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Preparation of enriched extracts from *Mentha longifolia* subsp. *typhoides* samples collected from different localities in terms of ursolic and oleanolic acids

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Preparation of enriched extracts from *Mentha longifolia* subsp. *typhoides* samples collected from different localities in terms of ursolic and oleanolic acids

Running title: Ursolic/oleanolic acids enrichment in *Mentha longifolia* extracts

Barış Reşitoğlu[1], Özge Tokul Ölmez[2], Nesrin İnceören[1], İsmail Yener[1], Mustafa Abdullah Yılmaz[1], Mehmet Fırat[3], Mehmet Akdeniz[4], Mehmet Öztürk[2,5], Ufuk Kolak[6], Abdulselam Ertaş[1]

[1]*Department of Analytical Chemistry, Faculty of Pharmacy, Dicle University, Diyarbakir, Türkiye*

[2]*Department of Chemistry, Faculty of Science, Mugla Sıtkı Kocman University, Mugla, Türkiye*

[3]*Department of Biology, Faculty of Education, Van Yuzuncu Yil University, Van, Türkiye*

[4]*Diyarbakir Group Chairmanship, The Council of Forensic Medicine, Diyarbakir, Türkiye*

[5]*Al-Farabi Kazakh National University, Faculty of Chemistry and Chemical Technology, Almaty-Kazakhstan*

[6]*Department of Analytical Chemistry, Faculty of Pharmacy, Istanbul University, Istanbul, Türkiye*

***Corresponding author:** A. Ertas, E-mail: abdulselamertas@hotmail.com, abdulselam.ertas@dicle.edu.tr, Phone: +90 412 248 8030/7536/+90505 628 3447.

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Abstract

In this study, the phytochemical profile of *Mentha longifolia* subsp. *typhoides*, a member of the Lamiaceae family with recognized medicinal and economic value, was investigated. The primary objective was to determine the optimal extraction conditions for triterpenic compounds, particularly ursolic acid (UA) and oleanolic acid (OA), and to prepare enriched extracts. To this end, 17 plant samples collected from 14 different localities in Eastern Türkiye were extracted using solvents of varying polarity (petroleum ether, dichloromethane, dichloromethane: acetone 9:1, acetone, ethyl acetate, ethanol, and 2% sodium hydroxide: ethanol) through maceration. GC-MS analyses revealed that dichloromethane and ethanol extracts were particularly rich in ursolic and oleanolic acids. LC-MS/MS results further demonstrated that ethanol extracts contained rosmarinic acid at a level of 67.31 mg/g extract, along with high concentrations of polyphenolic compounds such as acacetin, naringenin, and cynaroside. Among the solvents tested, ethanol yielded the highest extraction efficiency

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(6.70%), leading to the determination of optimal extraction conditions as 35 °C and pH 7 under these conditions, the 17 plant samples were fractionated into root, stem, leaf, and flower parts, and extracted with ethanol via maceration. GC-MS and TLC analyses indicated that the bioactive compounds were predominantly concentrated in the leaf and flower tissues. In the second phase of the study, based on GC-MS data, samples from the four richest localities were collected at different vegetative stages (pre-flowering, flowering, and post-flowering) and extracted under optimized conditions. The highest total triterpenic acid content (ursolic+oleanolic acids), amounting to 609.68 mg/g extract, was detected in the BH-5 (*Mentha longifolia*, Türkiye: Mardin, Nusaybin), flower extract collected from the Mardin-Beyazsu locality. Overall, the species was found to be rich in these compounds throughout its vegetative cycle, with the highest levels observed post-flowering. Additionally, PCA-based evaluation of soil elemental composition revealed an associative pattern between elevated Cu and Na concentrations and increased ursolic and oleanolic acid accumulation in plant tissues. These findings suggest that soil composition may contribute to triterpenoid distribution and underscore the potential of this species as a valuable natural resource for the cosmetic industry.

Keywords: *Mentha longifolia*; GC-MS; LC-MS/MS; Ursolic acid; Oleanolic acid; Rosmarinic acid

Introduction

Throughout history, plants obtained from nature have been indispensable resources for maintaining human health and treating diseases[1]. At the core of this effect lie secondary metabolites with high biological activity. To date, more than 100,000 secondary metabolites have been identified, and due to their structural diversity, they offer significant potential for pharmacology and drug discovery. Synthesized in plants through tissue- and organ-specific enzymes, these compounds are generally classified into three main groups: terpenes, phenolics, and nitrogen-containing compounds. They constitute a valuable source for the development of new therapeutic approaches in modern medicine[2]. Among plant families distinguished by their diversity of secondary metabolites, Lamiaceae is one of the largest and most established worldwide. Within this family, the genus *Mentha* L. is represented by approximately 25-30 species globally and 7 species in Türkiye[3]. Owing to its rich content of essential oils and bioactive compounds, this genus has found broad applications ranging from the food industry to pharmaceutical and cosmetic uses[4,5]. *Mentha* species are widely utilized in essential oil production, spice and herbal tea formulations,

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confectionery and bakery products, as well as in toothpaste, chewing gum, and beverages; moreover, they are also employed in the fields of cosmetics, pharmaceuticals, and biopesticide production[6].

Mentha longifolia L. subsp. *typhoides* (Briq.) Harley, commonly known as yarpuz, grows naturally in almost every region of Türkiye and is widely used in the treatment of respiratory and digestive disorders[7]. The essential oil composition obtained from the leaves and flowers of this species through hydrodistillation has been reported to be dominated by constituents such as piperitone oxide, piperitenone oxide, *p*-menthone, trans-dihydrocarvone, and linalool in different chemotypes. Such chemical diversity is significant in elucidating the underlying basis of the variations observed in the pharmacological activities of the species. Moreover, these constituents have been consistently highlighted in the literature as being associated with antimicrobial, antioxidant, and anti-inflammatory properties[8]. From this species, various bioactive secondary metabolites have been isolated, including flavonoids (apigenin, luteolin, hypolaetin), phenolic acids (rosmarinic acid), glycosides, terpenoids (ursolic acid, oleanolic acid), and β-sitosterol[9]. Among these, ursolic acid (UA) and oleanolic acid (OA) are particularly noteworthy due to their anti-inflammatory, antioxidant, anticancer, hepatoprotective, and enzyme-inhibitory properties[10]. UA and OA are constitutional isomers sharing the molecular formula $C_{30}H_{48}O_3$, distinguished by their pentacyclic skeletal types, ursane and oleanane respectively, which differ in the position of a methyl substituent at C-19 of the E ring[11]. In the literature, OA has been reported to exhibit strong natural antioxidant activity,[12,13] while UA has been shown to enhance antioxidant enzyme levels and reduce inflammatory markers[12,14]. Reliable evaluation of the biological activities of these compounds depends primarily on their efficient extraction from plant material and subsequent detection using robust analytical methods. Advanced analytical techniques such as GC-MS, LC-MS/MS, and UPLC-MS/MS are widely employed in the analysis of triterpenes like UA and OA[15-17].

In the existing literature on *M. longifolia*, studies have primarily focused on topics such as essential oil composition, geographical variation, solvent effects, and seasonal changes[7,18,19]. However, no comprehensive research has been found that addresses multidimensional fractionation, optimization of solvent/temperature/pH conditions, and comparative analyses across different localities with respect to ursolic acid and oleanolic acid. Özen et al.[20] comprehensively investigated the phytochemical composition and antioxidant capacity of *M. longifolia* subsp. *typhoides* var.

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typhoides using HPLC-TOF/MS. Their study reported high levels of phenolic compounds with strong antioxidant activity; however, no direct comparison between methodologies or compound classes was provided, and triterpenic acids were not specifically addressed. Similarly, Ertaş et al.[9] successfully isolated and characterized UA and OA from *M. longifolia* subsp. *noeana*. In addition, they evaluated the essential oils of this subspecies, demonstrating notable antioxidant, cholinesterase inhibitory, and antimicrobial properties, thereby highlighting its significant pharmacological potential. These findings underscore the relationship between *M. longifolia* subsp. *typhoides* and other subspecies, while also clarifying the originality and importance of the present study. Therefore, in this study, *M. longifolia* subsp. *typhoides* samples collected from the Eastern and Southeastern Anatolia regions of Türkiye were subjected to extractions using different solvents, temperatures, and pH values. The resulting extracts were analyzed using GC-MS, LC-MS/MS, and TLC techniques. Furthermore, a multivariate statistical approach based on Principal Component Analysis (PCA) was employed to evaluate the relationships between bioactive compounds in different plant organs and the chemical properties of the soils in which they grew. Considering the pharmacological effects and industrial applications of UA and OA, obtaining these compounds in high quantities from natural sources will become increasingly important in the coming years. The present study aims to provide detailed fundamental data necessary for the medium-scale production of these two compounds.

Materials and Methods

Plant material

The roots and aerial parts of *M. longifolia* L. subsp. *typhoides* (Briq.) Harley (yarpuz) was collected between June and August (2024) from the provinces of Batman, Mardin, Diyarbakır, Elazığ, and Van, located in the Eastern and Southeastern Anatolia regions of Türkiye. Taxonomic identification of the plant materials was carried out by Dr. Mehmet Fırat, Department of Biology, Faculty of Education, Yüzüncü Yıl University. Herbarium numbers of 17 specimens collected from 14 different localities of *M. longifolia* subsp. *typhoides* are presented in Table 1. The herbarium specimen was dried for storage in Van Yüzüncü Yıl University Faculty of Science herbarium. In order to evaluate the growth conditions of the species, preliminary field studies were conducted, during which samples from three localities were collected in the pre-flowering stage. Additional sampling was performed during the flowering stage from the same localities. Accordingly, specimens BH-1 (*M. longifolia*, Türkiye: Mardin, Sultan Şeyhmus) and BH-4 (*M.*

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longifolia, Türkiye: Mardin, Sultan Şeyhmus), BH-2 (*M. longifolia*, Türkiye: Mardin, Nusaybin) and BH-5 (*M. longifolia*, Türkiye: Mardin, Nusaybin), as well as BH-3 (*M. longifolia*, Türkiye: Diyarbakır, Silvan) and BH-6 (*M. longifolia*, Türkiye: Diyarbakır, Silvan) represent the pre-flowering and flowering stages, respectively. In 2025 (June-August- September), considering both the richness of content and the natural population density among 14 localities, the samples BH-4, BH-5, BH-7 (*M. longifolia,* Türkiye: Yeni Köprü, Batman) and BH-8 *(M. longifolia,* Türkiye: Ergani, Diyarbakır) were selected. These samples were recollected in three distinct phenological stages: pre-flowering, flowering, and post-flowering.

(Table 1.)

Plant material collection and optimization of extraction conditions

Seventeen plant specimens were collected from 14 different localities in the Eastern and Southeastern Anatolia regions of Türkiye. The collected plants were dried in the shade, and their root and aerial parts were separated. The plants obtained from different localities were coded, and subsequently their root and aerial parts were combined to form homogeneous samples. The plant materials were ground into powder. Preliminary trials were conducted to determine solvent selection and optimal extraction conditions. For this purpose, homogeneous samples (10 g) were subjected separately to maceration with different solvents (50 mL) including petroleum ether, dichloromethane, dichloromethane: acetone (9:1), acetone, ethyl acetate, ethanol, and 2% sodium hydroxide: ethanol. Each maceration cycle lasted 8 hours. The maceration process was repeated five times, and the filtrates obtained were combined. Solvents were removed under reduced pressure by evaporation. Content analyses revealed that ethanol provided the highest extraction yield in the extraction processes. Moreover, among the solvents used, ethanol was found to have the lowest toxicity. Therefore, to determine the optimum conditions, 10 g samples of the species were subjected to varying temperatures (25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100 °C) and pH values (6, 7, 8, 9, 10) using ethanol as the solvent (50 mL). pH adjustments were carried out using a diluted acetic acid solution to achieve pH 6, while the other pH values were obtained by dissolving solid NaOH in 96% ethanol. All pH measurements were performed directly in the extraction solution with a pre-calibrated glass electrode pH meter. This methodological clarification enhances reproducibility and ensures the reliability of the extraction conditions. Content analyses indicated that the optimum extraction conditions were 35 °C and pH 7. In the second phase of the study, four localities with the highest chemical content and dense natural populations, identified from the first-year analyses, were selected.

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Plant samples were collected from these localities at different phenological stages: pre-flowering, flowering, and post-flowering. The collected specimens were again dried in the shade, separated into underground and aerial parts, and ground into powder for extraction. Prepared dry samples (10 g) were subjected to maceration in ethanol (50 mL) under the optimal conditions (35 °C, pH 7). This process was repeated five times. At the end of extraction, the solutions were filtered, and the solvents were removed using a rotary evaporator (40 °C, 175-800 mbar). Thus, crude extracts were obtained and prepared for subsequent content analyse.

Quantitative GC-MS analysis

To enhance the volatility of moronic acid, *α*-amyrin, oleanonic acid, oleanolic acid, betulinic acid, ursolic acid, and ursonic acid, both standards and samples were derivatized using N, O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS). For this purpose, 100 µL of sample or standard solution was transferred into a glass vial, evaporated to dryness, and subsequently treated with 100 µL BSTFA+TMCS (99:1, v/v). Different temperatures and reaction times were tested to optimize derivatization conditions, and the most suitable parameters were determined as 70 °C for 120 min. Analyses were performed using an Agilent Technologies 7890A GC system coupled with a 5977B MS detector. Chromatographic separation was achieved on an HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm). The GC oven temperature was programmed from 200 °C to 300 °C at a rate of 10 °C/min, held at 300 °C for 15 min., and then increased to 310 °C at 5 °C/min with a final hold of 2 min. Helium was used as the carrier gas at a constant flow rate of 0.8 mL/min. Injections were performed in splitless mode with a volume of 2.0 µL, and both the injector and transfer line temperatures were set at 300 °C. The mass spectrometer operated in electron ionization mode at 70 eV, with an ion source temperature of 230 °C, and data were acquired in full scan mode over the *m/z* 50–650 range. Compound identification was based on retention times and comparison of mass spectra with authentic standards. Quantitative analysis was carried out using two or three high-intensity characteristic fragment ions for each compound. Method validation included evaluation of linearity, recovery, precision, repeatability, reproducibility, LOD, and LOQ parameters. Retention times for *α*-amyrin-TMS, moronic acid-TMS, oleanonic acid-TMS, oleanolic acid-2TMS, betulinic acid-2TMS, ursolic acid-2TMS, and ursonic acid-TMS were determined as 17.99, 20.71, 20.96, 21.55, 21.90, 22.55, and 22.91 minutes, respectively. The molecular ions were recorded at *m/z* 498 for *α*-amyrin-TMS, *m/z* 527 for moronic acid-TMS, oleanonic acid-TMS, and

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ursonic acid-TMS, and *m/z* 601 for oleanolic acid-2TMS (the first three fragment ions: 203, 189, and 320), betulinic acid-2TMS, and ursolic acid-2TMS (the first three fragment ions: 203, 189, and 320). Calibration curves were constructed using linear regression equations, with correlation coefficients (r^2) ranging from 0.9991 to 0.9997. Relative standard deviation (RSD%) values ranged between 0.015 and 0.029, recovery values between 0.938 and 0.986, while LOD/LOQ values were calculated as follows: α-amyrin-TMS (335.71/389.85 mg/L), moronic acid-TMS (484.57/575.49 mg/L), oleanonic acid-TMS (493.52/569.16 mg/L), oleanolic acid-2TMS (504.40/589.80 mg/L), betulinic acid-2TMS (484.45/546.40 mg/L), ursolic acid-2TMS (459.09/506.43 mg/L), and ursonic acid-TMS (484.09/572.27 mg/L)[21].

LC-MS/MS analysis

LC-MS/MS analyses were performed using a Shimadzu Nexera UHPLC system coupled with a Shimadzu LC-MS 8040 triple quadrupole mass spectrometer. The liquid chromatography system consisted of an LC-30AD gradient pump, DGU-20A3R degasser, CTO-10ASvp column oven, and SIL-30AC autosampler. Chromatographic separation was achieved on an Inertsil ODS-4 C18 column (100 mm × 2.1 mm, 2 µm). Mobile phase consisting of water (0.1% formic acid) and acetonitrile (0.1% formic acid) was employed. The gradient program was initiated with a high proportion of the aqueous phase and gradually shifted towards the organic phase. The flow rate was maintained at 0.3 mL/min, and the injection volume was set to 5 µL. Appropriate method parameters were determined during method validation. Extracts prepared for analysis were first diluted to a concentration of 250 mg/L and then filtered through a 0.22 µm PTFE membrane prior to injection. The triple quadrupole mass spectrometer was equipped with an electrospray ionization (ESI) source operating in both positive and negative ion modes. LC-ESI-MS/MS data were processed using LabSolutions software (Shimadzu, Kyoto, Japan). Analyses were conducted with reversed-phase C18 chromatography and electrospray ionization. Ion transitions were monitored in Multiple Reaction Monitoring (MRM) mode. Calibration curves were constructed using external standards[22].

TLC analysis

Thin-layer chromatography (TLC) was performed using pre-coated silica gel plates (20 × 20 cm). Prepared stock solutions of the extracts were applied onto the TLC plates. For comparison purposes, solutions of ursolic acid and oleanolic acid were also spotted onto the plates. The TLC plates were

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placed in a chamber saturated with a dichloromethane: acetone (9:1) solvent system, allowing the compounds to migrate. Separation of the mixture components occurred based on their adsorption mechanisms on the silica gel surface. After the solvent development process, the chromatographic spots were visualized under UV light. Subsequently, ceric sulfate reagent was applied, and the plates were heated in an oven at 110 °C for 5 min., resulting in the appearance of distinct and well-defined spots[9].

Soil quality assessment and characterization of components

Soil pH and saturation levels were determined according to the methods described by Tüzüner[23]. The phosphorus content of the soil samples was measured using the Olsen method developed by Olsen et al[24]. Carbonate (lime) determination was generally performed using a calcimeter; in this study, the total lime content of the soil samples was measured with a Scheibler calcimeter[25]. Soil texture analyses were conducted to determine the proportional distribution of sand, silt, and clay fractions. The quantities of these fractions were measured using the Bouyoucos hydrometer method[26]. Organic carbon and organic matter contents of the soil samples were analyzed using the modified Walkley-Black wet combustion method[27]. Macro- and micro-element analyses of the soil samples were performed using Atomic Absorption Spectrometry (AAS), following the procedures described by Yener et al[28]. For soil characterization, samples were collected at approximately 10 cm root depth. From each locality, three separate subsamples were taken and homogenized in the laboratory to obtain a composite sample. The soil samples were stored in a dry environment at room temperature until analysis. The relationships among soil chemical properties and bioactive compounds in plant organs were evaluated using Principal Component Analysis (PCA), a multivariate statistical method.

Statistical analysis

In this study, statistical evaluations were conducted using a multivariate approach based on Principal Component Analysis (PCA), performed with the Minitab 17 software package. PCA was applied to explore the relationships between bioactive compounds in plant organs and the chemical properties of the soils in which they were grown. In addition, comparative analyses were carried out across seven solvents, eleven temperature levels, five pH levels, four organ types, seventeen localities, and three phenological stages. Pearson correlation analyses were conducted to evaluate associations

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between individual soil elemental variables and UA/OA contents in roots and leaves.

Results and Discussion

TLC and GC-MS characterization of M. longifolia subsp. *typhoides extracts obtained using different solvents*

The data presented in Table S1 demonstrate that the extraction yields obtained from different parts of *M. longifolia* using various solvents at room temperature showed considerable variation. The highest yield (6.70%) was achieved with ethanol. This finding indicates that ethanol is the most suitable solvent due to its broad solubility for bioactive compounds and low toxicity profile. Among solvents of intermediate polarity, ethyl acetate provided a yield of 4.36%, suggesting a potential advantage in the selective extraction of phenolic and flavonoid compounds. In contrast, dichloromethane resulted in a lower yield (2.90%), yet remains noteworthy for its effectiveness in extracting lipophilic compounds such as triterpenic acids. Although dichloromethane afforded the highest ursolic and oleanolic acid content per gram of extract, considering that equal amounts of plant material (10 g) were used in the initial extractions, the higher extraction yield of ethanol led to a greater total UA+OA content. Therefore, while dichloromethane appears superior in terms of concentration, ethanol is more advantageous in terms of total content. Taking into account its low toxicity profile as well, ethanol was selected as the solvent in our study. In conclusion, when extraction yield, phytochemical composition, and toxicity profile are collectively evaluated, ethanol is identified as the most appropriate solvent for the high-yield, safe, and broad-spectrum extraction of *M. longifolia*

Numerous studies in the literature have emphasized that solvent polarity plays a decisive role in extraction yield and phytochemical content. For instance, Wakeel et al.[29] reported that an increase in polarity index enhanced the total phenolic and flavonoid content, although this effect was limited at excessively high polarity levels. Similarly, Tourabi et al.[30] demonstrated that hydroethanolic extracts of *M. longifolia* provided the highest values in terms of phenolic compounds and antioxidant capacity. In contrast, low-polarity solvents (e.g., petroleum ether, n-hexane) were insufficient in dissolving biologically active compounds, resulting in lower extraction yields. These findings are consistent with the results obtained in our study, highlighting that ethanol is the most suitable solvent for the extraction of biologically active compounds from *M. longifolia*, owing to its high extraction yield and low toxicity profile.

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TLC analyses of extracts prepared with different solvents were carried out in comparison with UA and OA standards. Examination of the chromatograms, considering spot area and intensity values, revealed that extracts prepared with pure dichloromethane, dichloromethane: acetone (9:1, v/v), and pure ethanol contained relatively high levels of both triterpenic acids (Fig. 1).

(Fig. 1)

The data presented in Table 2 show the GC-MS results for the amounts of UA and OA in extracts of the studied species prepared with different solvents (Fig. 2). Consistent with the TLC findings, GC-MS analysis revealed that the dichloromethane (DCM) extract contained the highest levels of both UA and OA. In the DCM extract, the OA content was determined to be 76.27 mg/g extract, while the UA content reached 310.89 mg/g, resulting in a total OA+UA content of 387.15 mg/g. The results also indicated that ethanol extracts exhibited a rich profile of UA and OA, with values comparable to those of the DCM extract. In contrast, petroleum ether extracts contained the lowest amounts of triterpenic acids, with OA measured at 18.60 mg/g and UA at 23.89 mg/g. These findings highlight the decisive role of solvent polarity in the extraction efficiency of triterpenic acids. While the medium-polarity solvent DCM enabled efficient extraction of both UA and OA, the low-polarity solvent petroleum ether was limited in dissolving these compounds. Similarly, more polar solvents such as ethanol facilitated high levels of UA and OA extraction. Considering the polarity of ethanol, the extraction process appears to occur not only through dissolution of the compounds in the solvent but also through their transfer into the solvent phase, indicating that ethanol functions both as a solvent and as an effective carrier for separating target compounds from the plant matrix. Notably, sediment formation was observed at the bottom of ethanol extracts after resting, and subsequent analyses revealed that this precipitate was highly enriched in UA and OA. Ertas et al[9]. reported that the subspecies *M. longifolia* subsp. *noenae* contained high levels of UA and OA during isolation and purification studies; however, quantitative analyses of these triterpenes were not performed, and no data on quantitative content analysis of this species are available in the literature. In comparison, Fu et al[31]. aimed to optimize the extraction of UA and OA from pomegranate flowers (*Punica granatum* L.). In their study, different solvents were evaluated, and ethanol was selected for safety reasons. Furthermore, ultrasound-assisted extraction was reported to provide higher yields in shorter times compared to conventional methods. As a result, the extraction yields of UA and OA were reported as 12.6 mg/g and 9.7 mg/g, respectively.

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(Table 2**)**, (Fig. 2)

LC-MS/MS characterization of M. longifolia subsp. *typhoides extracts obtained using different solvents*

Table 3 presents the LC-MS/MS analysis results of extracts prepared from all parts of the studied species using different solvents (Fig. 3). The findings clearly demonstrate that the type of solvent employed had a significant and pronounced effect on the quantities of extracted compounds. Among the compounds identified, rosmarinic acid emerged as the most abundant, with its concentration in the ethanol extract reaching 67.315 mg/g extract. In addition to rosmarinic acid, notable levels of acacetin, naringenin, and cynaroside were also detected, with maximum concentrations in the ethanol extract measured at 23.614, 13.643, and 12.727 mg/g extract, respectively. These results unequivocally indicate that ethanol is highly effective in extracting phenolic and flavonoid compounds. Furthermore, the data strongly support the decisive role of solvent polarity and solvent-matrix interactions in determining extraction efficiency[32,33]. The findings emphasize that optimizing solvent selection is a critical parameter not only for extraction efficiency but also for the bioavailability of biologically active compounds. In the literature, only one study has quantitatively assessed the phenolic content of this species using HPLC-TOF/MS[20]. Consistent with the present results, that study also reported that the species is rich in phenolic compound.

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(Table 3), (Fig. 3)

TLC and GC-MS characterization of M. longifolia subsp. *typhoides under different extraction conditions*

As shown in Table S2, comparison of ethanol extracts prepared at different temperatures revealed a general trend of increasing yield with rising temperature. This observation suggests that elevated temperatures enhance the diffusion coefficient and solubilizing capacity of the solvent, thereby facilitating the dissolution of target compounds from the plant matrix[34,35]. Similar findings in the literature report that increasing extraction temperature accelerates mass transfer and strengthens solvent-matrix interactions[35]. However, it is well established that secondary metabolites-particularly terpenoids and certain volatile compounds-may undergo thermal degradation at temperatures above 50 °C[36]. Thus, although higher temperatures may increase extraction yield, there is a risk of partial loss of biologically valuable compounds. At lower temperature ranges, the highest yield was obtained at 35 °C. Consistent with this, several studies have

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reported that moderate temperatures (30-40 °C) are optimal for preserving phenolic and terpenoid compounds while ensuring high extraction efficiency[37,38]. Taken together, these results indicate that 35 °C represents the optimal extraction temperature for *M. longifolia* ethanol extracts, balancing both yield and compound stability.

TLC analyses revealed that ethanol extracts of *M. longifolia* prepared at different temperatures possessed a rich component profile in terms of UA and OA (Fig. S1). In all temperature groups, both triterpenic acids produced distinct bands, indicating that the extracts contained high levels of these compounds. Notably, extracts prepared at 35 °C exhibited more pronounced band intensities compared to those obtained at other temperatures. This finding suggests that moderate thermal conditions may enhance the extraction efficiency of the targeted triterpenic acids. Thus, 35 °C can be considered to provide a balanced extraction environment, ensuring both solubility and compound stability.

Table S3 presents the GC-MS results for the amounts of UA and OA in ethanol extracts of *M. longifolia* subsp. *typhoides* prepared at different temperatures (Fig. S2). Consistent with the TLC findings, the results showed that the total UA+OA content decreased at temperatures of 50 °C and above. In contrast, within the 25-35 °C range, an increase in the amounts of these compounds was observed with rising temperature. Previous studies have reported that *M. longifolia* is rich in volatile oils and secondary metabolites, particularly phenolic and flavonoid compounds as well as triterpenic acids[9]. The biological activities of these compounds are highly sensitive to extraction conditions, especially temperature. At elevated temperatures, processes such as thermal degradation, oxidation, and isomerization may occur, leading to a reduction in compound levels[36,39]. The findings of this study demonstrate that extraction temperature is a critical factor influencing the stability of triterpenic acids in this species, with the optimal temperature range being around 35 °C. The decline observed at higher temperatures is likely attributable to thermal degradation mechanisms such as decomposition and/or isomerization. Therefore, for the efficient recovery of UA and OA from *M. longifolia*, low to moderate temperature ranges are recommended to preserve the existing levels of these bioactive compounds.

As shown in Table S4, the ethanol extract yields of *M. longifolia* did not exhibit significant variation across different pH values. Ethanol extracts prepared under acidic, neutral, and basic conditions were analyzed by TLC in comparison with UA and OA standards. The resulting chromatograms revealed that extracts prepared under all pH conditions contained high

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levels of both triterpenic acids (Fig. S3). Importantly, the absence of a marked reduction in these compounds under varying pH conditions suggests that the triterpenes display structural stability within the plant matrix. This finding highlights the robustness of UA and OA against pH fluctuations during extraction.

GC-MS analyses revealed the amounts of UA and OA in *M. longifolia* subsp. *typhoides* ethanol extracts prepared at different pH values (pH 6-10) (Table S5, Fig. S4). The results indicated that pH variation did not cause a significant difference in the overall UA+OA content of the extracts. However, the highest total triterpenic acid content was measured in extracts prepared at pH 6 and 7, with values of 367 mg/g and 364.52 mg/g extract, respectively. This increase observed under mildly acidic conditions may be attributed to the enhanced extraction efficiency of triterpenic acids such as UA and OA in such environments. These findings demonstrate that the pH of the extraction medium plays a critical role in the efficiency and stability of triterpenic acids. In particular, the high yields observed at pH 6–7 can be explained by the protonation equilibrium of carboxylic acid groups[40]. Within this range, the compounds maintain molecular integrity and structural stability, thereby enhancing extraction efficiency. Conversely, at higher pH values, complete ionization of carboxyl groups disrupts chemical stability and markedly increases the risk of oxidative degradation under alkaline conditions[41]. Consistent with these observations, previous studies have reported that the optimum extraction of triterpenic acids occurs under mildly acidic conditions, whereas elevated pH values negatively affect structural stability[31,42]. These mechanistic explanations support the high UA and OA contents observed in our study at pH 6 and 7. Therefore, it can be concluded that mildly acidic conditions should be preferred to preserve the functional component richness of *M. longifolia* extracts.

TLC and GC-MS characterization of M. longifolia subsp. *typhoides plant parts from different locality*

Evaluation of ethanol extract yields from roots, stems, leaves, and flowers of 17 specimens collected from 14 different localities revealed that extracts obtained from roots generally exhibited lower yields, whereas those from flowers and leaves showed comparatively higher yields. Among all samples analyzed, specimen BH-17L exhibited the highest extract yield at 15.27%, followed by BH-17F with 14.25% (Table S6). When extract yields were compared across localities, only limited variation was observed for the same plant organ. In contrast, significant differences were detected among

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different organs of the plant. Specifically, extracts from flowers and leaves consistently demonstrated higher yields, while those from roots remained relatively low. These findings suggest that focusing on the flower and leaf parts of the plant represents a more rational scientific and practical approach for enhancing extraction efficiency. Moreover, harvesting only these organs contributes positively to the sustainability of natural populations of the species.

Plant specimens coded BH-1 to BH-17, collected from different localities, were separated into roots, stems, leaves, and flowers, and ethanol extracts were prepared. These extracts were analyzed by TLC in comparison with UA and OA standards (Fig. S5-S8). The results revealed that leaf and flower extracts contained high levels of UA and OA, whereas root extracts exhibited relatively low levels. This finding clearly demonstrates that the distribution of UA and OA varies among different plant organs (Fig. 4). The high abundance of these compounds in leaf and flower tissues is consistent with reports in the literature indicating that triterpenic compounds are frequently stored in the aerial parts of plants[43]. Such localization suggests that these triterpenic acids are deposited in above-ground organs, where they play important roles in defense mechanisms and the regulation of key metabolic processes[38]. Overall, the data highlight that both locality and plant organ exert significant influence on triterpenic acid content. This underscores the critical importance of selecting the appropriate plant organ and collection site when targeting these pharmacologically valuable compounds. The findings further demonstrate that metabolite distribution within the plant is not homogeneous, with certain organs being particularly enriched in specific bioactive constituents.

(Fig. 4)

GC-MS analyses of ethanol extracts prepared from roots, stems, leaves, and flowers of specimens coded BH-1 to BH-17 revealed marked differences in UA and OA contents (Tables S7-S9, Fig. S9-S11). In general, root and stem extracts contained lower levels of UA and OA, whereas leaf and flower extracts exhibited substantially higher amounts. Among BH-1–BH-6 specimens, the BH-5F extract was the richest, with a total UA+OA content of 609.68 mg/g extract. Within BH-7–BH-11 specimens, BH-11F reached the highest value at 426.91 mg/g extract. A similar trend was observed in BH-12–BH-17 specimens: root and stem extracts showed relatively low levels, while leaf and flower extracts contained higher and comparable amounts of UA+OA. In this group, BH-12L extract was the richest with 409.73 mg/g

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extract, while BH-12S also presented a notable content of 273.71 mg/g extract. Across all specimens, UA levels consistently exceeded OA levels, indicating that UA biosynthesis is dominant in this species. This trend is consistent with findings reported for other members of the Lamiaceae family[44,45]. In conclusion, ethanol extracts of *M. longifolia* obtained from leaves and flowers were particularly rich in UA and OA, while the content levels varied depending on the locality. These results emphasize the importance of both plant organ and collection site in determining triterpenic acid yields.

TLC and GC-MS characterization of M. longifolia subsp. *typhoides plant parts at different vegetative stages*

Table S10 presents the ethanol extract yields of *M. longifolia* subsp. *typhoides* specimens collected from different localities and phenological stages (pre-flowering, flowering, and post-flowering), considering only above- and below-ground parts. According to the content analysis results, specimens coded BH-4, BH-5, BH-7, and BH-8 were recollected at different phenological stages. Overall, extracts obtained from pre-flowering samples exhibited higher yields compared to those collected during and after the flowering stage. The yields of extracts from the flowering and post-flowering stages were found to be similar. In addition, the above-ground parts of the species consistently showed higher extraction yields than the below-ground parts. These findings demonstrate that extraction efficiency is influenced not only by locality but also by vegetative stage and plant organ. Notably, the highest extraction yield (7.04%) was obtained from the above-ground parts of specimens collected before flowering in the Sultan Şeyhmus/Mardin locality.

Ethanol extracts obtained from the aerial and root parts of specimens coded BH-4, BH-5, BH-7, and BH-8, collected at different phenological stages, were analyzed by TLC in comparison with UA and OA standards. The results revealed that extracts from the above-ground parts of BH-4 (flowering stage) and BH-7 (post-flowering stage) exhibited particularly high intensities for both UA and OA (Fig. S12-13). These findings indicate that the aerial organs of *M. longifolia* during flowering and post-flowering stages are especially rich in triterpenic acids, highlighting the influence of phenological stage and plant organ on metabolite accumulation.

Based on the content analyses of the samples collected in 2024 and the population status of the species in the relevant localities, BH-4, BH-5, BH-7, and BH-8 were selected from among 14 localities in 2025. These samples were recollected at three distinct phenological stages pre-flowering,

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flowering, and post-flowering and content analyses were conducted across vegetative periods. The GC-MS results of OA and UA quantities in the ethanol extracts of the aboveground and underground parts collected at different vegetative stages are presented in Table 4 (Figure S14). In line with ITK results, the aboveground parts were found to be considerably richer in OA and UA compared to the underground extracts in all samples. Overall, the content of samples collected at different vegetative stages varied substantially depending on locality. For instance, the pre-flowering samples of BH-4 exhibited the lowest content, whereas BH-5 in the pre-flowering stage showed the highest OA and UA levels. Previous studies have reported that triterpenic acid levels in species of the Lamiaceae family vary according to developmental stage, reaching maximum levels particularly in the post-flowering period[46]. In contrast, the present study concluded that vegetative stage did not generate significant variability in OA and UA content for this species.

(Table 4)

Soil profiles of M. longifolia subsp. *typhoides localities*

Evaluation of the macro-element contents of soils from the BH series revealed that calcium (Ca), potassium (K), magnesium (Mg), and sodium (Na) reached their highest concentrations in specific samples (Table S11). According to the analyses, Ca content was highest in BH-10 at 5067.0 ± 22.1 mg/kg, K in BH-16 at 503.6 ± 4.0 mg/kg, Mg in BH-13 at 316.8 ± 1.6 mg/kg, and Na also in BH-13 at 61.4 ± 0.2 mg/kg. Overall, the BH soil samples exhibited high macro-element levels, indicating that these soils are rich in mineral nutrients. Assessment of micro-element contents showed that certain elements were present at significantly higher concentrations in specific samples (Table S12). Cadmium (Cd) reached its highest level in BH-1 at 0.099 ± 0.001 mg/kg, copper (Cu) in BH-11 at 10.060 ± 0.010 mg/kg, iron (Fe) in BH-7 at 48.650 ± 0.009 mg/kg, manganese (Mn) in BH-1 at 18.040 ± 0.116 mg/kg, lead (Pb) in BH-7 at 1.692 ± 0.008 mg/kg, and zinc (Zn) in BH-5 at 12.090 ± 0.117 mg/kg. These results demonstrate that BH soils are also rich in micro-elements. Soil fertility analyses indicated that BH-7 belonged to the heavy clay texture class with a saturation value of 112.46. pH analysis revealed that BH-9 exhibited a slightly alkaline reaction with a value of 7.75. All BH samples were classified as non-saline in terms of total salt content. Lime contents varied across samples, ranging from calcareous to moderately and highly calcareous classes. Organic matter levels ranged from very low to high. Phosphorus (P_2O_5) contents were classified as very low, low, and high, while potassium (K_2O, kg/da) contents

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were generally found to be high (Table S13). In conclusion, soils from the BH series were determined to be rich in both macro and micro elements, while also exhibiting variability in fertility parameters.

Chemometric evaluation of soil analysis and content analysis results

The data were evaluated using PCA, one of the multivariate statistical methods. Since roots are in direct contact with the soil and high levels of UA and OA were detected, PCA was performed using both root and leaf results. The PCA score plot illustrates the distribution of *M. longifolia* root samples (BH-coded) according to GC-MS data (UA, OA) and the macro-element, micro-element, and soil fertility parameters of the soils from which they were collected. The first two components explained 59.9% of the total variance. Although four principal components with eigenvalues greater than 1 were identified, together they accounted for 82.7% of the variance. The first principal component (PC1) indicated similarities among dominant variables such as K, Mg, Na, Cd, Cu, Fe, Mn, Pb, and Zn, as well as soil parameters including saturation, pH, total salinity, lime content, and organic matter. Loading values for root samples are presented in Table 14, and score values in Table 15 (Tables S14 and S15 highlighted). The score values show which samples exhibited higher dominance of these components. Root samples BH-5-R, BH-2-R, BH-16-R, BH-4-R, BH-1-R, and BH-10-R clustered together, reflecting similarities in macro- and micro-element contents of their soils. Fig. 5a presents the PCA score plot, while Fig. 5b shows the loading plot. Examination of the score plot revealed that samples (Mardin samples; purple triangles: BH-1, BH-2, BH-4, BH-5, BH-16) collected from similar geographical regions tended to cluster together, possibly reflecting regional similarities in soil composition and environmental conditions. In addition, BH-7 and BH-10 (blue circles), collected from Batman, grouped together due to similarities in soil macro- and micro-element contents. The Mardin samples displayed a highly homogeneous distribution and were similar to the Batman samples. Joint evaluation of the score and loading plots suggested an association between higher soil Cu and Na levels and increased UA and OA contents in root extracts. These compounds were particularly abundant in BH-13, BH-6, BH-8, and BH-11. UA and OA are noteworthy due to their significant pharmacological effects and therapeutic potential in treating various diseases[47,48]. Therefore, investigating the factors that enhance UA and OA accumulation in the same plant species is of considerable importance. Wang et al[49]. reported that the addition of Na_2CO_3 during cultivation significantly increased OA and UA levels in *G. longituba*. Similarly, Wu et al[50].

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demonstrated that soil applications of Pb, Cu, and Cd in *Prunella vulgaris* influenced metabolite accumulation, with Cu enrichment leading to a significant increase in UA content. In our study, plants grown in soils from Diyarbakır, having relatively higher Na and Cu levels, also influenced UA and OA contents in their roots. While this association is consistent with the findings of Wang et al[49]. and Wu et al[50]., our results are based on naturally occurring variation in field-collected soils. This suggests that the observed relationships may partially reflect regional environmental conditions such as geology, climate, and soil type, rather than the direct effects of individual elements. Grouping samples by province in PCA further supports the influence of common regional environmental characteristics. In this context, similar Cu and Na contents observed in samples from the same provinces may contribute to corresponding increases in UA and OA levels; however, these relationships may reflect combined regional environmental influences rather than the independent effects of individual elements. Samples BH-12, BH-3, BH-15, BH-14, BH-17, and BH-9 were positioned in the upper left region of the score plot. The formation of this group was primarily influenced by soil pH levels. Unlike the Mardin and Batman groups, this cluster did not display homogeneity and included specimens collected from different provinces.

In our study, PCA was applied to investigate the relationships between UA and OA contents in *M. longifolia* subsp. *typhoides* leaf samples and the macro and micro element composition as well as fertility parameters of the soils in which these plants were grown. This approach highlighted the connections between leaf metabolites and soil characteristics. The first two principal components explained 53.3% of the total variance, while the first four components together accounted for 80.6%. For the first principal component (PC1), dominant variables included soil minerals and nutrients such as Cd, Mn, P, organic matter, K, Zn, and Fe. These variables were particularly prominent in samples BH-2L, BH-5L, BH-16L (Mardin), and BH-7L (Batman) (Tables S16 and S17 highlighted). The analysis revealed that samples from Mardin and Batman exhibited similar soil properties and formed a homogeneous cluster, as shown in the score plot (Fig. 5c). For the second principal component (PC2), the dominant variables were UA, OA, total UA+OA, Na, and Cu. Leaf samples from Diyarbakır (BH-8L, BH-11L) and Elazığ (BH-13L) showed higher levels of these variables compared to other samples, whereas BH-14L (Diyarbakır) and BH-16Y (Mardin) exhibited lower levels. In BH-3Y, BH-6Y, BH-14L, BH-15L, and BH-17L, both soil mineral contents and triterpenic acid levels were found to be low, reflecting a heterogeneous distribution across the region. In the score plot (Fig. 5c),

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low-value samples were positioned in the lower left quadrant, while Figure 5d illustrates the loading plot, showing that soil pH was the key variable influencing these samples.

(Fig. 5)

Pearson correlation analyses were performed using Minitab 17 to evaluate the relationships between soil elemental composition and triterpenoid contents (UA, OA) in the roots and leaves of *M. longifolia* subsp. *typhoides*. In root samples (Table S18), a strong positive correlation was observed between UA and OA contents (r = 0.923), indicating a similar accumulation pattern of these triterpenoids. Among the soil elements, Na showed a strong positive correlation with both OA (r = 0.623) and UA (r = 0.608), while Cu exhibited a particularly strong positive correlation with UA (r = 0.759) and a moderate positive correlation with OA (r = 0.595). These correlations were statistically significant ($p < 0.05$), suggesting that elevated Na and Cu levels may be associated with increased triterpenoid accumulation in roots. In addition, K and Mn showed a strong positive correlation (r = 0.805), whereas Cd and Mn were also highly correlated (r = 0.896), reflecting possible co-accumulation trends among soil elements. In leaf samples (Table S19), UA and OA again displayed a very strong positive correlation (r = 0.933). Positive correlations were also detected between Na and both OA (r = 0.369) and UA (r = 0.475), as well as between Cu and OA (r = 0.295) and UA (r = 0.208). However, these relationships were weaker compared to those observed in roots. Nevertheless, Pearson analysis indicated statistically significant associations ($p < 0.05$) between Na/Cu levels and triterpenoid accumulation patterns in leaf tissues. Furthermore, strong positive correlations were identified between K and Zn (r = 0.814), K and Mn (r = 0.805), and Mn and Zn (r = 0.771), indicating close relationships among these mineral elements in the soil matrix. Overall, the correlation analyses supported the PCA findings and suggested that Na and Cu may be among the soil factors associated with UA and OA accumulation in *M. longifoli*a subsp*. typhoides.*

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Conclusion

This study evaluated the UA and OA contents of *M. longifolia* subsp. *typhoides* specimens collected from different localities in Türkiye under varying extraction parameters. In addition, soil samples from these localities were analyzed for macro and micro element composition and fertility parameters, and their relationships with UA and OA contents were assessed chemometrically. The findings revealed that extraction yield varied significantly depending on solvent type and temperature. Ethanol was

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identified as the most effective solvent, providing the highest extract yield. Increasing temperature generally enhanced extraction efficiency (e.g., 9.94% at 100 °C). However, GC-MS analyses clearly demonstrated that high temperatures led to degradation of triterpenic structures. In contrast, pH variation did not produce significant differences in UA and OA contents. Based on these results, ethanol is recommended as the preferred solvent for the extraction of these compounds, with optimal conditions being 35 °C and the natural pH of the solvent. This combination ensures both high extraction yield and preservation of triterpenic acid stability.

It has been determined that the species is particularly rich in UA and OA contents, especially in its leaf and flower parts. This finding is considered a critical result in terms of the sustainability of the species when collection activities are carried out from natural populations. Cultivated *Mentha* species are known to become ready for harvest up to five times a year under suitable climatic conditions. Moreover, the similarity of the contents of samples collected at different vegetative stages indicates that the leaves, which constitute the majority of the plant, provide a significant advantage for the utilization of these compounds. Although the UA and OA contents vary among samples collected from different localities, the fact that all samples are considerably rich in these components demonstrates that the species can be cultivated under diverse climatic and soil conditions. This situation reveals a noteworthy potential for the incorporation of the species into agricultural production.

PCA and Pearson correlation analyses, consistent with each other, identified links between soil fertility, elemental composition, and UA/OA levels in samples from various locations. Notably, plants grown in soils with higher Cu and Na levels tended to accumulate more of these triterpenoids in their roots and leaves. Samples BH-8 and BH-11 from Diyarbakır, along with BH-12 and BH-13 from Elazığ, showed higher Cu and Na contents, which correlated with increased UA and OA levels. Although not all Diyarbakır samples exhibited these traits, the results imply that soil characteristics may influence triterpenoid buildup. In conclusion, *M. longifolia* subsp. *typhoides* can be considered a highly suitable natural source of economically valuable UA and OA under appropriate extraction conditions. The species' ability to grow across different localities, the high compound content in its leaves and flowers, and its potential for sustainable production when cultivated highlight its promise as an important raw material for pharmaceutical and industrial applications.

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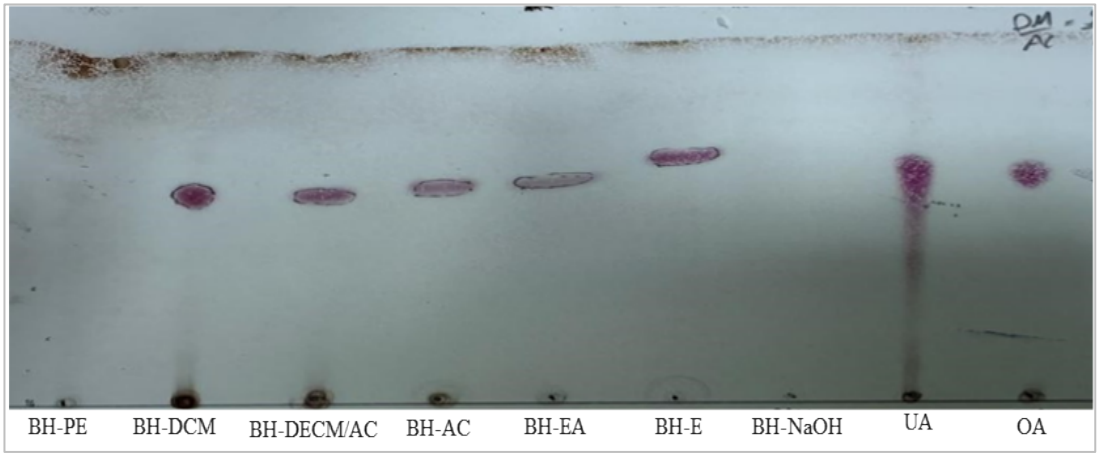
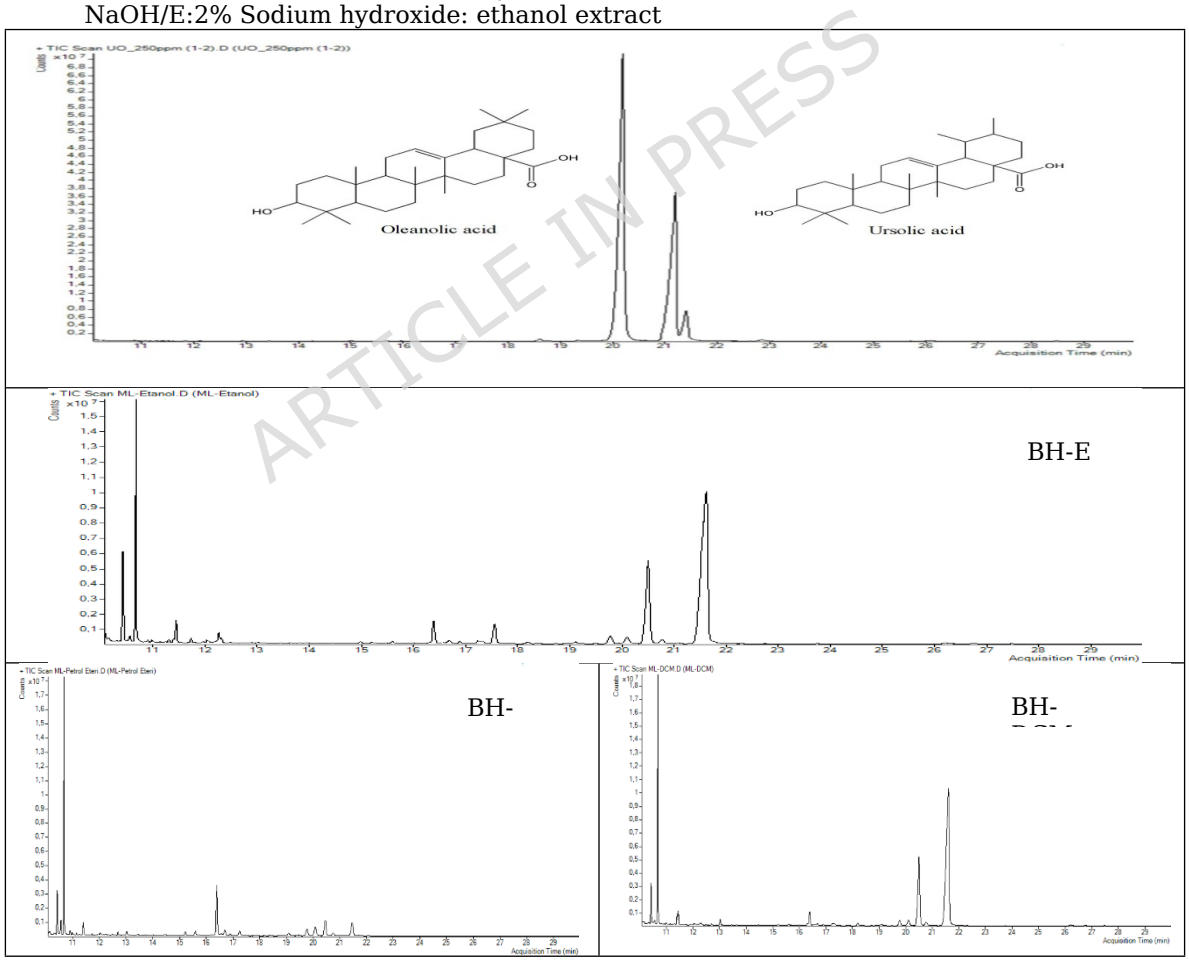


Fig. 1. Comparative TLC analyses of extracts obtained from mixtures of aerial part and root of *M. longifolia* subsp. *typhoides* prepared with different solvents, in reference to ursolic and oleanolic acid standards. BH-PE: Petroleum ether extract, BH-DCM: dichloromethane extract, BH-DCM/AC: dichloromethane: acetone:9:1 extract, BH-AC: acetone extract, BH-EA:ethyl acetate extract, BH-E: ethanol extract, BH-NaOH/E:2% Sodium hydroxide: ethanol extract



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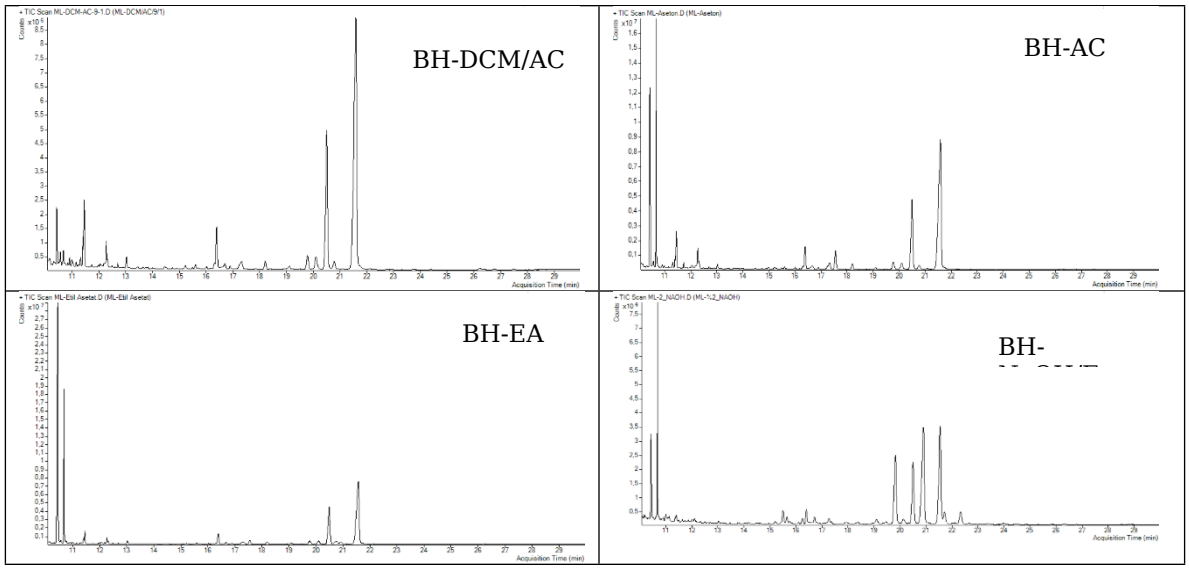
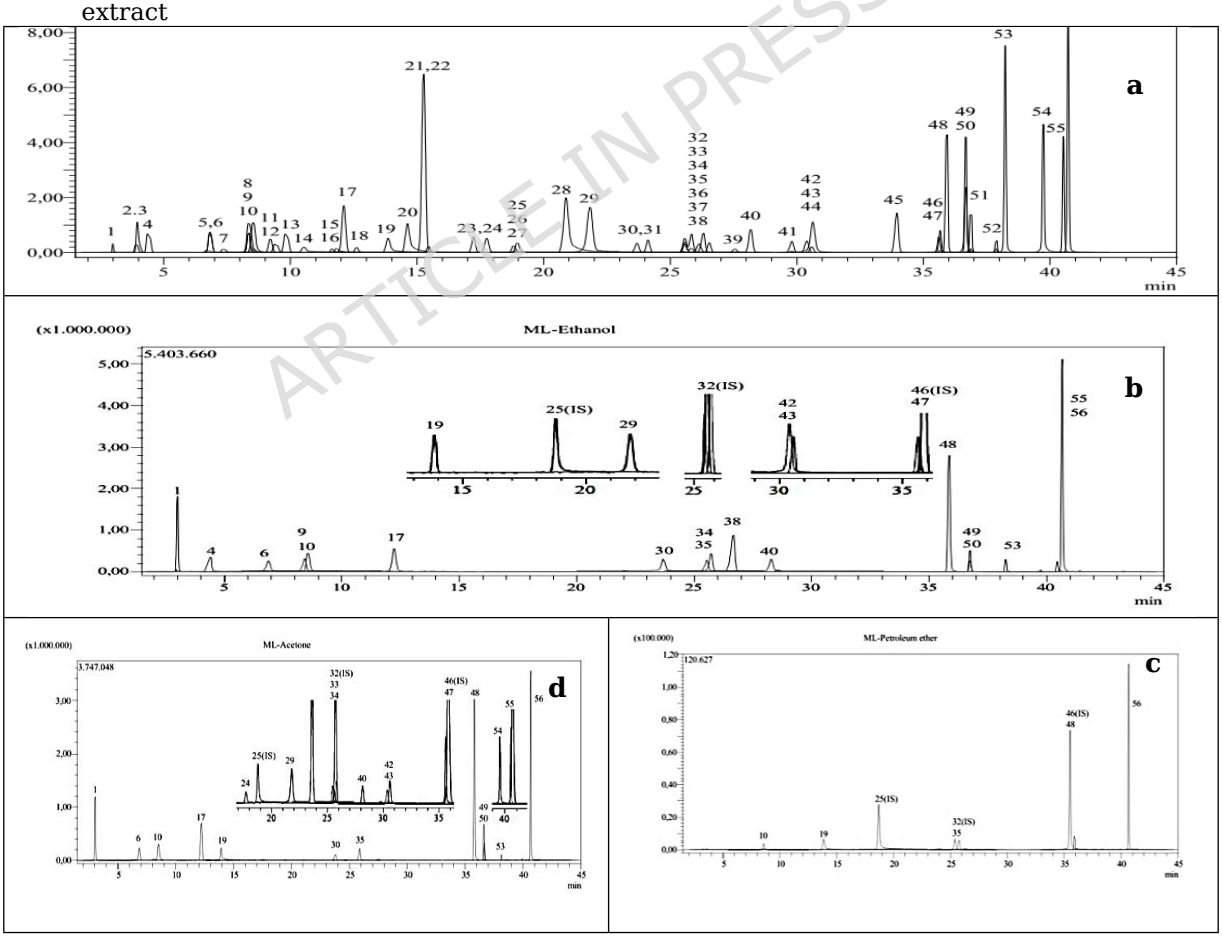


Fig. 2. GC-MS chromatograms of the extracts obtained from the mixtures of aerial and root of *M. longifolia* subsp*. typhoides* prepared with different solvents, along with those of the standard ursolic and oleanolic acids. BH-PE: Petroleum ether extract, BH-DCM: dichloromethane extract, BH-DCM/AC: dichloromethane: acetone:9:1 extract, BH-AC: acetone extract, BH-EA: ethyl acetate extract, BH-E: ethanol extract, BH-NaOH/E:2% Sodium hydroxide: ethanol extract



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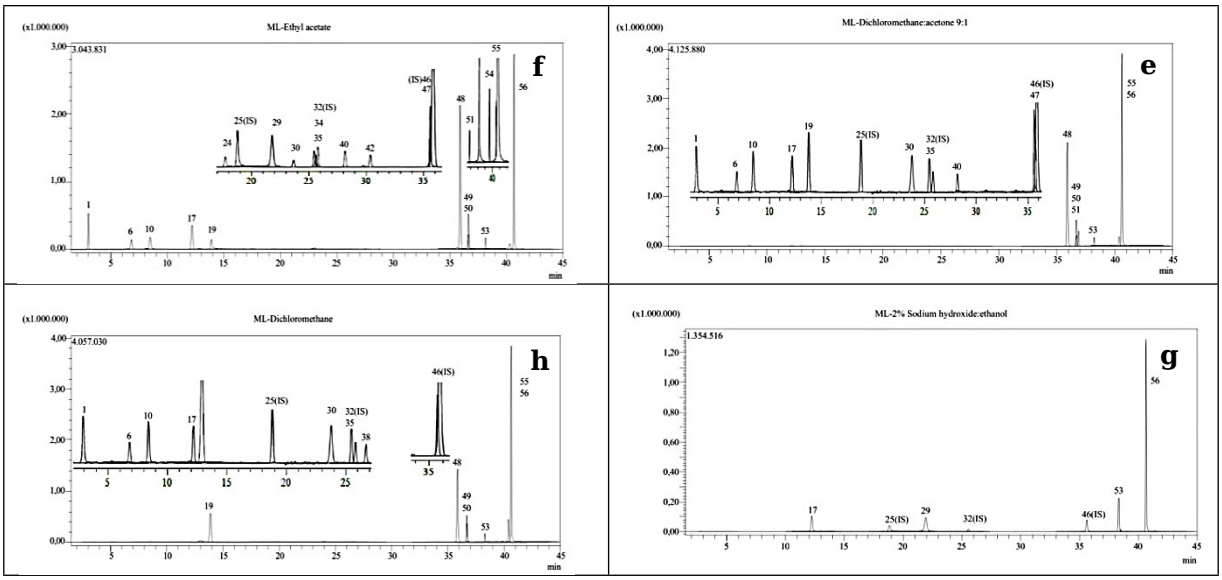

Fig. 3. a) TIC chromatogram of the standard (1 µg/mL) analysed by LC-MS/MS; LCMS/MS chromatograms of *M. longifolia* subsp. *typhoides* **b)** ethanol extract **c)** petroleum ether extract **d)** acetone extract **e)** dichloromethane: acetone 9:1 extract **f)** ethyl acetate extract **g)** 2% sodium hydroxide: ethanol extract **h)** dichloromethane extract

Fig. 4. Comparative TLC analyses of ethanol extracts obtained from different parts (root, leaf, stem, and flower) of *M. longifolia* subsp. *typhoides* samples coded BH9 to BH11 from various localities, in reference to ursolic and oleanolic acid standards

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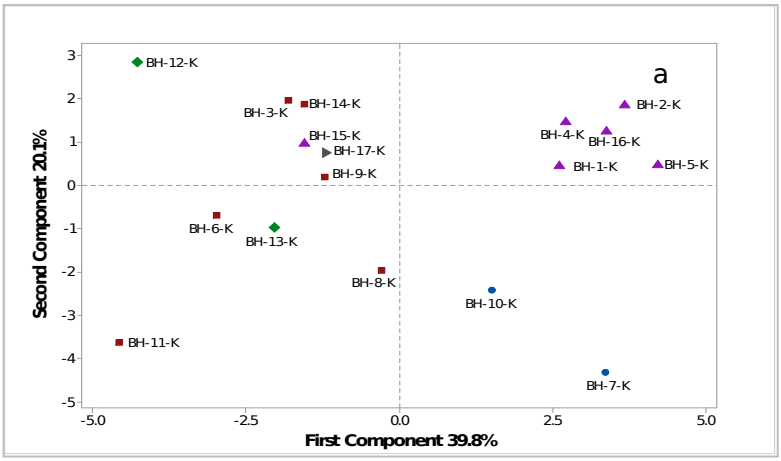

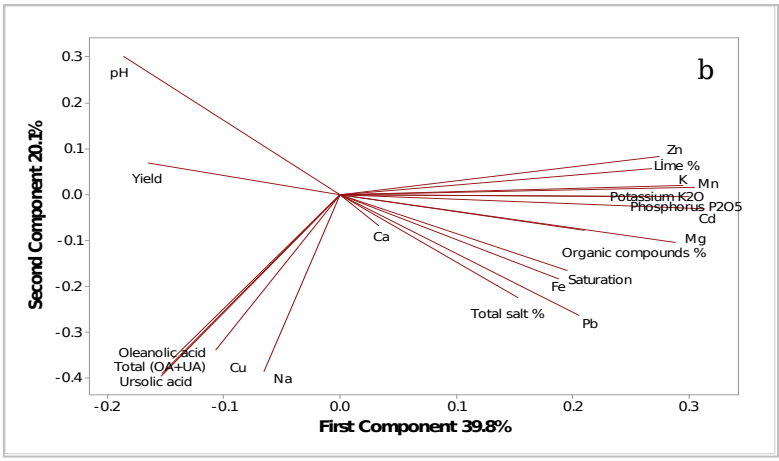

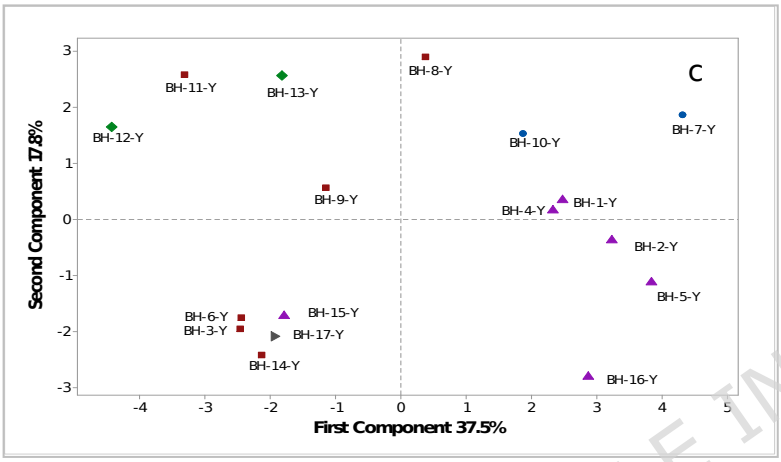

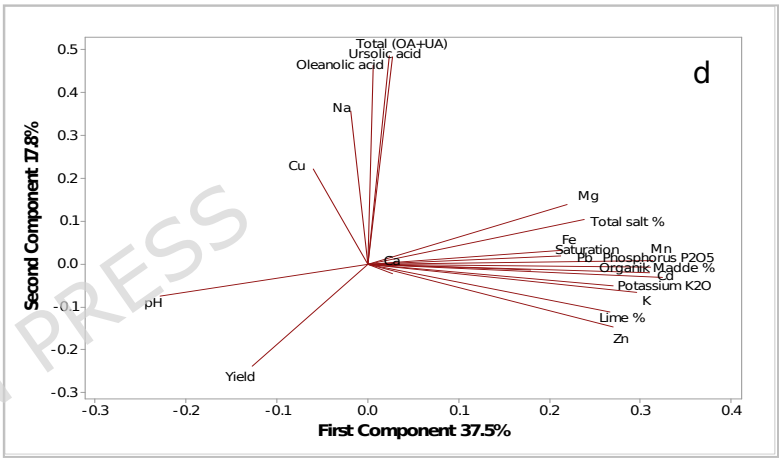

Fig. 5. **a)** Score graph of *M. longifolia* subsp. *typhoides* root samples ▲Mardin, ■Diyarbakır, ◆ Batman, ●Elazığ, ▸ Van **b)** Loading graph for root. **c)** Score graph of *M. longifolia* subsp. *typhoides* leaves samples Mardin, ■Diyarbakır, ◆ Batman, ●Elazığ, ▸ Van **d)** Loading graph for leave

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Table 1. Herbarium number, collection time, and locality information of *M. longifolia* subsp. *typhoides* samples gathered from different region

Code	Collection Locality	GPS coordinates	Collection Date	Collection periods	Herbarium number
BH-1	Türkiye: Mardin, Center, Sultan Şeyhmus locality, riverside, humid site, 650 m	37°27'46.4"N 40°37'07.8"E	11 June 2024	Pre-flowering stage	M.Fırat 40241 (VHLV)
BH-2	Türkiye: Mardin, Nusaybin, Beyaz Su locality, humid site, 513 m	37°08'27.0"N 41°14'25.4"E	11 June 2024	Pre-flowering stage	M.Fırat 40242 (VHLV)
BH-3	Türkiye: Diyarbakır, Silvan, roadside, 35th km, humid site, 840 m	38°09'06.9"N 41°00'19.2"E	12 June 2024	Pre-flowering stage	M.Fırat 40243 (VHLV)
BH-4	Türkiye: Mardin, Merkez, Sultan Şeyhmus locality, riverside, humid site, 650 m	37°27'46.4"N 40°37'07.8"E	09 August 2024 June-August-September-2025	Flowering stage Pre-flowering, Flowering, Post-flowering	M.Fırat 40316 (VHLV)
BH-5	Türkiye: Mardin, Nusaybin, Beyaz Su locality, humid site, 513 m	37°08'27.0"N 41°14'25.4"E	09 August 2024 June-August-September-2025	Flowering stage Pre-flowering, Flowering, Post-flowering	M.Fırat 40317 (VHLV)
BH-6	Türkiye: Diyarbakir, Silvan, roadside, 35th km, humid site, 840 m	38°09'06.9"N 41°00'19.2"E	14 August 2024	Flowering stage	M.Fırat 40320 (VHLV)
BH-7	Türkiye: Batman, Yeni Köprü locality, humid site, 580 m	37°53'16.3"N 41°02'02.9"E	17 August 2024 June-August-September-2025	Flowering stage Pre-flowering, Flowering, Post-flowering	M.Fırat 40326 (VHLV)
BH-8	Türkiye: Diyarbakir, Ergani, roadside, 24th km, humid site, 920 m	38°05'59.5"N 39°44'55.4"E	14 August 2024 June-August-September-2025	Flowering stage Pre-flowering, Flowering, Post-flowering	M.Fırat 40321 (VHLV)
BH-9	Türkiye: Diyarbakir, Bismil, roadside, 24th km, humid site, 630 m	37°50'45.7"N 40°35'51.9"E	14 August 2024	Flowering stage	M.Fırat 40322 (VHLV)
BH-10	Türkiye: Batman, Bismil, Bıçakçılar village, humid site, 630 m	37°50'45.7"N 40°35'51.9"E	17 August 2024	Flowering stage	M.Fırat 40325 (VHLV)
BH-11	Türkiye: Diyarbakir, Eğil, Dam locality, humid site, 860 m	38°15'36.6"N 40°05'22.2"E	14 August 2024	Flowering stage	M.Fırat 40323 (VHLV)
BH-12	Türkiye: Elazig, Maden, roadside, 80th km, humid site, 1100 m	38°23'53.4"N 39°40'38.9"E	18 August 2024	Flowering stage	M.Fırat 40327 (VHLV)
BH-13	Türkiye: Elazig, Sivrice, Keban Dam locality, humid site, 820 m	38°47'04.2"N 38°45'15.3"E	18August 2024	Flowering stage	M.Fırat 40328 (VHLV)
BH-14	Türkiye: Diyarbakir, Kulp, roadside, humid site, 1000 m	38°27'40.7"N 41°02'21.4"E	14 August 2024	Flowering stage	M.Fırat 40324 (VHLV)
BH-15	Türkiye: Mardin, Kızıltepe, Dikmen village locality, humid site, 470 m	37°10'57.2"N 40°34'19.4"E	09 August 2024	Flowering stage	M.Fırat 40318 (VHLV)
BH-16	Türkiye: Mardin, Nusaybin, Avaşin village, humid site, 520 m	37°11'26.3"N 41°17'59.2"E	09 August 2024	Flowering stage	M.Fırat 40319 (VHLV)
BH-17	Türkiye: Vân, Bahçesaray, Köşk village locality, humid site, 1890 m	38°05'37.8"N 42°48'05.8"E	20 August 2024	Flowering stage	M.Fırat 40329 (VHLV)

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Table 2. GC-MS analysis results of *M. longifolia* subsp. *typhoides* samples prepared with different solvents

Botanical Name	Code	Oleanolic acid (OA)	Ursolic acid (UA)	Total (OA+UA)
		(mg component/g extract)		
M. longifolia subsp. *typhoides*	BH-Petroleum ether (BH-PE)	18.60	23.89	42.49
	BH-Dichloromethane (BH-DCM)	76.27	310.89	387.15
	BH-Dichloromethane: acetone: 9:1 (BH-DCM/AC)	69.64	244.46	314.09
	BH-Acetone (BH-AC)	64.54	241.54	306.09
	BH-Ethyl acetate (BH-EA)	63.09	198.30	261.39
	BH-Ethanol (BH-E)	69.84	303.31	373.16
	BH-2% Sodium hydroxide: ethanol (BH-NaOH/E)	32.91	79.04	111.95

Table 3. LC-MS/MS content results of *M. longifolia* subsp. *typhoides* extracts prepared with different solvents

No	Analytes	RT[a]	M.I. (*m/z*)[b]	F.I. (*m/z*)[c]	BH-PE	BH-DCM	BH-DCM/AC	BH-AC	BH-EA	BH-E	BH-NaOH/E
1	Quinic acid	3.0	190.8	93.0	N.D.	0.039	0.033	0.100	0.038	3.887	N.D.
4	Gallic acid	4.4	168.8	79.0	N.D.	N.D.	N.D.	N.D.	N.D.	0.035	N.D.
6	Protocatechuic acid	6.8	152.8	108.0	N.D.	0.032	0.065	0.371	0.274	0.997	N.D.
9	Chlorogenic acid	8.4	353.0	85.0	N.D.	N.D.	N.D.	N.D.	N.D.	0.329	N.D.
10	Protocatechuic aldehyde	8.5	137.2	92.0	0.008	0.084	0.266	0.494	0.312	1.560	N.D.
17	Caffeic acid	12.1	179.0	134.0	N.D.	0.024	0.103	2.495	0.855	6.323	0.024
19	Vanillin	13.9	181.0	151.1	0.157	1.228	0.377	0.52	0.497	0.232	N.D.
24	*p*-Coumaric acid	17.8	163.0	93.0	N.D.	N.D.	N.D.	0.093	0.061	N.D.	N.D.
29	Salicylic acid	21.8	137.2	65.0	N.D.	N.D.	N.D.	0.020	0.012	0.074	0.031
30	Cynaroside	23.7	447.0	284.0	N.D.	0.094	0.046	4.543	0.954	12.727	N.D.
33	Rutin	25.6	608.9	301.0	N.D.	N.D.	N.D.	0.266	N.D.	N.D.	N.D.
34	Isoquercitrin	25.6	463.0	271.0	N.D.	N.D.	N.D.	0.498	0.114	1.206	N.D.
35	Hesperidin	25.8	611.2	449.0	0.005	0.039	0.053	2.907	0.316	5.085	N.D.
38	Rosmarinic acid	26.6	359.0	197.0	N.D.	0.055	N.D.	N.D.	N.D.	67.315	N.D.
40	Cosmosiin	28.2	431.0	269.0	N.D.	N.D.	0.017	0.406	0.113	1.111	N.D.
42	Astragalin	30.4	447.0	255.0	N.D.	N.D.	N.D.	0.108	0.023	0.222	N.D.
43	Nicotiflorin	30.6	592.9	255.0/284.0	N.D.	N.D.	N.D.	0.050	N.D.	0.197	N.D.
47	Quercetin	35.7	301.0	272.9	N.D.	N.D.	0.011	0.041	0.035	0.043	N.D.
48	Naringenin	35.9	270.9	119.0	0.026	6.714	10.059	13.336	9.268	13.643	N.D.
49	Hesperetin	36.7	301.0	136.0/286.0	N.D.	0.509	0.531	0.688	0.432	0.982	N.D.
50	Luteolin	36.7	284.8	151.0/175.0	N.D.	0.030	0.441	0.916	0.558	1.347	N.D.
51	Genistein	36.9	269.0	135.0	N.D.	N.D.	0.003	N.D.	0.003	N.D.	N.D.
53	Apigenin	38.2	268.8	151.0/149.0	N.D.	0.111	0.270	0.156	0.099	0.325	0.050
55	Chrysin	40.5	145.0/119.0	40.5	N.D.	N.D.	N.D.	0.004	0.008	N.D.	N.D.

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56	Acacetin	40.7	239.0	40.7	N.D.	0.020	0.023	0.022	0.018	0.044	N.D.

[a]RT.: Retention time, [b]MI (*m/z):* Molecular ions of the standard analytes (*m/z* ratio), [c]FI (*m/z):* Daughter ions, [d]Not detected, Unidentified compounds: 2: Fumaric asit, 3: Aconitic acid, 5: Epigallocatechin, 7: Catechin, 8: Gentisic acid, 11: Tannic acid, 12: Epigallocatechin gallate, 13: 1,5-dicaffeoylquinic acid, 14: 4-OH Benzoic acid, 15: Epicatechin, 16: Vanilic acid, 18: Syringic acid, 20: Syringic aldehyde, 21: Daidzin, 22: Epicatechin gallate, 23: Piceid, 25: Ferulic acid-D3-IS, 26: Ferulic acid, 27: Sinapic acid, 28: Coumarin, 31: Miquelianin, 32: Rutin-D3-IS, 36:*o*-Coumaric acid, 37: Genistin, 39: Ellagic acid, 41: Quercitrin, 44: Fisetin, 45: Daidzein, 46: Quercetin-D3-IS, 52: Kaempferol 54: Amentoflavone. BH-PE: Petroleum ether extract, BH-DCM: dichloromethane extract, BH-DCM/AC: dichloromethane: acetone: 9:1 extract, BH-AC: acetone extract, BH-EA: ethyl acetate extract, BH-E: ethanol extract, BH-NaOH/E: 2% Sodium hydroxide: ethanol extract

Table 4. GC-MS content results of ethanol extracts obtained from the aerial parts and roots of *M. longifolia* subsp. *typhoides* samples coded BH4, BH5, BH7 and BH8, collected at different vegetative stages

Botanical Name	Code	Oleanolic acid (OA)	Ursolic acid (UA)	Total (OA+UA)
		(mg component/g extract)		
M. longifolia subsp. *typhoides*	BH-4-AP/PreF	20.65	76.39	97.04
	BH-4-AP/F	41.28	161.34	202.63
	BH-4-AP/PostF	34.71	127.72	162.43
	BH-4-R/PreF	10.37	7.03	17.40
	BH-4-R/F	11.71	7.94	19.66
	BH-4-R/PostF	9.43	7.16	16.60
	BH-5-AP/PreF	37.81	165.56	203.37
	BH-5-AP/F	33.27	127.66	160.93
	BH-5-AP/PostF	24.03	77.75	101.78
	BH-5-R/PreF	23.66	9.99	33.66
	BH-5-R/F	15.31	9.62	24.93
	BH-5-R/PostF	16.60	10.99	27.60
	BH-7-AP/PreF	32.74	150.24	182.98
	BH-7-AP/F	36.22	153.38	189.59
	BH-7-AP/PostF	43.13	172.25	215.38
	BH-7-R/PreF	16.93	11.05	27.99
	BH-7-R/F	47.00	22.93	69.93
	BH-7-RPostF	28.78	14.51	43.30
	BH-8-AP/PreF	35.12	136.96	172.08
	BH-8-AP/F	26.26	108.89	135.14
	BH-8-AP/PostF	32.74	134.15	166.88
	BH-8-R/PreF	26.40	12.61	39.02
	BH-8-R/F	35.83	15.73	51.56
	BH-8-RPostF	16.36	8.64	25.01

AP=Aerial parts; R=Root; PreF=Pre-flowering; F=Flowering; PostF=Post-flowering

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