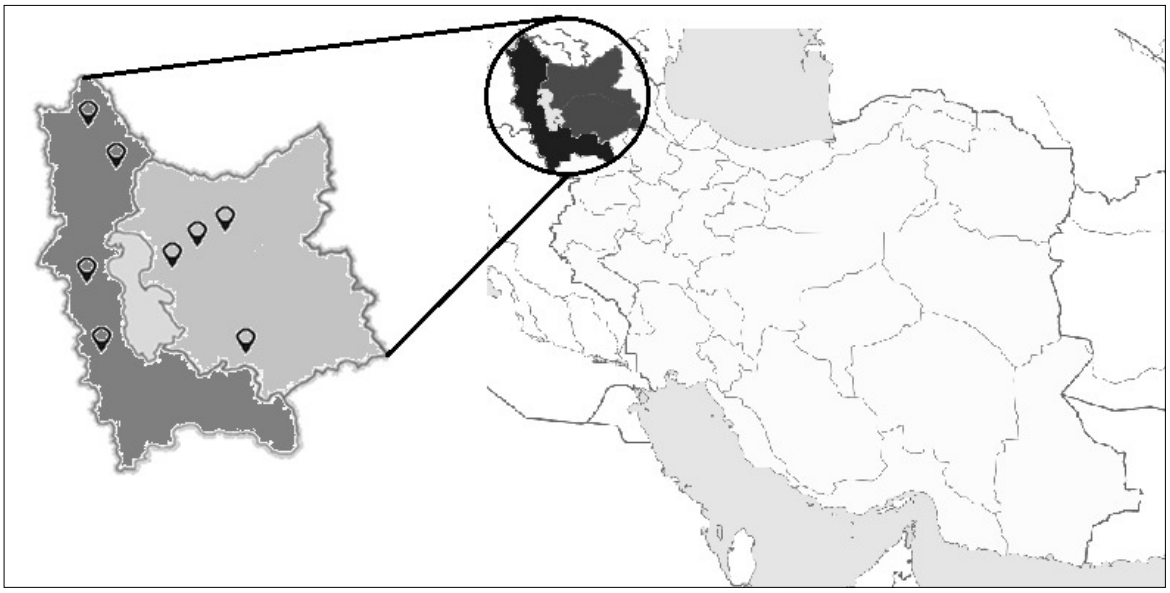

Fig. 1. Natural distribution of *M. longifolia* in Azerbaijan.

analysis by GC-MS.

GC-MS analysis : The GC-MS profile of the essential oil compounds was determined using a gas chromatography system (6890-5973) coupled with a mass spectrometer. The system was equipped with an HP-5MS column (60 m length, 0.22 mm inner diameter and 0.25 µm film thickness) for the analysis. The most volatile chemical compounds in the essential oil were identified by comparing their retention indices (KI) with those of n-alkanes (C_6-C_{24}).

HPLC system : The HPLC method utilized a Kromasil C18 column due to its excellent ability to separate polar and semi-polar compounds. It is ideal for analyzing many phytochemicals commonly found in plant extracts. The mobile phase, composed of methanol and phosphate buffer, was chosen for its ability to effectively dissolve various compounds while maintaining stable pH conditions to ensure reliable retention times and separation. A 0.5 mL/min flow rate was selected to balance efficient separation and reasonable analysis time. At the same time, the UV detector was set to 214 nm to maximize sensitivity for compounds such as phenolics and flavonoids, which absorb strongly at this wavelength. A sample volume of 10 µL was injected to prevent column overloading while ensuring detectable peaks. Finally, the analysis was performed at around 20-25°C, as it was sufficient for effective separation without the risk of compound degradation or unnecessary complexity, ensuring optimal resolution and reproducibility.

Determination of chlorophyll : Chlorophylls were extracted using an acetone-water solution in a 20 %-80 % (v/v) ratio. Two grams of homogenized Mentha tissue were mixed with 25 mL of 80 % acetone solution and blended in a laboratory blender for 2 min. The extracts were then centrifuged at 2700 rpm for 10 min and the supernatant was collected. Absorbance was recorded at 663, 645 and 470 nm using a Cary 50 UV-Vis Spectrophotometer. The chlorophyll concentrations (a, b) and total carotenoids were reported as mg/g of fresh weight. The chlorophyll a, b and carotenoid levels were determined using the Equation 1-3 formula (21).

$$Ch\text{-}a=12.25A_{663} - 2.79A_{646} \quad \text{(Eqn. 1)}$$

$$Ch\text{-}b=21.5A_{646} - 5.1A_{663} \quad \text{(Eqn. 2)}$$

$$C\ x+c= (1000A_{470} - 1.82C_a - 85.02C_b)/198 \quad \text{(Eqn. 3)}$$

A = Absorbance, Ch-a = Chlorophyll a, Ch-b = Chlorophyll b, C x+c = Carotenoids (ß Caroten+ Xanthophyll)

Determination of flavonoids : The flavonoid content was determined following the standard method (22). Leaf samples were ground in a mortar and pestle with 3 mL of a 1 % acetic acid-ethanol solvent (1:99, v/v). The homogenate was transferred into centrifuge tubes and centrifuged at 18000 g for 30 min. The supernatant was then incubated in a water bath at 80 °C for 10 min and then cooled to room temperature. After cooling, the flavonoid content was determined by measuring the absorbance at 300 nm. The flavonoid content was expressed as µmol/g of fresh weight (FW) and the concentration was calculated using the extinction coefficient for flavonoids (⊠ = 33,000 per mol/cm).

Determination of total phenolic content in the essential oils : The total phenolic content in the leaf extract was determined using the Folin-Ciocalteu method. 0.5 mL of the extract and 7 mL of deionized water were added to a test tube, followed by adding 0.5 mL of Folin-Ciocalteu reagent. After 3 min, 1 mL of saturated sodium carbonate solution was added to the sample and the volume was adjusted to 10 mL with distilled water. The mixture was allowed to incubate for 1 hr, after which the absorbance was measured at 725 nm using a Cary 50 UV-Vis Spectrophotometer (USA). The total phenolic content of the extract was quantified using a standard curve based on the amount of caffeic acid (mg) per gram of extract (23). The control tubes contained distilled water and the reagents (Fig. 2).

Oleanolic acid and ursolic acid content detection

Sample preparation : The samples were extracted with 100 mL of methanol using an ultrasonic extraction device for 30 min. After filtration, the extracts were transferred to a measuring flask. A 10 mL calibration sample was prepared using methanol and filtration through a 0.45 µm membrane filter.

Standard solution preparation : Stock standard oleanolic and ursolic acid solutions were prepared by dissolving an appropriate amount of each compound in methanol to achieve a final concentration of 2.0 mg/mL for both acids. To prepare the standard solutions, serial dilutions of each stock solution were

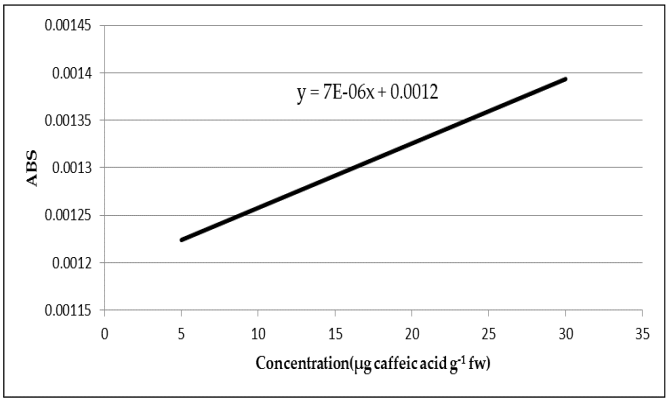

Fig. 2. Total phenolic standard curve.

made with methanol to achieve 50, 100, 200 and 300 µg/mL concentrations. Oleanolic acid and ursolic acid were sourced from Sigma-Aldrich (France). Pure-grade methanol and water were used in the experiments and all other chemicals employed were of analytical grade.

OA and UA precision : The two acids were combined with 10 µL of the four standard comparison solutions and analyzed under the specified chromatographic conditions.

OA and UA reproducibility : Following the prescribed sample preparation method, a 10.0 g sample of *M. longifolia* powder was used for each ecotype.

Recovery : For each ecotype, 5.0 g of *M. longifolia* with a known content was added to 3.80 mg of Oleanolic acid standard solution and 5.92 mg of Ursolic acid. The recovery percentage was calculated using the formula in Equation 4:

$$\text{Recovery } (\%) = (M-N)/O \times 100 \qquad \text{(Eqn. 4)}$$

M: result after addition; N: amount of sample without adding standards; O: added amount of the standards.

Statistical analysis

The experiments were conducted in triplicate and all data were analyzed using SPSS 23.0.0.0. A completely randomized design was applied for the phytochemical experiments, while a factorial design was used for the fungal experiments. The results are presented as the mean ± standard deviation (SD) of the triplicates.

Results and Discussion

Essential oil and phytochemical compounds of M. longifolia ecotypes

The results indicated that 20 active compounds were identified within 97.21 % of the essential oil of *M. longifolia* L. The essential oil yield ranged from 0.17 % (A1) to 0.52 % (A2) (v/w), as shown in Fig. 3. Previous studies have reported varying yields of essential oil extracted from *M. longifolia*. These yields include 0.39 %, 1.39-4.05 %, 0.9-1.8 % and 1.55-1.64 % (24). The discrepancies between the results can be attributed to variations in the environmental conditions of the target regions, which likely influenced the essential oil content of *M. longifolia*.

All samples contained (+) polegune, piperitenone oxide and menthone as the main components, as shown in Table 2. The essential oil contained the following elements in varying concentrations: Polegune (28.9 %), Piperitenone

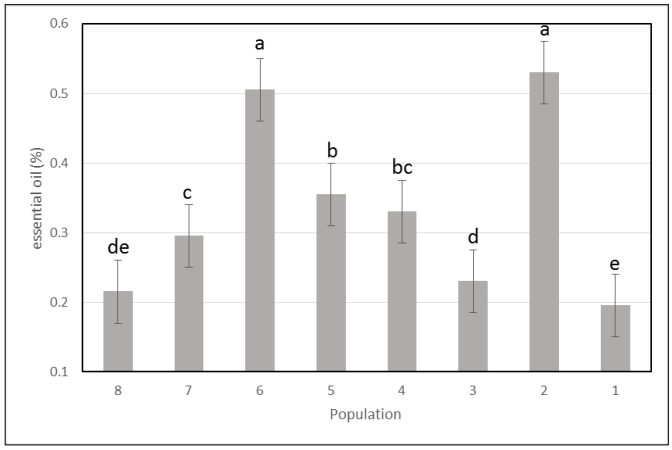

Fig. 3. Percentage of essential oil extracted from *M. longifolia* population ± SD. Means within each column followed by the same letter are not different according to the LSD test; Vertical bars indicate standard error.

epoxide (27.61 %), menthone (21.6 %), Piperitone (13.62 %), Dihydrocarvone (trans) (8.4 %), Iso-polegune (7.66 %), Piperitenone oxide (7.59 %), 1,8-Cineol (6.53 %), Menthol (5.41 %), β-Caryophyllene (4.55 %), Caryophyllene oxide (3.45 %), Spathulenol (2.23 %), β-Pinene (2.2 %), Sabinene (2.15 %), Myrcene (1.49 %), Isomenthyl acetate (1.54 %), Iso-D-hydrocarveol (1.36 %), Piperitenone (1.18 %), Menthyl acetate (1.17 %) and Pinene (0.93 %). Traore et al. (25) reported that the major compounds identified in *M. longifolia* from dried plants were polegune (42.4 %), menthone (21.2 %), 1,8-cineole (11.4 %) and isomenthone (13.2 %). In another study, it was shown that 97.8 % of the total oil consisted of polegune (32.3 %), Isopolegune (19.7 %), Isopolegune (8.9 %), menthone (13.8 %) and 1,8-Cineole (8 %) (26). Previous studies also identified 23 compounds, with the major components being Piperitenone oxide, pulegone and 1,8-Cineole. According to other research, the main components of the oil and their respective percentages were cis-piperitone epoxide (7.8-77.6 %), piperitenone oxide (1.5-49.1 %), carvone (0.0-21.5 %), polegune (0.3-5.4 %), menthone (0.0-16.6 %) and thymol (1.5-4.2 %) (27). The results of a phytochemical study indicated that polegune, constituting 31.54 %, is the most active ingredient of the plant (28). In contrast, the main component of *M. longifolia* L. native to Ilam was found to be beta-Phellandrene (29).

The study on *M. longifolia* essential oil composition reveals varying yields and concentrations of key compounds, such as pulegone, piperitenone oxide and menthone, when compared to other studies on Mentha species. The essential oil yield from *M. longifolia* ranged from 0.17 % to 0.52 %, which is lower than the yields reported in previous studies for this species (e.g., 0.39 %, 1.39-4.05 %, 0.9-1.8 % and 1.55-1.64 %) (30). Despite this variation, the major components like pulegone, menthone and piperitenone oxide were consistently present, albeit in different concentrations. For instance, pulegone accounted for 28.9 % and piperitenone oxide 7.59 %, similar to other studies reporting pulegone as the primary compound, but with different proportions. These discrepancies in yield and composition can be attributed to environmental conditions, such as climate, soil and altitude, which have been shown to influence the essential oil content (31).

There are several reports on the essential oils of

Table 2. The essential oil component of *M. longifolia*

No	Compound	KI*	Composition (%)							
			A_1	A_2	A_3	A_4	A_5	A_6	A_7	A_8
1	Polegune	1243	52.23	29.73	30.76	34.7	20.12	7.18	7.96	48.55
2	IsoPolegune(sic)	1163.32	7.81	-	-	-	-	-	-	7.52
3	Myrcene	981.91	1.49	-	-	-	-	-	-	-
4	ßpinen	979.47	1.67	1.77	-	-	-	-	-	3.17
5	Sabinen	971.74	0.87	0.57	-	-	-	2.3	-	-
6	αpinen	937.6	1.01	0.54	-	-	-	-	-	1.23
7	Menthon	1146.18	14.96	28.64	28.37	22.01	32.54	14.5	10.18	21.55
8	1,8cineol	1029.01	8.07	7.81	-	-	5.16	3.2	6.91	8.07
9	Caryophyllene oxide	1589.58	4.88	2.18	-	4.21	3.45	-	-	2.51
10	Menthol	1168.74	-	8.53	6.01	3.53	3.93	4.5	5.98	-
11	Spatholenol	1548	-	0.67	-	-	-	3.8	-	-
12	Piperitenon	1338.9	-	1.48	-	-	-	-	-	0.88
13	Piperitenon oxide	1354.78	0.77	4.44	15.74	10.04	3.51	9.02	16.01	1.14
14	Menthil acetate	1321	-	0.82	-	-	1.53	-	-	-
15	ßcaryophyllen	1433.2	-	1.92	6.59	7.86	-	2.17	4.22	-
16	Isomenthyl acetate	1307.54	-	1.47	-	-	-	1.5	1.66	-
17	Piperiton	1249.9	-	-	12.53	14.72	-	-	-	-
18	Dhydrocarvon(trans)	984.34	-	-	-	1.2	15.65	-	-	-
19	Pipriton epoxide	1253.75	-	8.46	-	-	3.71	51.83	46.46	
20	Iso-D-hydrocarvoeol	1583.73	-	-	-	-	1.36	-	-	-
21	Total (%)		93.76	99.03	100	98.27	99.79	92.82	99.38	94.62

M. longifolia from different geographical locations, including studies on menthone, piperitone oxide, cis-piperitone epoxide and menthol (27, 32- 34). Research identified the essential oil of *M. longifolia* as a primary source of key compounds, including piperitone, polegune, dihydrocarvone, cis-dihydrocarvone and piperitenone. These studies also highlighted that ecological factors, such as climate and soil conditions, significantly impact the essential oil content (5). This study suggests that genetic variation, growth stages, plant parts used and variations in maturity are key factors influencing the composition and yield of *M. longifolia* essential oil. The differences in essential oil content and composition of *M. longifolia* leaves can be attributed to environmental and geographical factors, such as temperature, rainfall and altitude. Consequently, our findings reveal that the chemical composition of the essential oil derived from the leaves and flowers of *M. longifolia*, collected from eight different regions in Iran, displays significant variation in qualitative and quantitative characteristics.

The observed variations in the essential oil profiles of *M. longifolia* can be attributed to a combination of environmental, genetic and ecological factors. Environmental conditions such as temperature, rainfall, altitude and soil type influence the composition and yield of the oil, as seen in the differences in polegune and iso- polegune concentrations across ecotypes. The plants' growth stage also plays a role, as secondary metabolites are produced in response to environmental stresses or developmental cues. Additionally, geographical differences in altitude and soil conditions can impact the chemical composition of the oil. These factors offer valuable insights for identifying and developing high-value ecotypes suited for medicinal and commercial applications.

Climatic factors such as temperature, rainfall and humidity significantly affect the concentration of key compounds like pulegone, menthone and piperitenone oxide in *M. longifolia* essential oil. These compounds are secondary metabolites, meaning they are often produced in response to environmental stressors or growth conditions and their levels can fluctuate based on climate. Higher temperatures generally promote the synthesis of volatile compounds like pulegone and menthone, as plants often produce more secondary metabolites to cope with heat stress or to protect themselves from excessive UV radiation. Warmer climates can increase the activity of enzymes involved in terpenoid biosynthesis, leading to higher concentrations of compounds like pulegone. In contrast, cooler temperatures might reduce the production of such compounds, as plants focus more on growth rather than chemical defences. This could explain variations in pulegone levels between ecotypes from different regions with varying temperature profiles. The amount of rainfall also significantly influences the production of essential oil components. Higher rainfall often leads to better plant growth and potentially higher essential oil yields; however, it can also dilute the concentration of terpenoid compounds like piperitenone oxide. Excessive water can reduce the synthesis of secondary metabolites if the plants' resources are diverted toward growth rather than defence mechanisms. On the other hand, limited rainfall or dry conditions can increase the production of secondary metabolites like piperitenone oxide as a stress response, enhancing the plants' defence mechanisms against water loss or herbivory. The higher concentration of piperitenone oxide in specific ecotypes might be linked to these stress-induced responses to drought. High humidity can alter the plants' physiological processes, including the transpiration rate, influencing the overall synthesis of secondary metabolites. Excessive air moisture can reduce essential oil quality and quantity by affecting the volatile nature of compounds like menthone and pulegone. Low humidity, in contrast, may stimulate the production of more aromatic compounds as part of the plants' response to drier air (35, 36).

In summary, the climatic factors of temperature, rainfall and humidity directly affect the biosynthesis of key terpenoids in *M. longifolia* by influencing plant stress responses, growth patterns and metabolic pathways. The varying concentrations of polegune, menthone and piperitenone oxide across different ecotypes reflect these

climatic influences and highlight the plants' adaptability to different environmental conditions.

Chlorophylls (a & b) and carotenoids content

Table 3 shows that the contents of chlorophylls (a, b) and carotenoids in *M. longifolia* range from 0.005 mg/g FW (A_2) to 0.025 mg/g FW (A_7) for chlorophyll a, 0.003 mg/g FW (A_2) to 0.015 mg/g FW (A_7) for chlorophyll b and 0.001 mg/g FW (A_2) to 0.006 mg/g FW (A_7) for carotenoids, respectively. Other studies have also indicated that genotype can significantly influence plants' color and chlorophyll content (37).

The highest chlorophyll content was found in the 'Bavarian' mint variety leaves. Research also indicated a chlorophyll content of 75.2 µg/g in dry *M. piperita* tea (38). The carotenoid yield in mint species ranged from 4.6 to 16.9 mg/100 g (39).

Flavonoids content

The flavonoid content of *M. longifolia* ranged from 0.5 µg/mL (A_1) to 4.5 µg/mL (A_3) (Table 3). Other researchers reported a flavonoid content of 6.52 mg/g FW (35). However, another study reported a lower flavonoid content, approximately 0.40 g (4 %) (40). Research indicates that the total flavonoid content in the ethanol extracts of *M. longifolia* was 23.68 ± 0.20 mg RE/g (41).

Total phenolic content

The total phenolic content of the tested *M. longifolia* ecotypes ranged from 1.54 mg/g FW (A_1) to 75.52 mg/g FW (A_4), based on a caffeic acid equivalent (Table 3. *Mentha piperita* had the highest total phenolic content (12.63 ± 0.878 µg GAE), followed by Mentha spicata with 9.41 ± 0.594 µg GAE (42). Other studies have reported total phenolic contents of 67.05 ± 0.85 GAE/g, 30.327 ± 1.4 g GAE/100g and 6.08 mg RE/g FW (38). Phenolic compounds play a significant role in enhancing plants' quality and nutritional value by influencing attributes such as colour, taste, aroma and flavour. These compounds also offer several health benefits, including antioxidant, anti-inflammatory and antimicrobial properties. Phenolic compounds also play a crucial role in plant defence mechanisms by counteracting reactive oxygen species (ROS), helping plants survive and protect themselves from damage caused by microorganisms, insects and herbivores. The high concentration of phenolic and flavonoid compounds in *M. longifolia* suggests a strong potential for health benefits, including antioxidant, anti-inflammatory and antimicrobial properties. Previous studies have also highlighted the presence of high phenolic and flavonoid content in *M. longifolia* as well as other Mentha species (43).

Evaluation of oleanolic acid and ursolic acid in plant materials OA and UA specification curve

The standard curve for oleanolic acid is represented by the equation y = 39406x - 2E+06 and for ursolic acid, it is y = 2630.3x + 659168, where x is the concentration and y is the peak area integral value of the respective acid. The flow rate was 0.5 mL/min and the column temperature was maintained at 25 °C. Under these HPLC conditions, the retention times for oleanolic acid and ursolic acid were 11-13 minutes and 13-15 min, respectively. The chromatogram peaks were identified based on their retention times and by comparison with authentic standards of the acids. The average concentrations of oleanolic acid and ursolic acid in the *M. longifolia* ecotypes ranged from 0.17 to 8.07 mg/g and 0.24 to 2.94 mg/g, respectively (Fig. 4-5). The results revealed variations in oleanolic and ursolic acid contents among the different ecotypes. The analytical results indicated that the A_2 ecotype had lower oleanolic and ursolic acid levels than the other ecotypes. The results also revealed the absence of ursolic acid in many ecotypes, which could be attributed to variations in climate and environmental conditions.

OA and UA precision

Oleanolic acid and ursolic acid were quantified using the integral values of their peak areas. The results presented in Table 4 demonstrate the high accuracy and reliability of the developed method.

OA and UA reproducibility

The peak area integral values for Oleanolic acid and Ursolic acid, determined under the specified chromatographic

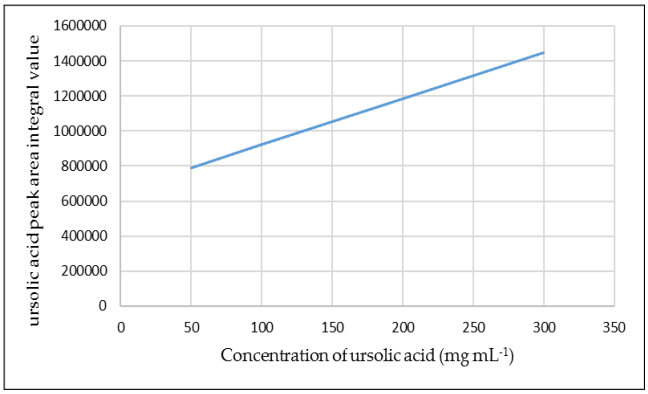

Fig. 4. Ursolic acid standard curve.

Table 3. Phytochemical properties of *M. longifolia* population: chlorophyll a, chlorophyll b, carotenoids, flavonoid and total phenolic content

Population	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Carotenoids (mg/g)	Flavonoid (mg mol/g FW)	Total phenolic (mg caffeic acid/g FW)
A_1	^{bcd}0.014±0.0000	^{c}0.008±0.0001	^{cd}0.003±0.0000	^{e}0.5±0.05	^{d}1.5±0.01
A_2	^{e}0.005±0.0001	^{d}0.003±0.0000	^{e}0.001±0.0001	^{d}0.9±0.02	^{c}11±0.26
A_3	^{de}0.011±0.0000	^{c}0.007±0.0000	^{de}0.002±0.0000	^{a}4.5±0.05	^{b}27.27±4.98
A_4	^{abc}0.02±0.0000	^{bc}0.01±0.0000	^{bc}0.004±0.0000	^{c}1.21±0.15	^{a}75.52±8.79
A_5	^{cd}0.013±0.0000	^{c}0.007±0.0000	^{cd}0.003±0.0001	^{d}0.8±0.01	^{d}3.3±0.03
A_6	^{de}0.012±0.0000	^{c}0.007±0.0000	^{de}0.002±0.0000	^{b}3.01±0.16	^{c}11.74±1.57
A_7	^{a}0.025±0.0003	^{a}0.015±0.0002	^{a}0.006±0.0001	^{c}1.07±0.06	^{d}1.2±0.01
A_8	^{ab}0.021±0.0110	^{b}0.01±0.0044	^{ab}0.005±0.0024	^{c}1.1±0.02	^{d}4.09±0.00

Note: Flavonoids and phenolic compounds are known for their antioxidant, anti-inflammatory and antimicrobial properties, which enhance plant bioactivity and help in stress resistance. These compounds also have potential health benefits for humans, such as disease prevention and inflammation reduction. Means followed by the same letter are not different according to the LSD test. FW: Fresh weight

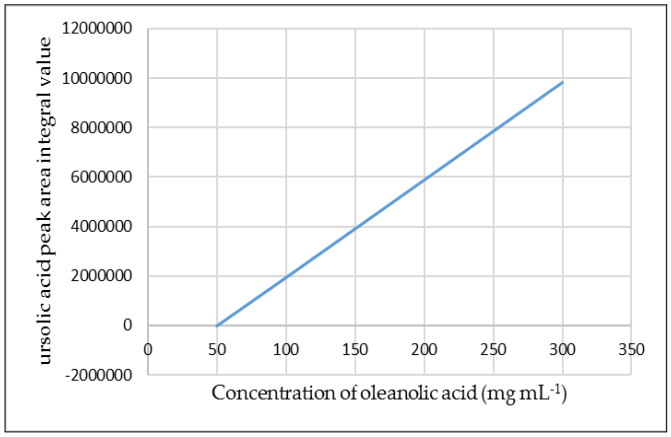

Fig. 5. The oleanolic acid standard curve.

Table 4. Precision for OA and UA shows the accuracy of the analytical method used to quantify oleanolic acid (OA) and ursolic acid (UA) in plant samples

Standard	Concentration (µg/mL)	RSD (%)
OA	50	1.45
	100	1.28
	200	1.32
	300	1.12
UA	50	1.53
	100	1.42
	200	1.26
	300	1.27

conditions, showed RSD percentages of 1.32 % and 0.49 %, respectively. These results indicate a high level of accuracy in the experiment.

Recovery

Using the sample preparation method and the specification curve, the average recovery (n=8) for oleanolic acid and ursolic acid was 98.59 % (RSD = 1.92 %) and 99.65 % (RSD = 0.44 %), respectively. The detailed results are provided in Tables 5 and 6. Recovery experiments are valuable for assessing the accuracy of the method used to quantify oleanolic and ursolic acid in *M. longifolia* L. samples. The levels of oleanolic acid and ursolic acid in *M. longifolia* Lam. are summarized in Table 7. This method provided relatively short retention times and high sensitivity and accuracy for quantifying both triterpenoids. Gas chromatography-mass spectrometry (GC-MS) analysis determined that some Lamiaceae members' oleanolic acid content in dried plant materials ranged from 0.09 % to 0.9 %. In comparison, the Ursolic acid content ranged from 0.09 % to 1.6 % (44). Other studies have shown that *Rosmarinus officinalis* leaves are a rich source of oleanolic acid and ursolic acid, containing high concentrations of these triterpenoids. Liang reported that oleanolic acid and ursolic acid in acetone extracts were 0.643 ± 0.034 mg/g and 2.930 ± 0.060 mg/g, respectively (45).

Oleanolic and ursolic acids are triterpenoid compounds renowned for their diverse pharmacological properties, including anti-inflammatory, hepatoprotective and anticancer

Table 5. Recovery rate of oleanolic acid presents the recovery rate of oleanolic acid from plant samples based on the extraction method used

Ecotype	Recovery %	Average %	RSD %
A1	94.65	98.59	1.92
A2	99.12		
A3	98.94		
A4	100.1		
A5	97.61		
A6	98.11		
A7	99.24		
A8	101.01		

Table 6. The recovery rate of ursolic acid conclusion presents the recovery rate of ursolic acid (UA) from plant samples using a specific extraction method

Ecotype	Recovery %	Average %	RSD %
A_1	-	99.65	0.44
A_2	100.12		
A_3	-		
A_4	-		
A_5	99.61		
A_6	-		
A_7	99.24		
A_8	-		

effects. Variations in their concentrations among different plant ecotypes can significantly influence their therapeutic potential. In some ecotypes, low concentrations of these acids may limit their efficacy in treating conditions such as liver disorders, where higher concentrations are typically required for optimal hepatoprotective effects. For instance, oleanolic acid has been marketed in China as an oral drug for human liver disorders, underscoring its importance in hepatoprotection (9).

Additionally, the anti-inflammatory properties of these compounds are dose-dependent. Lower concentrations might not achieve the necessary anti-inflammatory effects, potentially reducing their effectiveness in managing inflammatory diseases. Furthermore, the antimicrobial properties of oleanolic and ursolic acids are also concentration-dependent. Studies have shown that these compounds exhibit antimicrobial activity even at lower concentrations, but higher concentrations are generally more effective (47).

Therefore, ecotypes with low concentrations of oleanolic and ursolic acids may have limited applications in pharmacology, particularly in treatments where these compounds' therapeutic effects are concentration-dependent. This highlights the importance of selecting plant sources with optimal concentrations of these triterpenoids for pharmaceutical applications. So, the pharmacological efficacy of oleanolic and ursolic acids is closely linked to their concentrations within plant ecotypes. Ecotypes with low levels of these compounds may offer reduced therapeutic benefits,

Table 7. Oleanolic acid and ursolic acid in *M. longifolia* ecotypes present the concentrations of oleanolic acid (OA) and ursolic acid (UA) in different ecotypes

	A_1	A_2	A_3	A_4	A_5	A_6	A_7	A_8
Oleanolic acid (mg g^{-1})	1.99±0.06	0.17±0.005	8.07±0.05	1.08±0.034	1.06±0.05	1.84±0.26	0.45±0.1	0.73±0.04
Ursolic acid (mg g^{-1})	-	0.24±0.04	-	-	2.94±04	-	2.42±0.19	-

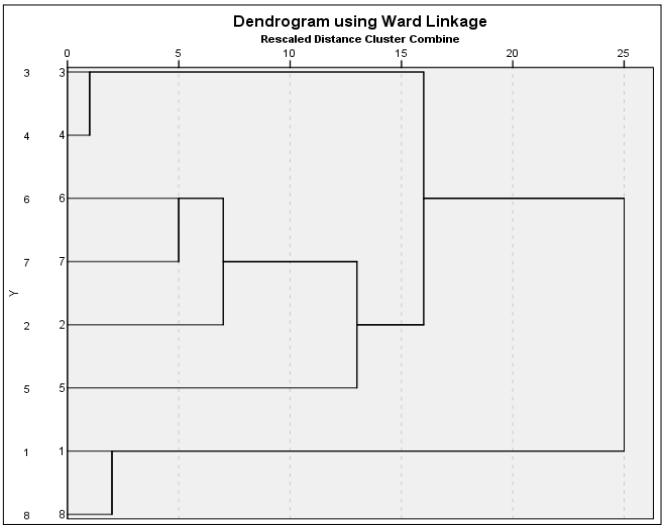

Fig. 6. Dendrogram of essential oil percentage + GC using Wards' minimum variance cluster analysis method. 1. West Azerbaijan-Khoy, 2. West Azerbaijan - Oroumieh, 3. West Azerbaijan-Sardasht, 4. East Azerbaijan-Maragheh, 5. East Azerbaijan-Oskoo, 6. East Azerbaijan-Bostan abad, 7. West Azerbaijan-Salmas, 8. East Azerbaijan-Azershahr.

emphasizing the need for careful selection and cultivation of plant varieties with higher concentrations for medicinal use.

Dendrogram of similarities

Fig. 6 presents the dendrogram illustrating the similarities among eight Mentha populations, based on Wards' minimum variance method of cluster analysis, which considered the essential oils' quantitative and qualitative properties. The study revealed two distinct groups: Cluster I, which included six ecotypes (A_2, A_3, A_4, A_5, A_6 and A_7) and Cluster II, which contained two ecotypes (A_1 and A_8). The difference between the two groups may be attributed to the lower percentage of essential oil and the higher concentrations of polegune and iso-pulegone components extracted from A_1 and A_8 compared to the other ecotypes.

Correlation of essential oil content with climatic characteristics

The heatmap shows the correlation between the essential oil content and the environmental factors (altitude, temperature and relative humidity) for the *M. longifolia* accessions. The values range from -1 to 1, where values closer to 1 indicate a strong positive correlation and values closer to -1 indicate a strong negative correlation. A value of 0 suggests no correlation. Polegune and Menthon show a strong positive correlation with temperature, suggesting that warmer conditions might enhance the essential oil content. Piperitenon oxide and piperiton exhibit negative correlations with altitude, indicating that higher altitudes might decrease their concentrations. Relative Humidity (RH) does not consistently correspond with most compounds, suggesting its influence may vary across different compounds (Fig. 7).

Table 8 underscores the diverse phytochemical profiles of *Mentha* species, each with unique strengths in essential oil composition, antioxidant activity and antimicrobial properties. This diversity makes them invaluable for various industrial, medicinal and therapeutic applications. Future research could optimize cultivation techniques and explore synergistic effects when combining different *M.* species for enhanced benefits. The comparative table between *M. longifolia* and other *M. species* highlights

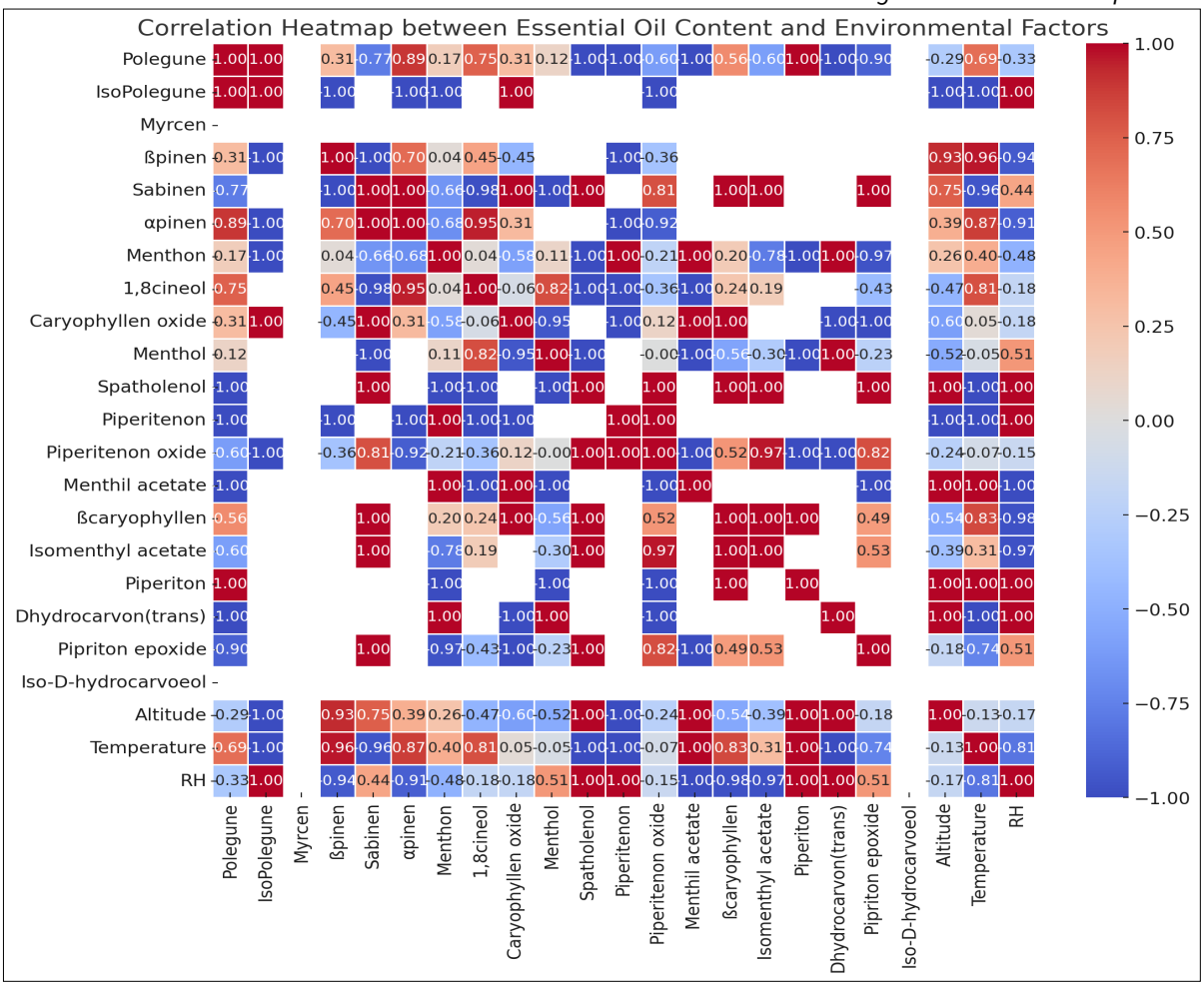

Fig. 7. Heat map correlation between essential oil content and components of the studied *M. longifolia* accessions with climatic and their habitats.

Table 8. A comparative overview of the phytochemical properties of *M. longifolia* and other *Mentha* species

Phytochemical compound	*M. longifolia*	*M. piperita*	*M. spicata*	*M. arvensis*	*M. viridis*	*M. × piperita* (Hybrid)
Essential oil composition	Rich in oxygenated monoterpenes, major components include piperitone oxide and 1,8-cineole. (48)	High in menthol (40.7 %) and menthone (23.4 %). (49)	Dominated by carvone (40.8 %) and limonene (20.8 %). (50)	Contains menthol and menthone as significant components. (51)	Characterized by high levels of carvone and limonene. (51)	Similar to *M. piperita*, it has high menthol and menthone content. (49)
Flavonoids	Contains flavonoids such as luteolin and apigenin.(52)	Rich in eriocitrin, hesperidin and kaempferol 7-O-rutinoside. (49)	Contains flavonoids like eriocitrin and luteolin. (52)	Flavonoid content includes luteolin derivatives. (52)	Rich in flavonoids, particularly eriocitrin. (52)	Contains similar flavonoids to *M. piperita*. (49)
Phenolic compounds	High in phenolic acids, including rosmarinic acid. (41)	Contains phenolic compounds like rosmarinic acid. (49)	Rich in phenolic compounds, notably rosmarinic acid. (41)	Contains phenolic acids such as caffeic acid. (52)	High in phenolic content, including rosmarinic acid. (52)	Similar phenolic profile to *M. piperita*. (49)
Antioxidant activity	Exhibits strong antioxidant activity due to high phenolic content. (41)	Demonstrates significant antioxidant properties.(49)	Shows notable antioxidant activity attributed to its phenolic compounds. (19)	Possesses antioxidant potential linked to its phytochemical constituents.(52)	Exhibits antioxidant activity due to its phenolic and flavonoid content.(41)	Comparable antioxidant activity to *M. piperita*.(49)
Antimicrobial activity	Displays significant antimicrobial properties against various pathogens. (46)	Effective against a range of microorganisms. (49)	Shows antimicrobial activity, particularly against *Staphylococcus aureus* and *Escherichia coli*.(19)	Demonstrates antimicrobial effects against certain bacteria. (19)	Possesses antimicrobial properties due to its essential oil composition.(19)	Similar antimicrobial efficacy to *M. piperita*. (49)

significant differences and similarities in their phytochemical compositions and biological properties. All species share key compounds in their essential oils, such as menthol, menthone and carvone, responsible for their medicinal and aromatic qualities. Among these, *M. piperita* stands out for its high menthol and menthone content, offering strong therapeutic properties. Meanwhile, *M. spicata* and *M. viridis* are rich in carvone and limonene, contributing to their distinct aroma and antifungal properties. Additionally, flavonoids and phenolic compounds, such as rosmarinic acid, are prevalent in *M. longifolia* and *M. piperita*, enhancing their antioxidant and anti-inflammatory effects (48-52).

These species' antioxidant and antimicrobial properties underscore their potential in preventing and treating various diseases. *M. longifolia* and *M. piperita* exhibit the most vigorous antimicrobial and antioxidant activities due to their higher levels of phenolic compounds and essential oils. In contrast, *M. spicata* and *M. viridis* show moderate effects compared to *M. piperita*. This phytochemical diversity within the Mentha species provides valuable applications in medicine, agriculture and industry, including herbal remedies, essential oil production and their use in food and cosmetics.

Conclusion

The present study suggests that Mint essential oil could serve as a potential source of natural antimicrobial compounds, owing to its phenolic components and robust antioxidant activity. These properties position mint oil as a promising candidate for use in the food, cosmetic and pharmaceutical industries, particularly for developing natural antimicrobial and antioxidant formulations. Further research is required to identify the biologically active compounds and characterize and purify Mentha species' crude extracts. In the second part of the present study, a simple and precise HPLC method with high accuracy is introduced for the quantification of oleanolic acid and ursolic acid in *M. longifolia* L. The study demonstrated that *M. longifolia* is a significant source of oleanolic acid and ursolic acid, compounds known for their anti-inflammatory, anticancer and hepatoprotective properties. These findings suggest that *M. longifolia* could be explored for therapeutic applications, particularly in developing nutraceuticals or as a source of bioactive ingredients for pharmaceutical formulations. In this context, selecting suitable wild plant varieties is essential to obtain high-yield triterpenoid cultivars to extract these pharmaceutically valuable compounds. It appears that additional studies on the compound variations within the wild populations of *M. longifolia* are needed. The variations in compound content observed in the studied *M. longifolia* suggest that environmental conditions play a crucial role in influencing the quality and quantity of these compounds, which may affect their potential industrial and therapeutic applications.

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Authors' contributions

NM, VA, VZ and AM planned the design of the study, carried out the experiments, performed statistical analysis and drafted the manuscript. NM and VA conceptualized the work and review the manuscript. All the authors approved and read the final manuscript.

Compliance with ethical standards

Conflict of interest : 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.

Ethical issues: None

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