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Silymarin Chemotype and Content in Wild Iranian Milk Thistle (*Silybum marianum* (L.) Gaertn.)

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ABSTRACT

Milk thistle (*Silybum marianum*) of the *Asteraceae* family is recognized as a valuable medicinal herb, primarily due to the presence of silymarin - a complex of flavonolignans concentrated in its fruits. Naturally occurring milk thistle populations can contain individuals of uniform chemotype or mixtures of multiple chemotypes, though only three (A, B, and C) have been documented so far. This research investigated the silymarin chemotype and content across 18 wild milk thistle populations gathered from various Iranian regions and cultivated under uniform environmental conditions. Eight populations were classified as chemotype B, while the remainder consisted of mixed chemotypes. The mean silymarin content was found to be 32.68 ± 2.46 mg/g dry weight, with population 23 exhibiting the highest level (47.94 mg/g) and population 21 the lowest (21.87 mg/g). Additionally, 16 individual plants from four heterogeneous populations were assessed, revealing the known chemotypes A, B, and C, and identifying a novel chemotype (designated E) for the first time. This finding highlights significant chemical diversity in Iranian milk thistle. Both A and B chemotypes were widespread, with no clear geographic pattern. Overall, these findings enrich the understanding of milk thistle chemodiversity and offer insights useful for breeding programs aimed at enhancing silymarin yield and quality.

Keywords: Marian thistle, Silybin, Chemotyping, Chemotaxonomy, Phytochemical variation
Abbreviations: wt.: weight

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INTRODUCTION

Milk thistle (*Silybum marianum* (L.) Gaertn.) is a spiny herbaceous plant that belongs to the *Asteraceae* family. A distinctive feature of most genotypes is the white marbling on the upper surface of the leaves [1-4]. In natural conditions, this species begins its growth cycle after autumn germination, staying in a vegetative rosette phase during winter. Flowering typically occurs the following summer, resulting in an overall life cycle of about 8-9 months, which is why it is usually regarded as a biennial. However, under cultivation, it is commonly grown as an annual, with spring sowing [1-3].

This species has been valued as a medicinal herb for more than two millennia, first noted by the Greek botanist Theophrastus in the 4th century B.C. [4]. Standardized extracts derived from milk thistle fruits have been marketed for around 50 years, mainly for their liver-protective properties as well as antioxidant, anti-inflammatory, and anti-fibrotic effects [5]. Milk thistle is used for treating liver diseases (cirrhosis and hepatitis) and for protecting the liver from toxic substances [6]. Milk thistle remains one of the top-selling herbal remedies worldwide, generating annual sales of over $2.6 million [7]. Its therapeutic benefits are mainly linked to silymarin - a complex mixture of flavonolignans concentrated in the fruit's tissue. The highest silymarin levels are found in the achene (technically a fruit, though often called a seed), particularly in the seed coat, with only minimal amounts in the surrounding pericarp [8]. The main components of silymarin include silybin A and B (SBA and SBB), isosilybin A and B (ISBA and ISBB), silychristin (SC), silydianin (SD), and the flavonoid taxifolin (TXF) [9]. In addition, minor constituents like silychristin B and isosilychristin have also been identified [9]. Notably, each of these flavonolignans exhibits unique biological activities that collectively contribute to the overall medicinal potential of silymarin [6]. Due to its significant pharmaceutical relevance and the increasing need for a reliable, standardized supply, various strategies have recently focused on optimizing silymarin yield and consistency [10].

Milk thistle is believed to have originated in the Mediterranean region and now spans southern Europe, Asia Minor, and North Africa, but today it can be found growing wild or cultivated worldwide. In Iran, wild milk thistle is naturally distributed in the northern provinces (Golestan, Mazandaran, Gilan, Ardabil) and the western provinces (Kermanshah, Ilam, Khuzestan, Bushehr, Hormozgan and Fars) [11]. Although populations from different regions have been thoroughly examined and are known to display significant variation in both total silymarin quantity and composition [12-14], information about Iran's wild populations remains scarce [11].

The silymarin content in milk thistle fruits varies widely. Generally, it ranges between 1% and 3% of the achene's dry weight but can exceed 8% in some cases [3, 15]. Therefore,

developing cultivars with reliably high silymarin content is a key goal [16, 2]. Mature fruits should contain at least 1.5% to 2% silymarin in their dry mass based on the European Pharmacopoeia and the United States National Formulary [17, 18]. However, silymarin levels are strongly influenced by the genetic makeup of the plant and the conditions under which it grows [15, 5].

Besides total quantity, silymarin quality can also differ significantly because the relative abundance of each flavonolignan may vary among genotypes [12]. Silymarin compositions are generally grouped into distinct profiles, resulting in a few recognized chemotypes [12]. Here, we use "silymarin chemotype" to describe specific silymarin profiles that occur either within individual plants or in populations where all individuals share the same profile, following Martinelli *et al.* 2021a [13]. However, many studies have analyzed bulk samples from mixed wild populations, which cannot capture single plant variation [12]. Notably, single plant analyses of cultivated and wild European accessions have so far revealed only three consistent chemotypes (A, B and C) [12], and a broader global survey confirmed this finding [19].

Investigating the phytochemical diversity of milk thistle may help identify additional chemotypes in wild populations. Effective genetic improvement depends on a thorough understanding of this natural diversity and how plants adapt to different conditions [16]. The primary aim of breeding efforts is to create productive varieties with enhanced and stable silymarin levels [16]. A challenge for breeders is the limited knowledge of milk thistle's genetic and chemical variability needed to develop cultivars for various uses [2]. The opportunity for selecting more productive, bioactive-rich lines remains open [16]. Wild populations, with their high adaptability to diverse conditions, are an essential gene pool for future improvement [16]. Therefore, broader investigations into wild genetic resources from various regions are strongly recommended [11].

In this study, 18 wild milk thistle populations collected from different regions in Iran were grown under the same environmental conditions to: 1) compare silymarin content across accessions under uniform cultivation; 2) examine silymarin composition at both the population and individual plant levels; and 3) explore whether chemotypes are linked to specific geographic patterns. Documenting chemotypes in Iran's wild milk thistle could provide valuable information for future breeding and help clarify the species' possible origin.

MATERIALS AND METHODS

Plant Material Collection

Seeds were collected randomly from 10 single plants per population in late June 2023 from milk thistle natural habitats and from various sources in Iran. Also, Population 23 was received from the Yazd Salt Research Center, 24 from the seed collection of Dr. Pirmoradi (Vali-e-Asr University of Rafsanjan, Kerman Province), 30 from the seed collection of Dr. Kohanmu (Persian Gulf University located in Borazjan city, Bushehr Province), 32 to 35 from the seed bank of the National Forests and Rangelands Research Institute (Peykan Shahr, Tehran Province) and 38 from the seed collection of Dr. Hosseini Monfared (Agricultural Research Center of Zanjan, Zanjan Province) (Table 1; Fig. 1a).

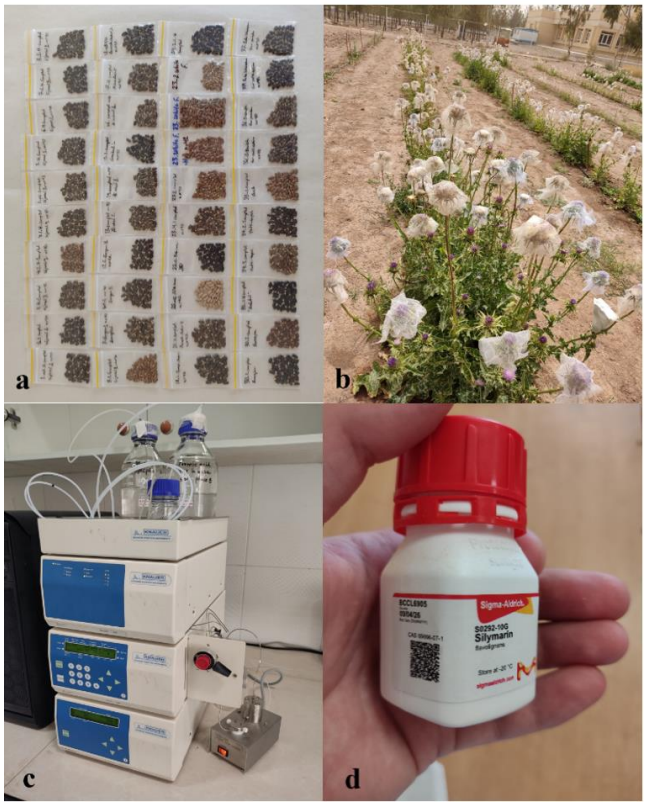

Fig. 1 Figures of (a) seeds, (b) farm, (c) HPLC, (d) Silymarin Standard (Sigma, S0292).

Table 1 Code, name and collection location of the studied milk thistle populations

Population Code	Population Name	Collection Location (ºN, ºE)	Altitude (m)	Province
Pop. 5	Sijaval-5	36°52'21.1"N, 54°09'31.3"E	-25	Golestan
Pop. 8	Sijaval-8	36°51'51.2"N, 54°09'33.4"E	-26	Golestan
Pop. 11	Gorgan-3	36°50'55.6"N, 54°29'19.4"E	92	Golestan
Pop. 12	Gorgan-4	36°52'46.1"N, 54°39'38.4"E	107	Golestan
Pop. 15	Gonbad-1	37°10'57.1"N, 55°10'06.7"E	53	Golestan
Pop. 17	Minoodasht-1	37°12'28.7"N, 55°22'42.6"E	153	Golestan
Pop. 19	Darreh Shahr-2	33°10'34.2"N, 47°23'27.5"E	622	Ilam
Pop. 21	Darreh Shahr-4	33°11'47.6"N, 47°23'03.9"E	570	Ilam
Pop. 22	Bileh Savar-1	39°19'53.8"N, 48°15'00.8"E	126	Ardabil
Pop. 23	Yazd-1	31°55'08.7"N, 54°16'54.0"E	1211	Yazd
Pop. 24	Budakalaszi-1	Hungary	-	-
Pop. 30	Borazjan-1	29°13'05.7"N, 51°14'37.1"E	101	Bushehr
Pop. 32	Ardabil-1 *(RIFR: 23330)	38°20'02"N, 48°15'10"E	1321	Ardabil
Pop. 34	Haft tapeh-1 *(RIFR:32655)	32°01'13"N, 48°31'33"E	43	Khuzestan
Pop. 35	Shush-1 *(RIFR: 32778)	32°11'59"N, 48°13'04"E	74	Khuzestan
Pop. 36	Orzuieh-1	28°20'31.6"N, 56°34'05.4"E	1081	Kerman
Pop. 37	Baba Kharazm-1	33°05'42.1"N, 47°32'48.8"E	617	Ilam
Pop. 38	Zanjan-1	36°43'51.4"N, 48°25'31.4"E	1658	Zanjan

*(RIFR): Research Institute of Forests and Rangelands Gene Bank Code

Cultivation and Harvest

The collected seeds were sown on December 23, 2023 in the research farm of Vali-e-Asr University of Rafsanjan (30°22'59.8"N, 55°55'30.0"E, 1524 m) (row spacing: 2 m × 30 cm, planting depth: 1 cm; Fig. 1b). Irrigation was done by drip tape once a week. Before flowering, the flower heads were closed with netting to prevent possible cross-pollination and seed shattering. All flower heads were harvested on June 11, 2024 (the 172nd day after planting and after the seeds dried on the flower heads) and were transferred to the laboratory in cardboard envelopes for seed separation and phytochemical analysis.

Silymarin Extraction

Extraction and analysis of silymarin were performed according to the method of Martinelli *et al*. (2016) with minor modifications [12]. At first, 1 g of seeds (approximately 40 seeds) was weighed and then thoroughly powdered with an electric coffee grinder for 5 minutes. Then, 40 mg of powder was poured into 2 ml Eppendorf microtubes and 1.5 ml of n-hexane (Merck, 104368) was added twice for defatting. Each time defatting was performed on a shaker (with rotation of 70 rpm) at 25 °C for 24 hours. After both extraction^{o} samples were centrifuged for 5 minutes at 15,000 rpm (equivalent to 20,600×g). After discarding the fatted hexane, the microtubes were placed at laboratory temperature (25 °C) to dry the sediments. Then, 1.5 ml of 75% methanol (Merck, 106007) was added and the samples were again placed on a shaker (70 rpm) for 24 hours and centrifuged (15,000 rpm, equivalent to 20,600×g for 5 minutes). Then, the methanolic extracts were passed through a 0.45 µm nylon syringe filter. The 75% methanol extracts were placed in a refrigerator at -20 °C before further HPLC analysis.

HPLC Analysis and Quantification

First, 100 µl of 75% methanol extract was diluted with 139 µl of 20% methanol to a final methanol concentration 43%. Then it was injected into a 20 µl loop using a Hamilton syringe. HPLC analysis was performed with a Knauer Smartline HPLC (Manager 5000, Pump 1000, Detector 2500 UV-VIS, Germany; Fig. 1c) using a Eurospher II (C18) reversed phase column (4.6 × 150 mm, 5µm, 100Å with precolumn (15VE181E2J), Germany).

To prepare the column, it was first washed with 75% methanol for 30 minutes and then equilibrated with 43% methanol for 15 minutes before injection. The injected methanol concentration was the same as the mobile phase concentration at the time of injection (43%) according to the protocol [12]. The column was then washed again after every 10 injections. Detector wavelength: 288 nm, column temperature: 23 °C, mobile phase gradient change: 0 to 3 min: isocratic mode 43% phase A and 57% phase B, 3 to 17 min: gradient mode 43% to 57% phase A and 57% to 43% phase B, 17 to 28 min: isocratic mode 57% phase A and 43% phase B. Phase A: chromatography grade methanol (Merck, 106007), phase B: chromatography grade water (Merck, 115333) with 0.1% formic acid (Merck, 100264) according to the protocol [12]. With a flow rate of 1 ml/min and a pressure of less than 240 bar, the absorbance changes per time unit were recorded and examined with ChromGate v.3.3.2 software and the corresponding chromatogram was drawn (Fig. 2).

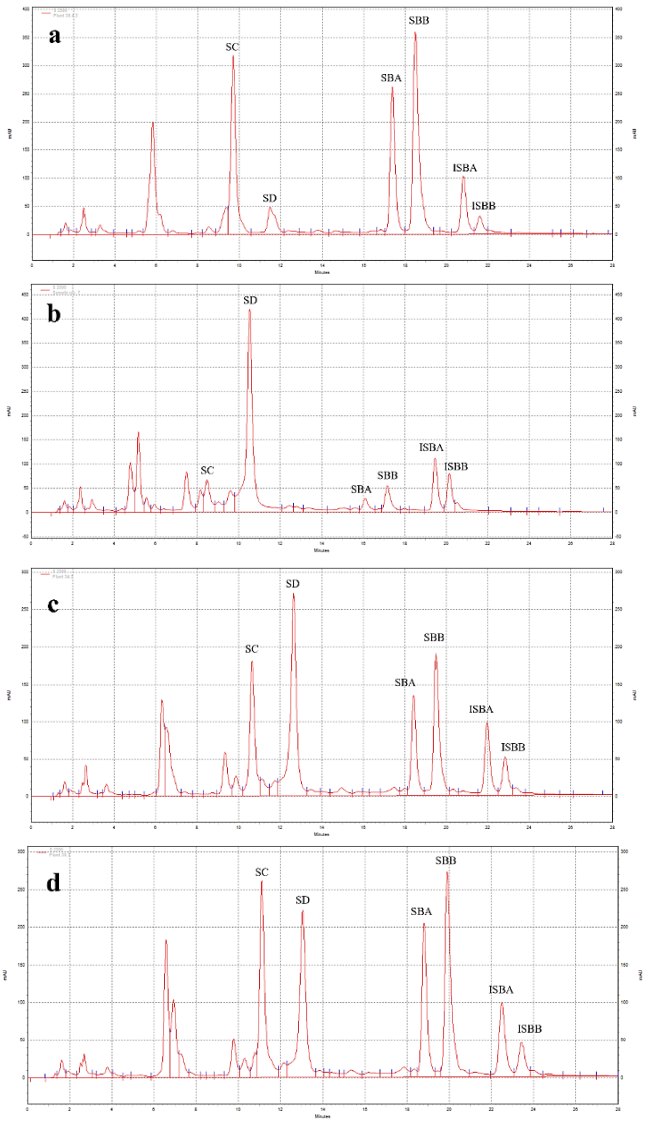

Fig. 2 Chromatograms of (a) Chemotype A, (b) Chemotype B, (c) Chemotype C, (d) Chemotype E (new) at the single plant level: SBA, silybin A; SBB, silybin B; ISBA, isosilybin A; ISBB, isosilybin B; SC, silychristin; SD, silydianin.

Silymarin standard was purchased from Sigma-Aldrich company (Sigma, S0292; Fig. 1d). Concentrations of 1000, 500, 250, 125, 62.5 and 31.25 µg/ml in 43% methanol were used to plot the calibration curve and obtain the area under the peak and concentration equation. The total silymarin content was also obtained from the sum of the individual components of silymarin (sum of single flavonolignans) (Fig. 3).

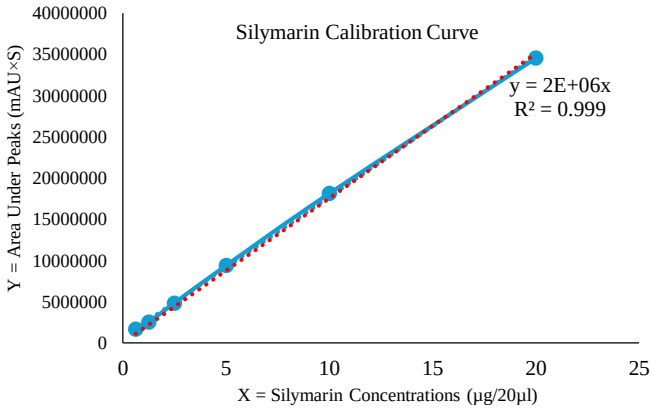

Fig. 3 Silymarin calibration curve.

RESULTS AND DISCUSSION

At the level of individual plants, previous studies have consistently identified only three distinct chemotypes. Chemotype A typically shows roughly equal amounts of silychristin, silybin A, and silybin B (around 30% each), with very low levels of silydianin (Table 2). In contrast, chemotype B is dominated by silydianin (about 75%) with lower amounts of the other compounds. Chemotype C displays an intermediate silymarin composition compared to A and B (Table 2 [13], Fig. 2). In this study, eight populations (5, 8, 12, 15, 17, 22, 35, and 37) were composed entirely of chemotype B plants, while ten populations (11, 19, 21, 23, 24, 30, 32, 34, 36, and 38) contained a heterogeneous mixture of plants with various chemotypes (Table 3).

Table 2 Silymarin chemotype and content at the single plant level [13].

		Total silymarin	SC	SD	SBA	SBB	ISBA	ISBB
	n	(mg/g fruit dry/wt)	(% of total silymarin)					
Chemotype A	29	40.27	29.99	trace	25.34	31.67	9.27	3.73
±*SD*		4.91	0.86	-	0.36	0.53	0.41	0.45
Chemotype B	42	34.69	2.89	74.35	2.36	4.9	8.14	7.36
±*SD*		8.56	0.18	1.87	0.57	0.63	0.47	0.58
Chemotype C	6	39.83	15.24	36.63	15.98	19.09	8.21	4.86
±*SD*		3.48	1.16	3.97	1.31	1.58	0.3	0.27

n, number of samples; SD, standard deviation; SBA, silybin A; SBB silybin B; ISBA, isosilybin A; ISBB, isosilybin B; SC, silychristin; SD, silydianin.

In general, the chemotype identified at the individual plant level matched the overall profile observed at the population level. Mixed populations contained plants with chemotypes A, B, and C, while those identified as chemotype B at the population level were made up exclusively of plants with chemotype B. No populations were found where all plants were chemotype A. Since chemotype C results from crosses between A and B and is genetically unstable [20], populations consisting solely of C-type plants do not occur.Silymarin content varied among populations, ranging from 21.87 to 47.94 mg/g dry/wt, with an average of 32.68 ± 2.46 mg/g. The highest levels were found in populations 23 (47.94 mg/g), 34 (42.51 mg/g) and 19 (40.48 mg/g), while the lowest were in populations 21 (21.87 mg/g), 24 (24.63 mg/g), and 17 (25.41 mg/g) (Table 3).

Table 3 Table of silymarin chemotype and content for the studied populations and single plants

Population No.	Population Chemotype	Silymarin Content (mg/g dry/wt)	Single Plant Chemotype	±SD
Pop. 5	B	35.56	-	± 2.37
Pop. 8	B	36.08	-	± 2.83
Pop. 11	Mix	33.83	-	± 1.39
Pop. 12	B	31.88	-	± 2.57
Pop. 15	B	28.68	-	± 2.81
Pop. 17	B	25.41	-	± 3.19
Pop. 19	Mix	40.48	-	± 2.41
Pop. 21	Mix	21.87	A, A, B, B	± 2.95
Pop. 22	B	30.08	-	± 3.16
Pop. 23	Mix	47.94	A, A, A, B	± 1.04
Pop. 24	Mix	24.63	-	± 2.98
Pop. 30	Mix	32.73	A, A, E, E	± 1.51
Pop. 32	Mix	37.96	-	± 2.65
Pop. 34	Mix	42.51	A, A, C, C	± 3.05
Pop. 35	B	28.26	-	± 2.31
Pop. 36	Mix	36.51	-	± 3.03
Pop. 37	B	28.36	-	± 2.73
Pop. 38	Mix	25.52	-	± 1.45

SD, standard deviation; A, chemotype A; B, chemotype B; C, chemotype C; E, chemotype E.

Because all plants were cultivated under the same environmental conditions, these differences are mainly due to genetic variation rather than local environmental factors. Similar findings were reported for plants grown under uniform conditions in Italy, where silymarin content ranged from 33.94 to 49.92 mg/g dry/wt, with an average of 41.89 mg/g [12]. This pattern aligns with Martinelli *et al*. (2021a), who found silymarin levels between 21.47 and 51.41 mg/g dry/wt (average 38.72 mg/g) in Italian wild populations, with no clear geographic clustering of high or low content [13]. By comparison, Greek wild populations showed a lower average (23.06-77.12 mg/g, average 33.11 mg/g) [21], while Pakistani populations had even lower levels (4.5-23.6 mg/g, average of 14.01 mg/g) [22].

Analysis at the individual plant level, covering 16 plants from 4 populations, showed silymarin contents between 27.09 and 51.54 mg/g dry/wt, with all three main chemotypes (A, B, and C) present (Table 3). Consistent with previous studies [13], no substantial differences in total silymarin content were found among these chemotypes. Notably, two individual plants from population 30 revealed a novel chemotype, described here for the first time and designated as chemotype E (Table 3, Fig. 2d). This designation follows Pasquariello *et al*. (2025), where silymarin chemotype D was assigned to *S. eburenum* [19]. The new E chemotype has a unique HPLC profile, distinct from A, B, and C, with relative contents of silychristin (21.5%), silydianin (22.3%), silybin A (15.9%), silybin B (26.0%), isosilybin A (9.6%), and isosilybin B (4.6%). Importantly, population 30 was not included in Pasquariello *et al*.'s study [19], and it remains to be studied whether chemotype E results from crosses of other chemotypes, like the case of chemotype C [20]. Further research on its progeny will help clarify this aspect.

In Iran, the various chemotypes are scattered across multiple regions, with no clear link between chemotype and geography (Fig. 4). This matches earlier reports of discontinuous chemotype distribution [13, 19]. Chemotype B was the most common (54.5% of cases), while the newly identified chemotype E was the rarest, appearing in about 12.5% of samples. Notably, Iran is unique in showing four distinct chemotypes within a single country. Other studies have only documented A, B, and their hybrid C [19]. This broader chemical diversity suggests higher overall biodiversity in Iran, supporting the idea that this region may be included in the species' center of origin. Further genetic studies are needed to confirm this.

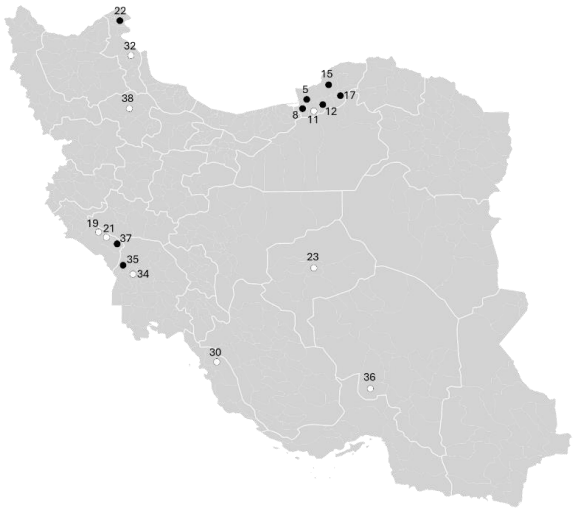

Fig. 4 Distribution map for the studied milk thistle populations in Iran. Black dots: Chemotype B, White dots: mix of plants with different chemotypes.

CONCLUSION

This research identified four distinct chemotypes (A, B, C, and the new E) in wild Iranian milk thistle populations. Stable chemotypes A and B appeared widely across regions, sometimes coexisting within the same population. No clear link was found between chemotype distribution and geography. The discovery of the new chemotype E highlights Iran's significant milk thistle diversity, suggesting the region could be part of the species' center of origin. These findings provide a foundation for further characterizing stable chemotypes and support breeding efforts to enhance the yield and quality of this valuable phytochemical.

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Authors Contributions:

Mirzaabolghasemi, M.A.: Conceptualization, Project administration, Data curation, Formal analysis, Resources, Writing - original draft preparation, review and editing, Investigation, Visualization, Software calculations, Funding.

Martinelli, T.: Methodology, Supervision, Advice, Validation, Editing.

Pirmoradi, M.R.: Supervision. Raghami, M.R.: Editing.

Conflict of Interest

The authors state that they have no financial or personal conflicts that could have affected the results or interpretation of this study.

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Data and Material Availability Statement

Most data generated or analyzed during this study are included in this article. All data will be made available upon request.

Ethics Approval and Consent for Publication

All the ethical protocols for working were followed and all authors consent to the publication of this article.

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