



Altitude Dependent Flavonoid Glycoside Profiles of Iranian *Morus nigra* L. Leaves Analyzed by UPLC-DAD-QTOF/MS

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Abstract

Morus nigra L. (Moraceae) is one of Iran's notable native species, valued for its medicinal and nutritional properties, particularly in its foliage. The species is widely distributed across Iran, from arid lowlands to high-altitude regions, where environmental conditions can influence plant metabolite composition. In this study, leaves from 15 Iranian *M. nigra* varieties were analyzed to investigate their flavonoid profiles and the effect of altitude on metabolite accumulation. Ultrahigh-performance liquid chromatography coupled with diode-array detection and quadrupole time-of-flight mass spectrometry (UPLC-DAD-QTOF/MS) was employed for precise qualitative and quantitative analysis. Seven flavonoids were identified, including isoquercitrin, kaempferol methyl ether glucuronide, quercetin, and rutin, confirming the reliability of the analytical method. Quantitative analysis revealed that flavonoid accumulation increased with altitude, with samples from the highest elevation exhibiting the greatest total flavonol content (1026.1 ± 79.4 mg/g). These findings highlight that geographical factors, particularly altitude, play a significant role in determining flavonoid content in *M. nigra* leaves. The study provides new insights into the distribution of bioactive flavonoids in mulberry leaves and underscores their potential as stable, industrially valuable ingredients with antioxidant and functional properties. The results also offer guidance for optimizing cultivation and harvesting strategies to maximize flavonoid yield, supporting the development of mulberry leaf extracts for food, pharmaceutical, and cosmetic applications.

Keywords: Altitude effect; Bioactive compounds; Flavonoid glycosides; *Morus nigra* L.; UPLC-DAD-QTOF/MS

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1. Introduction

Morus nigra L. is a deciduous tree belonging to the genus *Morus* in the family Moraceae, which comprises approximately 10 to 16 species and is widely distributed in tropical, subtropical, and temperate regions of the world

[1]. It is also considered one of the native species of Iran and is widely recognized for its medicinal and nutritional properties, particularly those associated with its leaves [2]. The medicinal use of mulberry is attributed to its variety of health-promoting properties, such as antihyperglycemic, anti-allergic, and immunomodulatory

activities [3,4,5]. Mulberry (*Morus* spp.) is recognized as a valuable source of functional food ingredients due to its diverse profile of secondary metabolites and bioactive compounds, including flavonoids (anthocyanins, rutin, quercetin, and isoquercetin), steroids, amino acids, polysaccharides, γ -aminobutyric acid (GABA), various vitamins, and 1-deoxynojirimycin (DNJ) [6,7,8,9]. Flavonoids, one of the major groups of polyphenols, are widely distributed in fruits, vegetables, and medicinal plants, where they play central roles in plant defense, antioxidant activity, and the modulation of biological processes associated with oxidative stress [10]. The genus *Morus* has been reported to be particularly rich in flavonoids. Quercetin-3-(6-malonylglucoside), rutin, and isoquercetin are among the predominant flavonoids identified in its tissues [11,12]. In addition, anthocyanins such as cyanidin-3-rutinoside and cyanidin-3-glucoside have been detected in both the leaves and fruits of various species [13]. These compounds exhibit considerable antioxidant potential and have been associated with a reduced risk of disorders linked to oxidative stress [13]. Extensive structural and analytical studies have been carried out on the flavonoid composition of mulberry leaves across multiple species and cultivars. Using high-performance liquid chromatography (HPLC), Lee et al. [14] and Wang et al. [8] characterized the molecular profiles of leaf flavonoids. Katsube et al. [11] further demonstrated that quercetin-3-(6-malonylglucoside) is the most abundant flavonoid in dried *Morus* leaves. In a comprehensive investigation, Tabti et al. [15] employed HPLC-DAD and HPLC-MS to identify and quantify a broad range of phenolic acids and flavonol glycosides in *M. alba* var. *alba*, *M. alba* var. *rosa*, and *M. rubra*. Their work provided the first report of kaempferol-7-O-glucoside, quercetin-3-O- β -glucoside-7-O- α -rhamnoside, and quercetin-3-O-rhamnoside-7-O-glucoside in mulberry leaves. Subsequently, Tabti et al. [15] reported that *M. rubra* exhibits the highest total flavonoid content among the species studied. Despite these advances, the flavonoid profile of several economically relevant yet less explored mulberry species such as *M. nigra* remains incompletely characterized, and the precise composition of their secondary metabolites is still poorly understood. Moreover, genetic improvement strategies, including interspecific hybridization to combine desirable traits particularly those related to enhanced vegetative performance and improved leaf quality remain long-term priorities in global mulberry breeding programs [9]. The flavonoid profiles of 15 mulberry species collected from diverse regions of Iran at varying altitudes were analyzed to assess the influence of elevation on flavonoid composition. Identification and characterization were performed using ultra-performance liquid chromatography coupled with diode-array detection and

quadrupole time-of-flight mass spectrometry (UPLC–DAD–QTOF/MS). Because ultraviolet (UV) radiation increases with altitude and poses a threat to plant tissues, high-altitude plants often develop enhanced protective mechanisms. Flavonoids, which absorb UV radiation and mitigate UV-induced reactive oxygen species, are key components of these protective systems [16]. Based on this, the levels of the flavonoids kaempferol and quercetin, which play important roles in health, beauty, and scientific research due to their antioxidant and anti-inflammatory properties, were systematically investigated across different altitudes in Iran in this study. This research provides fundamental data to inform mulberry breeding programs and facilitates chemical applications as well as practical utilization. The significance of this research lies in the fact that to the best of our knowledge, the effect of altitude on flavonoid content in *Morus nigra* L. has not previously been reported.

2. Experimental

2.1. Material

2.1.1. Leaves collection

Fresh leaves of the plant were collected during its fruiting season from 15 different regions across Iran. Table 1 and Fig. 1 present the precise collection sites and their corresponding elevations. Collected leaf samples were washed with distilled water and allowed to dry at ambient room temperature. Upon reaching constant weight, the leaves were pulverized into a fine powder for further analytical procedures.

2.1.2. Samples preparation and extraction

2.1.2.1. Determination of extraction yield

Finely ground dried plant material (2.00 g) was extracted with 20 mL of methanol using an ultrasonic cell crusher (Biosync Technology) for 15 min at 35 °C. The extract was filtered through Whatman No. 42 filter paper, and the filtrate was evaporated to dryness in an oven at 40 °C until constant weight. The extraction yield was calculated using the following equation:

$$\text{Extraction yield (\%)} = (\text{Weight of dried extract} / \text{Initial dry weight of plant material}) \times 100 \text{ [18].}$$

All extractions were performed in triplicate.

2.1.2.2. Preparation of Leaf Extract for Flavonoid Analysis

A separate extraction procedure was used for flavonoid analysis.

Table 1. The experimental material of 15 *Morus nigra* L

NO.	Sample	Locality	Latitude	Longitude	Altitude (m)	Yield (%)
1	Alborz	Purkan village	35°52'57.3"N, 51°02'24.0"E		1494	20.8
2	Bushehr	Dashtestan, Doroudgah	29°35'68"N, 51°10'87" E		35	23.9
3	Fars	Shiraz, Maharlo lake	29°27'09.3"N, 52°43'26.9"E		146	12.7
4	Golestan	Estunabad	36°42'44.1"N, 53°56'28.2"E		122	23.2
5	Hamedan	Tuyskeran, Serkan, Ellu waterfall	34°37'06.2"N, 48°28'59.7"E		2581	17.3
6	Hormozgan	Bandar Abbas, Geno	27°24'24.2"N, 56°14'53.8"E		50	24.4
7	Kurdistan	Sanandaj, Sarab Quamish	35°26'14.0"N, 46°58'35.6"E		1548	18.4
8	Mazandaran	Amol, Nava	35°51'16.5"N, 52°12'40.3"E		2282	16.8
9	Qom	Golestan village	34°39'10.3"N, 50°28'49.2"E		1199	22.7
10	Qazvin	Traditional Garden	36°14'44.9"N, 50°00'02.4"E		1280	19.7
11	Semnan	Bastam	36°29'08.1"N, 54°58'27.3"E		1408	18.9
12	Sistan and Baluchestan	Zahaedan, Lar river	29°40'25.3"N, 60°54'10.0"E		1195	21.6
13	South Khorasan	Qayen	33°41'16.4"N, 59°11'40.6"E		1514	22
14	Tehran	Tajrish, Baghe Angiri	35°49'00.9"N, 51°26'17.4"E		1697	19.1
15	Yazd	Chak chak	32°22'26.1"N, 54°24'00.6"E		1776	15.76

Details of the diverse geographical areas and their geographic coordinates from which the 15 *M.nigra* L. samples were gathered for further analysis and investigation in this study.



Figure 1. Leaves of 15 samples were collected from their natural habitats along the five different natures of Iran (North, west, east, south and center) [17]. The red marker pins (📍) on the map indicate the sites from which the plant specimens were obtained for analysis; Tehran, Qazvin, Golestan, kurdistan, Fars, Alborz, Hamedan, Qom, Semnan, Mazandaran, Yazd, South Khorasan, Hormozgan, Bushehr, Sistan & Baluchestan

Briefly, 1.00 g of powdered dried leaf material was homogenized with 10 mL of methanol: water: formic acid (50:45:5, v/v/v) containing galangin (100 ppm) as an internal standard. The mixture was vortexed for 1 min and shaken on an orbital shaker at 200 rpm for 5 min. The extract was centrifuged at $2000 \times g$ for 15 min at 10 °C. The supernatant was collected and filtered through a 0.45 µm PTFE syringe filter (Whatman, Maidstone, UK). An aliquot (0.5 mL) of the filtrate was diluted with distilled water to a final volume of 5 mL prior to solid-phase extraction (SPE). For purification, a Sep-Pak C18 cartridge (Waters, Milford, MA, USA) was preconditioned sequentially with 2 mL methanol and 2 mL water. The diluted extract was loaded onto the cartridge, and non-retained compounds were removed by washing with 2 mL water. The retained flavonoids were eluted with 3 mL methanol. The eluate was evaporated to dryness under a gentle nitrogen stream and reconstituted in 0.5 mL of methanol: water: formic acid (50:45:5, v/v/v) without internal standard prior to instrumental analysis. Each experimental sample was prepared and analyzed in triplicate.

2.2. Method

2.2.1. Analysis Conditions for UPLC-DAD-QTOF/MS

An ultra-high-performance liquid chromatography (UPLC) system equipped with a diode array detector (DAD) set at 280 and 320 nm, based on the method described by Ogidigo et al. [20], and modified for UPLC-DAD-QTOF/MS analysis, was employed for the chemical profiling of the samples. UV-Vis spectra were acquired over the range of 210–600 nm.

Chromatographic separation was achieved under the following conditions: A Luna Omega C18 column (1.6 µm, 150 × 2.1 mm; Phenomenex, USA) coupled with a Security Guard ULTRA UHPLC C18 guard cartridge (2.1 mm i.d.; Phenomenex) was used. The mobile phases consisted of (A) water with 0.5% formic acid and (B) acetonitrile with 0.5% formic acid. The flow rate was maintained at 0.3 mL/min, and the column temperature was set to 30°C. The injection volume was 5 µL, and the total run time was 60 min. The gradient elution program for solvent B was as follows: 7% (0–2 min), 15% (2–4 min), 30% (4–10 min), 60% (10–15 min), 90% (15–20 min), and re-equilibration to 7% (20–30 min) [21] with slight modification.

Mass spectrometric detection (QTOF/MS) was performed using positive electrospray ionization (ESI⁺) under the following conditions: sampling cone voltage 40 V, ion source temperature 120°C, desolvation temperature 400°C, desolvation gas flow 1000 L/h, cone gas flow 30

L/h, and capillary voltage 3500 V. Data were collected over an m/z range of 50–800 [22] and with minor adjustments for QTOF/MS.

3. Result and discussion

3.1. Identification and Extraction of Flavonoid Constituents from *M. nigra* L. Leaves

The analysis of 15 leaves from various Iranian *M. nigra* mulberry varieties using UPLC-DAD-QTOF/MS led to the identification of seven flavonoid compounds, four of which are natural flavonoids, including Kaempferol, and three plant pigments, notably Quercetin. The UPLC-DAD-QTOF/MS chromatograms for the primary flavonols detected in SIS. and FAR. are presented in Fig. 2. Through this analysis, seven flavonol compounds, including derivatives of quercetin and kaempferol, were tentatively identified in positive ionization mode ([M+H]⁺). Compound identification was based on factors such as retention time (t_R), accurate molecular mass, molecular formula, and characteristic MS/MS fragmentation patterns (as detailed in Table 2). All detected flavonols exhibited diagnostic aglycone fragment ions at m/z 303 (quercetin) and m/z 287 (kaempferol), which are well-recognized markers for flavonol identification in LC-MS studies [23,24]. The observed neutral losses of 162 m/z and 308 m/z corresponded to the cleavage of glucose and rutinose moieties, respectively, confirming the presence of mono- and diglycosylated flavonols. These fragmentation behaviors are consistent with previously reported UPLC-QTOF/MS analyses of flavonoid glycosides in plant extracts [25,26]. Among the quercetin derivatives, quercetin 3,7-di-O-glucoside, quercetin 3-O-rutinoside (rutin), and quercetin 3-O-glucoside (isoquercitrin) were identified. Kaempferol derivatives included kaempferol 3-O-glucoside (astragalol), kaempferol 3-O-rutinoside (nicotiflorin), kaempferol 3,7-di-O-glucoside, and kaempferol 3-O-(6''-O-malonyl) glucoside. The detection of a malonylated flavonol was confirmed by its characteristic mass increase and fragment ions, in agreement with reported data for acylated flavonoids [22,27].

3.2. Structural Classification of Identified Flavonols

Based on aglycone type, degree of glycosylation, and acylation pattern, the identified compounds were categorized into mono-glycosides, di-glycosides, and acylated glycosides (Table 3). Only quercetin and kaempferol were detected as aglycone cores, underscoring their prominence in the flavonol biosynthetic pathway of the analyzed samples. Glycosylation is known to enhance

flavonoid stability, solubility, and cellular storage, whereas acylation such as malonylation plays a role in metabolic regulation and intracellular sequestration [28]. The observation of similar distribution patterns of

quercetin and kaempferol glycosides across various plant matrices further supports the reliability of the identification method employed in this study [29, 30].

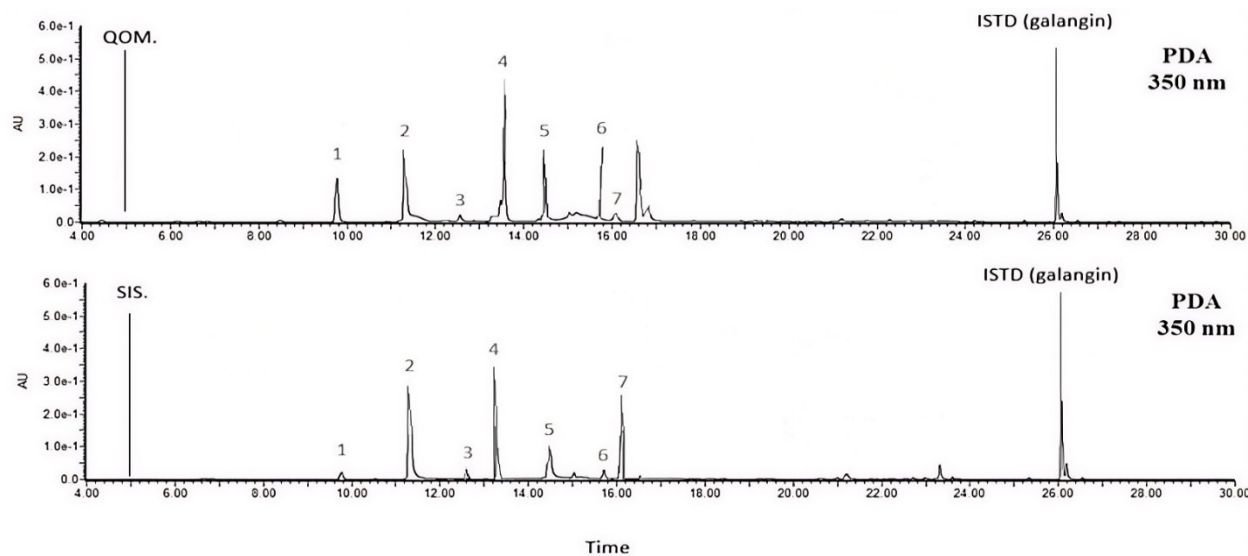


Figure 2. Liquid chromatography (LC) chromatograms of flavonoids detected at 350 nm using a photodiode array (PDA) detector in *Morus nigra* L. samples. Chromatograms correspond to the cultivars QOM. (top) and SIS. (bottom). Peaks are numbered according to identified flavonoid compounds, Galangin was used as the internal standard (ISTD)

Table 2. Flavonoids identified of the 15 Iranian *Morus nigra* L. (black mulberry) specimens leaf extracts using UPLC-DAD-QTOF/MS

P No.	t_R	MS	MS/MS	Molecular Weight	Molecular Formula	Identification
1	9.152	627.5	649,627,465,303	626.52	$C_{27}H_{30}O_{17}$	Quercetin 3,7-di-O-glucoside
2	11.628	609	633,611,465,449,303	610.52	$C_{27}H_{30}O_{16}$	Quercetin 3-O-rutinoside (rutin)
3	12.314	593.2	633,611,449,287	610.52	$C_{27}H_{30}O_{15}$	Kaempferol 3,7-di-O-glucoside
4	13.628	465	487,465,303	464.38	$C_{21}H_{20}O_{12}$	Quercetin 3-O-glucoside (isoquercitrin)
5	14.314	593.2	617,595,449,287	594.52	$C_{17}H_{30}O_{15}$	Kaempferol 3-O-rutinoside(nicotiflorin)
6	15.910	447.2	471,449,287	448.38	$C_{21}H_{20}O_{11}$	Kaempferol 3-O-glucoside (astragaline)
7	16.141	533	557,535,287	534.43	$C_{24}H_{22}O_{14}$	Kaempferol 3-O-(6''-O-malonyl) glucoside

All samples were analyzed in positive ion mode ($[M+H]^+$) using UPLC-DAD-QTOF/MS. Quantitative values are presented as the mean \pm SD of three replicates, with galangin used as the internal standard.

*P No. = Peak Number

Table 3. 7 flavonoids were isolated from the leaves of 15 Iranian *Morus nigra* L. (black mulberry) specimens, and their masses were determined

Aglycones	Glycosides	Acylation	Peak Number	Individual flavonols	MW	Fragment ions (m/z)
Kaempferol (m/z 287)	Mono	Mal	7	Kaempferol 3-O-(6''-O-malonyl) glucoside	534.43	557, 535, 287
			6	Kaempferol 3-O-glucoside (astragaline)	448.38	471, 449, 287
	Di	Mal	5	Kaempferol 3-O-rutinoside (nicotiflorin)	594.52	617, 595, 449, 287
			3	Kaempferol 3,7-di-O-glucoside	610.52	633, 611, 449, 287
Quercetin (m/z 303)	Mono	Di	4	Quercetin 3-O-glucoside(isoquercitrin)	464.38	487, 465, 303
			2	Quercetin 3-O-rutinoside (rutin)	610.52	633, 611, 465, 449,303
	1	Quercetin 3,7-di-O-glucoside	626.52	649, 627, 465, 303		

All samples were analyzed in positive ion mode ($[M+H]^+$) using UPLC-DAD-QTOF/MS

3.3. Quantitative Distribution Across Sampling Sites and Effect of Altitude on Flavonoid Content

Quantitative analysis revealed pronounced spatial variability in total flavonol content among the investigated sites (Table 4). HAM. showed the highest total flavonol concentration (1026.1 ± 79.4), followed by MAZ. (754.9 ± 70.9) and YAZ. (460.3 ± 65.1). These sites consistently exhibited elevated values across multiple sampling points (PN1–PN7), indicating enhanced accumulation of flavonol glycosides. In contrast, BUS., GOL., and HOR. displayed flavonol levels below the limit of detection (LOD) at most sampling points, suggesting either minimal biosynthesis or unfavorable conditions for flavonoid accumulation. Sites such as QOM., SIS., and QAZ. contained comparatively low concentrations, which may reflect differences in environmental stress, metabolic activity, or degradation processes. Comparable variability has been reported in previous studies, where flavonol accumulation was strongly influenced by environmental and physiological factors such as light intensity, temperature, and soil conditions [31,32]. The dominance of glycosylated flavonols across all detectable sites corroborates results reported in earlier UPLC-based investigations of plant secondary metabolites. [33]. The relationship between altitude and total flavonoid content of the samples is presented in Fig. 3. As shown, total flavonoid content (mg/100 g dry weight) increased with increasing altitude. Linear regression analysis revealed a positive correlation between altitude and total flavonoid content, indicating that samples collected from higher altitudes tended to contain higher levels of flavonoids. This trend suggests that altitude plays an important role in influencing flavonoid accumulation in the studied samples. The observed correlation was statistically significant ($p < 0.05$), confirming the effect of altitude on total flavonoid content. The quantitative distribution of total flavonoid content across the sampling sites showed a clear dependence on altitude. As illustrated in Fig. 4, total flavonoid content (mg/100 g dry weight) increased progressively with increasing altitude, indicating that samples collected from higher altitudes accumulated greater amounts of flavonoids. This trend suggests that altitude-related environmental factors may enhance flavonoid biosynthesis or accumulation. The positive relationship between altitude and total flavonoid content was further supported by linear regression analysis, confirming altitude as an important factor influencing flavonoid levels across the sampling sites. In addition, principal component analysis (PCA) provided complementary insight into the variation in phenolic composition. The PCA score plot revealed a structured distribution of samples corresponding to differences among sampling sites, while the loading plot indicated

that several phenolic compounds contributed strongly to this differentiation. Taken together, the univariate and multivariate analyses demonstrate that altitude affects not only the total flavonoid content but also the overall phenolic profile of the samples, highlighting its key role in shaping the quantitative distribution of flavonoids across different sampling sites. As described above, elevation significantly influences flavonoid accumulation in plant leaves, with higher altitudes promoting increased flavonoid content and variability in response to environmental stress conditions such as enhanced UV radiation [34]. In addition, lower temperatures at elevated sites stimulate antioxidant pathways and the expression of key genes involved in flavonoid biosynthesis, leading to the production of protective secondary metabolites [35]. These observations further suggest that the consistency of our results may be attributed to altitude driven physiological and molecular responses in plants.

3.4 Industrial-Relevant Profiling of Quercetin and Kaempferol Glycosides Using UPLC-DAD-QTOF/MS

The present investigation confirms earlier LC–MS-based studies identifying quercetin and kaempferol glycosides as the predominant flavonols in plant-derived extracts [36,37,38]. The strong agreement between the flavonol profiles obtained in this work and those previously reported demonstrates the reliability and robustness of UPLC-DAD-QTOF/MS as an analytical platform for industrially relevant phytochemical characterization. From an industrial chemistry perspective, the dominance of glycosylated flavonols is of particular importance. Glycosylation significantly improves the physicochemical properties of flavonols, including aqueous solubility, thermal stability, and resistance to oxidative and hydrolytic degradation during processing and storage [39]. These characteristics enhance process compatibility and broaden the applicability of flavonol-rich extracts in food, pharmaceutical, cosmetic, and nutraceutical formulations. The identification of major flavonol glycosides such as rutin and astragalol further supports the industrial value of the investigated extracts. Given their well-documented antioxidant activity and functional properties, these compounds represent valuable bioactive ingredients for commercial applications [40]. Due to their renewable supply of phytochemicals, plants are valuable resources for industrial applications [41,42]; therefore, their prevalence suggests that the extracts analyzed in this study may serve as suitable natural sources of stable flavonoid-based ingredients. Quantitative analysis revealed noticeable site-dependent variations in flavonol content, indicating that environmental and geographical factors may influence

flavonol accumulation. From an industrial standpoint, such variability highlights the importance of raw material selection, sourcing strategy, and standardization to ensure consistent product quality. The consistency of MS/MS fragmentation patterns with literature data confirms reliable compound identification, while the observed quantitative differences provide valuable insight for optimizing cultivation and extraction strategies. Overall,

the combined qualitative and quantitative findings demonstrate that the analyzed extracts are characterized by a flavonol profile dominated by quercetin and kaempferol glycosides with favorable industrial attributes. These results support the potential utilization of flavonol-rich plant extracts as stable, functional ingredients and provide a scientific basis for their further development in industrial chemistry applications.

Table 4. Content of 7 flavonoids were isolated from the leaves of 15 Iranian *Morus nigra* L. (black mulberry). (Values are expressed as mg/100 g dry weight)

Samples	PN1	PN2	PN3	PN4	PN5	PN6	PN7	Total
1. ALB.	12.4±0.9	18.1±1.8	13.3±0.6	15.1±1.5	14.4±1.3	12.4±10.0	9.5±0.7	95.2±16.8
2. BUS.	ND	ND	ND	ND	ND	ND	ND	<LOD
3. FAR.	ND	0.3±0.0	ND	0.3±0.0	ND	0.5±0.1	ND	1.1±0.01
4. GOL.	ND	ND	ND	ND	ND	ND	0.5±0.0	0.5±0.0
5. HAM.	203.2±13.1	120.8±8.8	122.3±5.5	146.2±16.1	118.1±5.9	207±17.6	108.5±12.4	1026.1±79.4
6. HOR.	ND	ND	ND	ND	0.3±0.0	ND	ND	0.3±0.0
7. KUR.	23.3±2.0	45.7±1.3	22.5±1.0	21.7±0.7	31.6±0.1	16.7±0.7	25.8±1.7	187 ±7.5
8. MAZ	102.3±12.5	121.5±7.0	132.4±21.3	112.9±12.8	95.1±7.5	101.2±6.2	89.5±3.6	754.9±70.9
9. QAZ.	7.7±0.5	6.3±0.7	4.9±0.2	5.3±0.2	8.7±0.9	7.9±0.6	6.4±0.5	47.2±3.6
10. QOM.	1.4±0.3	2.2±0.1	0.8±0.0	4.3±0.2	1.7±0.1	4.5±0.2	0.8±0.1	15.7±1.0
11. SEM.	14.1±1.3	11.4±0.8	12.3±1.7	19.1±1.9	11.3±0.8	10.4±0.6	16.3±1.8	94.9±8.9
12. SIS.	0.5±0.1	2.3±0.1	0.7±0.1	3.0±0.4	1.2±0.1	0.3±0.0	2.1±0.1	10.1±0.9.0
13. SOU.	18.6±2.1	21.8±0.7	19.0±0.9	23.1±1.1	37.0±1.6	24.1±3.2	24.0±1.1	167.6±10.7
14. THE.	65.1±3.1	70.9±0.5	26.0±2.1	45.6±2.1	76.8±4.5	64.6±7.0	32.9±9.8	381.9±29.1
15. YAZ.	90.9±12.7	71.1±9.4	30.9±9.8	63.7±9.1	38.7±10.1	72.0±6.4	93.0±7.6	460.3±65.1

*Abbreviations: Alborz=ALB, Bushehr=BUS, Fars=FAR, Golestan=GOL, Hamedan=HAM, Hormozgan=HOR, kurdistan=KUR, Mazandaran=MAZ, Qom=Qom, Qazvin= QAZ, Semnan=SEM Sistan & Baluchestan=SIS, South Khorasan=SOU, Tehran= TEH, Yazd=YAZ, PN=Peak Number, ND= Not Detected. Samples were subjected to UPLC-DAD-QTOF/MS analysis in positive ion mode ($[M+H]^+$). Reported values correspond to the mean \pm SD of triplicate measurements, employing galangin as the internal standard. Peak1: Quercetin 3,7-di-O-glucoside, Peak2: Quercetin 3-O-rutinoside(rutin), Peak3: Kaempferol 3,7-di-O-glucoside, Peak4: Quercetin 3-O-glucoside(isoquercitrin), Peak5: Kaempferol 3-O-rutinoside(nicotiflorin), Peak6: Kaempferol 3-O-glucoside(astragalinal), Peak7: Kaempferol 3-O-(6"-O-malonyl).

Relationship between altitude and total flavonoid content

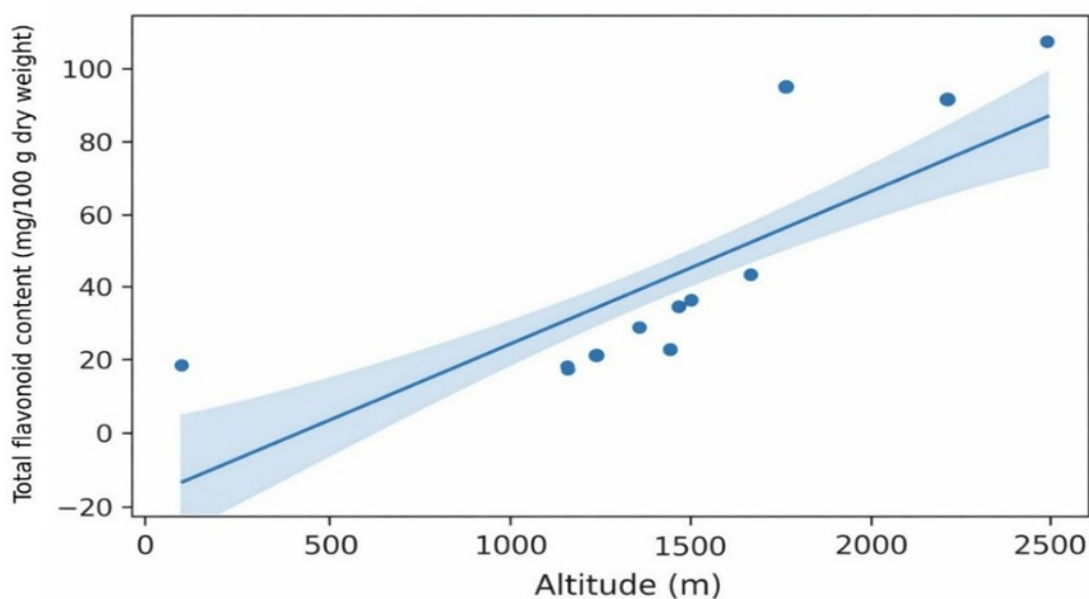


Figure 3. Relationship between altitude and total flavonoid content (mg/100 g dry weight) of the samples. A positive linear correlation was observed ($p < 0.05$)

Principal component analysis (PCA) of phenolic compounds

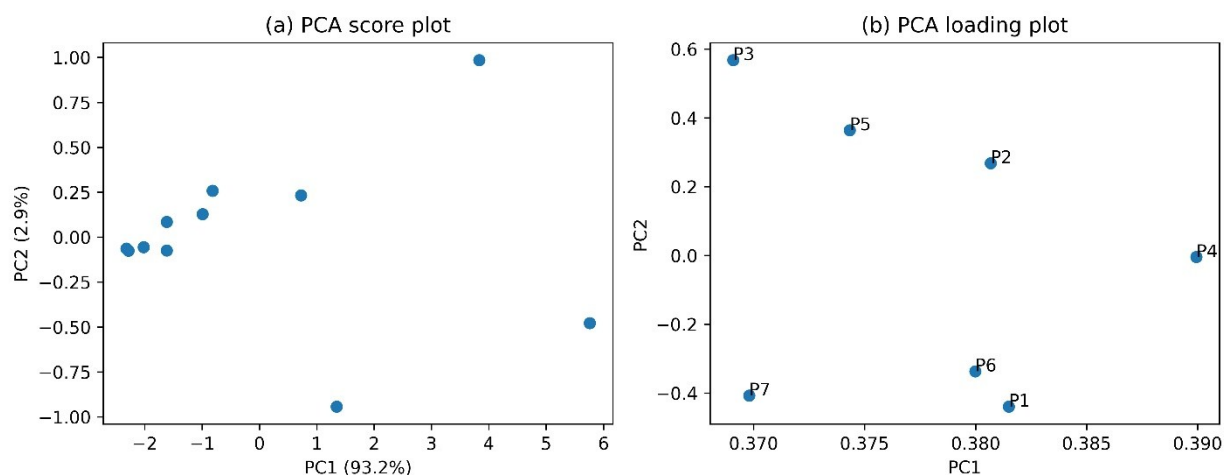


Figure 4. Principal component analysis (PCA) of phenolic compounds in the samples: (a) score plot showing the distribution of samples based on PC1 and PC2, and (b) loading plot illustrating the contribution of individual phenolic compounds to the principal components

4. Conclusion

Flavonoid richness: Iranian *Morus nigra* L. leaves are rich in flavonoid glycosides, mainly quercetin and kaempferol derivatives, consistent with previous UPLC–HR–ESI–MS/MS studies.

Analytical reliability: UPLC-DAD-QTOF/MS enabled precise identification of seven flavonoids, including isoquercitrin, kaempferol methyl ether glucuronide, quercetin, and rutin.

Altitude effect: The results indicate that altitude is a decisive factor in flavonoid accumulation, with higher elevations promoting increased total flavonoid content as well as significantly influencing the overall phenolic composition of the samples.

Industrial potential: *M. nigra* L. leaf extracts are bioactive and stable ingredients suitable for industrial applications.

Cultivation considerations: Optimization of flavonoid yield depends on environmental and cultivation conditions, highlighting the importance of raw material sourcing.

Authors Contribution

All the authors have participated sufficiently in the intellectual content, conception and design of this work or the analysis and interpretation of the data (when applicable), as well as the writing of the manuscript.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Conflict of interests

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

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