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RESEARCH PAPERS

Ecophysiological and Phytochemical Insights in to *Silybum marianum*: Geographic Variations in Antioxidant Activity and Silybin Content

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Abstract—*Silybum marianum* (L.) Gaertner, commonly known as Milk Thistle, is a medicinal plant of the Asteraceae family, valued for its bioactive flavonolignans, particularly silybin (A + B). Despite its widespread distribution, limited research has explored the impact of diverse climatic conditions on its phytochemical properties especially on wild populations that evolved under diverse climatic conditions. This study examined the effects of climatic parameters on the phytochemical composition of *S. marianum* seeds collected from various geographical regions. The findings revealed substantial variation in silybin (A + B) content among wild populations. Seeds from Gorgan, characterized by high soil temperatures and moderate rainfall, exhibited the highest silybin content (0.06 mg/g dry weight), whereas Ardabil seed population, with its cold climate and high altitude, had the lowest content (0.018 mg/g dry weight). In contrast, phenolic and flavonoid compounds and antioxidant activity were highest in Ardabil seeds population, indicating enhanced biosynthesis of secondary metabolites in response to cooler temperatures and higher altitudes. Fatty acid composition also varied, with Ardabil seeds population containing more saturated fatty acids and Gorgan seeds population showing a higher proportion of unsaturated fatty acids. Additionally, drought stress significantly increased silybin content (A + B) while reducing phenol and flavonoid levels, highlighting an adaptive response to environmental stressors. This study underscores the significant influence of climatic factors on the phytochemical profiles of *S. marianum*. These insights are crucial for optimizing its cultivation and maximizing its medicinal potential.

Keywords: *Silybum marianum*, phenolic content, silymarin, HPLC

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INTRODUCTION

The hepatoprotective properties of milk thistle (*S. marianum*) seeds are primarily attributed to the presence of flavonolignans, particularly silymarin (SM), which has been utilized for over 2000 years [1]. Among the plant's organs, seeds exhibit the highest concentrations of SM, with its biosynthesis closely associated with specific growth stages. Silymarin is a complex mixture of flavonolignans, including silybin A and B, isosilybin A and B, silydianin, silychristin, isosilychristin, and the isoflavonoid taxifolin [2]. Silybin, the most bioactive component of SM, has demonstrated therapeutic potential in liver detoxification, skin protection, cardiovascular health, diabetes management, anticancer activity, and cholesterol regulation. It enhances liver regeneration by activating RNA polymerase in hepatocyte nuclei, thereby promoting ribosomal protein synthesis [3].

Abiotic stressors significantly influence the production and accumulation of secondary metabolites in plants, as they affect biochemical and physiological pathways [4]. Seed coat is the main site of silymarin synthesis and significantly influenced by environmental conditions [5]. The results of previous studies confirm that, the content of silybin (as the main compound of SM) in natural conditions was much greater than plants grown in greenhouse. The highest concentration of SM typically found in moist soil conditions with approximately 60–65% humidity [6], and biosynthesis of SM in plants exposure to drought and water logging stresses, significantly increased [5].

Climatic variations across different regions influence the synthesis of secondary metabolites in plants. Environmental parameters such as temperature, humidity, altitude, and solar radiation significantly affect the uptake, synthesis, and accumulation of

these compounds. The same species cultivated in diverse climatic zones often exhibit variability in secondary metabolite concentrations [7]. For example, changes in rainfall patterns have profound impacts on pasture conditions, which in turn influence plant growth and metabolite production. Unlike motile organisms, plants must adapt to environmental fluctuations, making them particularly vulnerable to climate change [8]. Temperature, a critical environmental factor, exerts multifaceted effects on metabolic processes. Low temperatures induce stress responses in plants, leading to the accumulation of osmoprotectants such as sugar alcohols and soluble sugars. Similarly, altitude-related stresses, including reduced temperatures and elevated UV radiation, enhance the production of phenolic and flavonoid compounds. Gupta et al. [9] reported increased phenolic and flavonoid content with decreasing temperatures and increasing altitudes. Additionally, UV stress at higher elevations promotes the synthesis of secondary metabolites like alkaloids, anthocyanins, carotenoids, phytosterols, and saponins, which function as reactive oxygen species scavengers.

Recent studies have highlighted the regulatory role of environmental stressors on the expression of genes encoding key enzymes in phenolic biosynthesis, such as phenylalanine ammonia-lyase (PAL), cinnamate-4-hydroxylase (C4H), 4-coumarate-CoA ligase (4CL), and chalcone synthase (CHS). These findings underscore the importance of environmental adaptation in optimizing secondary metabolite production in medicinal plants [10].

Unsaturated fatty acids play a crucial role in maintaining membrane stability and resistance under various stress conditions [11]. Seeds of *S. marianum* are a rich source of essential phospholipids, unsaturated fatty acids, such as linoleic and oleic acid, and vitamin E. Among these, linolenic acid (C18:3) is particularly important for preserving chloroplast membrane fluidity and resistance under cold stress [12].

The concentrations of silybin (A + B) and fatty acids in milk thistle seeds are influenced by genetic factors and environmental conditions, including climate (e.g., rainfall and temperature), soil characteristics, fertilization practices, and plant phenology [10]. Milk thistle's robust root system enables it to grow in a wide range of soil types, from sandy to clay soils, even those low in micronutrients and moisture. However, optimizing the growth and phytochemical yield of this plant remains challenging due to the complexity of selecting suitable populations for diverse environmental conditions [13].

Despite extensive research on the growth, development, and phytochemical properties of *S. marianum*, comparative analyses of the pharmacological traits of different populations adapted to varying climatic conditions remain limited. Therefore, this study focuses on evaluating the quantitative and qualitative phytochemical characteristics and antioxidant activity of wild *S. marianum* populations collected from various geographical regions in Iran, comparing them with a population from Hungary, serving as the control.

MATERIAL AND METHODS

Collection of *S. marianum* fruit samples. Seeds of *S. marianum* were collected from five different regions, including northern, northwestern, and northeastern Iran (native regions), and one from Hungary (as control) in April–September 2021. The population was confirmed according to a voucher in the herbarium of Ferdowsi University, Mashhad, Iran (no. 48355-FUMH).

Climatic data. Meteorological data were obtained for 30 years from the I. R. of Iran Meteorological Organization (IRIMO) and for Hungary from https://www.mapsofworld.com.

Extraction of seed sils. For seed oil extraction, the seed powder (5 g of each sample) was defatted in a Soxhlet device (Hanon instrumrnt, China) with 70 mL of n-hexane (Merck, United States) at 70°C for 8 h.

Silymarin extraction. The defatted material was subsequently extracted with 150 mL of acetone (Merck) in a shaker machine (Wise Cube, WIS-20R, Ukrain) at room temperature for 24 h. The acetone extracts were evaporated to dryness on a rotary evaporator (IKA RV 10 digital V, Germany) at 45°C to obtain the dried extract.

HPLC analyses. The content of silybin (A + B) was determined by high-performance liquid chromatography (HPLC) with a Nucleosil C_{18} 5 μm (250 × 4.6 mm) column, a Knauer K2600A UV detector DAD 2.1 L and Chromgate software (Germany) for peak integration. The solvents phosphoric acid, methanol, and water (0.5 : 25 : 65; v/v/v) were used as the mobile phase. The run time and flow rate were 40 min and 1 mL/min, respectively, and the seven peaks of SM detected and the average of silybin (A + B) diastereomers calculated at 288 nm [14]. The content of silybin (A + B) was expressed as mg/g dry weight (DW).

Fatty acid analysis. Gas chromatography of fatty acid methyl esters (F.A.M.Es) was performed according to [15]. The oil was analyzed by gas chromatography with flame ionization detector (GC–FID) using an Agilent model 7890A gas chromatograph (Agilent, India) equipped with a DB-WAX-fused polyethylene glycol (PEG) (Merck) column (60 m × 0.25 mm, film thickness 0.25 μm) (P/N 122-7062 (J & W scientific company, Agilent). Preparation of triglyceride fatty acid methyl esters were according to the ISO 12966-2: 2011 [16]. F.A.M.E are a type of fatty acid ester that is derived by transesterification of fats with methanol for analysis of fatty acids by gas chromatography technique. Since the majority of herbal fatty acids are in triglyceride form, we used a process to prepare methyl esters from triglycerides. Gas chromatography of samples was performed by injection of 0.5 μL of *n*-octane solution of fatty acid methyl esters using ISO 12966-1:

2014 [17]. The oven temperature was programmed as follows: the initial temperature was held at 170°C for 5 min and then increased to 220°C at a rate of 1°C/min. The detector (FID) temperature and injector temperature were 220°C. Nitrogen was used as the carrier gas with a linear velocity of 1 mL/min. Quantification data were obtained from GC-FID area percentages without the use of correction factors. The content of fatty acids in each region is expressed as a percentage (%). The components of oils were identified based on the basis of their retention indices. Their identification was confirmed by co-injection of some commercial F.A.M.E. Mix Merck company standards as: 18916-1AMP, F.A.M.E. Mix, C18:0–C20:0, certified reference material, pkg of 100 mg (Neat); CRM18918, F.A.M.E. Mix, C8–C24, certified reference material, ampule of 100 mg (Neat); and CRM1891, F.A.M.E. Mix GLC-10, certified reference material, pkg of 100 mg (Neat).

Seed extraction. For methanolic extraction, 1 g of powdered seeds was macerated in 10 mL methanol (Merck) for 24 h. After centrifugation with 12000 rpm for 15 min the supernatants were separated and then the resulting dry powder was used for subsequent measurements.

Total Extractable Phenolic Content (TPC). The TPC was tested by the colorimetric method with the Folin–Ciocalteu reagent (Sigma-Aldrich, United States) [18]. Briefly, 200 μL of the reagent (10%) was added to 100 μL of each methanolic extracts (1 mg/mL) and then mixed with 500 μL of dH_2O and 700 μL of 7% sodium bicarbonate. After 120 min of standing at room temperature in the dark room, the absorbance was measured at 765 nm wavelength by optical spectroscopy (Japan, Jasco, VIS/UV7800). Gallic acid (Merck) (0–1000 μg/mL) was used as a standard ($y = 0.0011x + 0.1624$; $R^2 = 0.9444$) and is expressed as mg gallic acid/g DW.

Total Flavonoid Content (TFC). Briefly, 0.2 mL of the methanolic extracts (1 mg/mL) were added to 40 μL of 10% aluminum chloride (Merck) (w/v), 40 μL of 1 M potassium acetate, 0.6 mL of 80% methanol, and 1.12 mL dH_2O. The mixture was stored at room temperature for 30 min, and the absorbance was measured at 415 nm wavelength [19]. Quercetin (Sigma-Aldrich) (0–120 mg/mL) was used to construct a standard curve ($y = 0.0049x - 0.0033$; $R^2 = 0.9997$), and the results are reported as mg quercetin/g dry weight.

Total Phenolic Acid Content (TPAC). The TPAC was measured by the Arnow reagent [20]. Briefly, 500 μL of each methanolic extracts (1 mg/mL) was added to 2.5 mL dH_2O, followed by 500 μL of HCl (0.1 M), 500 μL of Arnow reagent (10% sodium molybdate and 10% sodium nitrite; w/v), and 500 μL of 1 M NaOH. Then, the mixture was added to a volumetric flask, and the absorbance was recorded quickly at 490 nm wavelength. To calculate the TPAC, caffeic acid (Sigma-Aldrich) was used as a curve (1–120 μg/mL) ($y = 0.0036x + 0.0287$; $R^2 = 0.99$). The results are expressed as mg caffeic acid/ g DW.

DPPH radical scavenging assay. DPPH (2,2'-diphenyl-1-picrylhydrazyl) (Sigma-Aldrich) radical scavenging activity was determined by the DPPH assay according to Hatano et al [21]. One milliliter of a methanolic solution of DPPH (0.1 mM) was added to 100–500 μL of each methanolic extract (1 mg/mL), and subsequently, 1500 μL of methanol was added. Then, the samples were kept in a dark chamber at room temperature for one hour. The absorbance was read at 517 nm. Methanol was used as a blank for optical spectroscopy. The radical-scavenging activities (%) of the tested samples were measured by comparison with those of a control (2 mL of DPPH solution and 1 mL of methanol). DPPH radical scavenging activity was calculated by Eq. (1):

$$\text{DPPH IC}_{50},(\%) = \left[(A_c - A_s)\right]/A_c \times 100 \qquad (1)$$

where A_c is the control absorption and A_s is absorption of the tested sample after 1 h.

Statistical analysis. All experiments were performed in triplicate, and statistical significance was calculated by one-way ANOVA at $P \leq 0.05$ and $P \leq 0.01$. Statistical analysis was performed with SPSS version 16, and the graphs were drawn with Excel. We used Pearson's correlation to determine the relationships between the data by using Graph Pad Prism 9. Multivariate statistical analysis was performed by using PAST ver. 3.14 [22]. The quantitative data for every phytochemical characteristic in the five populations were evaluated via a principal component analysis (PCA) biplot to test the proportion of each variable to the whole variety using the following variables [23]. Only principal components (PCs) with an eigenvalue greater than 1 were considered.

RESULTS

Agro-Climatic

The mean meteorological data for 30 years (1991–2021) from the IRIMO for different Iranian regions are given in Table 1. The data were collected from the west, northwest, north, and northeast of Iran. Among the five regions of Iran, the highest rainfall and humidity respectively occurred in Lorestan (60.41 mm) and Moghan (78.37%), and the lowest respectively occurred in Moghan (25.92 mm) and Lorestan (55.83%). The highest air and soil temperatures are related to Gorgan (14.37°C, 6.14°C), and the lowest are related to Ardabil (5.6°C, −1.57°C). In terms of altitude, Ardabil and Moghan, which are 1335.2 and 72.6 m above sea level, respectively, are the highest and lowest among the different regions of Iran, but in general, the growing area of *S. marianum* in Hungary has the highest rainfall, soil temperature and altitude.

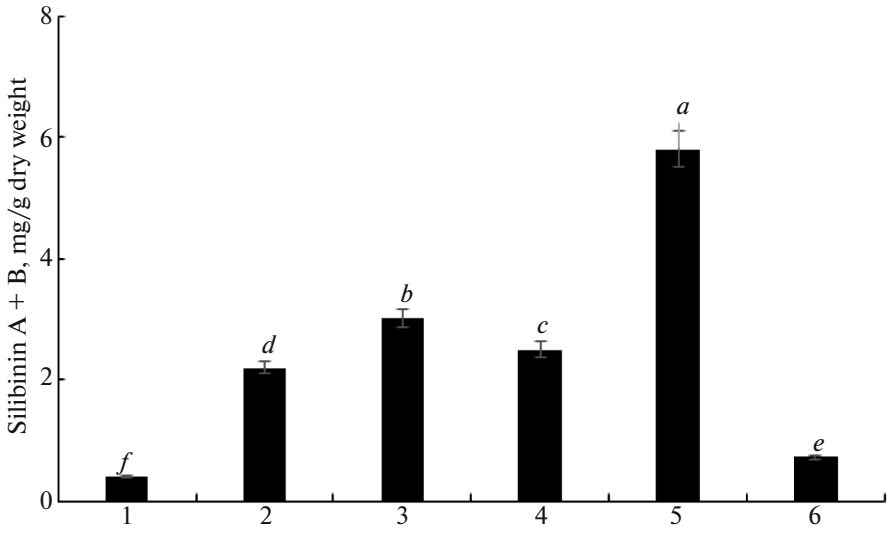

Fig. 1. Average silibinin content (mg/g dry weight) determined by HPLC from *Silybum marianum* seeds related to native populations of different geographical regions of Iran (*1*) Ardabil; (*2*), Bojnord; (*3*), Gorgan; (*4*), Lorestan; (*5*), Hungary; (*6*), Moghan desert.

Silibinin Content

The data analysis revealed that climate had a significant effect ($P \leq 0.05$) on seed silibinin content. The quantitative data related to the chromatographic profile of the acetonic solutions for the six extract samples belonging to each region are shown in Fig. 1 and the HPLC choromatogram of the standard is presented in Fig. 2. The lowest and highest silibinin (A + B) contents were detected in Ardabil and Hungary, respectively, but in the Iranian population, the lowest and highest silybin (A + B) contents were detected in Ardabil and Gorgan, with values of 0.4 and 3.02 mg/g DW, respectively. The results of the heatmap Pearson's correlation coefficient confirmed that soil temperature ($P \leq 0.05$), rainfall and temperature ($P \leq 0.01$), which are metrological data, were significantly positively correlated with the silibinin content (Fig. 3).

Oil Content

Seed oil percentage and fatty acids are mentioned as the most significant factors in diets and medical regimes. The quantity and quality of *S. marianum* seeds differ among geographical regions. The fatty acid compositions of the *S. marianum* seeds are shown in Table 2 and gas chromatogram of seed oil fatty acid composition in Hungary seed (as control) was performed by GC-FID (Fig. 4). Gas chromatography (GC-FID) confirmed the presence of two unsaturated fatty acids, oleic acid (C18:1), linoleic acid (C18:2), and palmitic acid (saturated). The results of the fatty acid analysis showed that the greatest and lowest percentages of oleic acid were related to the Moghan and Bojnord regions, respectively. In general, the seeds of Gorgan's population were superior to those of other populations in terms of linoleic and oleic acid fatty acids (80.79%).

The linoleic acid content increased with increasing temperature, while the oleic acid content decreased. The highest palmitic acid content was detected in the Ardabil population, and the lowest was detected in the Hungary and Bojnord populations. The results of heatmap Pearson's correlation coefficient showed that there was a positive correlation between saturated fatty

Table 1. An average of 30 years of meteorological data for five growing regions of *Silybum marianum* populations in Iran and Hungary

Station	Longitude	Latitude	Altitude, m	rrr24, mm	Umm, %	tm_m, °C	tsoilm_m, °C
Ardabil	48.33	38.22	1335.2	29.72	73.32	5.6	−1.57
Bojnord	57.3	37.49	1065	27.34	66.16	8.16	0.27
Gorgan	56.86	37.5	890	46.84	73.80	14.37	6.14
Lorestan	48.28	33.44	1147.8	60.41	55.83	11.7	1.71
Hungary	20	47	143	600	75	10.7	11.1
Moghan	47.8	39.6	72.6	25.92	78.37	9.9	3.8

Abbreviations: rrr24, rainfall, umm, relative humidity, tm_m, temperature, tsoilm_m, soil temperature.

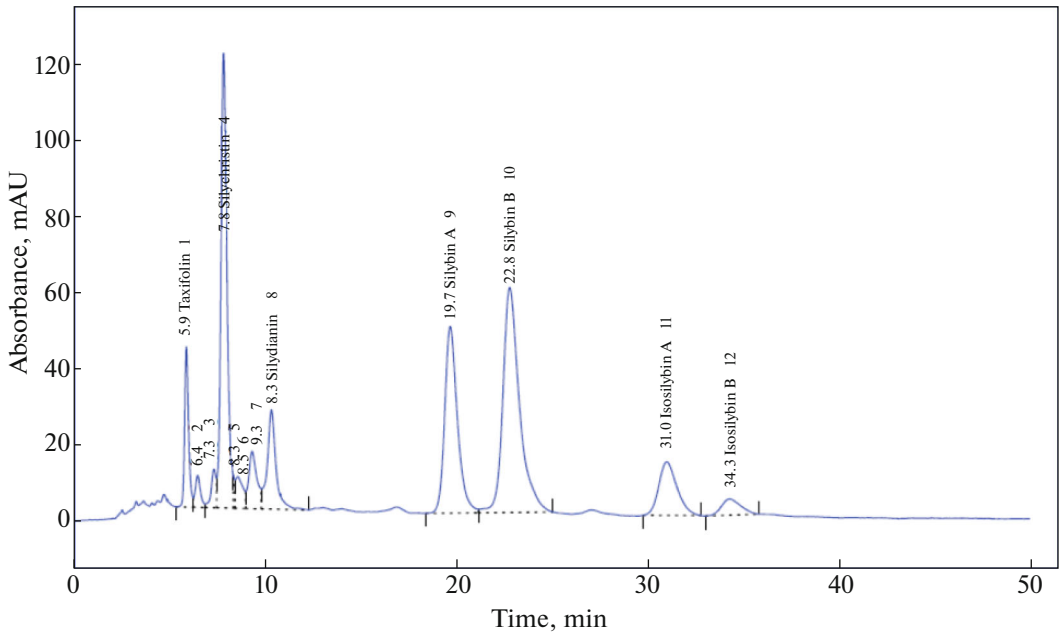

Fig. 2. The HPLC choromatogram of the standard solution (Silymarin) at 288 nm.

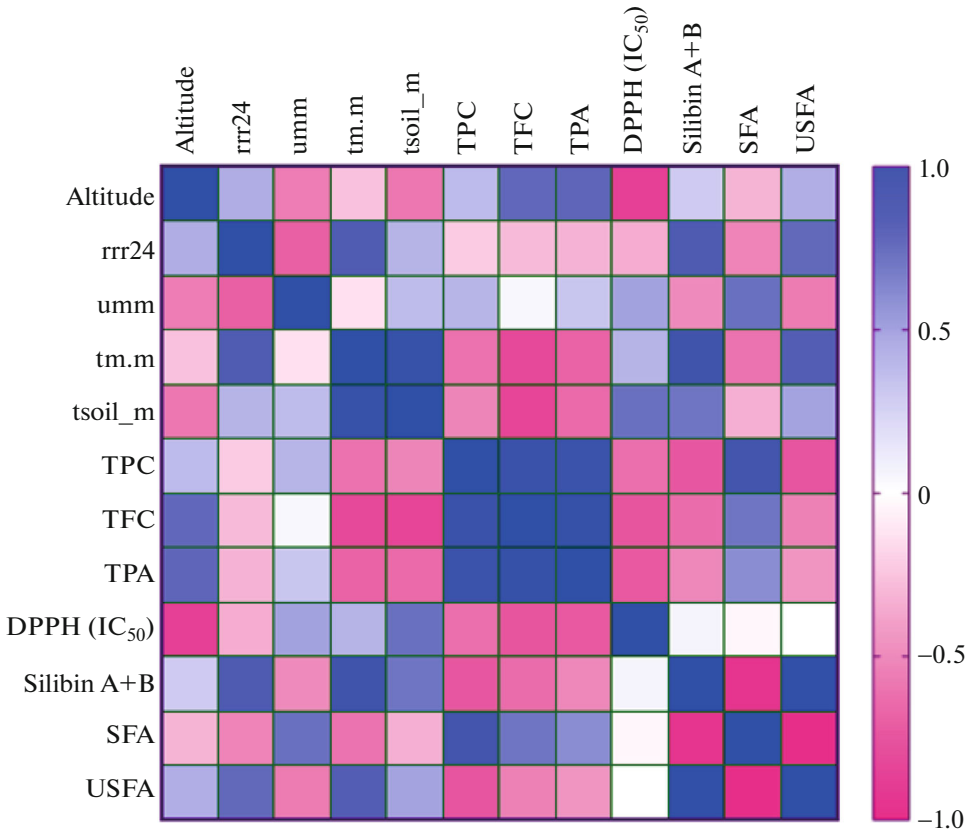

Fig. 3. Heatmap of Pearson's correlation coefficient between ecological factors and metabolite content in wild seed populations of *Silybum marianum*. *Abbreviations:* rrr24, rainfall, umm, relative humidity, tm_m, temperature, tsoil_m, soil temperature, TPC, total phenol content, TFC, total flavonoid content; TPA, total phenolic acid, DPPH IC_{50}, free radicals conferring, SFA, saturated fatty acid, USFA, unsaturated fatty acid.

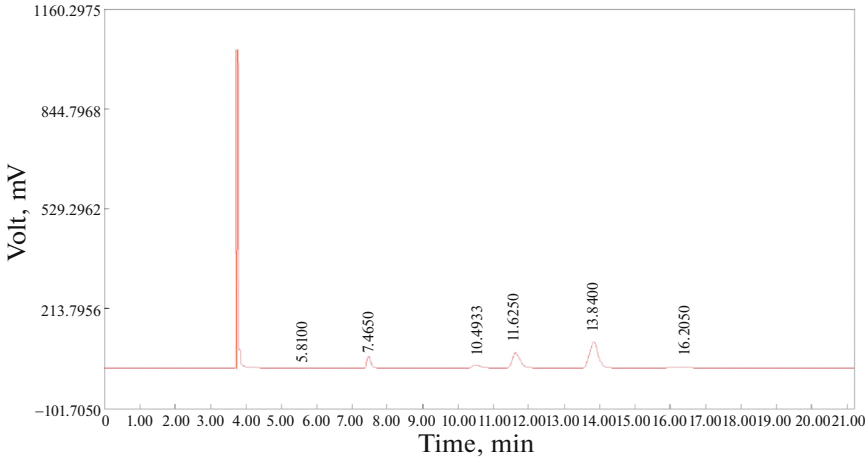

Fig. 4. The gas chromatogram of seed oil fatty acid composition in Hungary seed (as control) of *Silybum marianum* via GC-FID.

acid content and total phenol content ($P \leq 0.01$) and between relative humidity and total flavonoid content ($P \leq 0.05$); in contrast, unsaturated fatty acid content was significantly positively correlated with air temperature and silibinin content ($P \leq 0.01$) (Fig. 3).

Total Phenolic and Flavonoid Contents

The data analysis revealed that weather features had significant effects ($P \leq 0.05$) on seed phenolic and flavonoid contents. The highest total phenol content was detected in the seeds of the Ardabil population (49.45 mg/g DW), and the lowest was detected in the Boujnurd (16.61 mg/g DW) (Fig. 5a). The seeds of Hungary were close to moderate (26.83 mg/g DW) in this respect. The highest (4.44 mg/g DW) and lowest (1.7 mg/g DW) flavonoid contents were detected in the Ardabil and Gorgan populations, respectively. The average flavonoid content in Hungaryan seeds was also low (Fig. 5a). The correlation analysis confirmed that there was no significant correlation between altitude (0.286) or relative humidity (0.307) and phenolic compound content, while a positive and significant correlation (0.593) was found between altitude and flavonoid content. Pearson's test revealed a strong negative correlation between the TPC (–0.611), TFC (–0.824), and TPA (–0.683) and air temperature. Similar results were observed between the mentioned compounds and soil temperature (Fig. 3). According to the heatmap of Pearson's correlation coefficient, there was a positive and strong correlation between TPC and TFC (0.858). We observed a strong negative correlation between TPC (–0.624) and TFC (–0.745) with IC_{50} values. In conclusion, the TPC and TFC decreased with increasing air and soil temperatures (Fig. 3).

Phenolic Acid Contents

Our results revealed that metrological factors had significant effects ($P \leq 0.05$) on the PAC. The highest and lowest phenolic acid contents were detected in Ardabil (10.77 mg/g DW) and Moghan (3.49 mg/g DW), respectively (Fig. 5a). Pearson's test revealed a negative correlation between several environmental factors, such as altitude (–0.298), air temperature (–0.683), and soil temperature (–0.648), and the TPA (Fig. 3).

Table 2. Results of the determination of the fatty acid composition (%) of *Silybum marianum* seeds from different geographical regions via *GC-FID* analysis

		Regions					
		1	2	3	4	5	6
C16	Palmitic acid	17.9	11.123	12.14	12.53	10.9	15.9
C16:1	Palmitoleic acid	0.16	0.2	0.12	No, recognize	No, recognize	No, recognize
C18	Stearic acid	8.13	6.83	5.8	6.9	6.4	9.92
C18:1	Oleic acid	38.07	24.76	24.84	26.65	27.6	40.49
C18:2	Linoleic acid	34.28	53.98	55.95	50.44	53.9	32.4
C18:3	Linolenic acid	1.7	2.3	1.18	3.5	0.97	1.3

Designations: 1, Ardabil, 2, Bojnourd, 3, Gorgan, 4, Lorestan, 5, Hungary, 6, Moghan desert.

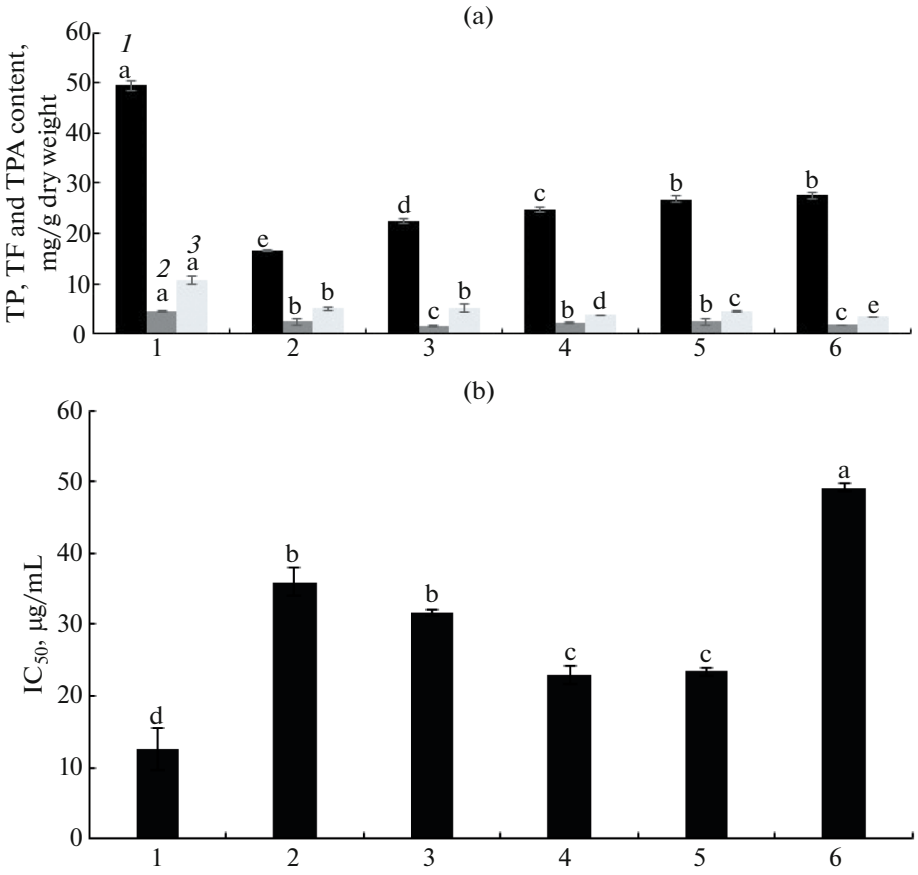

Fig. 5. The means of phenolic, flavonoid, and phenolic acid contents (a) and antioxidant activity determined by DPPH IC_{50} values (b) of wild *Silybum marianum* seeds from different geographical regions of (1) Iran, Ardabil; (2) Bojnord; (3) Gorgan; (4) Lorestan; (5) Hungary; (6) Moghan desert. *Abbreviations:* (*1*), black column, TPC, (total phenol content), (*2*), dark gray column, TFC (total flavonoid content); (*3*), light gray column, TPA (total phenolic acid).

IC_{50} Values of Antioxidant

The IC_{50} values (inhibitory concentration (50% inhibition)) demonstrated that all the seed extracts blocked free radicals, conferring remarkable antioxidant activity ($P \leq 0.05$). The lowest and highest antioxidant activities were related to Moghan seed population (49.2 μg/mL) and Ardabil seed population (12.68 μg/mL), respectively, according to the IC_{50} values (Fig. 5b). In this study, the highest TPC, TFC, and antioxidant capacity were related to the Ardabil seed population.

Correlation of Ecological Factors and Metabolite Content

The correlation analysis confirmed that increasing altitude and rainfall lead to increased antioxidant activity via a decrease in the IC_{50} value (Fig. 3) the increase in altitude was −0.880** ($P \leq 0.01$), and the increase in rainfall was −0.350 ($P \leq 0.05$). Our results revealed a negative linear correlation between the total phenolic content ($P \leq 0.05$) and flavonoid content ($P \leq 0.01$) and the DPPH free radical scavenging activity (IC_{50} value). Correlation analysis revealed that there was a negative correlation between the IC_{50} and the total phenolic ($r = -0.6$) and flavonoid contents ($r = -0.73$) (Fig. 6).

Principal Component Analysis

PCA was performed to study the communication among *S. marianum* from different geographical regions of Iran, using Pearson's correlation coefficient to display the degree of their similarity (Table 3; Fig. 7). Because of the high correlations among all variables, as shown in the heatmap of Pearson's correlation, there were 4 important factors for separating the regions where *S. marianum* was collected and two principal components explaining 99.147% of the overall variance (76.155 and 22.992% of the variance for PC1 and PC2, respectively), and the analyzed clusters were divided into 4 separate parts. The first principal component is related to Ardabil seed population, which has the highest amount of phenol because of its high altitude and low temperature, and the second is related to DPPH IC_{50} (PC2, 22.992%), which

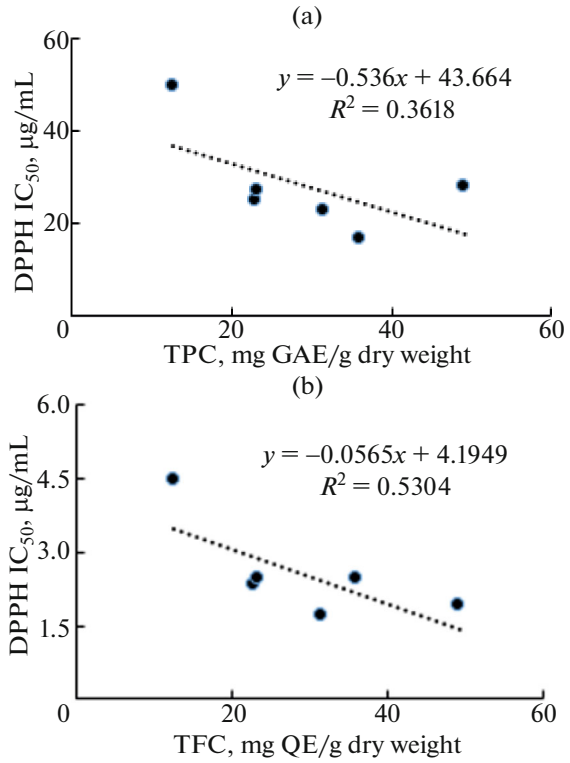

Fig. 6. Regression of antioxidant activity in *Silybum marianum* based on phenolic compound (a) and flavonoid contents (b).

depends on Moghan seed population. Interestingly, a negative correlation between the antioxidant activity according to the DPPH IC_{50} value and the phenol and flavonoid contents was also detected by the PCA biplot. The highest antioxidant activity was related to the lowest DPPH IC_{50} values and depended on the highest phenol and flavonoid contents. PCA revealed that Gorgan seed population had the most USFAs, and Ardabil seed population had the most SFAs.

DISCUSSION

Silibinin Content

Secondary metabolites play a key role in the relationship between the plant and the surrounding environment. Silybin is one of the most active components of SM. The results showed that silybin content in seeds of different populations were very different from each other. This difference may be attributed to variations in rainfall, air and soil temperatures. There is evidence that silymarin content in seeds can be strongly variable according to genotype and growth conditions, so the difference in the amount of flavonolignans in *Silybum marianum* depends on the different climatic conditions and habitat [24].

In this study, the content of silybin (A + B) in the Gorgan population was 7.5 times greater than that in the Ardabil population, indicating the influence of environmental factors such as rainfall, temperature, and altitude [25]. The highest content of silybin (A + B) was observed in rainy regions such as Gorgan due to higher air and soil temperatures, as well as rainfall and humidity, which promote silybin (A + B) production. The CHS and CHI genes overexpression and consequently increased flavonolignans in moderate climates indicated that climate condition contributed significantly to the production of silybin [26].

Svobodova et al. [27] reported that a lack of UV light may reduce the flavonolignan content because flavonoids used as antioxidants for scavenging reactive oxygen species (ROS). The CHS enzyme and mRNA levels of CHS change during developmental stages and in response to environmental stimuli, leading to changes in the biosynthesis of final compounds in the pathway. CHS promoters with cis-elements can regulate flavonoid biosynthesis during development and in response to various environmental stimuli [28]. The adaptability of *S. marianum* to diverse habitats underscores the significance of selecting populations with elevated flavonolignan levels for pharmacological studies.

Table 3. Eigenvalues and % of the variance for factors obtained from the PCA based on several variables of five groups of native Iranian seeds harvested in April-September 2021

Variable	Component			
	PC1	PC2	PC3	PC4
TPC	0.66326	0.55325	0.11194	0.45194
TFC	0.057791	0.014678	0.11836	−0.37704
TPA	0.15315	0.030098	0.86479	−0.33142
DPPH IC_{50}	−0.71402	0.63652	0.20949	0.17998
Silybin (A + B)	−0.030513	−0.10067	0.0078763	0.34758
SFA	0.11298	0.37947	−0.34591	−0.29963
USFA	−0.098904	−0.3654	0.249	0.54843
Eigenvalues	295.809	89.306	2.20153	0.56678
% of variance	76.155	22.992	0.56678	0.28614

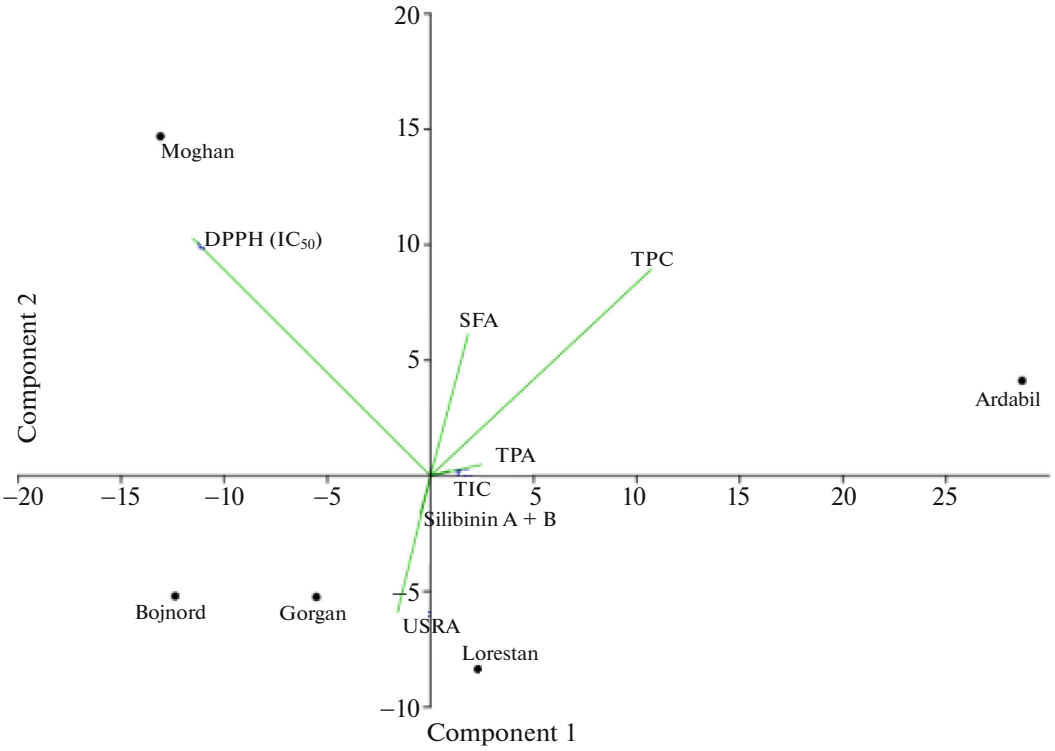

Fig. 7. Principal component analysis (PCA) of *Silybum marianum* based on the covariance matrix of regions (PC scores) and plant metabolites, including TPC, TFC, TPA, DPPH (IC_{50}), SFA, and USFA, was performed. The first and second components had significant positive and strong correlations with the TPC and IC_{50}, respectively. *Abbreviations*: TPC, total phenol content; TFC, total flavonoid content; TPA, total phenolic acid; IC_{50}, free radicals conferring; SFA, saturated fatty acid; USFA, unsaturated fatty acid.

Fatty Acids

Fatty acid profiles varied significantly among populations, influenced by genetic and environmental factors. Seeds from Ardabil contained higher saturated fatty acids, whereas Gorgan seeds exhibited a greater proportion of unsaturated fatty acids, particularly linoleic and oleic acids, which were associated with the region's moderate rainfall and higher temperatures. Genetic factors, environmental conditions, irrigation practices significantly influence the quantity of oil and fatty acids [29]. Notably, drought stress has been shown to enhance oil content and unsaturated fatty acid levels, underscoring its potential role in selecting stress-tolerant genotypes [15].

Phenol and Flavonoid Contents

Phenolic and flavonoid content displayed marked regional differences, with Ardabil population seeds having the highest levels due to the region's low temperatures and high altitude. Conversely, seeds population of Bojnord and Gorgan exhibited lower phenolic and flavonoid content, possibly due to higher temperatures that suppress biosynthetic enzyme activity. Climatic factors such as temperatures, radiation, UV absorption, drought and salinity, as well as seasonal variations influenced by latitude and longitude [30]. These factors are known to activate biosynthetic enzymes like phenylalanine ammonia-lyase (PAL), leading to increased production of phenolic compounds, which strengthen cell walls through lignin and suberin deposition under stress conditions [31, 32].

Phenolic and flavonoid compounds exhibit high antioxidant activity because they can donate electrons to ROS, thereby preventing the oxidation of biological molecules and protecting plants against free radical [33]. Lee and oh [34] reported that chilling stress induces the synthesis of phenolic compounds through the induction of PAL, which aligns with the results observed in Ardabil population seeds. Conversely, elevated temperatures (30 to 40°C) can halt the synthesis of phenolic and flavonoid compounds by suppressing gene expression and enzyme activity [35]. Flavonoids also play a crucial role in UV protection at high altitudes, acting as radiation absorbers to mitigate oxidative damage [36].

Phenolic Acids

Phenolic acids were most abundant in Ardabil seed population, while the lowest phenolic acid content was found in Moghan seed population. Phenolic acids are critical for cell wall integrity and act as precursors in the phenyl propanoid pathway, especially under

stress conditions [37]. Phenolic acids a subset of phenolic compounds, correlating with higher antioxidant potential and greater resistance to environmental stress [1]. Golbeiowska-Pikania et al. [38] observed in winter triticale that exposing to cold stress leads to an increase in phenolic acids, confirming our results.

DPPH IC_{50} Values

The highest DPPH radical scavenging activity, indicating superior antioxidant capacity, was observed in Ardabil seed population, which also had the lowest IC_{50} values. Thus, the lowest IC_{50} value corresponded to the highest DPPH antioxidant activity and high antioxidant capacity [39]. This activity aligns with the high phenolic content and reflects the role of ecological factors such as temperature, rainfall, radiation and humidity in modulating antioxidant defenses. The antioxidant activity of plants is required for the prevention of oxidative stresses [30].

Correlation of Ecological Factors and Antioxidant Activity

The study highlights a significant inverse relationship between altitude and IC_{50} values, indicating that higher altitudes enhance antioxidant activity. Similarly, increased rainfall showed a weaker but significant negative correlation. These findings suggest that environmental stressors such as lower temperatures at higher altitudes and increased water availability can stimulate the biosynthesis of antioxidant compounds.

Heatmap Analysis

The heatmap underscores complex interactions between ecological variables and metabolite concentrations. Notably, altitude and temperature emerge as key determinants of phenolic and flavonoid content.

Principal Component Analysis

The negative correlation between DPPH IC_{50} and phenolic/flavonoid content observed in PCA biplots reaffirms the strong antioxidant potential of phenolic-rich seeds. This suggests that environmental stressors such as high altitude and low temperature optimize antioxidant metabolite production. However, ecological factors also influence fatty acid profiles. The trade-off between SFAs and USFAs across regions may reflect metabolic adaptations to environmental conditions. Finally, this study demonstrates how integrating ecological data with biochemical analyses can provide a nuanced understanding of the factors influencing plant metabolite profiles and their antioxidant capacities.

CONCLUSIONS

This study highlights the significant influence of climatic factors such as altitude, rainfall, humidity, and temperature on the phytochemical composition and antioxidant activity of wild *S. marianum* populations. The Gorgan seed population, characterized by higher rainfall and moderate temperatures, exhibited the highest levels of silybin (A + B) and unsaturated fatty acids. In contrast, the Ardabil seed population, with its high altitude and low temperatures, showed the highest phenolic content and antioxidant activity. These findings underscore the importance of geographical and climatic factors in shaping the pharmacological and phytochemical profiles of *S. marianum*. In this experiment, sometimes we encountered conflicting results, so more efforts and expand studies to additional regions are needed to obtain novel information on how climatic and edaphic factors and their interactions influence physio-biochemical mechanisms related to the synthesis of phytochemical compounds.

ABBREVIATIONS AND NOTATION

CHS	chalcone synthase
DPPH	2,2'-diphenyl-1-picrylhydrazyl
F.A.M.Es	fatty acid methyl esters
FID	flame ionization detector
FRAP	ferric reduction activity potential
FRSA	free radical-scavenging activity
GC-FID	gas chromatography with flame ionization detector
HPLC	high-performance liquid chromatography
IC_{50}	inhibitory concentration (50% inhibition)
IRIMO	I.R IRAN of Meteorological Organization
PAL	phenylalanine ammonia-lyase
PCA	principal component analysis
PCs-	principle components
PEG	polyethylene glycol
ROS-	reactive oxygen species
SM	silymarin
TFC	total flavonoid content
TPAC	total phenolic acid content
TPC	total extractable phenolic content

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This work does not contain any studies involving human and animal subjects.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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