Determination of flavonoids from *Cirsium japonicum* var. *maackii* and their inhibitory activities against aldose reductase

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**Abstract** The therapeutic activities of flavonoids from natural plant sources were investigated. The ethanol extracts from the aerial parts of *Cirsium japonicum* var. *maackii* (CJM) were tested for aldose reductase inhibition (ARI). Additionally, stepwise polarity fractions and flavonoids from CJM were evaluated for ARI. The ethyl acetate (EtOAc) fraction from CJM showed significant inhibitory effects. The compounds in the EtOAc fraction were identified as the flavonoids-cirsimaritin (1), hispidulin (2), and cirsimarin (3). Based on an ARI assay, the EtOAc fraction and hispidulin (2) exhibited good AR inhibitory activity (IC\(_{50}\) values of 0.21 μg/mL and 0.77 μM, respectively). An HPLC quantitative analysis of different parts of CJM showed that the aerial part collected in the spring season (CJL1) contains the highest total flavonoid content. These results serve as a basis for maximizing the flavonoid yield and for the efficient usage of various parts of CJM. Our results also suggest that CJM could be a useful ARI material for the treatment of various diabetic complications.

**Keywords** Aldose reductase · *Cirsium japonicum* var. *maackii* · Diabetic complications · Flavonoid · Hispidulin

**Introduction**

Diabetes mellitus is a metabolic disorder wherein patients fail to produce or respond to insulin which could result to hyperglycemia [1]. It is a chronic disease that affects approximately 382 million individuals in 2013 and expected to increase by over 590 million by 2035 [2]. This is a result of urbanization, changing lifestyle, lack of physical activities, and aging population [3]. Diabetes is known to be associated with complications due to chronic hyperglycemia. This includes retinopathy, nephropathy, sexual dysfunction, and cardiovascular disease [4]. These complications are influenced by the production of non-enzymatic glycation end products which are proteins and lipids glycated after exposure to aldose sugars [5]. However, the reduction in these aldose sugars also produces alcoholic sugars under the polyol pathway. Accumulation of polyol is also considered the leading cause of cataract formation in diabetic patients which is the leading cause of blindness [6].

Polyol pathway is a minor pathway in glucose metabolism activated by an increase in sugar levels. In this pathway, glucose is reduced to sorbitol by the action of aldose reductase (AR) using nicotinamide adenine dinucleotide phosphate (NADPH), cofactor. Sorbitol is metabolized, by sorbitol dehydrogenase, to fructose using NAD\(^+\), cofactor. The sorbitol pathway has been implicated in the
pathogenesis of sugar cataracts characterized by osmotic stress due to sorbitol accumulation [6]. Previous studies have suggested that it is related in cataract formation and the inhibition of AR could prevent the formation of cataracts [7]. Several studies have suggested the potential use of natural plant sources for the development of drugs to reduce diabetic complications, especially plants that contain high quantities of flavonoids and have strong in vivo AR inhibitory activity [8]. Various flavonoids including quercitrin, luteolin, kaempferol, quercetin, and afzelin exhibit AR inhibitory activity [9, 10].

Cirsium japonicum var. maackii (CJM) is a perennial herb distributed in many areas of China, Korea and Japan [11]. It is considered as traditional Chinese medicine and is used as an anti-hemorrhagic, anti-hypertensive, anti-hepatitis, and uretic agent. It has been prescribed as a possible treatment for cancer [12]. Phytochemical studies have identified various flavonoids in C. japonicum, i.e., apigenin, acacetin, diosmetin, pectolinarin, hispidulin-7-neohesperidoside, 5,7-dihydroxy-6,4′-dimethoxyflavone, linarin, and luteolin [8, 12–15]. Other compounds have also been isolated from the genus Cirsium including silybin [16]. Previous reports have shown that these flavonoids have pharmacological importance. For example, linarin has anti-cancer activity, luteolin has anti-inflammatory effects, and pectolinarin has anti-diabetic activity [11, 17, 18].

In the present study, the AR inhibitory activity of CJM was assessed. The extract, different fractions, and flavonoids isolated from the aerial parts of CJM were subjected to an AR assay. A simultaneous determination of isolated flavonoids by HPLC–UV was conducted to evaluate the flavonoid content from various parts of CJM. The results of this study could serve as basis for maximizing the flavonoid content and for the efficient usage of various parts of CJM.

### Materials and methods

#### Plant material

The aerial part of CJM (ICF-1) collected in the spring (CJL1) and fall (CJL2), seeds (CJS), roots (CJR) heat-treated through hot blast, and flowers (CJF) were obtained from Imsil Herbal Medicine, Republic of Korea.
Instruments, reagents, and chemicals

Isolated compounds were identified by mass spectrometry (MS) and nuclear magnetic resonance (NMR), using a JEOL JMS-600 W mass spectrometer (Tokyo, Japan) and a Bruker AVANCE 500 NMR spectrometer (Rheinstetten, Germany), respectively. Reagents and solvents, including nicotinamide adenine dinucleotide 2'-phosphate (β-NADPH), D,L-glyceraldehyde, sodium phosphate buffer, potassium phosphate buffer, 3,3-tetramethylene glutaric acid (TMG), and dimethylsulfoxide (DMSO), were obtained from Sigma-Aldrich (St. Louis, MO, USA).

HPLC analyses were performed using a Waters Breeze System with a 1525 Binary HPLC Pump and a 2489 System Ultraviolet–Visible Detector (Milford, MA, USA). Standard compounds (e.g., acacetin, apigenin, diosmetin, linarin, luteolin, pectolinarin, and silybin B) were acquired from Sigma-Aldrich.

Extraction, fractionation, and isolation

Dried CJL1 (5.71 kg) was extracted with EtOH for 3 h (7 L × 3) at 65–75 °C under reflux. The solution was filtered, and the EtOH extract (667.2 g) was obtained after the solvent was removed in vacuo. The extract was supplemented with water and partitioned sequentially with n-hexane (213.6 g), CHCl₃ (39 g), EtOAc (67.6 g), and n-BuOH (47 g). The EtOAc fraction was divided into 19 factions (Fr.1–Fr.19) by column chromatography on a silica gel (6 × 80 cm, No. 7734) with step gradient elution by CHCl₃–MeOH (100:0 → 0:100). Among the fractions, Fr. 3 (CJ2E27), Fr. 7 (CJ2E34), and Fr. 15 (CJ2E56) led to the isolation of compounds 1–3, respectively, with MeOH recrystallization.

| Compound | Description | MS Data | 1H- and 13C-NMR Details |
|----------|-------------|---------|------------------------|
| 1        | Yellow powder; C₁₇H₁₄O₆ | 314 [M]+ (100), 299 (82.8), 285 (19.7), 271 (25.4), 254 (4.2), 239 (2.4), 227 (1.0), 200 (2.1), 195 (1.8), 181 (14.3), 167 (2.1), 153 (23.7), 135 (4.6), 128 (4.6), 119 (9.4), 93 (2.5), 69 (9.0); 1H- and 13C-NMR (500 MHz, DMSO-d₆) (see Table 1). |
| 2        | Yellow powder; C₁₆H₁₂O₆ | 300 [M]+ (100), 285 (67.0), 271 (6.7), 257 (40.3), 242 (1.3), 228 (1.8), 181 (1.1), 167 (9.2), 153 (3.9), 139 (8.9), 129 (4.3), 119 (9.8), 91 (1.6), 69 (16.2); 1H- and 13C-NMR (500 MHz, DMSO-d₆) (see Table 1). |
| 3        | Yellow powder; C₂₃H₂₄O₁₁ | 477 [M+H]+; 1H- and 13C-NMR (500 MHz, DMSO-d₆) (see Table 1). |

Measurement of AR activity

Based on previous studies [10, 19], lenses from Sprague–Dawley rats were removed and were preserved in a freezer until the experimental assay. Each sample, including the EtOH extract, n-hexane, CHCl₃, EtOAc, n-BuOH fractions and compounds 1–3, was dissolved in DMSO for the AR assay.

Preparation of standards and samples for HPLC

Stock standard solutions were obtained by dissolving compounds 1–3 in MeOH to obtain a 1.0 mg/mL solution. Each compound was subjected to serial dilution to obtain various concentrations (1, 0.1, 0.01, 0.001, and 0.0001 mg/mL) for the calibration curve. The CJS, CJL1, CJL2, CJR, and CJF samples (each 25 g) were extracted with EtOH (each 1000 mL) under reflux and were evaporated. Each extract was melted with MeOH and filtered using a syringe filter (0.45-µm).

Table 2  IC₅₀ of the EtOH extract and fractions from CJL1on rat lens AR

| Samples | Concentration (µg/mL) | AR inhibition (%) | IC₅₀ (µg/mL) |
|---------|-----------------------|-------------------|--------------|
| EtOH ext. | 10 | 49.40 | – |
| n-Hexane fr. | 10 | 12.65 | – |
| CHCl₃ fr. | 10 | 68.98 | 1.16 |
|  | 1 | 64.60 |  |
|  | 0.1 | 10.65 |  |
| EtOAc fr. | 10 | 82.83 | 0.21 |
|  | 1 | 65.96 |  |
|  | 0.1 | 42.17 |  |
| n-BuOH fr. | 10 | 43.37 | – |
| TMG | 10 | 83.28 | 0.28 |
|  | 1 | 62.21 |  |
|  | 0.1 | 40.13 |  |

* Inhibition rate was calculated as a percentage of the control value
* IC₅₀ calculated from the least-squares regression line of the logarithmic concentrations plotted against the residual activity
* TMG was used as a positive control
The quantitative analysis of flavonoids (compounds 1–3, acacetin, apigenin, diosmetin, linarin, luteolin, and pectolinarin) was conducted using a reverse phase HPLC system. An INNO C18 column (25 cm × 4.6 mm, 5 μm) was used. The mobile phase was a gradient of water containing 0.5% acetic acid (solvent A) and acetonitrile (ACN, solvent B). Solvent A was decreased from 83 to 70% for 10 min, maintained for 15 min, decreased from 70 to 20% for 5 min, decreased from 20 to 0% for 5 min, and maintained for 5 min, and 0 to 83% for 10 min and maintained 5 min. The flow rate of mobile phase was 1 mL/min. The injection volume was 10 μL, and the detector was set at a UV absorbance of 270 nm. The column temperature was maintained at 30 °C. However, silybin B was recorded at UV 287 nm and a total of 50 min running time using a gradient elution. Solvent A was set at 70% and maintained for 10 min. It was decreased to 20% after 15 min and later to 0 after 5 min. It was maintained for another 5 min before it was increased to 70% for 10 min. The elution was maintained for another 5 min.

### Calibration curve

Compounds 1–3 were dissolved in MeOH, and stock solutions (0.1–1000 μg/mL) were prepared. The contents of compounds 1–3 in the samples were determined from the corresponding calibration curves. The calibration functions of compounds 1–3 were determined based on the peak area (Y), concentration (X, μg/mL), and mean values (n = 5) ± standard deviation.

### Results

#### Identification of isolated compounds

The separation of EtOAc soluble fraction from CJL1 was conducted by repeated rounds of open column
chromatography using a silica gel and recrystallization, resulting in the isolation of compounds 1–3. The typical flavonoid signals of compounds 1–3 were detected in the 1H-NMR spectra. The presence of singlet signals at δ 12.85–13.07 of compounds 1–3 showed 5-OH of an A-ring in the structure. The 1H- and 13C-NMR data were compared with values from previous studies [20–23]. Consequently, compounds 1–3 were identified as cirsimaritin, hispidulin, and cirsimarin, respectively, and isolated for the first time from CJL1 (Table 2). The chemical structure for each isolated compound is shown in Fig. 1.

AR inhibitory activity

The EtOH extract and fractions of CJL1 were tested for AR inhibition. The results are summarized in Table 2. The EtOAc fraction showed significant inhibition on the rat lens (IC50 value 0.21 μg/mL). Both n-Hexane and n-BuOH fractions showed less than 50% inhibition therefore their IC50 was not further determined. Compounds 1–3 were tested for rat lens AR inhibition (Table 3). Among them, compounds 1 and 2 (IC50 values of 2.83 and 0.77 μM, respectively) exhibited greater inhibitory effects than those of TMG (IC50 value of 3.91 μM), used as a positive control, against AR.

Quantitative determination of compounds 1–3

HPLC separation of compounds 1–3 was conducted for quantitative analyses using a reverse phase system and elution with solvents A and B as mobile phases. The standard calibration curves for compounds 1–3 are shown in Table 4. The amounts of compounds 1–3 in various parts of CJM were simultaneously determined (Fig. 2) using the optimized analytical methods. The flavonoid content was higher in flowers and aerial parts than in roots, and seeds. In a previous study, the flavonoid content was higher in flowers and leaves than in roots [23]. For compounds 1–3, the content of cirsimaritin (1) was higher than those of hispidulin (2) and cirsimarin (3). CJL1 contained the highest amount of cirsimaritin (1) (13.143 mg/g). The total flavonoid content of various parts was highest in CJL1 (22.58 mg/g) (Table 5). The presence of acacetin, apigenin, diosmetin, linarin, luteolin, pectolinarin, and silybin B on the EtOH extracts from the different parts of CJM was also determined. The chemical structures for all mentioned

| Table 3 | IC50 of compounds 1–3 from CJL1 on rat lens AR
| Compound | Concentration (μg/mL) | AR inhibitiona (%) | IC50 (μM)
| Cirsimaritin (1) | 10 | 84.19 | 2.83 |
| | 1 | 45.26 | |
| | 0.1 | 25.08 | |
| Hispidulin (2) | 10 | 89.35 | 0.77 |
| | 1 | 63.61 | |
| | 0.1 | 57.19 | |
| | 0.01 | 4.28 | |
| Cirsimarin (3) | 10 | 63.97 | 3.35 |
| | 1 | 33.46 | |
| | 0.1 | 10.29 | |
| TMGc | 10 | 87.46 | 3.91 |
| | 1 | 58.64 | |
| | 0.1 | 32.54 | |

a Inhibition rate was calculated as a percentage of the control value
b IC50 calculated from the least-squares regression line of the logarithmic concentrations plotted against the residual activity
c TMG was used as a positive control

| Table 4 | Calibration curves for compounds 1–3
| Compound | tR Calibration equation a Correlation factor, r2b
| Cirsimaritin (1) | 32.21 Y = 3000000X + 9045 1 |
| Hispidulin (2) | 30.19 Y = 4000000X + 7131.8 1 |
| Cirsimarin (3) | 17.41 Y = 2000000X + 2219.7 1 |

a Y = peak area, X = concentration of standard (mg/mL)
b r2 = correlation coefficient for three data points in the calibration curve
compounds are shown in Fig. 1. However, HPLC–UV results in Figs. 2 and 3 suggest that these compounds were not detected based on comparison with retention times and spike tests for individual compounds.

Discussion

The accumulation of polyol in the lens membrane could result to osmotic stress. Previously, it has been suggested that AR is directly involved in this pathological condition [7]. Extracts and isolated compounds from natural plant source have being widely considered as an alternative to synthetic drugs [8, 24]. Jung et al. [8] evaluated the MeOH extract and fractions of different parts of C. maackii to determine AR inhibitory and anti-oxidant activity and found that leaves have 0.54 µg/mL AR inhibitory activity (IC50). They concluded that the polar fractions, including EtOAc and BuOH, have greater anti-oxidant potential than that of the other fractions. This suggests that the aerial parts of CJM and its polar fractions are functionally important and accordingly should be examined in further studies. Previous studies have indicated that various parts of Cirsium extracts show good

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Fig. 2 HPLC chromatograms of flavonoids (A) and EtOH extracts of CJS (B), CJL1 (C), CJL2 (D), CJR (E), and CJF (F) (linarin: 14.68, pectolinarin: 15.02, luteolin: 15.27, cirsimarin: 17.41, apigenin: 26.95, diosmetin: 28.57, hispidulin: 30.19, cirsimaritin: 32.21, and acacetin: 33.12 min)
anti-oxidant activity [25] and anti-microbial activity [26], among others.

Flavonoids are considered as strong active constituents against AR [27]. The studied compounds are identified as flavones. Flavones have shown more activity than flavonols and flavanones [28, 29]. Liao et al. [18] have also suggested that flavones from *C. japonicum* exhibit high glucose uptake. This leads to the modulation of the

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Table 5 Contents of compounds 1–3 in the EtOH extracts of CJM

| Sample | Content (mg/g DW) | Cirsimarin (1) | Hispidulin (2) | Cirsimarin (3) | Total       |
|--------|-------------------|----------------|----------------|----------------|-------------|
| CJS    | 0.183 ± 0.010     | 0.025 ± 0.002  | 0.026 ± 0.001  | 0.375 ± 0.014  |
| CJL1   | 13.143 ± 0.106    | 1.402 ± 0.007  | 7.920 ± 0.044  | 22.580 ± 0.161 |
| CJL2   | 9.347 ± 0.043     | 0.661 ± 0.009  | 7.230 ± 0.084  | 17.280 ± 9.130 |
| CJR    | 0.387 ± 0.003     | 0.018 ± 0.001  | 0.377 ± 0.002  | 0.782 ± 0.006  |
| CJF    | 0.845 ± 0.016     | 1.447 ± 0.010  | 2.104 ± 0.005  | 5.721 ± 0.040  |
insulin signaling pathway which could be beneficial for those diabetic patients. Hispidulin (2) exhibited significant activity against AR. There are limited reports on the rat lens AR inhibitory activity of hispidulin (2). However, Matsuda et al. have suggested that the presence of hydroxyl group in R2 could suggest higher AR activity [29]. In a study by Kowluru and Kennedy, they suggested that supplementation of anti-oxidant inhibited retinal metabolic abnormalities [30]. Further studies are required to understand the mechanism on how it inhibits retinopathy. Compound 2 from Artemisia capillaris has exhibited a potent inhibitory effect against bovine lens AR [31]. Compound 3 had no AR inhibitory activity. The presence of sugar substituents affects ARI activity and the presence of O-glucoside moiety in R4 position reduces ARI [28, 29]. This could suggest the inactivity of compound 3 as ARI.

Several previous studies have also evaluated the chemical composition of plants in the genus Cirsium and have isolated flavonoids, including apigenin [32], acacetin, diosmetin [33], linarin [13], luteolin [8, 14], and pectolinarin [26, 28]. In the present study, compounds 1 and 3 were considered as major constituents of CJL1 based on content analysis using HPLC (Fig. 2). In addition, more flavonoids were identified in flowers and leaves than in roots and stems including luteolin, apigenin, and hispidulin.
Park et al. [21] also identified hispidulin, cirsimaritin, and acacetin from the leaves of *C. japonicum* var. *usuriense*. This suggests that CJL1 and CJL2 contain high flavonoid contents. However, in previous studies [23, 25, 33], other flavonoids, including acacetin, apigenin, diosmetin, linarin, luteolin, pectolinarin, and silybin B, were isolated from Genus *Cirsium*. However, silybin B was not detected from the different parts of CJM (Fig. 3).

Our results suggest that the aerial part of CJM could be a useful ARI material for various diabetic complications. Analyses of flavonoids in aerial parts compared to other parts will provide useful data for various applications. The results of this experiment could be used as standard data for each part of CJM.

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