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HPLC 분석을 통한 한국산 밀크씨슬 중 실리마린과 실리빈의 정량

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Determination of Silymarin and Silybin Diastereomers in Korean Milk Thistle using HPLC/UV Analysis

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Abstract – Silymarin (SM) and silybin diastereomers (SD) in milk thistle (*Silybum marianum*) were determined using high-performance liquid chromatography and quantified using a reverse-phase column in a gradient elution system. UV detection was performed at 288 nm. The content of SM and SD in milk thistle was 3.236 and 0.553 mg/g DW, respectively. Determining the presence and quantifying the content of SM and SD in milk thistle are vital for the pharmaceutical industry to identify optimal sources for developing health supplements or therapeutics.

Keywords – HPLC, Silybin diastereomer, *Silybum marianum*, Silymarin

Milk thistle (*Silybum marianum* L.) is an annual or biannual plant of the Asteraceae family. It was originally native to Southern Europe and Asia; but, now it is found worldwide. Milk thistle also has other common names, such a Marian thistle, blessed milk thistle, Mary thistle, Carduus Marianus, and Saint Mary's thistle.[1)] It is one of the most valuable medicinal plants for the pharmaceutical industry. Since ancient times, milk thistle has been used in traditional medicine mainly for treating liver disease.[2,3)] Its seeds have been reported to possess anti-inflammatory, immunomodulatory, anti-viral, and other therapeutic properties.[4)]

Silymarin (SM) derived from milk thistle has been used extensively for centuries to protect the liver from toxins.[5)] It is useful in treating or preventing liver diseases.[6)] In addition, SM exhibits significant anti-oxidant activity as observed from several *in vitro* assays. It can prevent or minimize lipid oxidation, retard the formation of toxic oxidation products,[7)] and possess hypopigmentary effects.[8)] Recent reports suggest SM inhibits the progression of Alzheimer's disease symptoms.[9)] SM principally consists of a mixture of active flavonolignans, including silydianin, silychristin, two diastereomers of silybin (silybins A and B), two diastereomers of isosilybin (isosilybins A and B), and taxifolin.[10,11)] Among them, flavonolignans have anti-inflammatory, anti-fibrotic, hypolipidemic, and neuroprotective effects.[12)] In addition, silybins A and B are silybin diastereomers (SD). SD also has anti-oxidant, hepatopro-tective,[13)] anti-inflammatory, and anti-fibrogenic effects.[14)]

Determining and quantifying the content of SM and SD in milk thistle is vital for the pharmaceutical industry to identify optimal sources for developing health supplements or therapeutics. In this study, the quantitative analysis of SM and SD was performed using high-performance liquid chromatography (HPLC).

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Materials and Methods

Plant Materials – Milk thistle seeds (Fig. 1) were provided by the Imsil Herbal Medicine Association (2020), Imsil 55955, Korea and identified by Dr. C. G. Park, National Institute of Horticultural and Herbal Science, South Korea. A voucher specimen (No. LEE 20-01) was deposited at the herbarium of the Department of Plant Science and Technology, Chung-Ang University, Korea.

Instruments, Chemicals, and Reagents – Chromatographic analysis was performed using an HPLC system (Agilent technology 1290 Infinity II) equipped with a pump, an auto-sampler, and a UV detector (Santa Clara, CA, USA) with an INNO C18 column (25 cm × 4.6 mm, 5 μm). SM and SD (Fig. 2) were obtained from the Natural Product Institute of Science and Technology (www.nist.re.kr), Anseong 17546, Korea. Solvents used for HPLC (acetonitrile (ACN) and water) were purchased from J. T. Baker (Avantor, Radnor, PA, USA). Acetic acid (99.7%) was purchased from Samchun Pure Chemicals (Pyeongtaek, Korea).

Fig. 1. Korean milk thistle.

Sample Preparation and Stock Solution – Dried milk thistle seeds (20 g) were extracted in distilled water under reflux for 5 h. The samples were dried using a freeze dryer to obtain 1.6 g of extract. The experimental stock solution was prepared by dissolving 1 mg of milk thistle extract in 70% ACN, sonicated for 20 min, and filtered using a 0.45-μm PVDF membrane filter. For the standard stock solution, 1 mg of each of SM and SD was dissolved in 70% CAN, sonicated for 20 min, and filtered using a 0.45-μm PVDF membrane filter.

HPLC Conditions – Quantitative analyses of SM and SD were performed in a gradient elution HPLC system using a reverse-phase INNO C18 column (4.6 mm × 25 cm, 5 μm). The injection volume was 10 μL, and the UV detection wavelength was 288 nm. The column temperature was maintained at room temperature and the flow rate was set at 1 mL/min. The mobile phase gradient elution system consisted of 0.5% acetic acid in water (A) and acetonitrile (B). The elution system 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 Curves – Standard stock solutions of SM and SD were prepared by dissolving the compounds in 70% ACN (1 mg/mL). The working solutions used to construct the

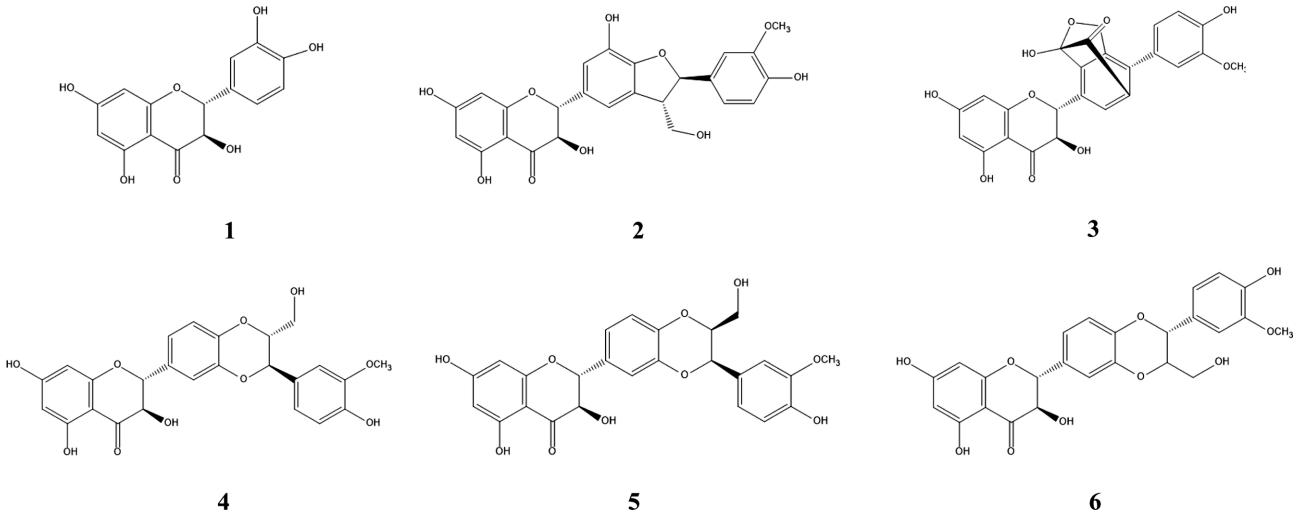

Fig. 2. Chemical structures of SM [taxifolin (**1**), silychristin (**2**), silydianin (**3**), silybin A (**4**), silybin B (**5**), and isosilybin (**6**)].

calibration curve were prepared by serially diluting the selected stock solutions to the desired concentrations. The calibration functions of SM and SD were calculated using the peak area (Y) and concentration (X, mg/mL), and represented as mean values ± standard deviation (n = 3).

Results and Discussion

Quantitative analysis of SM and SD was performed using HPLC/UV with a reverse phase column and gradient elution of solvents A and B in the mobile phase. The HPLC method showed good separation, and a wavelength of 288 nm was found to be optimal for the detection of SM and SD. The calibration curves of standard SM and SD are shown in Table I. The calibration curves were constructed by plotting the peak area against the prepared concentrations and were analyzed using linear regression. The linear regression coefficients (r^2) for SM and SD were 0.9997 and 0.9996, respectively.

Chromatographic peaks of SM i.e. taxifolin, silychristin, silydianin, silybins A and B, and isosilybin showed good separation with retention times 11.835, 15.898, 16.467, 27.661, 28.463, and 30.148 min, respectively (Table II). Chromatograms of SM, SD, and the milk thistle extract are shown in Fig. 3. Table III shows the content of SM and SD in milk thistle extracts.

Table I. Calibration curves of silymarin (SM) and silybin diastereomer (SD)

Compound	Calibration equation[a]	Correlation factor, r^{2b}
SM	Y = 13.627X + 77.771	0.9997
SD	Y = 13.559X + 87.762	0.9996

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

The primary active component of milk thistle, SM, acts mainly as a hepatoprotective and an anti-oxidant activity.[15,16] SM in milk thistle is a potential source for functional foods and cosmetics. Further, because of its high bioavailability, it can also be developed into drugs.[17,18] Tayoub *et al.* (2018) conducted a quantitative analysis of milk thistle harvested from four different locations.[19] They reported that the total

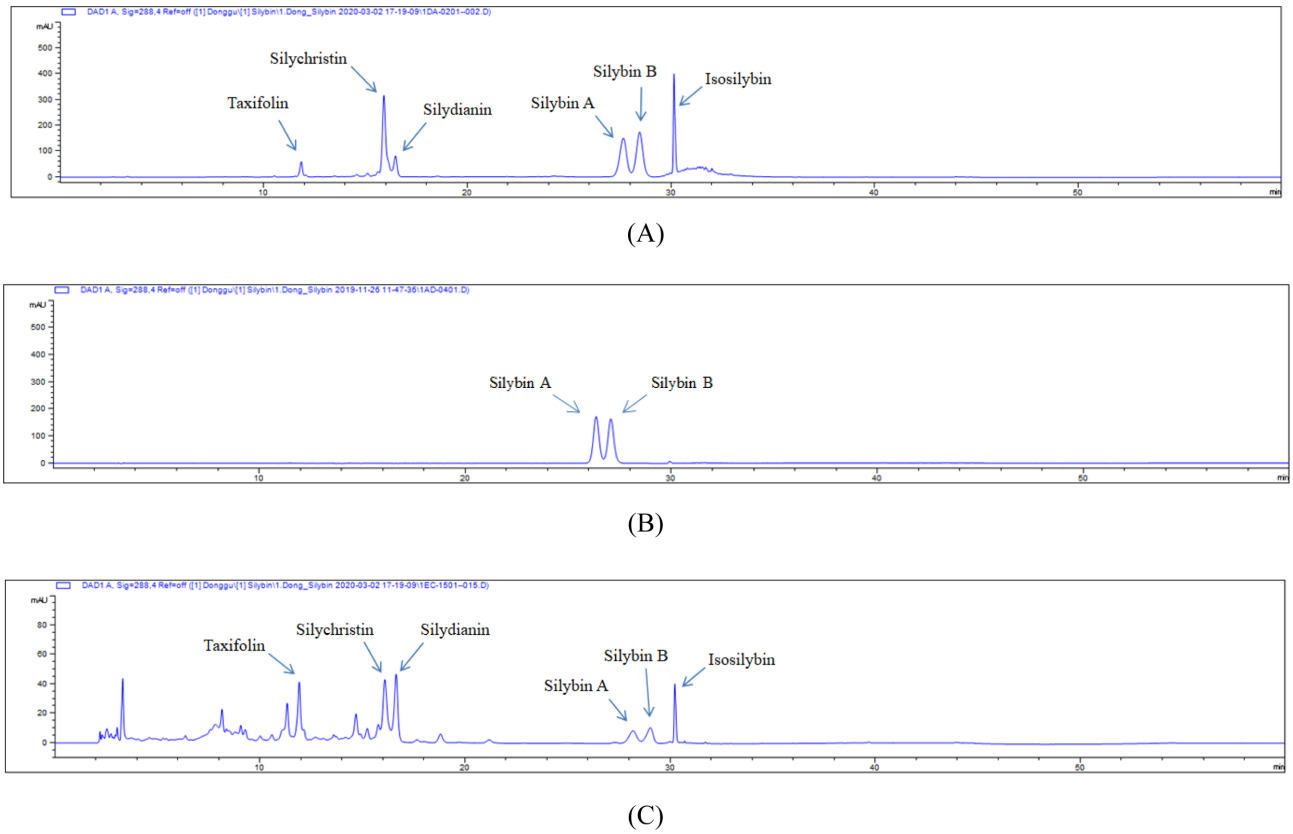

Fig. 3. HPLC chromatograms of SM (A), SD (B), and milk thistle extract (C).

Table II. Components of SM in milk thistle

SM	t_R
Taxifolin	11.835
Silychristin	15.898
Silydianin	16.467
Silybin A	27.661
Silybin B	28.463
Isosilybin[a]	30.148

[a]Sum of isosilybin diastereomers (isosilybin A and isosilybin B)

Table III. Content of SM and SD in milk thistle

Compound	Content (mg/g FD)	Content (mg/g DW)
SM	40.792 ± 0.409	3.236 ± 0.033
SD	6.915 ± 0.479	0.553 ± 0.038

SM content in seeds ranged from 0.54% to 2.91% for the tested accession sites, indicating relatively higher content than that reported in the present study. This finding may be due to differences in the locations of the milk thistle accessions. The results of quantitative analysis by Radjabian *et al*. (2008) showed that the amount of total SM varied from 23.98% to 45.46% in four different ecotypes.[20] The SM content in the four ecotypes was not significantly different. Likewise, the Radjabian study is similar to the present study. They found that the highest amounts of SD in two populations of the four ecotypes were 24.86% and 19.74%, respectively. Rodriguez *et al*. (2018) quantified SD and provided milk thistle from the same association as the present study.[21] But they reported that the SD content in seeds was 7.434 mg/g DW, indicating significantly higher content than that reported in the present study. This finding may be due to differences in the solvents for extraction and experimental stock solution. Zhang *et al*. (2018) quantified the total SM and each compound in milk thistle from different origins. The results showed that the SM content varied by places of origin, and was found to be between 1.64 and 4.97 g/100g. Silybin B content was generally higher than other compounds. Comparing with Zhang study, it can be seen that the SM content in the present study is sufficiently high.

In conclusion, SM and SD were detected in milk thistle seeds using HPLC/UV. Quantitative analyses of SM and SD could help identify possible sources of drugs that can be developed by the pharmaceutical industry.

Acknowledgments

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