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Analysis of silybin A and silybin B in different accessions of *Silybum marianum* seeds

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Abstract *Silybum marianum* more commonly known as milk thistle is one of the most well-researched medicinal plants, particularly for the treatment of liver disease. Silbyin A and silybin B are compounds found in the silymarin complex, the phytochemical responsible for the bioactivity of *S. marianum*. Silymarin is currently widely used as a dietary supplement. In this study, the contents of the silybin mixture (silybin A and silybin B) in different accessions of *S. marianum* seeds were determined using high-performance liquid chromatography (HPLC). The aqueous extracts of *S. marianum* were evaporated and resuspended with HPLC-grade 70% acetonitrile for the analysis. The silybin mixture was quantified using a reverse-phase column with a gradient elution system and a wavelength of 288 nm. Among a total of 14 samples (samples S1-S14) of *S. marianum* from different accessions, samples S4, S1, and S7 showed particularly high concentrations of silybin A and B, ranging from approximately 19 to 27 mg/g ext. Determining the presence and quantifying the content of the silybin mixture in *S. marianum* seeds is crucial to identify alternative and optimal sources of therapeutic compounds, thereby increasing the number of available medicines.

Keywords Silybin A · Silybin B · *Silybum marianum* · Quantitative analysis

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Introduction

Silybum marianum (L.) Gaertn is an annual or biennial plant belonging to the family Asteraceae [1]. This plant species is also commonly known by various names such as milk thistle, Mary thistle, Marian thistle, Saint Mary's thistle, Mediterranean milk thistle, variegated thistle, and Scotch milk thistle [2]. Originally native to southern European regions and Asia, this plant has become a cosmopolitan species found in Africa, North and South America, and Australia [3]. In addition to its reddish-purple flowers, this species is characterized by the white patches, or marbling, found along the veins of its dark green leaves [4].

S. marianum is valued worldwide for its bioactive properties [5], including antioxidant [6], immunomodulatory [7], and anticancer [8] activities. In particular, it is widely recognized for its hepatoprotective effects. Used since ancient times to treat various liver and gallbladder disorders such as hepatitis, cirrhosis, and jaundice, milk thistle has also been widely used to protect the liver against the toxic effects of snake bites, insect stings, mushroom poisoning, and alcohol [9].

Silymarin, a polyphenolic complex of various flavonolignans, is responsible for these bioactive effects [10]. Among the flavonoids in silymarin, silybin is considered the most active compound and is primarily responsible for its documented benefits [11]. Silybin consists of two diastereomeric compounds, silybin A and silybin B, which occur in a 1:1 ratio [12]. These compounds have iron chelating properties with contribute to their hepatoprotective

effects. Silymarin, derived from milk thistle, consists of several flavonolignans, including silychristin, silydianin, silybin A, silybin B, isosilybin A, and isosilybin B [13]. Among these, the two diastereoisomeric compounds silybin A and B are the major constituents of silymarin, accounting for approximately 70% of all silymarin compounds, and are the most significant contributors to its biological effects [14].

In recent years, a number of herbal supplements containing silymarin have been on the market. However, the production costs of these supplements are prohibitively high, as silymarin only accounts for approximately 1.5%-3% of the chemical composition of milk thistle [15]. Notably, the silymarin content is more concentrated in the seeds than in other parts of the plant [16]. However, the phytochemical profile of plants can vary depending on their source. This variation may be due to the environmental conditions in the habitat where the plants were grown.

Therefore, our study aimed to differentiate and quantify the silybin A and silybin B content in different accessions of *S. marianum* seeds. The results of this study contribute to the rigorous breeding of *S. marianum* for the selection of optimal silymarin sources.

Materials and Methods

Plant materials

A total of *S. marianum* seeds (samples S1-S14) from different accessions were provided by the National Institute of Agricultural Sciences, Rural Development Administration (RDA), Korea, and EL&I Co., Ltd., Korea (Table 1). Among them, S1-S5 were provided by RDA, Korea, while S6-S14 were provided by EL&I Co., Ltd. and collected from Pyeongtaek Herbal Farm (latitude: 36.9954425º; longitude 126.9456º), Korea (Fig. 1A). All seeds were cultivated in Hwaseong, Gyeonggi-do, Korea by EL&I Co., Ltd. (2019) (Fig. 1B). A voucher specimen was deposited at the herbarium of the National Institute of Agricultural Sciences, RDA, Korea.

Instruments, chemicals, and reagents

Chromatographic analysis was performed using a high-performance liquid chromatography (HPLC) system (Agilent technology 1260 Infinity II, Santa Clara, CA, USA) equipped with a pump, autosampler, and UV detector. The solvents used for HPLC {water and acetonitrile (ACN)} were purchased from J. T. Baker (Phillipsburg, PA, USA). Acetic acid was purchased from Samchun Pure Chemicals (Pyeongtaek, Korea). The silybin mixture was obtained from the Natural Product Institute of Science and Technology (www.nist.re.kr), Anseong, Korea (Fig. 2).

Sample preparation

All *S. marianum* seed samples (S1-S14) were dried prior to extraction. Next, two hundred grams of dried *S. marianum* seeds (S1-S14) were extracted using distilled water under reflux for 5 h. The samples were then evaporated and dried in a lyophilizer to a weight of 1.6 g. Next, 1 mg of *S. marianum* extract was dissolved in 70% ACN under sonication for 20 min as an experimental stock solution and filtered using a 0.45-μm PVDF membrane filter. Similarly, 1 mg of silybin mixture was dissolved in 70% ACN under sonication for 20 min and filtered using a 0.45-μm PVDF membrane filter.

HPLC condition

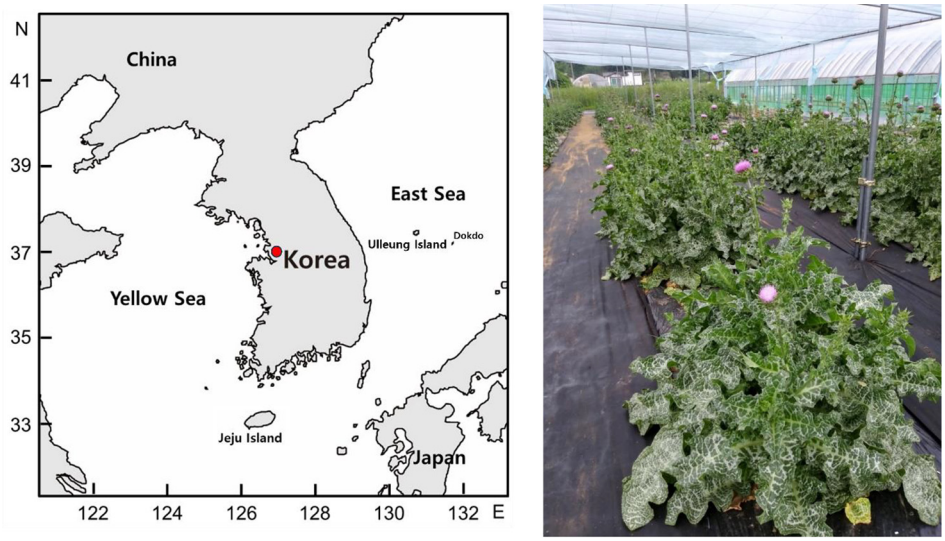
Quantitative analysis of the silybin mixture was performed using a reverse-phase HPLC system with an INNO C18 column (25 cm ×4.6 mm, 5 μm). The injection volume was 10 μL and was monitored at 288 nm. The column was kept at room temperature and the flow rate was set at 1 mL/min. The mobile phase of the gradient elution system consisted of 0.5% acetic acid in water (A) and ACN (B). The composition of the elution system throughout the analysis process was as follows: 83% A at 0 min, 70% A at 10 min, 70% A at 25 min, 20% A at 30 min, 100% B at 35 min, 100% B at 40 min, 83% A at 50 min, and 83% A at 55 min.

Calibration

Standard stock solutions were prepared by dissolving the reference

Table 1 Plant characteristics in different accessions of *S. marianum* seeds

Sample	Provider	Collection site	Characteristics
S1	RDA	South Korea	relatively long spine in the involucre
S2		Canada	relatively early bolting
S3		Germany	early bolting
S4		North Korea	tall plant height
S5		Moldova	wide involucre width, short spine in the involucre
S6	EL&I Co., Ltd.	South Korea (Pyeongtaek)	short plant height
S7			long spine in the involucre, shiny dark-colored seeds
S8			simultaneously ripening, low number of flower
S9			shiny and dark-colored seeds
S10			relatively high yield
S11			relatively late ripening
S12			high yield, low one hundred seed weight
S13			soft bract, short spine in the involucre
S14			late bolting, light-colored seeds



(A) (B)

Fig. 1 Collection site of S6-S14 (A) and cultivation of different accessions of *S. marianum* seeds (B) by EL&I Co., Ltd.

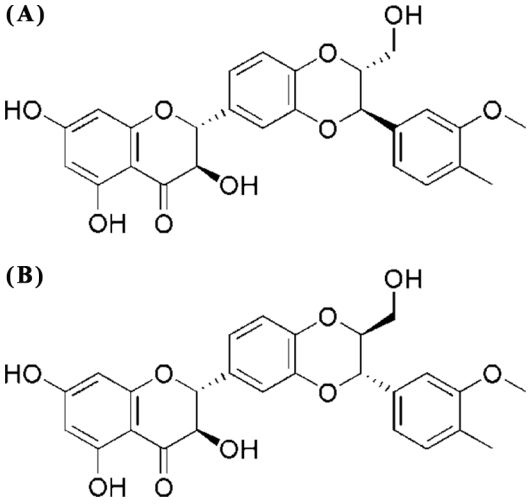


Fig. 2 Chemical structures of silybin A (**A**) and silybin B (**B**)

compound in 70% ACN (1 mg/mL). The working solutions used to construct the calibration curve were prepared by serial dilution of the selected stock solutions to the desired concentrations. The sample was also dissolved in 70% ACN (10 mg/mL). Both the standard and sample solutions were filtered using a 0.45-µm PVDF filter prior to their use. Calibration functions for the silybin mixture were calculated based on peak areas (Y), concentrations (X, µg/10 µL), and mean values ± standard deviation (SD) (n =5).

Statistical analysis

Results are expressed as mean ± SD and all analyses were performed in triplicate. All data were analyzed via one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. All statistical tests were performed using the GraphPad Prism 8.0.2 software (GraphPad Software, Boston, MA, USA). *p*-Values <0.05 were considered statistically significant.

Results and Discussion

HPLC analyses were conducted to quantify the silybin mixture content in different accessions of *S. marianum* seeds. Quantitative analyses were performed using a reverse-phase system and gradient elution of the silybin mixture in the mobile phase. Standard calibration curves for the silybin mixture are shown in Table 2. A wavelength of 288 nm was determined to be effective for the detection and quantification of the silybin mixture. The HPLC conditions were optimized to examine the quantitation parameters of the compounds. In addition, the calibration curve of the separated compounds was established by linearly plotting the peak area of the prepared concentrations and evaluated using linear regression analysis. The mixture of compounds studied showed an excellent regression coefficient (r^2) of 1.0000. The results showed a good separation, with retention times detected at 26.448 and 27.150 min, corresponding to silybin A and silybin B, respectively (Fig. 3).

Furthermore, the silybin mixture was also detected in the samples, as shown by the chromatographic peaks in Fig. 4. As shown in Table 3, the silybin mixture content of the samples varied greatly, leading to the formation of different groups based on the concentrations detected. Particularly, samples S4, S1, and S7 showed uniquely high silybin concentrations ranging from roughly 19.2 to 27.6 mg/g ext. The silybin concentrations of all three of the aforementioned samples were statistically different

Table 2 Calibration curve of silybin mixture

Compound	Calibration equation [a]	Correlation factor, r^2 [b]
Silybin mixture	Y = 23.229X + 98.385	1.0000

[a] Y = peak area, X = concentration of standard (μg/mL)
[b] r^2 = correlation coefficient for five data points in the calibration curve

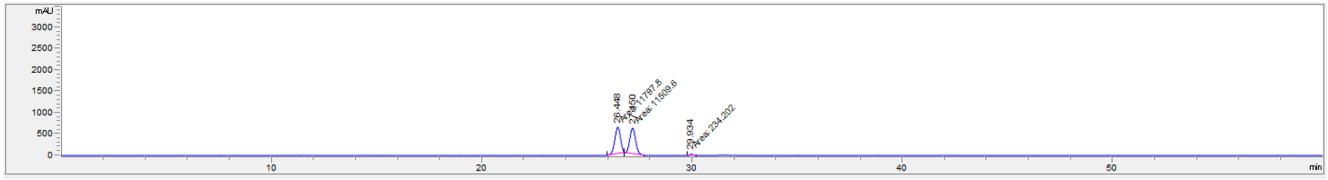
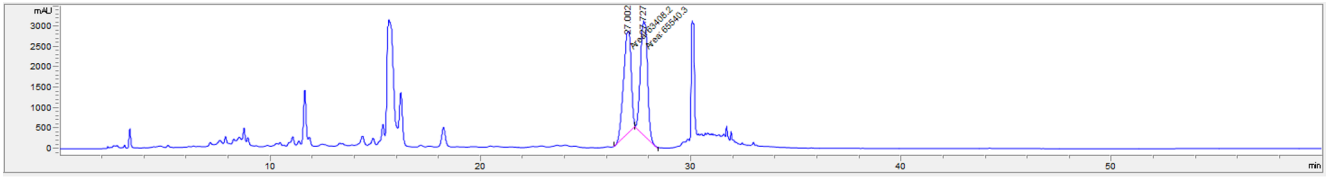
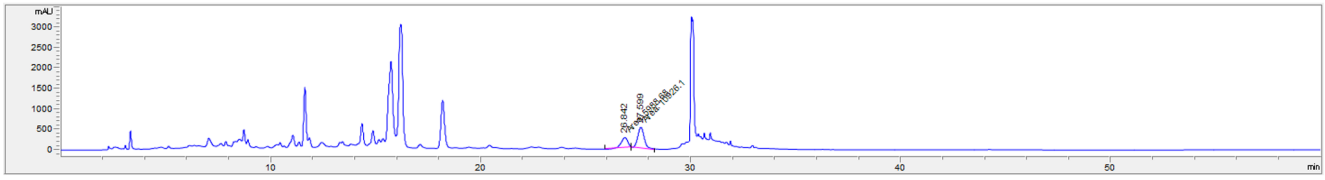


Fig. 3 HPLC chromatogram of silybin mixture (silybin A: 26.448 min, Silybin B: 27.150 min)



(A)



(B)

Fig. 4 HPLC chromatograms of S4 (A) and S10 (B)

from each other and from the rest of the samples. These three samples probably represent setups or processes that yield exceptionally high silybin mixtures. In contrast, samples S2, S3, S10, and S11, had the lowest silybin concentrations in the group, ranging from approximately 2.3 to 4.0 mg/g ext. These results suggest that variations in treatment settings or extraction techniques influence the silybin content of milk thistle seeds.

Considerable variation was observed in the silybin mixture contents of the examined samples examined (S5, S9, S13, and S14), even within the same accessions. This suggests that there may be slight variations in the experimental protocols. Furthermore, samples S6, S12, and S8, which fall between the higher and lower concentration groups, showed a moderate level of silybin mixture. However, all samples except for S4, S1, and S7 were not significantly different from each other. These findings underline the complexity of the variables affecting silybin mixture content and highlight the importance of careful experimental design and interpretation in studies aimed at quantifying silybin mixture.

A similar study investigated the chemical diversity of *S. marianum* from different locations in Egypt, examining the silymarin content of fruits based on fruit age, variety, and location [17]. Using HPLC with qNMR-controlled reference standards, the authors found significant substantial differences in silymarin content between samples, and different clusters were identified according to silymarin composition. The highest silymarin content was found in samples from the Nile Delta, Egypt, with no discernible relationship between fruit development stage and silymarin levels. However, their study quantified more compounds in the silymarin complex, whereas our study only quantified the silybin mixture. Additionally, the levels of silybin A and silybin B in all *S. marianum* samples collected in the aforementioned study were significantly different from those in the present study. Specifically, the silybin A and silybin B content in their samples ranged from 0.14 to 1.90 mg/g, whereas the silybin A and silybin B content in our study ranged from 2.3 to 27.6 mg/g in ext. and 0.5 to 5.9 mg/g in dry weight (DW).

Another study also attempted to differentiate the silymarin content of the same plant from different locations in Syria [18]. Their results showed considerable regional heterogeneity. Similar to the previous study, they also quantified more compounds from the silymarin complex. The authors reported lower levels of silybin A and silybin B (ranging from 0.10 to 0.39 mg/g DW and

Table 3 Contents of silybin mixture in different accessions of *S. marianum* seeds

Sample	Content	
	mg/g ext.	mg/g DW
S1	19.23±0.05^{c}	4.28±0.02^{c}
S2	2.27±0.02^{d}	0.46±0.00^{d}
S3	2.49±0.01^{d}	0.48±0.00^{d}
S4	27.55±0.11^{a}	5.85±0.03^{a}
S5	3.43±0.72^{d}	0.63±0.19^{d}
S6	4.95±0.46^{d}	0.93±0.12^{d}
S7	25.45±0.86^{b}	3.89±0.18^{b}
S8	4.08±0.51^{d}	0.61±0.11^{d}
S9	3.52±0.83^{d}	0.62±0.21^{d}
S10	2.57±0.22^{d}	0.40±0.05^{d}
S11	3.97±0.53^{d}	0.73±0.14^{d}
S12	4.38±0.59^{d}	1.07±0.20^{d}
S13	3.70±0.40^{d}	1.23±0.19^{d}
S14	3.51±0.51^{d}	0.69±0.14^{d}

All data were analyzed using a one-way ANOVA followed by Tukey's post hoc test. Values with p <0.05 were considered to be statistically significant.

0.05 to 1.10 mg/g DW, respectively) than in the present study, which were even lower than those reported by Abouzid et al. [18]. These differences in the results of different studies highlight the influence of environmental conditions on silymarin synthesis and the usefulness of the plant as a source of this pharmacologically active chemical, suggesting possible ecotypic differences in *S. marianum*.

This also supports the notion that the levels of phytochemical constituents in S. *marianum* vary in an accession-dependent manner. The concept of ecotype may explain for the significant variation in silybin A and silybin B levels. Ecotypes are populations of plants adapted to specific environmental conditions [19]. Different ecotypes can be accurately identified by genetic means [20-21]. For example, samples from the Nile Delta, which has distinct environmental characteristics, had highest silymarin content, suggesting that *S. marianum* ecotypes may have adapted locally to the ecological niche of the delta.

The quantification of silybin A and silybin B in *S. marianum* seeds is of vital importance in many industries. Pharmacologically, these substances are the major bioactive components of silymarin, which is known for its potent antioxidant and hepatoprotective properties. Accurate quantification therefore provides vital information on the medicinal potential of *S. marianum* seeds in the treatment of conditions such as liver disease. Furthermore, silybin A and silybin B serve as essential markers in the field for quality control, ensuring the consistency and legitimacy of herbal products derived from *S. marianum*.

From an economic point of view, the measurement of silybin A and silybin B in silymarin will allow an accurate assessment of the value of *S. marianum* seeds, facilitating informed decision-making and optimizing their commercial potential in the nutraceutical, cosmetic, and pharmaceutical sectors.

Finally, the results of this study can contribute to research to support agricultural operations by guiding cultivation strategies and cultivar selection for maximum silymarin yield. The measurement of these chemicals thus underscores their importance in advancing medicinal plant research, product development, and sustainability from an economic perspective.

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