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Antioxidant activities and silymarin content of *Silybum marianum* using different extraction methods

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Abstract Silymarin in the leaves, roots, and seeds of *Silybum marianum* (milk thistle) were analyzed by reversed-phase high-performance liquid chromatography and an ultraviolet detector. The content of silymarin and its constituents (taxifolin, silydianin, and silybin) was compared using water and ethanol (EtOH) extracts. Silymarin was only detected in the seeds of the extracts, whereas the silymarin content in the EtOH extract was higher than in the water extract. The antioxidant activity via 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^+$) radical scavenging activities of milk thistle and its constituents was evaluated. The EtOH extract of seeds, which contained high level of taxifolin and silybin, showed higher radical scavenging activity than other parts of the EtOH extract and water extract. In addition, silymarin and its individual constituents (taxifolin, silydianin, and silybin) showed antioxidant activity as DPPH and $ABTS^+$ radical scavengers. In particular, taxifolin showed a higher radical scavenging activity than other constituents from the seeds. Therefore, it has been suggested that *S. marianum* and its active constituents could be a useful material for antioxidant-related various diseases.

Keywords 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid · Antioxidant · 1,1-Diphenyl-2-picrylhydrazyl · *Silybum marianum* · Silymarin

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Introduction

Silybum marianum L. (milk thistle) is an annual or biennial plant of the Asteraceae family [1] and is well known for its hepato-protective properties [2]. Milk thistle is native to southern Europe and Asia, but it is now found all over the world. The scientific name of milk thistle, "*S. marianum*", comes from the belief that the white veins in the leaves contain the milk of the Virgin Mary. Milk thistle has been used since ancient times to treat liver and gallbladder disorders, including cirrhosis [3] and hepatitis [4], and to protect the liver from poisoning by chemical and environmental toxins such as insect stings, snake bites, and mushroom poisoning [1]. Milk thistle may also be effective in the treatment of alcoholic, cholestatic, viral, and primary malignant liver diseases [5]. In addition, it also has anti-diabetic, immunomodulatory, antiviral, anti-inflammatory, and other therapeutic properties [6,7]. Several studies have analyzed the active constituents of milk thistle, such as apigenin, betaine, and silymarin [8]. Milk thistle seeds consist of oil (26%), crude fiber (5%), moisture (4%), carbohydrates (87%), ash (2%) and total protein (23%) [9]. Milk thistle seeds contain a variety of constituents, but the key

constituent is silymarin.

In particular, silymarin is well known for its antioxidant and hepatoprotective properties [10] and has also been shown to provide some protection against breast, prostate, and ectocervical cancers [11,12]. Silymarin consists mainly of a flavanol (taxifolin), isomer flavonolignans (silydianin and silychristin), two diastereomers of silybin (silybin A and silybin B), and two diastereomers of isosilybin (isosilybin A and iso-silybin B) [13,14]. Silymarin has been shown to minimize lipid oxidation and inhibit the formation of toxic oxidation products [15]. It has also been found to stabilize cell membranes, preventing toxins from entering and releasing toxic chemicals [16,17]. Silybin also acts as an aldose reductase inhibitor and may reduce diabetic complications such as diabetic nephropathy, diabetic neuropathy, and steatohepatitis [7]. Recently, some of the other beneficial properties of silymarin, such as inhibiting the progression of Alzheimer's disease, have also been investigated [18]. Although silymarin is found throughout the entire milk thistle plant, it is particularly concentrated in the seeds [19]. In addition, many more studies have been conducted on the silymarin content of milk thistle seeds compared to other parts of the plant, such as the leaves and roots.

Oxidative stress has been implicated in the pathogenesis of diseases such as cancer, cardiovascular and Alzheimer's diseases [20]. Free radicals are unpaired electrons that are widely known to cause oxidative stress [21]. The overproduction of free radicals can damage biological molecules such as proteins, lipids, and DNA, resulting in oxidative stress [22]. Under normal conditions, free radicals are generated by many systems such as aerobic cellular metabolism [23], which means that the body needs to balance oxidation and antioxidants to defend against oxidative stress [24]. Natural product-derived antioxidants are known to exhibit antioxidant activity by donating or transferring electrons to scavenge radicals [25,26]. Therefore, many studies have investigated the antioxidant activities of natural products and their derived active compounds such as phenolic metabolites [25,26]. Previous studies have reported on the antioxidant activity of milk thistle and its active constituents. Dietary supplements of milk thistle and natural silymarin may increase antioxidant capacity [27]. In addition, milk thistle seed oil and its active constituent, silymarin, exert antioxidant activity in vitro [15]. However, the comparison of the in vitro antioxidant activity of the milk thistle varied by different parts and extracts has not yet been investigated.

In this study, the aim of our research was to quantify the content of silymarin and its constituents from water and ethanol (EtOH) extracts of milk thistle leaves, roots, and seeds using high-performance liquid chromatography (HPLC) and an ultraviolet (UV) detection. To evaluate the antioxidant activity of milk thistle and silymarin, the in vitro antioxidant activities of different parts of milk thistle from water and EtOH extract, and its constituents (silymarin and its individual constituents such as taxifolin, silydianin, and silybin) were investigated by measuring radical scavenging activities.

Materials and Methods

Plant materials

The leaves and roots of *S. marianum* L. were obtained from the vegetative growth stage 90 days after sowing. Each dried part of leaves, roots, and seeds of *S. marianum* was provided by EL&I Co., Ltd., Hwaseong, Korea and voucher specimens (No. LEE22-011, No. LEE22-012, and No. LEE22-013, respectively) were deposited at the herbarium of the Department of Plant Science and Technology, Chung-Ang University, Anseong, Korea.

Instruments, chemicals, and reagents

Silymarin was analyzed by HPLC using a Flexar Quaternary Pump (PerkinElmer Life and Analytical Sciences Inc., Waltham, MA, USA), a PDA LC UV detector (PerkinElmer), and an autosampler. Standards of silymarin (mixture of six constituents of taxifolin, silychristin, silydianin, silybin A, silybin B, and isosilybin) and its individual constituents (taxifolin, silydianin, and silybin) were obtained from the Natural Product Institute of Science and Technology (www.nist.re.kr), Anseong, Korea (Fig. 1). HPLC-grade water and acetonitrile (ACN) were purchased from J. T. Baker (Avantor, Radnor, PA, USA), acetic acid (99.7%) and EtOH were purchased from Samchun Pure Chemicals (Pyeongtaek, Korea), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^+$) were purchased from Alfa Aesar (London, UK) and Roche (Mannheim, Germany), respectively.

Extraction methods and preparation of samples

Dried milk thistle leaves, roots, and seeds (5 g samples) were extracted with distilled water and EtOH (100 mL each) under reflux for 5 h. Samples were filtered and dried using a freeze dryer to obtain the water extracts, while other samples were filtered and evaporated using a rotary evaporator to obtain EtOH extracts. Extraction yields were calculated by dividing the weight of extract after extraction by the dry weight. The experimental stock solutions were prepared by dissolving each milk thistle extract in 70% ACN under sonication for 20 min and before conduction filtration using a 0.45-μm polyvinylidene difluoride membrane. The water and EtOH seed extracts were prepared at concentrations of 6.8 and 3.7 mg/mL, respectively. Leaf and root extracts were prepared at a concentration of 20 mg/mL.

DPPH radical scavenging activity

The radical scavenging activity of DPPH was determined according to the method described by Hatano et al. with slight modification [28,29]. DPPH radical has tendency to interact primarily with lipid-soluble constituents, and DPPH radical scavenging activity is commonly used to represent the antioxidant activity of lipid-soluble constituents [28,29]. The sample extract was prepared by dilution to a final concentration of 5, 25, 50, 100 μg/mL. Each sample was dissolved in 50% EtOH, before being

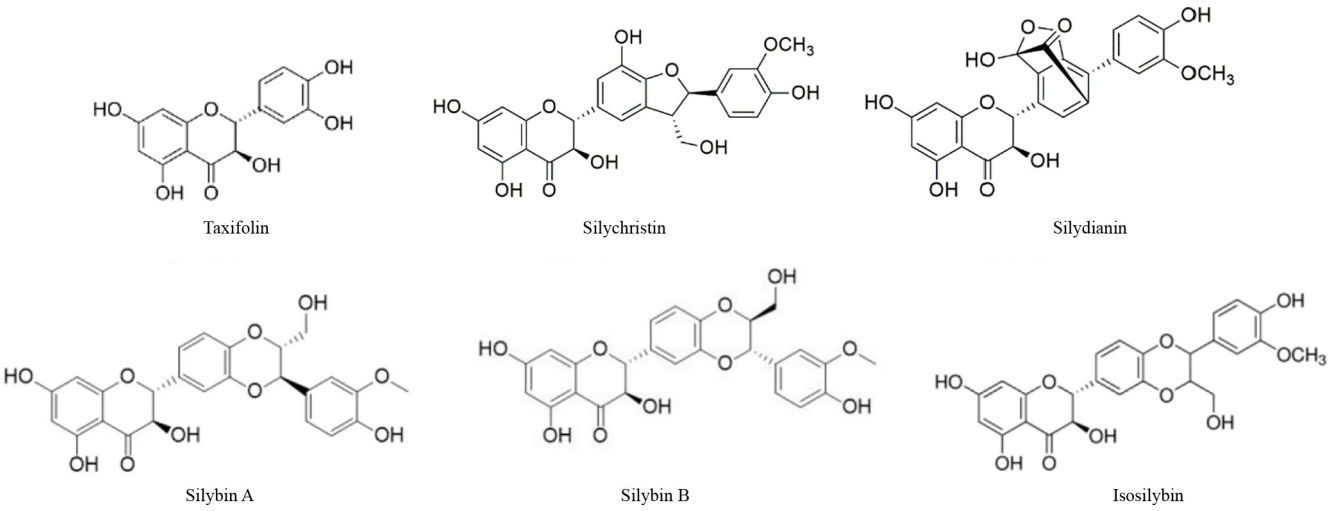


Fig. 1 Chemical structures of silymarin

added to 60 µM DPPH solutions in a 96-well plate. These reactions were then incubated for 30 min at room temperature, and the absorbance of each well was measured at 540 nm using a microplate reader (Multiskan skyhigh microplate spectrophotometer, Thermo Fisher, Waltham, MA, USA). The control was prepared in the same manner, except that 50% EtOH was used instead of the sample. The DPPH radical scavenging activity was expressed as EC_{50}, which is the concentration in µg/mL required to inhibit the formation of DPPH radicals by 50%.

$$\text{DPPH radical scavenging activity (\%)} = [A_{control} - A_{sample}]/A_{control}] \times 100$$

A_{sample} and $A_{control}$ means the absorbance of samples and the control, respectively

ABTS$^+$ radical scavenging activity

The ABTS$^+$ radical scavenging activity was measured using the modified method of Re et al. [30,31]. ABTS$^+$ radical has a tendency to interact primarily with water-soluble constituent and is therefore commonly used to represent the antioxidant activity of water-soluble constituents [30,31]. The 7.4 mM ABTS reacted with 2.6 mM potassium persulfate for 16 h, this working ATBS$^+$ solution was used in this experiment. The sample extract was prepared by dilution to a final concentration of 5, 25, 50, 100 µg/mL. Each sample was added to a working ABTS$^+$ solution in a 96-well plate and incubated for 30 min. The absorbance of each well was then read at 600 nm using a microplate reader. The control was prepared in the same manner, except that 50% EtOH was used instead of the sample. Again, the scavenging activity of ATBS$^+$ was expressed as EC_{50}.

$$\text{ABTS}^+ \text{ radical scavenging activity (\%)} = [A_{control} - A_{sample}]/A_{control}] \times 100$$

A_{sample} and $A_{control}$ means the absorbance of samples and the control, respectively

HPLC conditions

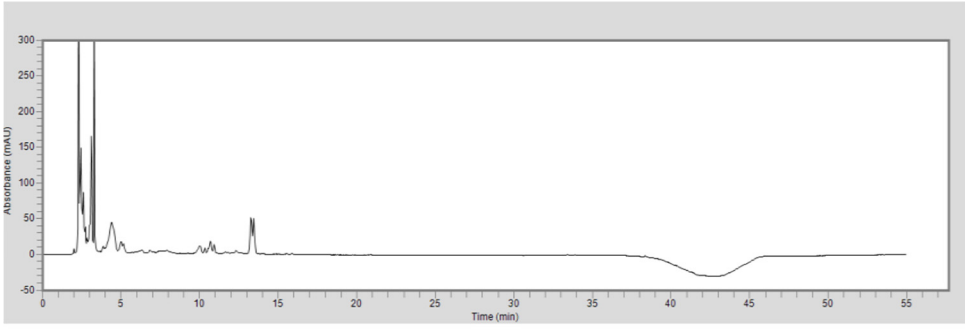
Quantitative analyses of the milk thistle were performed in a gradient elution HPLC system using a reversed-phase INNO C18 column (4.6 mm × 25 cm, 5 µm). The injection volume was 10 µL, and the UV detection wavelength was 288 nm. The column temperature was maintained at 30ºC and the flow rate was set at 1 mL/min. The elution system consisted of 0.5% acetic acid in water (A) and ACN (B). The elution system was 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. All injections were performed three times.

Calibration curves

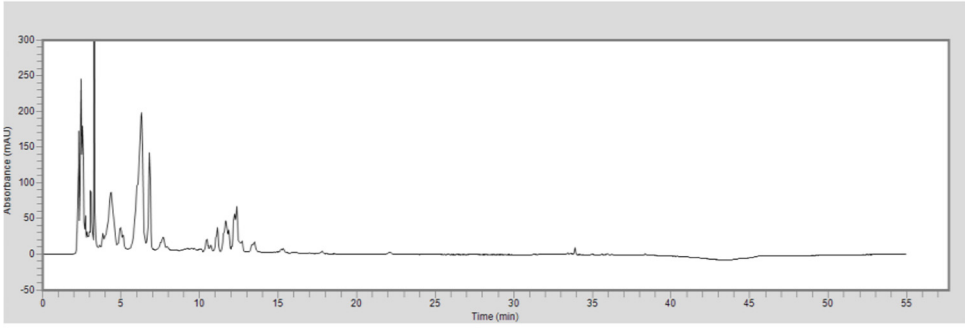
Standards of silymarin and its individual constituents (taxifolin, silydianin, and silybin) were prepared. Each stock standard solution was prepared by dissolving the constituent in 70% ACN (1 mg/mL). The working solutions used to construct the calibration curve were prepared by serially dilution of the selected stock solutions (1000, 500, 250, 125, 62.5, 31.25, 15.625, 7.813, and 3.906 µg/mL) to the desired concentrations (silymarin: 62.5-1000 µg/mL, taxifolin and silydianin: 3.906-62.5 µg/mL, and silybin (mixture of silybin A and silybin B): 15.625-250 µg/mL). Calibration functions of the standards were calculated using the peak area (Y) and concentration (X, µg/mL), represented as mean value ± SD (n =3).

Statistical analysis

ABTS$^+$ and DPPH radical scavenging activities were expressed as the mean ± SD. Statistical significance (p <0.05) between group differences was calculated using analysis of variance (ANOVA), followed by Duncan's multiple range test among same extract or concentration.



(A)



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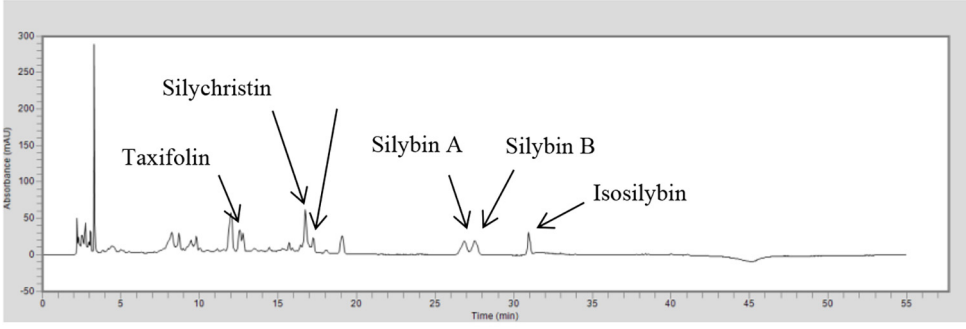

(C)

Fig. 2 HPLC chromatograms of the water extract of the leaves (A), roots (B), and seeds (C)

Results and Discussion

HPLC analysis

Quantitative analysis of silymarin was performed in each extract of milk thistle using HPLC-UV with a reversed-phase column and gradient elution system. The HPLC method showed good separation, and a wavelength of 288 nm was found to be optimal for the detection of silymarin. The retention times of silymarin (taxifolin, silychristin, silydianin, silybin A, silybin B, and isosilybin) were 12.5, 16.6, 17.1, 26.8, 27.4, and 30.9 min, respectively. HPLC chromatograms of the water and EtOH extracts of milk thistle are shown in Figs. 2 and 3, respectively. The contents of silymarin and extraction yields for water and EtOH extracts of milk thistle are shown in Table 1, and calibration curves of standards are shown in Table 2. In general, the extraction yield of EtOH extract was higher yield than that of the water extract. Extraction yields were calculated by dividing the weight of extract with all solvent evaporated after extraction of milk thistle by the dry weight. Silymarin is known to be particularly concentrated in the fruits and seeds of the whole plant. In this study, silymarin was only detected in the seeds of the water and the EtOH extract of milk thistle. Each constituent was tested by spiking test. The EtOH extract of milk thistle seeds had a higher concentration of silymarin, including taxifolin and silybin, than the water extract. There was little difference in the level of silydianin between the water and EtOH extracts. In addition, among the constituents of silymarin, the ratio of silybin was higher than that of the other constituents in water and EtOH extracts of milk thistle.

Aziz et al. evaluated the antioxidant activities of milk thistle and quantified silymarin content using two extraction methods

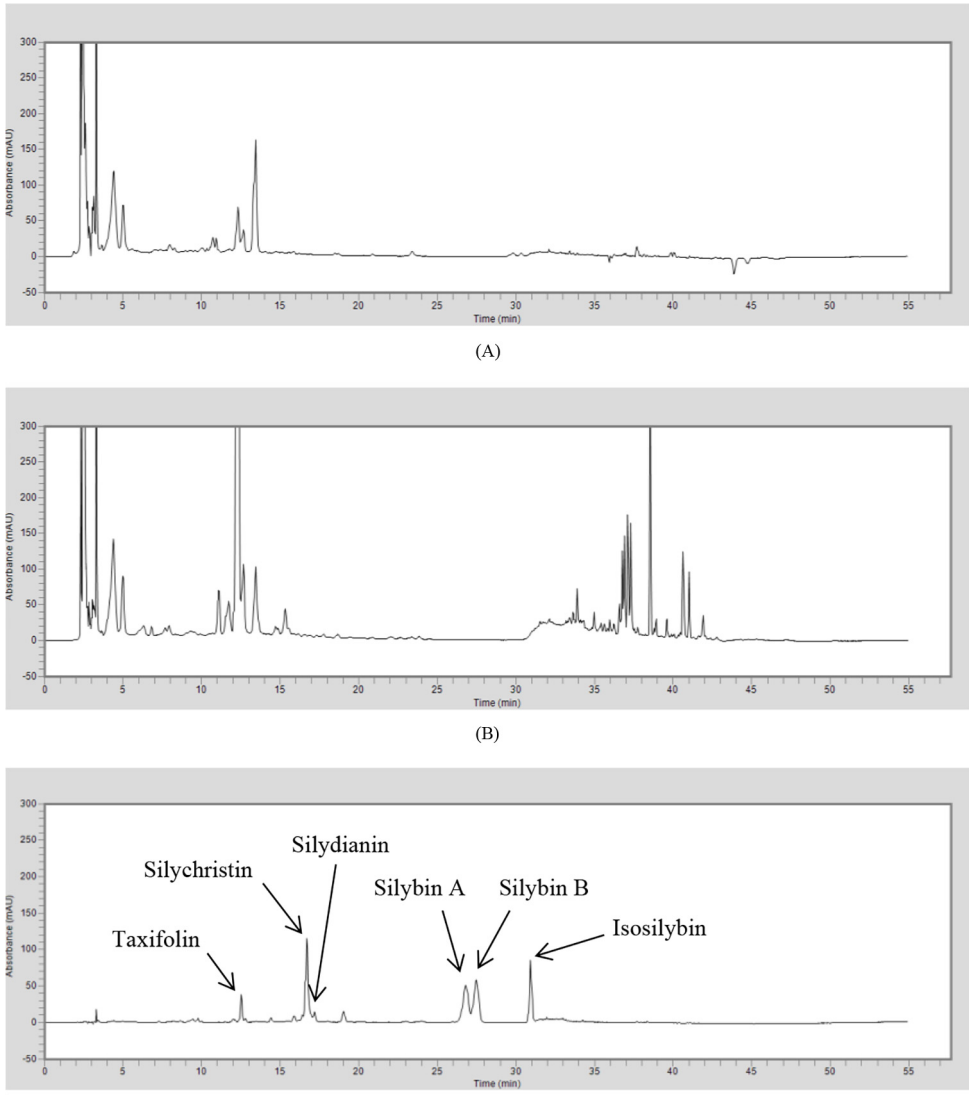

Fig. 3 HPLC chromatograms of the EtOH extract of the leaves (A), roots (B), and seeds (C)

(Soxhlet extraction and microwave-assisted extraction) [32]. Reflux extraction was used, and the extraction yield was up to 8.9 and 13.4% for water and EtOH extract, respectively, which can be considered as good efficiency. Ibrahim et al. determined the effect of extraction methods on silymarin content from the leaves and roots of artichokes (*Cynara scolymus* L.) with the highest silymarin and silybin content found in artichoke leaves using infusion methods (25.6 and 5.2 μg/g, respectively) [33]. In terms of silymarin content, the infusion extraction method using boiling water was more efficient than the other two methods (water and methanol extraction methods) in a shorter time. In addition, Ibrahim et al. found that more silymarin could be obtained by extending the extraction time [33].

Studies have shown that milk thistle seeds contain the highest concentration of silymarin. In our experiment, silymarin was found in the seeds, but not in the leaves or roots. In addition, it appears that milk thistle leaves have not previously been evaluated for their bioactive constituents. Recently, however, other studies have analyzed other parts of the milk thistle plant in addition to the seeds. For example, Balian et al. reported that methanol extract from milk thistle leaves showed an anti-inflammatory activity in rat paw oedema models [34]. In addition, Omar et al. quantified the content of silymarin in the leaves of milk thistle during different growth stages and compared it with milk thistle seeds [35]. Although the content of silymarin in the milk thistle seeds was higher than in the leaves per same weight, they suggested the possibility of using the leaves of milk thistle in the pre-flowering stage as a source for silymarin production. Elwekeel et al. evaluated the content of individual flavonolignans (silychristin, silydianin, silybin, and isosilybin) of milk thistle fruits at different

Table 1 Content of silymarin in the water and the EtOH extracts of milk thistle

Sample		Extraction Yield (%)	Content (mg/g ext.)			
			Silymarin[a]	Taxifolin	Silydianin	Silybin
Water extract	Leaves	8.9	ND	ND	ND	ND
	Roots	6.8	ND	ND	ND	ND
	Seeds	3.1	27.17±0.16	0.65±0.01	1.53±0.03	4.43±0.03
EtOH extract	Leaves	8.0	ND	ND	ND	ND
	Roots	8.1	ND	ND	ND	ND
	Seeds	13.4	121.47±0.14	2.63±0.01	1.53±0.02	23.87±0.13

[a]Silymarin contains the compounds taxifolin, silychristin, silydianin, silybin A, silybin B, and isosilybin
ND; not detected (below the LOD value)

Table 2 Calibration curves of standards

Constituent	Calibration equation[a]	Correlation factor, r^2 [b]
Silymarin	Y = 9435.5X + 37837	1
Taxifolin	Y = 31317X + 5491.3	1
Silydianin	Y = 13455X + 509.12	0.9994
Silybin	Y = 21624X + 35912	0.9999

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

stages of maturity using HPLC, with the highest content found in fully ripe fruits [36].

Radical scavenging activities of different parts and extracts of milk thistle

The DPPH and the radical cations derived from ABTS act as stable free radicals, while the antioxidants react with the DPPH and $ABTS^+$ radicals [37]. A change in the color of the DPPH and $ABTS^+$ radicals can predict the antioxidant activities of the particular samples [32,33], which has led to DPPH and $ABTS^+$ radical scavenging activity being widely used in the evaluation of natural products [28, 38-44]. DPPH radical scavenging activity is typically measured by observing color changes at 517 nm [29]. However, in this study, absorbance was measured at 540 nm. This approach was considered suitable for comparing samples under the same experimental conditions in this study. Furthermore, it has been reported in other studies that the antioxidant activity of different samples was compared by DPPH radical experiments at 540 nm under the same experimental conditions [45]. Ferryl myoglobin oxidizes ABTS to ABTS cation radical and its formation can be monitored at absorbance at 600 or 735 nm [31,46].

To evaluate the antioxidant activity of milk thistle, the DPPH and $ABTS^+$ radical scavenging activities of different parts and extracts of the milk thistle plant were investigated. The antioxidant activity was determined by the EC_{50} values, which indicate the concentration of the samples required to inhibit 50% of the DPPH and $ABTS^+$ radicals. Therefore, the lower the EC_{50} value, the higher the antioxidant activity of the sample. As shown in Table 3, the EC_{50} value of DPPH radical scavenging activity in the seeds of milk thistle water extract was 689.14 μg/mL, which was lower than the EC_{50} values for the roots (5578.62 μg/mL) and leaves (1327.26 μg/mL) of milk thistle water extract. In addition, the EC_{50} value of $ABTS^+$ radical scavenging activity in the seeds of milk thistle water extract showed 78.09 μg/mL, which was the significantly lower EC_{50} values of $ABTS^+$ radical scavenging activities among roots (102.55 μg/mL) and leaves (191.75 μg/mL) of milk thistle water extract. This results indicated that seed of milk thistle water extract showed a higher DPPH and $ABTS^+$ radical scavenging activity among leaves and roots of milk thistle water extract. In addition, the seeds of milk thistle EtOH extracts showed lower EC_{50} values, resulting in higher DPPH and $ABTS^+$ radical scavenging activities compared to other parts of the milk

Table 3 DPPH and $ABTS^+$ radical scavenging activities of the water and the EtOH extracts of milk thistle

Sample		EC_{50} (μg/mL)	
		DPPH	$ABTS^+$
Water extract	Leaves	1327.26±278.83[b]	191.75±9.12[a]
	Roots	5578.62±1378.93[a]	102.55±2.50[b]
	Seeds	689.14±9.59[b]	78.09±1.05[c]
EtOH extract	Leaves	248.38±7.10[a]	93.48±4.74[a]
	Roots	64.40±1.63[b]	85.59±4.02[a]
	Seeds	8.53±0.95[c]	64.56±1.77[b]

Values are means ± standard deviation (SD). Different letters (a-c) are significantly different (p <0.05) among the same extract and same experiment by Duncan's Multiple Range Test (DMRT). Mean values with same letters are not significantly different (p <0.05) among same extracts.

thistle EtOH extracts. In other studies, the DPPH radical scavenging activity of milk thistle seeds was greater than that of other parts of the plant such as leaves, stems, and roots [47]. Our result indicated that the milk thistle seeds also showed the strongest radical scavengers compared to other parts of the leaves and roots from milk thistle.

In our results, the leaves, roots, and seeds of milk thistle EtOH extract showed lower EC_{50} values of DPPH and $ABTS^+$ radical scavengers than leaves, roots, and seeds of milk thistle water extract. These results indicate that the radical scavenging activity of the EtOH extract was higher than that of the water extract in all parts of the milk thistle. Nowak et al. showed that the EtOH extract of milk thistle had higher antioxidant activity than other extractions including methanol, acetone, and petroleum ether [48]. They also showed that the EtOH extract had a higher radical scavenging activity than the water extraction. Previous studies have reported the solubility of active constituents from milk thistle. Silymarin is a mixture containing taxifolin, silychristin, silydianin, silybin A, silybin B, and isosilybin, and is widely known to be the major constituent from milk thistle as antioxidants [10,49]. Silymarin contains phenolic hydroxyl groups. However, the presence of hydroxyl groups does not necessarily make silymarin highly water-soluble. In particular, taxifolin is slightly soluble in water rather than insoluble [50]. In addition, the solubility of constituents can vary depending on factors such as temperature and pH [51]. Silymarin exhibits hydrophobicity and limited water solubility despite the presence of hydroxyl groups. Therefore, it is likely that the higher antioxidant activity of milk thistle EtOH extract compared to the water extract from milk thistle is due to the solubility of the active constituents such as silymarin. Nevertheless, the leaves and roots of the milk thistle also exhibited DPPH and $ABTS^+$ radical scavenging activity. According to Padma et al., the leaves of milk thistle contain common and bioactive constituents such as (+)-2-bornanone, 9,12,15-octadecatrienal, α-santoline alcohol, dodecane, 2,6,11-trimethyl-dodecane, hexadecane, D-mannose, undecanoic acid, 9-octadecenoic acid, and oleic acid [52]. In particular, 9,12,15-octadecatrienal has been reported to have antioxidant activity. However, research on the active constituents in the leaves and roots of milk thistle is limited, so further studies are needed to identify substances with antioxidant activity in both the leaves and roots of milk thistle.

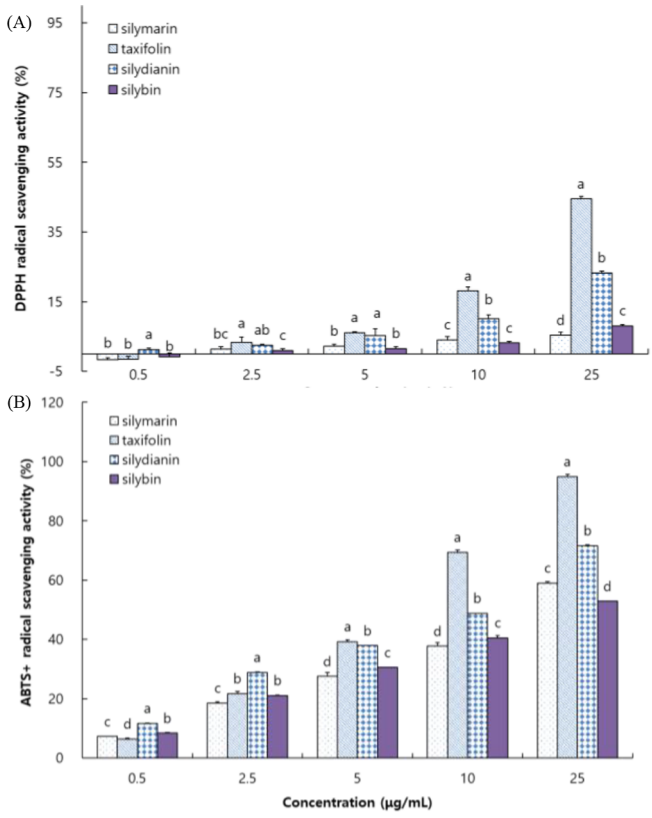

Fig. 4 The DPPH (A) and $ABTS^+$ (B) radical scavenging activities of constituents from milk thistle. Silymarin contains the compounds taxifolin, silychristin, silydianin, silybin A, silybin B, and isosilybin. Values are means ± SD. Different letters (a-d) are significantly different (p <0.05) among the same concentration by DMRT. Mean values with same letters are not significantly different (p <0.05) among same concentration

Radical scavenging activities of silymarin, taxifolin, silydianin, and silybin

The DPPH and $ABTS^+$ radical scavenging activities of constituents from milk thistle such as silymarin and its constituents (taxifolin, silydianin, and silybin) were investigated (Fig. 4). The DPPH and $ABTS^+$ radical scavenging activities of silymarin, taxifolin, silydianin, and silybin at concentrations of 0.5, 2.5, 5, 10, and 25 μg/mL increased in a dose-dependent manner. The taxifolin at concentrations of 10 and 25 μg/mL showed significantly higher DPPH radical scavenging activity among other constituents. This result indicated that taxifolin has a strong in vitro antioxidant activity than the other constituents. In the $ABTS^+$ radical scavenging activities of constituents of milk thistle, silymarin, taxifolin, silydianin, and silybin at a concentration of 25 μg/mL were 59.04, 94.92, 71.61, and 52.87%, respectively. Therefore, constituents of milk thistle suggest a promising role as free radical scavengers. In addition, taxifolin showed significantly higher $ABTS^+$ radical scavenging activities at dose of 5, 10, and 25 μg/mL, among constituents from milk thistle. Therefore, taxifolin showed the higher DPPH and $ABTS^+$ radical scavenging activities than other constituents from milk thistle, such as silydianin and silybin.

Taxifolin showed the higher antioxidant activity among other constituents from milk thistle. The IC_{50} values for DPPH and $ABTS^+$ radical scavenging activities of taxifolin were 77.00 and 0.83 μg/mL, respectively [53]. The potent antioxidant activity of the molecule is further supported by its conjugated structures and the resonance stability of both phenolic rings [53]. The antioxidant activity of taxifolin is primarily due to its unique molecular

structure, which includes two aromatic rings with phenolic groups positioned in the meta- and para-positions relative to each other [50,51]. This specific arrangement enhances its radical scavenging ability [53,54]. In addition, flavonoids with the dihydroxy functionality are the most active in donating an H atom, so taxifolin is expected to act as a hydrogen donor [54]. Taxifolin is a type of flavonol and is found in Citrus fruits, olive oil, and milk thistle seeds [54]. A previous study reported that of taxifolin showed the lower EC_{50} value (32 μM) for DPPH radical scavenging activity, among other constituents from milk thistle such as silydianin, silybin A, silybin B, isosilybin A, isosilybin B, and silymarin [46]. Similar to our results, taxifolin was a more effective free radical scavenger than other milk thistle constituents such as silychristin, silydianin, silybin A, silybin B, isosilybin A, and isosilybin B in a DPPH assay [55]. Furthermore, the antioxidant activity of silymarin was confirmed, as its radical scavenging activity has also been reported in other studies [56-58]. Liu et al. reported that administration of taxifolin attenuated oxidative stress by reducing reactive oxygen species (ROS) and lipid peroxidation levels and activating antioxidant proteins such as nuclear heme oxygenase-1 and NADH dehydrogenase quinone 1 in a D-galactose-induced ageing mouse model [59]. Algefare showed that oral administration of taxifolin attenuated oxidative stress by inhibiting nitric oxide and increasing nuclear factor erythroid-2-related factor 2 (Nrf2) signaling in nephrotoxicity-induced mouse model [60]. In addition, taxifolin has a neuroprotective effect by inhibiting ROS and up-regulating antioxidant proteins such as signal transducer and activator of transcription 3 in hippocampal cells, thereby taxifolin could be promising antioxidant materials [61]. Therefore, many studies reported that the pharmacological effect of taxifolin such as anti-cancer, anti-inflammation, prevention of cardiovascular disease, and the others, due to the antioxidant activity of taxifolin [57].

Silymarin is known to the major constituent of milk thistle and has the most health beneficial effects such as hepatoprotective effects, anti-cancer, and anti-diabetic effects [10,11]. A previous study reported that the EC_{50} values for silymarin on DPPH radical were found to be 20.8 μg/mL and 280 μM, respectively [15]. The many studies reported that silymarin attenuated oxidative stress by regulating various pathways such as increasing antioxidant enzymes, activating Nrf2 signaling, and mitochondrial enzymes [62]. In particular, the structure of silymarin effectively inhibits ROS, thereby it has been classified as an official medicine and widely used in clinical hepatoprotective agent [10,11]. Silybin is the major constituent of milk thistle [63] and has antioxidant properties, immune-enhancing effects, and antiviral activity [7]. Hepatic steatosis and fibrosis, cerebral ischemia-reperfusion injury and diabetic nephropathy have been ameliorated by the antioxidant activities of silybin [64-66]. In addition, silydianin inhibited oxidative stress by regulating oxidative products such as superoxide radical [67]. Silydianin protected oxidative stress-induced skin damage by upregulating glutathione levels and inhibiting carbonylated proteins [68]. Therefore, the active constituents of milk thistle may have health benefits through antioxidant activity.

This study investigated the comparison of the content of active constituents and antioxidant activity between water and EtOH extraction solvents. In addition, the comparison of the antioxidant activity of active constituents, silymarin such as taxifolin, silydianin, and silybin, was investigated by radical scavenging activities. Comparing of in vitro antioxidant activity and content of active constituents, including silymarin, taxifolin, silydianin, and silybin, in milk thistle extracts was focused on between water and ethanol extraction solvents. In particular, many previous studies have only looked at the antioxidant activity of milk thistle extract without comparing these different extraction solvents; this study clearly elucidated the differences between the two solvents. The antioxidant activity of active constituents such as silymarin, taxifolin, silydianin, and silybin was also compared. Such research comparing the antioxidant activity of these active constituents is uncommon, and our finding that taxifolin exhibited higher radical scavenging activities further highlights the originality of this study. Furthermore, our study suggests the need for further research to understand the antioxidant activity and mechanisms of action of the active constituents isolated from milk thistle. In particular, further research is needed to understand the antioxidant activity and mechanisms under *in vivo* system for active constituents isolated from the milk thistle. By proposing new research directions to deepen the under-standing of antioxidant activity, our study underlines its originality.

Silymarin was detected only in the seeds of the extracts, whereas the silymarin content was higher in the EtOH extract than in the water extract. The EtOH extract of seeds containing high levels of taxifolin and silybin showed higher radical scavenging activity than other parts of the EtOH and water extracts. In addition, silymarin and its individual constituents (taxifolin, silydianin and silybin) showed antioxidant activity as DPPH and $ABTS^{+}$ radical scavengers. In particular, taxifolin showed higher radical scavenging activity than other seed constituents. Therefore, it was suggested that *S. marianum* and its active constituents could be a useful material for antioxidant-related various diseases.

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