Quantitative HPLC analysis of two key flavonoids and inhibitory activities against aldose reductase from different parts of the Korean thistle, *Cirsium maackii*

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ABSTRACT

Previously, the correlation between antioxidant activity and HPLC profiles of several Korean thistles has been recognized. In our ongoing study of the comparative evaluation of Korean thistles, we chose *Cirsium maackii* to assess the antioxidant and aldose reductase (AR) inhibitory activities of its varying parts, including the leaves, roots, stems, and flowers, along with two major components, luteolin 5-0-p-glucopyranoside (1) and the aglycone luteolin (2). In order to determine selective and efficient usage of the individual parts, HPLC quantitative analysis of the two key flavonoids of each *C. maackii* part has been performed for the first time. From the results of the comparative evaluation between the oxidative stress-related diabetic complications and quantitative phytochemical analysis from various parts of *C. maackii*, the content of 1 and 2 might contribute to the antioxidant and AR inhibitory activities of this thistle, clearly suggesting their potential for use in the development of therapeutic or preventive agents for diabetic complications and oxidative stress-related diseases. Furthermore, our present study will pave the way of the guidelines for the efficacy and differentiation and standardization of individual parts of this herbal material, and as such, this bioactive thistle could serve as an alternative source of 1 and 2.

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1. Introduction

The thistle comprises a variety of the genera, including *Carduus*, *Cirsium*, and *Silybum*. Among them, milk thistle (*Silybum marianum*), has been very intriguing to numerous researchers and pharmaceutical companies in view of the fact that this medicinal plant is a rich source of the active components silymarin and silibinin, harboring various bioactivities directed towards various hepatic diseases (Pradhan and Girish, 2006; Wu et al., 2009), along with anti-cancer (Singh and Agarwal, 2004), antioxidant (Wallace et al., 2008), neuroprotective (Nencini et al., 2007), and anti-diabetic activities (Detaille et al., 2008; Velussi et al., 1997).

Likewise, *Cirsium maackii*, a member of the Asteraceae family, is a perennial thistle that grows abundantly in Korea. The plant can attain a height of 50–100 cm, and its upright stems are covered with white feathers. Its leaves are oblong, with sharp needles, and are 15–30 cm long and 6–15 cm wide. It exhibits reddish-purple flowers, 3–5 cm in diameter, and blooms between June and August (Lee, 2002). In particular, the roots or whole plants of *Cirsium* species (Cirsii Radix et Herba) have been used as a folk medicine in the treatment of hemorrhaging, inflammation of the liver and kidney, and a variety of abdominal and intestinal disorders (Kim, 1997). The *Cirsium* species have also been determined to exert various kinds of bioactivities, including antimicrobial (Nazaruk and Jakoniuk, 2005), anti-diabetic (Perez et al., 2001), antioxidant (Jeong et al., 2008; Yoo et al., 2008), hepatoprotective (Yoo et al., 2008; Park et al., 2004; Ku et al., 2008), anti-inflammatory (Lim et al., 2008), vasorelaxant (Kim et al., 2008), and anti-cancer activities (Liu et al., 2007). In addition, numerous phytochemicals, including flavonoids, phenolic acids, lignans, polyacetylenes, acetylenes, sterols, triterpenes, sesquiterpene lactones, and alkaloids have been thus far isolated from these species (Jeong et al., 2008; Jordan-Thaden and Louda, 2001; Lee et al., 1994; Yim et al., 2003; Chung et al., 2002).

Since the *Cirsium* and *Silybum* genera that belong to the thistle, exhibit similar bioactivities, including hepatoprotective and
antioxidant activities, and are thus consumed as hepatoprotective agents, and the best-known groups of their secondary metabolites are flavonoids. *C. maackii* was selected to develop promising inhibitory agents with aldose reductase (AR) through comparative evaluation between the bioactivity potential and the quantitative HPLC studies. The antioxidant capacities were determined by the 1,1-diphenyl-2-picrylhydrazyl (DPPH), peroxynitrite (ONOO⁻), and total reactive oxygen species (ROS) assays, and the AR inhibition potential was determined by the rat lens AR (RLAR) and human recombinant AR (HRAR) assays. The quantitative HPLC experiments of two major isolated flavonoids, luteolin 5-O-β-D-glucopyranoside (1) and its aglycone luteolin (2) in the individual parts were performed for the first time, offering valuable information on part-specific preponderance of active constituents in this thistle. The relationships between the contents of 1 and 2, and the intensity of their antioxidant and AR inhibitory activities, were also assessed in this study. Considering the results of these above experiments, we will evaluate the possibility of this Korean thistle as not only a therapeutic or preventive agent for diabetic complications and oxidative stress-related diseases, but also a rich source of 1 and 2.

2. Materials and methods

2.1. General

The 1H and 13C NMR spectra were determined using a JEOL JNM ECP-400 spectrometer (400 MHz for 1H NMR and 100 MHz for 13C NMR) in dimethylsulfoxide (DMSO)-d6. The chemical shifts were referenced to residual peaks aromatic proton (2.49 ppm for 1H NMR and 39.50 ppm for 13C NMR). Column chromatography was performed using silica (Si) gel 60 (20–230 mesh) and TLC was performed on precoated Merck Kieselgel 60 F 254 plates (20 × 20 cm, 0.25 mm), using 50% H2SO4 as a spray reagent.

2.2. Chemicals and reagents

DPPH, L-ascorbic acid, α,α′-dimino-3-mercapto-3-methylbutanamide (4-phenylalanine), phenylmethylsulfonyl fluoride (PMSF), ethylendiaminetetraacetic acid (EDTA), nicotinamide adenine dinucleotide phosphate (NADPH), NADPH, DL-glyceraldehyde 3-phosphate dehydrogenase (GAPDH), cytochrome c oxidase, and diethylstilbestrol in DMSO (DTPA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA) and dihydrorhodamine 123 (DHR 123) were of high quality and were purchased from Molecular Probes (Eugene, OR, USA). Hydrocortisone (Hydrocortisone), hydroxyl radical (•OH), and total reactive oxygen species (ROS) were determined by the 1,1-diphenyl-2-picrylhydrazyl (DPPH), peroxynitrite (ONOO⁻), and total reactive oxygen species (ROS) assays, and the AR inhibition potential was determined by the rat lens AR (RLAR) and human recombinant AR (HRAR) assays. The quantitative HPLC experiments of two major isolated flavonoids, luteolin 5-O-β-D-glucopyranoside (1) and its aglycone luteolin (2) in the individual parts were performed for the first time, offering valuable information on part-specific preponderance of active constituents in this thistle. The relationships between the contents of 1 and 2, and the intensity of their antioxidant and AR inhibitory activities, were also assessed in this study. Considering the results of these above experiments, we will evaluate the possibility of this Korean thistle as not only a therapeutic or preventive agent for diabetic complications and oxidative stress-related diseases, but also a rich source of 1 and 2.

2.3. Plant materials

Whole plants of *C. maackii* were collected in Ulsan, Republic of Korea, in August 2007. This plant was authenticated by specialist of *Cirsium* taxonomy, Dr. Y. Kadota at Department of Botany, National Museum of Nature and Science in Tsukuba, Japan. Each of whole plant was collected, followed by separating into flowers, leaves, roots, and stem and then dried in the shade for a week. A voucher specimen as a whole plant registered and deposited at the herbarium of National Museum of Nature and Science in Tsukuba, Japan as well as the Division of Food Science and Biotechnology, Pukyong National University (Professor Choi, J.S.).

2.4. Extraction, fractionation, and isolation of *C. maackii*

The dried leaves (1.28 kg) were extracted with hot MeOH (2.5 L × 3 times). After filtration, the MeOH extract (316.3 g) was obtained via in vacuo concentration at 40 °C. The MeOH extract (310 g) was then dissolved with a mixture of CH2Cl2/MeOH/H2O (10:1:9), after which the CH2Cl2 soluble fraction was concentrated to yield a CH2Cl2 fraction (68.8 g). The aqueous fraction was successively partitioned to yield the EtOAc (80.6 g), r-ButOH (30.7 g), and H2O soluble fractions (125.8 g). Repeated column chromatography of the EtOAc fraction (80.6 g) from the leaves extract with a mixture solvent of CH2Cl2 and MeOH (20:1–1:1, gradient) can afford 1 (23.25 g) and 2 (10.76 g) (Scheme 1). The roots, flowers, and stems were extracted and fractionated via procedures identical to those employed with the leaves, and the yields of each of the fractions are shown in Table 1. Two isolated flavonoids (1 and 2) were then identified by comparisons with published spectral data (Agrawal, 1989), as well as TLC and HPLC analysis.

![Scheme 1. Extraction, fractionation, and isolation of *C. maackii* leaves.](image-url)
2.6. Evaluation of bioactivities

2.6.1. Assay for DPPH radical scavenging activity

According to the modified method, which was first employed by Blois (1958), the DPPH radical scavenging activity was evaluated. Briefly, a 160 (μl) of methanolic solution of various sample (final concentration (f.c.) of 320 μg/ml for extract, fractions, and flavonoids) was added to 40 μl DPPH methanolic solution (1.5 × 10^-4 M). After vortexing thoroughly and leaving for 30 min at room temperature, the optical density was measured at 520 nm using a microplate reader spectrophotometer VERSAmax ( Molecular Devices, CA, USA). i-Ascorbic acid was employed as a positive control. The DPPH radical scavenging activity of each sample was expressed in terms of IC50 (μg/ml required to scavenge DPPH free radicals by 50%) and calculated from the log–dose inhibition curve.

2.6.2. Assay for ONOO- scavenging activity

ONOO- scavenging activity was assessed by the modified Kooys’s method (Kooys et al., 1994), which involved the monitoring of highly fluorescent rhodamine 123, which was rapidly produced from non-fluorescent DHR 123 in the presence of ONOO-. In brief, the rhodamine buffer (pH 7.4) consisted of 50 mM sodium phosphate dibasic, 50 mM sodium phosphate monobasic, 90 mM sodium chloride, 5 mM potassium chloride, and 100 mM DTPA. The final DHR 123 concentration was 5 μM. The buffer in this assay was prepared prior to use and placed on ice. The samples were dissolved in 10% DMSO (f.c. 100 μg/ml for the extracts/fractions and 10 μg/ml for the isolated flavonoids). The background and final fluorescent intensities were measured 5 min after treatment with and without the addition of authentic ONOO-, dissolved in 0.3 N sodium hydroxide. The fluorescence intensity of the oxidized DHR 123 was evaluated using a microplate fluorometer FL 500 (Bio-Tek Instruments Inc., Winoski, VT, USA) at excitation and emission wavelengths of 480 and 530 nm, respectively. ONOO- scavenging activity values were calculated as the final fluorescence intensity minus the background fluorescence intensity, via the detection of DHR 123 oxidation. l-Penicillamine was employed as a positive control.

2.6.3. Assay for the total ROS inhibitory activity

ROS generation was assessed using the ROS-sensitive fluorescence indicator DCFH-DA (Lebel and Bondy, 1990). The kidney or liver homogenates of lipopolysaccharide (LPS)-treated rats injected (5 mg/kg, intraperitoneal injection) were used as the total ROS sources. Following the guidelines of Aging Tissue Bank at Pusan National University (Busan, Republic of Korea), male Wistar rats weighing 150–200 g were sacrificed by decapitation and the kidneys were quickly removed and rinsed in ice cold buffer (100 mM Tris, 0.2 mM PMSE, 1 mM peptatin, 2 μM leupeptin, 80 μg/ml trypsin inhibitor, 20 μM β-glycerophosphate, 20 mM NaF, 2 mM sodium orthovanadate (pH 7.4)). The kidneys were immediately frozen in liquid nitrogen, and stored at −80 °C. Ten microliter of each test sample (f.c. 40 μg/ml for the extracts/fractions and 10 μg/ml for the isolated flavonoids) was added to 190 μl of kidney postmitochondrial fraction in a 50 mM potassium phosphate buffer. The samples were loaded with 50 μl of DCFH-DA (12.5 mM in a potassium phosphate buffer) and shaken for 5 min. Finally, the fluorescence of 2,7′-dichlorodihydrofluorescein (DCF) oxidation, the detection of DHR 123 oxidation, fluorescence intensity of the oxidized DHR 123 was evaluated using a microplate fluorometer FL 500 (Bio-Tek Instruments Inc., Winoski, VT, USA) at excitation and emission wavelengths of 480 and 530 nm, respectively. ONOO- scavenging activity values were calculated as the final fluorescence intensity minus the background fluorescence intensity, via the detection of DHR 123 oxidation. l-Penicillamine was employed as a positive control.

2.6.4. Assay for OH inhibitory activity

The crude extracts and fractions at a concentration of 40 μg/ml were added to 1 mM H2O2 and 0.2 mM FeSO4, and incubated at 37 °C for 5 min. Then esterase treated 2 μM DCFH-DA was added and the changes in fluorescence intensity were monitored on a microplate fluorescence spectrophotometer (Bio-Tek Instruments Inc., Winoski, VT, USA) with excitation and emission wavelengths at 460 and 530 nm, respectively (Jo et al., 2006). The percent inhibition (%) was obtained by the following equation: % Inhibition = 1 - (S0min - S30min)/(S0min - S30min) × 100, where S0 is the fluorescence of the control (enzyme, buffer, and substrate) after 30 min of incubation, and S30min was the initial fluorescence of the control, S was the fluorescence of the tested samples (enzyme, sample solution, and substrate) after 30 min of incubation, and S0min was the initial fluorescence of the tested samples. Trolox was used as a positive control.

2.6.5. Assay for RLAR inhibitory activity

Rat lens homogenates were used as AR sources. In the experiment, we followed the guidelines for Care and Use of Laboratory Animals as approved by Pukyong National University (Busan, Republic of Korea). According to the modified method of Hayman and Kinoshita (1985), rat lens homogenate was prepared. Briefly, the lens were removed from the eyes of Sprague–Dawley rats (Samtaco BioKorea, Inc.) weighing 250–280 g. The rat lens were homogenized in phosphate buffer (pH 6.2). The supernatant was obtained by centrifugation of the homogenate at 10,000 rpm at 4 °C for 20 min and was frozen until use. A crude AR, with a specific activity of 6.5 U/mg, was used in the evaluations for enzyme inhibition. The partially purified material was separated into 1.0 ml aliquots, and stored at −80 °C. Each 1.0 ml cuvette contained equal units of enzyme, 100 mM sodium phosphate buffer (pH 6.2), and 1.6 mM NADPH, both with and without 50 mM of the substrate, α-glycerophosphate, and an inhibitor (f.c. 10 μg/ml for the extract, and 100 μM for the isolated flavonoids, dissolved in 100% DMSO). The final concentration of DMSO is 1%. The AR activity was determined by measuring the decrease in NADPH absorption at 340 nm over a 1 min period on an Ultraspec®2100pro UV/Visible spectrophotometer with SWIFT II Applications software (Amersham Biosciences, New Jersey, USA). Quercetin and epalrestat, well known aldose reductase inhibitors (ARIs) were used as positive controls. The inhibition percentage (%) was calculated as 1−(AA sample/min− AA blank/min)/(AA control/min− AA blank/min) × 100, where AA sample/min represents the reduction of absorbance for 4 min with the test sample and substrate, respectively, and AA control/min represents the same, but with 100% DMSO instead of a sample.

2.6.6. Assay for HRAR inhibitory activity

The HRAR inhibitory activities were examined according to the method of Nishimura et al. (1991). The reaction mixture was prepared as follows: 100 μl of 0.15 min NADPH, 100 μl of 10 mM α-glycerophosphate, as a substrate, 5 μl of the HRAR, and various concentrations of the samples in a total volume of 1.0 ml of 100 mM sodium phosphate buffer (pH 6.2). The AR activity was determined by measuring the decrease in NADPH absorption at 340 nm over a 1 min period on an Ultraspec®2100pro UV/Visible spectrophotometer with SWIFT II Applications software (Amersham Biosciences, New Jersey, USA). Quercetin and epalrestat, well known aldose reductase inhibitors (ARIs) were used as positive controls. The inhibition percentage (%) was calculated similar to the RLAR assay, except that AA sample/min represents the reduction of absorbance for 1 min with the test samples and substrate.

2.6.7. Statistics

The Kruskal–Wallis test and the Mann–Whitney U test were used to determine the statistical significance of differences between values for various experimental and control groups. Data were expressed as the mean ±SEM in triplicate.

3. Results

3.1. Antioxidant capacities of different parts of C. maackii

As summarized in Table 1, and Figs. 1 and 2, characterization of the antioxidant activities of the MeOH extracts was attempted, along with the CHCl3, EtOAc, n-BuOH, and H2O fractions of the leaves, flowers, roots, and stems of C. maackii, using the in vitro DPPH free radical and authentic ONOO- scavenging assays, as well as the ·OH and total ROS inhibitory assays. As shown in Table 1, differences were noted in the antioxidant potentials of the crude MeOH extracts among the tested assays, depending on the part of the thistle assayed. The IC50 values of the DPPH radical scavenging capacity increased in the following order: roots (10.2 μg/ml); stems (11.9 μg/ml); flowers (51.9 μg/ml); leaves (84.9 μg/ml), as compared with a positive control, l-ascorbic acid (IC50 0.8 μg/ml). However, the ONOO- scavenging activity of the leaves (IC50 1.78 μg/ml) was significantly more profound than observed for other components of the plant, including the stems (IC50 8.27 μg/ml), roots (IC50 8.81 μg/ml), and flowers (IC50 11.27 μg/ml). As illustrated in Table 1, the IC50 values for the total ROS inhibition of the crude extracts were demonstrated, an order identical to that of the ONOO- assay: leaves (3.46 μg/ml); stems (8.13 μg/ml); roots (17.36 μg/ml); flowers (33.42 μg/ml). In particular, the MeOH extract of the leaves had substantial antioxidant activity, comparable with that of the positive controls, l-penicillamine (IC50 2.60 μg/ml) in the ONOO- assay, and Trolox (IC50 7.24 μg/ml) in the total ROS assay (Table 1). Among the several solvent soluble fractions obtained from different parts of the plant, the EtOAc and n-BuOH fractions had excellent antioxidant potential with IC50 values of 3.4–17.1 μg/ml for the DPPH assay (a positive control, l-ascorbic acid, IC50 0.8 μg/ml), and with IC50 values of 0.85–5.80 μg/ml for the ONOO- assays (a positive control, l-penicillamine, IC50 2.60 μg/ml) (Table 1). The EtOAc fraction obtained from all parts of the Ca. maackii also had predominant and concentration-dependent inhibitory activities with IC50 values ranging from 0.59 to 2.47 μg/ml, comparable to that of the positive control, Trolox (IC50 7.24 μg/ml), in the total ROS assay (Table 1 and Fig. 1).
However, the H2O fraction of the leaves manifested a significant 9.41\% inhibition of ONOO− generation in 1.0 mM H2O2 and 0.2 mM FeSO4, at a concentration of 40 µg/ml. Values were expressed as the mean ± SEM in triplicate.

Table 1

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Fraction</th>
<th>Yield (%)</th>
<th>DPPH (IC50 µg/ml)</th>
<th>ONOO− (% inhibition)</th>
<th>Total ROS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flowers</td>
<td>MeOH</td>
<td>9.17</td>
<td>51.9</td>
<td>11.27 ± 0.24</td>
<td>33.42 ± 0.95</td>
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<td></td>
<td>CHCl3</td>
<td>60.20</td>
<td>&gt;320</td>
<td>13.94 ± 0.19</td>
<td>&gt;40</td>
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<tr>
<td></td>
<td>EtOAc</td>
<td>5.70</td>
<td>17.1</td>
<td>1.37 ± 0.10</td>
<td>1.79 ± 0.14</td>
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<tr>
<td></td>
<td>n-BuOH</td>
<td>11.90</td>
<td>12.8</td>
<td>2.09 ± 0.04</td>
<td>7.33 ± 0.86</td>
</tr>
<tr>
<td></td>
<td>H2O</td>
<td>20.16</td>
<td>78.4</td>
<td>12.13 ± 0.13</td>
<td>&gt;40</td>
</tr>
<tr>
<td>Leaves</td>
<td>MeOH</td>
<td>24.71</td>
<td>84.9</td>
<td>1.78 ± 0.07</td>
<td>3.46 ± 0.20</td>
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<td></td>
<td>CHCl3</td>
<td>21.75</td>
<td>294.9</td>
<td>&gt;100</td>
<td>&gt;40</td>
</tr>
<tr>
<td></td>
<td>EtOAc</td>
<td>25.48</td>
<td>6.8</td>
<td>5.80 ± 0.04</td>
<td>0.59 ± 0.01</td>
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<tr>
<td></td>
<td>n-BuOH</td>
<td>9.71</td>
<td>5.9</td>
<td>1.49 ± 0.05</td>
<td>13.22 ± 1.48</td>
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<tr>
<td></td>
<td>H2O</td>
<td>39.77</td>
<td>83.9</td>
<td>9.41 ± 0.52</td>
<td>&gt;40</td>
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<tr>
<td>Roots</td>
<td>MeOH</td>
<td>16.21</td>
<td>10.2</td>
<td>8.81 ± 0.34</td>
<td>17.36 ± 0.61</td>
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<tr>
<td></td>
<td>CHCl3</td>
<td>14.29</td>
<td>132.9</td>
<td>10.04 ± 0.33</td>
<td>&gt;40</td>
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<tr>
<td></td>
<td>EtOAc</td>
<td>8.23</td>
<td>3.4</td>
<td>0.85 ± 0.03</td>
<td>2.47 ± 0.27</td>
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<td>n-BuOH</td>
<td>14.38</td>
<td>6.2</td>
<td>1.68 ± 0.05</td>
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<td>H2O</td>
<td>62.33</td>
<td>97.5</td>
<td>32.43 ± 0.28</td>
<td>&gt;40</td>
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<tr>
<td>Stems</td>
<td>MeOH</td>
<td>14.90</td>
<td>11.9</td>
<td>8.27 ± 0.22</td>
<td>13.81 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>CHCl3</td>
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<td>176.7</td>
<td>25.58 ± 0.71</td>
<td>25.29 ± 3.94</td>
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<tr>
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<td>EtOAc</td>
<td>6.89</td>
<td>7.9</td>
<td>0.41 ± 0.01</td>
<td>1.60 ± 0.12</td>
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<tr>
<td></td>
<td>n-BuOH</td>
<td>11.26</td>
<td>9.8</td>
<td>1.45 ± 0.14</td>
<td>14.91 ± 0.41</td>
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<tr>
<td></td>
<td>H2O</td>
<td>52.98</td>
<td>53.1</td>
<td>21.12 ± 0.74</td>
<td>&gt;40</td>
</tr>
</tbody>
</table>

\* Yields (%) of the MeOH extracts are expressed as the crude MeOH extract weight (g) per 100 g of dried plant materials, and that of each fraction as the fractions weight (g) per 100 g of the crude MeOH extract. These values were calculated by the equation [(1 − respective weight/total weight) × 100].

\*\* 50% inhibitory concentrations (IC50 µg/ml).

\*\*\* Positive controls. Values were expressed as the mean ± SEM in triplicate.

In conclusion, the antioxidant principles may exist in the relatively polar fractions, including the EtOAc and n-BuOH fractions.

3.2. RLAR inhibitory activities of different parts of C. maackii

In order to evaluate the AR inhibitory activities, different parts of C. maackii were determined via the RLAR inhibition assay. Each part of this Korean thistle showed a concentration-dependent inhibition. As shown in Table 2, the leaf extract exerted the most potent RLAR inhibitory activity with an IC50 value of 0.54 µg/ml, compared with the positive control, quercitin, with an IC50 value of 0.22 µg/ml. The root and stem extracts followed with IC50 values of 2.55 and 3.22 µg/ml, respectively, then the flower with an IC50 value of 7.98 µg/ml. These RLAR inhibitory results coincided with the order of the ONOO− scavenging and total ROS inhibitory data.

3.3. Quantitative analysis of two active flavonoids from different parts of C. maackii

By virtue of repeated column chromatography of the EtOAc fractions from the leaves, luteolin 5-O-β-D-glucopyranoside (1) and luteolin (2) were obtained as major constituents in substantial addition, the respective EtOAc and n-BuOH fractions had potent antioxidant activities with % inhibitions from 53.12 to 69.75, comparable to that of the positive control, Trolox (69.36\%), in the -OH assay (Fig. 2). Conversely, the CHCl3 fractions of the individual parts exhibited no activities in the -OH assay, and most of the H2O fractions tended to exhibit only minimal antioxidant activity. However, the H2O fraction of the leaves manifested a significant degree of ONOO− scavenging activity with an IC50 value of 9.41 µg/ml and an -OH % inhibition of 51.40 (Table 1 and Fig. 2). Considering the above antioxidant results of the extracts and fractions from the C. maackii flowers, leaves, roots, and stems, it can be

![Fig. 2](image-url)
amounts of 23.25 g and 10.76 g, respectively (Scheme 1). In order to determine the part-specific preponderance of 1 and 2, HPLC quantitative analysis of the C. maackii flowers, leaves, roots, and stems was performed (Fig. 3). The MeOH extract of the flowers had clear differences from the crude extracts obtained from other parts of the plant. The flower MeOH extract exhibited peaks smaller than those seen in the extracts of the leaves, stems, and roots, corresponding to 1 and 2 at retention times of 27.40 and 40.50 min, along with two other larger peaks at 33.27 and 43.92 min (Fig. 3A), which were confirmed to be apigenin 7-O-β-D-glucuronide and apigenin, respectively. In the MeOH extracts of the leaves, roots, and stems, however, the peaks corresponding to 1 and 2 were the primary peaks (Fig. 3B–D). The relative content of 1 in the flowers, leaves, roots, and stems MeOH extracts from C. maackii were 5.98%, 45.18%, 68.80%, and 55.12%, respectively. In the case of the leaves, the yield of the EtOAc fraction from the MeOH extract was 25.48%, indicating that the leaves might be a great source of 1 (Table 1). The contents of 2 in the flowers, leaves, roots, and stems MeOH extracts were 14.94%, 31.54%, 10.83%, and 22.79%, respectively (Fig. 4). The contents of 1 and 2 were also low in the crude flower extracts, exerting relatively weak antioxidant activities, indicating that the two flavonoids were crucial contributors to bioactivity.

3.4. Antioxidant and AR inhibitory activities of two active flavonoids

In this study we have evaluated the antioxidant and AR inhibitory properties of 1 and 2 in DPPH free radical, total ROS, ONOO−, RLAR, and HRAR assays (Table 3). Our results demonstrated diverse antioxidant potentials between the DPPH radical, ONOO−, and total ROS assays. Compound 1, which harbors a glucose moiety at the 5 position, exerted antioxidant activities more potent than observed in its aglycone (2) in the total ROS system. Compound 1 at a concentration of 40 μg/ml, was measured to have a % inhibition of 72.33, comparable to the positive control, Trolox (72.87%). Compound 2, however, had an activity higher than 1 in the DPPH radical and ONOO− systems. Compound 2 exhibited an ONOO− scavenging % of 73.78 at a concentration of 10 μg/ml, comparable to that of the positive control, l-penicillamine (75.08%). Also, 2 clearly possessed a DPPH radical scavenging activity with an IC50 value of 0.06 μM, more potent than the DPPH radical scavenging activities of the well-known antioxidant, l-ascorbic acid (IC50 1.74 μM). In both AR assays, 1 and 2 exhibited outstanding AR inhibitory activities with respective IC50 values of 0.33 and 0.52 μM for RLAR, and 6.07 and 9.13 μM for HRAR, as compared with well-known AR inhibitors, quercetin (IC50 1.53 μM for RLAR and IC50 14.79 μM for HRAR) and epalrestat (IC50 0.07 μM for RLAR and HRAR). In particular, 1 exerted as good an inhibitory activity as 2, an AR inhibitor, including RLAR and HRAR (Matsuda et al., 2002; Kato et al., 2008).
4. Discussion

AR is a NADPH-dependent oxidoreductase, and acts as a key enzyme in the polyol pathway. Under diabetic conditions, there is excessive glucose uptake in tissues, resulting in an increased rate of the AR-related polyol pathway. Accelerating the AR-polyol mechanism augments the reduction of glucose to sorbitol, followed by nicotinamide adenine dinucleotide (NADH)-dependent sorbitol dehydrogenase-catalyzed fructose production (Kaneko et al., 2005). These excessive glucose reduction and sorbitol oxidation are reported to result in consumption and depletion of NADPH, leading to augmentation of cellular oxidative stress and ROS production in cells and tissues (Chung et al., 2003; Srivastava et al., 2005). Excessive free radicals, ROS and RNS, result in the oxidation of cellular molecules, proteins, lipids, and DNA, ultimately culminating in cell death or tissue injury. This phenomenon has been implicated in a variety of human degenerative phenomena, including aging, diabetes (Srivastava et al., 2005), inflammation (Azad et al., 2008), and diabetic complications (Pacher et al., 2005; Brownlee, 2001). Considering this intimate relationship between antioxidant and AR inhibitory activities, potent antioxidants might take advantage of the development of promising anti-diabetic complications agents in addition to ARIs. In particular, flavonoids have been shown to exert their antioxidant activities by virtue of both their excellent metal chelation and radical scavenging properties (Rice-Evans et al., 1996). As a result of their antioxidant and free radical scavenging activities against ROS and RNS, the flavonoids are believed to function as anti-diabetic complications agents (Jung et al., 2008a,b; Manzano et al., 2006; Park et al., 2004). Since antioxidant activity is highly associated with AR inhibitory activities, and flavonoids might be the key contributors to both activities, the present study on antioxidant and AR inhibitory activities of C. maackii was of great value.

In our consecutive research on the relevance of the phytochemical content and bioactive potency of Korean thistles, we have focused on C. maackii. As milk thistle is popular worldwide due to its major components that harbor various bioactivities, silymarin, its major components that harbor various bioactivities, and silibinin, its major components that harbor various bioactivities, silymarin was verified previously from the leaves of C. sieboldii (Iwashina et al., 1995, 1989; Harborne, 1967). Interestingly, 1 and 2 were characterized and isolated as predominant compounds from the leaves of C. maackii in the present study (Scheme 1). Except for the flower MeOH extract, HPLC chromatograms of the other parts of the C. maackii bore two identical peaks at 27.40 and 40.50 min, corresponding to 1 and 2, respectively (Fig. 3B–D). Although 1 and 2 occurred in all parts of the C. maackii, there was little difference in relative content: 1 was 5.98%, 45.18%, 68.80%, and 55.12%, and 2 was 14.94%, 31.54%, 10.83%, and 22.79%, in the leaves, roots, stems, and flowers, respectively (Fig. 4). Considering the yield (%) of the extracts and fractions from each part (Table 1), the leaf MeOH extract and its EtOAc fraction were expected to harbor bioactive components (1 and 2) in large quantities, indicating that the leaves exerted the most effective part of the antioxidant and anti-diabetic complications among various parts of C. maackii.

Based on our antioxidant results, the MeOH extracts and polar fractions of each part of the C. maackii were found to possess significant antioxidant activity. The leaves, roots, and stems exerted excellent antioxidant capacities in the ONOO\(^{-}\) and total ROS assays, as well as predominant inhibitory activities in the RLAR assay. In particular, the EtOAc and n-BuOH fractions from the leaves, stems, and roots exhibited significant DPPH radical scavenging activities, as well as total ROS and -OH inhibitory activities (Table 1; Figs. 1 and 2). Although all parts of the C. maackii exhibited good inhibitory activities against RLAR, the leaves exhibited outstanding AR inhibitory activities (Table 2). While the antioxidant capacity of 1 and 2 showed more or less difference of the intensity in respective assays, two flavonoids exhibited significant antioxidant activities and AR inhibitory activities in test assays (Table 3). The presence of an ortho-hydroxyl group (catechol) within the B-ring was associated with marked antioxidant activities in the DPPH, ONOO\(^{-}\), and total ROS assays, and the presence of a hydroxyl group at the C-5 position may exert an even more significant activity in the total ROS assay than in other assays. Although studies on the inhibitory activities of the Cirsium species against diabetes and diabetic complications were limited, various activities of luteolin and its glycosides were reported: luteolin and its commonly occurring glycoside have been reported to possess significant antiviral, hepatoprotective (Park et al., 2004), antioxidant (López-Lázaro, 2009), anti-AR (Kato et al., 2008; Jung et al., 2007; Matsuda et al., 1995), and anti-diabetes activities (Kim et al., 2000; Reddy et al., 2005). In particular, luteolin 5-O-β-rutinoside was reported to possess anti-diabetes activities (Zarruolo et al., 1996). Although there are several AR inhibitory studies on luteolin (2) (Kato et al., 2008), the AR inhibitory activities of luteolin 5-O-β-rutinoside (1) and Cirsium species via the RLAR and HRAR assays were revealed in this study for the first time. To the best of our knowledge, the AR inhibitory activities of thistles were also first displayed in the present work. The differences in the RLAR and HRAR inhibitory potency of two flavonoids might be attributed to the chemical structures of the ARIs, as well as the differences of AR sources, such as the species, site, and tissue, leading to the different degrees of bulk tolerance for the various enzymes (Nishimura et al., 1991; Brownlee, 2001; Kador et al., 1980). Also there are several reports that some flavones exhibited a non-competitive AR

### Table 3

Antioxidant and Anti-AR inhibitory activities of two flavonoids from C. maackii.

<table>
<thead>
<tr>
<th></th>
<th>DPPH(^{a})</th>
<th>ONOO(^{-})(^{b})</th>
<th>Total ROS(^{c})</th>
<th>RLAR(^{d})</th>
<th>HRAR(^{e})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.56 ± 0.15</td>
<td>21.88 ± 1.39</td>
<td>72.33 ± 0.77</td>
<td>0.33 ± 0.00</td>
<td>6.07 ± 0.05</td>
</tr>
<tr>
<td>2</td>
<td>0.06 ± 0.01</td>
<td>73.78 ± 1.19</td>
<td>48.43 ± 0.71</td>
<td>0.52 ± 0.05</td>
<td>9.18 ± 0.10</td>
</tr>
<tr>
<td>1-Ascobic acid(^{f})</td>
<td>1.74 ± 0.19</td>
<td>75.08 ± 1.40</td>
<td>72.87 ± 1.31</td>
<td>1.53 ± 0.20</td>
<td>14.79 ± 0.35</td>
</tr>
<tr>
<td>1-L-Penicillamine(^{g})</td>
<td></td>
<td></td>
<td></td>
<td>0.07 ± 0.00</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>2-L-Penicillamine(^{g})</td>
<td></td>
<td></td>
<td></td>
<td>0.06 ± 0.01</td>
<td>73.78 ± 1.19</td>
</tr>
<tr>
<td>0.06 ± 0.01</td>
<td>73.78 ± 1.19</td>
<td>48.43 ± 0.71</td>
<td>0.52 ± 0.05</td>
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<td>73.78 ± 1.19</td>
</tr>
</tbody>
</table>

\(^{a}\) Inhibitory (50%) concentrations (IC\(_{50}\) µM).

\(^{b}\) % Inhibition at respective concentrations of 10 µg/ml and 40 µg/ml.

\(^{c}\) Positive controls. Values were expressed as the mean ± SEM in triplicate.
inhibition with DL-glyceraldehyde as the variable substrate (Okuda et al., 1982).

In other words, the larger the quantity of 1 and 2 in the extract and fractions from various thistle parts, the more effective the intensity of the antioxidant activities in the ONOO−, total ROS, and −OH assays, and the AR inhibitory activities in the RLAR and HRAR assays, supporting the findings that these two major flavonoids might be employed as key components for further characterization in the antioxidant and anti-diabetic complications potential of C. maackii.

In conclusion, comparative evaluation upon the AR inhibitory activities and HPLC quantitative analysis of C. maackii might be of use to the development of therapeutic or preventive agents for oxidative stress-related diabetic complications. Moreover, this plant is a rich source of 1 and 2, taking crucial roles in its bioactivities of this thistle. Therefore, this Korean thistle might be utilized as a medicinal and alternative herb source with a vast range of bioactivities and two active flavonoids (1 and 2). In addition, our present results will pave the way of the guideline for the efficacy, as well as preparation, differentiation, and standardization of this medicinal thistle, in that the bioactivities of the respective parts of this plant were evaluated. Further investigations into the bioactivity of these natural flavonoids may prove useful in the prevention of a variety of radical-mediated injuries and pathologic situations in vivo.

Conflict of interest

The authors declare that there are no conflicts of interest.

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