Determination of flavonoids from *Perilla frutescens* var. *japonica* seeds and their inhibitory effect on aldose reductase

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**Abstract** *Perilla frutescens* var. *japonica* (PF) is an annual aromatic herb has been consumed as a food ingredient and medicinal crop in Asian countries. To evaluate the therapeutic efficacy of aldose reductase (AR) inhibition, we tested the PF seeds. The stepwise polarities of PF were tested for AR inhibition, and we determined the CH$_2$Cl$_2$ and EtOAc fractions to be good inhibitors (5.81 and 3.99 µg/mL, respectively). Compounds 1–3 were isolated from the CH$_2$Cl$_2$ and EtOAc fractions and identified as luteolin (1), apigenin (2), and diosmetin (3) by physico-chemical and spectroscopic data. Among them, luteolin (1) and apigenin (2) had high AR inhibitory activity (1.89 and 4.18 µM). Deulsaem, a variety of PF, was determined to have the highest flavonoid content among ten PF seeds tested (2.10 mg/g). This study suggests that PF could be utilized as a natural source to treat diabetic complications.

**Keywords** Aldose reductase · Diabetic complications · *Perilla frutescens* var. *japonica*

**Introduction**

Aldose reductase (AR), part of aldo–keto superfamily, is the key enzyme in the polyol pathway, which catalyzes the reduction of glucose to fructose [1, 2]. AR can be found in almost all mammalian cells; however, its accumulation in the lens, sciatic nerves, and retina can cause diabetic complications [3]. An influx in the polyol pathway can lead to the increased sorbitol levels, which results in the generation of osmotic stress and blindness [4, 5]. Thus, preventing AR accumulation is vital.

*Perilla frutescens* var. *japonica* (PF) is an annual aromatic herbaceous plant that belongs to the Lamiaceae family. It has been consumed as a food ingredient and medicinal crop in Asian countries for many years [6]. It is grown primarily in East Asia, India, Japan, and Korea. In Korea, oil extracted from the seeds of PF by pressing is used. Presently, PF is used to extract oil and as an ornamental plant in Europe [7]. Previous research revealed that PF has antioxidant [8], anti-allergic [9], and anti-inflammatory properties; it also promotes anti-tumor effects [10], and effects on gastrointestinal motility [11]. Therefore, its oil could be a good supplement for improving blood flow [12].

PF oil contains a high amount of omega-3 fatty acids such as α-linolenic acid (ALA), which constitutes approximately 60% of the total. Other researchers have reported that dietary intake of ALA can protect against bladder cancer [13] and limit the risk of coronary artery disease [14, 15] and prevent diseases such as cardiovascular disorders, cancer, inflammatory, and rheumatoid arthritis [16]. Because of this, it is widely used as a functional food with pharmaceutical and nutritional value. Previous studies have reported on additional biological compounds of PF. Therefore, we investigated the chemical composition of PF.
The objective of this work was to investigate the chemical profile of PF. We isolated compounds from an EtOH extract, and their structures were elucidated by EI-MS, as well as $^1$H- and $^{13}$C-NMR. Additionally, fractions and compounds of PF were tested for AR inhibition.

### Materials and methods

#### Plant materials

PF was obtained in 2014 from Department of Southern Area Crop Science, National Institute of Crop Science, Rural Development Administration, Miryang, Korea. Voucher specimens of PF were deposited at our Department.

#### Apparatus and chemicals

EI-MS analyses were recorded on a JEOL JMS-600W mass spectrometer (Tokyo, Japan). $^1$H- and $^{13}$C-NMR spectra were determined on a Bruker AVANCE 500 NMR spectrometer (Rheinstetten, Germany). HPLC semi-prep was conducted on an Agilent 1260 infinity (Santa Clara, USA). Samples were analyzed using an Agilent 7890A gas chromatographer (Agilent, CA, USA) equipped with an FID and DB-wax column (Agilent, 30 mm × 0.25 mm, 0.25 μm). Reagents and solvents, including EtOH, n-hexane, CH$_2$Cl$_2$, EtOAc, and n-BuOH, were obtained from Sigma–Aldrich (St. Louis, MO, USA).

#### Extraction and fractionation of PF

Dried seeds of PF (6 kg) were finely powdered and extracted with EtOH for 3 h (21 L × 3) under reflux at 65–75 °C. After filtration and evaporation in vacuo, the EtOH extract residue (285.3 g) was collected. Collected extracts were suspended in distilled water and partitioned successively with n-hexane (222.8 g), CH$_2$Cl$_2$ (9.3 g), EtOAc (10.4 g), and n-BuOH (11.4 g).

#### Isolation of compounds 1–3 from PF

EtOH extract from PF seeds was subjected to chromatographic separation on a silica gel and Sephadex LH-20. A portion of the EtOAc fraction (3.4 g) was chromatographed by silica gel column chromatography (6 × 80 cm, No. 7734) using a CH$_2$Cl$_2$–MeOH elution system to yield eight fractions (E$_1$–E$_8$). Subfraction E$_4$ afforded compounds 1 and 2, which were isolated by semi-prep HPLC. A portion of the CH$_2$Cl$_2$ (3.1 g) fraction from PF was separated by silica gel column chromatography (6 × 80 cm, No. 7734) eluted with a stepwise gradient of the n-hexane–EtOAc system and then repeatedly by the EtOAc–MeOH system. The CH$_2$Cl$_2$ fraction yielded 18 additional fractions (M$_1$–M$_{18}$). Fraction M$_{12}$ yielded compound 3 by recrystallization using MeOH.

**Compound 1:** EI-MS m/z: 286 (100) [M]$^+$, 278 (16), 258 (14), 153 (28), 134 (7), 129 (13); $^1$H- and $^{13}$C-NMR (500 MHz, DMSO-$d_6$): See Table 1.

**Compound 2:** EI-MS m/z: 300 (51) [M]$^+$, 270 (100), 257 (7), 242 (13), 229 (4), 153 (20), 121 (9), 96 (2), 69 (5); $^1$H- and $^{13}$C-NMR (500 MHz, DMSO-$d_6$): See Table 1.

**Compound 3:** EI-MS m/z: 300 (100) [M]$^+$, 272 (11), 257 (17), 229 (12), 213 (2), 153 (22), 136 (9), 115 (8), 69 (5); $^1$H- and $^{13}$C-NMR (500 MHz, DMSO-$d_6$): See Table 1.

#### Measurement of AR activity

Rat lenses were removed from Sprague–Dawley rats and preserved by freezing until use. Each sample of the EtOH extract, n-hexane, CH$_2$Cl$_2$, EtOAc, n-BuOH fractions and compounds 1–3 were dissolved in DMSO for the AR activity assay [17].

#### Sample preparation for HPLC

For analysis of compounds 1–3 using HPLC, the PF seeds were extracted with EtOH. Extracts were then filtered with a syringe filter (0.45 μm), and the solution was used for HPLC analysis.

#### Quantitative analysis of PF compounds 1–3

HPLC analysis of PF compounds 1–3 was conducted. A Waters Spherisorb® INNO C18 (4.6 × 250 mm, 5 μm) column was used for their simultaneous determination. The mobile phase included 0.5% acetic acid (reagent A) and MeOH (reagent B). The gradient system was initially set at 70 (A):30 (B), increased in linear gradient to 45:55 for 20 min, and then increased to 0:100 for 30 min and kept for 5 min at 0.5 mL/min. Finally, the gradient was increased to 70:30 for 10 min. The total analysis was conducted over 65 min. Flavonoids were detected at 340 nm. The limits of detection and quantification (LOD and LOQ, respectively) for flavonoids as standard compounds were used to validate the HPLC method.

#### Calibration curves

Flavonoid stock solutions (0.1–10 μg/mL) were prepared in MeOH. The flavonoid content of the samples was determined using the corresponding calibration curves. The
calibration curve for flavonoids was calculated using the peak area (Y), concentration (X, mg/mL), and mean values (n = 3) ± standard deviation (SD), which is shown in Fig. 2.

**Results and discussion**

The PF EtOH extracts were tested for AR inhibition. The results are summarized in Table 2. The EtOAc and CH$_2$Cl$_2$ fractions significantly inhibited AR in rat lens (IC$_{50}$ = 3.99 and 5.81 µg/mL, respectively). Previous researchers also studied the various biological activities of PF. Extracts from PF seeds have been shown to have antioxidant [18] and antimicrobial activity [19]. However, there are still limited reports regarding the biological activity of PF. Our results demonstrate that PF seed extracts inhibit AR.

The EtOAc and CH$_2$Cl$_2$ fractions were chosen to be isolated and identified because they showed superior AR inhibitory properties. Specifically, they were chromatographed on a silica gel and Sephadex LH-20 column. Thus, three flavonoids (compounds 1–3) were isolated. The chemical structures were identified using $^1$H- and $^{13}$C-NMR and EI-MS. Compounds 1–3 were observed to have a typical flavonoid pattern. Spectrums of a singlet signal at δ 12.96 and 12.97 were observed in the presence of a 5-OH in an A-ring structure; $^1$H-NMR spectra data are shown in Table 1. The four compounds were verified based on previous literature [20, 21, 22]. Additionally, spectroscopic NMR data were obtained. As shown in Fig. 1, the chemical structures of compounds 1–3 were identified as luteolin (1), apigenin (2), and diosmetin (3).

Table 1 $^1$H- and $^{13}$C-NMR spectral data for compounds 1–3 from PF

| No. | 1 | 2 | 3 |
|-----|---|---|---|
| δH | δC | δH | δC | δH | δC |
| 2 | – | 163.8 | 163.6 | – | 163.6 |
| 3 | 6.67 (s) | 102.8 | 102.7 | 6.88 (s) | 103.2 |
| 4 | – | 181.5 | 181.6 | – | 181.8 |
| 5 | – | 157.2 | 157.3 | – | 157.3 |
| 6 | 6.19 (s) | 98.8 | 98.8 | 6.19 (d$_2$) | 98.8 |
| 7 | – | 164.1 | 164.4 | – | 164.1 |
| 8 | 6.44 (s) | 93.8 | 93.9 | 6.50 (d$_2$) | 94.0 |
| 9 | – | 161.4 | 161.1 | – | 161.4 |
| 10 | – | 103.6 | 110.2 | – | 110.2 |
| 1’ | – | 118.9 | 121.1 | – | 121.5 |
| 2’ | 7.40 (d, 2.0) | 113.3 | 7.92 (d, 8.5) | 128.3 | 7.54 (br s) | 120.3 |
| 3’ | – | 145.7 | 6.92 (d, 8.5) | 115.8 | – | 148.0 |
| 4’ | – | 149.7 | 161.4 | – | 150.7 |
| 5’ | 6.89 (d, 8.5) | 115.9 | 6.92 (d, 8.5) | 115.8 | 6.92 (d, 8.0) | 115.9 |
| 6’ | 7.42 (d, 8.5, 2.0) | 121.4 | 7.92 (d, 8.5) | 128.3 | 7.56 (dd, 8.0, 1.5) | 128.5 |
| 5-OH | 12.98 (s) | 12.96 (s) | 12.96 (s) | 5.89 (s) | 5.89 (s) |
| 4’-OCH$_3$ | 3.89 (s) | 55.9 |

Compounds 1–3 were tested for inhibition of AR in rat lens. Data are shown in Table 3. Luteolin (1) exhibited a greater inhibitory effect than TMG, the positive control. The percent inhibition for luteolin (1) and TMG was 1.89 and 2.52 µM, respectively. Compounds 1 and 2 were isolated from the EtOAc fraction, whereas compound 3 was isolated from the CH$_2$Cl$_2$. The structures of compounds 1 and 2 are similar, except for the presence of additional hydroxyl group in B-ring. Compounds 1 and 2 have a di- and monohydroxy group, respectively. The effects of compound 1 were greater than those of compound 2. The structures of compounds 1 and 3 were found to be except for B-ring; the structure of compound 3 contains a methoxy group. The AR inhibitory activity of compound 1 is greater than that of compound 3. These data demonstrate that a dihydroxyl in B-ring exhibited greater effect than a monomoiety, methoxy group at the B-ring, and positions at C-3 of skeleton do not affect the AR inhibitory activity. Generally, flavonoids are potent AR inhibitors. Previous studies demonstrated that the AR inhibitory activity of flavonoids is related to their structure [23, 24, 25]. They also have antioxidant and anti-inflammatory properties [26, 27]. Consequently, PF seed extracts could be used to inhibit AR. Further studies are required to understand the mechanisms of inhibition.
Four flavonoids showed significant AR inhibitory effects. HPLC/UV analysis was conducted to determine the concentration of compounds 1–3 in ten PF seeds (Dami, Danjo, Deulsam, Daesil, Anyu, Yujin, Dayu, Yupseol, Hyangim, and Hwahong). The flavonoid contents are shown in Fig. 2 and Table 5; HPLC chromatograms are shown in Fig. 2. In all samples, luteolin (1) and apigenin (2) were found in higher amounts among the four compounds tested (1.45 and 5.17 mg/g extract, respectively). Among the ten PF seeds, Deulsam contained the highest total concentration of flavonoids (2.1 mg/g extract). In contrast, Danjo contained no flavonoids. Almost PF seed has much amount of apigenin (2) and demonstrates that

### Table 2 IC₅₀ of PF extracts and fractions on rat lens AR

| Samples        | Concentration (µg/mL) | AR inhibitiona (%) | IC₅₀ (µg/mL) |
|----------------|-----------------------|-------------------|-------------|
| EtOH ext.      | 10                    | 57.70 ± 2.09      | –           |
| n-Hexane fr.   | 10                    | 56.24 ± 2.87      | –           |
| CH₂Cl₂ fr.     | 10                    | 76.54 ± 1.04      | 5.81        |
|                | 1                     | 21.26 ± 1.56      |             |
|                | 0.1                   | 11.76 ± 0.90      |             |
| EtOAc fr.      | 10                    | 90.70 ± 1.75      | 3.99        |
|                | 1                     | 32.52 ± 1.04      |             |
|                | 0.1                   | 20.93 ± 1.65      |             |
| n-BuOH fr.     | 10                    | 46.82 ± 0.90      | –           |
|                | 1                     | 60.70 ± 1.98      | 0.45        |
|                | 0.1                   | 32.93 ± 2.89      |             |

a Inhibition rate was calculated as a percentage of the control value

b IC₅₀ was 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 3 IC₅₀ of compounds 1–3 from PF on rat lens AR

| Compound | Concentration (µg/mL) | AR inhibitiona (%) | IC₅₀ (µM) |
|----------|-----------------------|-------------------|----------|
| 1        | 10                    | 79.75 ± 3.16      | 1.89     |
|          | 1                     | 63.24 ± 2.57      |          |
|          | 0.1                   | 28.05 ± 2.71      |          |
| 2        | 10                    | 90.58 ± 1.84      | 4.18     |
|          | 1                     | 44.66 ± 3.30      |          |
|          | 0.1                   | 8.03 ± 0.68       |          |
| 3        | 10                    | 66.48 ± 1.82      | –        |
|          | TMG                   | 87.32 ± 1.11      | 2.52     |
|          | 1                     | 57.23 ± 1.69      |          |
|          | 0.1                   | 32.49 ± 1.22      |          |

a Inhibition rate was calculated as a percentage of the control value

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

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Fig. 1 Structures of compounds 1–3
Apigenin (2) is major compound of PF. Ha et al. [28] and Guan et al. [29] also studied the content of phenolic compounds including flavonoids in PF using HPLC/PDA and HPLC-ESI/QTOF/MS/MS. Quantitative analyses were conducted using a calibration curve. The linearity of standard curves and correlation ($r^2$) for compounds 1–3 are shown in Table 4. The LOD and LOQ of compounds 1–3 were 0.000–0.001 mg/mL and 0.001–0.005 mg/mL, respectively (Table 6).

We evaluated the ability of PF seed extracts to inhibit AR; the EtOAc fraction and compound 1 had significant inhibitory activity. Deulsaem was determined to have the
highest total flavonoid content among ten PF seeds tested. This sample could be used to increase the flavonoid yield. The results of this study show that PF can be used in the development of preventative agents against diabetic complications. However, further investigations on the mechanisms of AR inhibition are needed.
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Table 4 Linearity of standard curves for compounds 1–3

| Compound | tR | Calibration equation \(a\) | Correlation factor, \(r^2 \) \(b\) |
|----------|----|---------------------------|-------------------------------|
| 1        | 21.66 | \(Y = 4000000X + 1427.3\) | 0.9999                       |
| 2        | 23.68 | \(Y = 6000000X + 2319.4\) | 0.9999                       |
| 3        | 23.84 | \(Y = 3000000X + 7595.6\) | 1                            |

\(a\) \(Y\) = peak area, \(X\) = concentration of standards (mg/mL)

\(b\) \(r^2\) = correlation coefficient for three data points in the calibration curves (\(n = 5\))

Table 5 Flavonoid content of PF

| PF  | Contents (mg/g extract) |
|-----|-------------------------|
|     | 1            | 2          | 3          | Total       |
| Dami | ± 0.00       | 0.19 ± 0.01| 0.02 ± 0.00| 0.22 ± 0.01|
| Danjo| ND           | ND         | ND         | ND          |
| Deulsaem | 0.47 ± 0.00 | 1.45 ± 0.12| 0.18 ± 0.03| 2.10 ± 0.15|
| Deulsaem | 0.33 ± 0.01 | 1.37 ± 0.04| 0.13 ± 0.00| 1.83 ± 0.05|
| Annyu | 0.09 ± 0.00 | 0.17 ± 0.00| 0.05 ± 0.00| 0.31 ± 0.00|
| Yujin | 0.15 ± 0.01 | 0.57 ± 0.00| 0.07 ± 0.00| 0.79 ± 0.01|
| Dayu  | 0.14 ± 0.02 | 0.46 ± 0.07| 0.04 ± 0.01| 0.64 ± 0.10|
| Yujseol | 0.07 ± 0.00 | 0.30 ± 0.01| 0.06 ± 0.00| 0.43 ± 0.01|
| Hyangim| tr           | 0.01 ± 0.00| tr          | 0.01 ± 0.00|
| Hwahong| 0.19 ± 0.00 | 0.65 ± 0.01| 0.04 ± 0.00| 0.88 ± 0.01|

ND none detected, tr trace

Table 6 Linearity of standard curves for compounds 1–3

| Compound | Calibration equation \(a\) | \(r^2 \) \(b\) | Linear range (mg/mL) | LOD (mg/mL) | LOQ (mg/mL) |
|----------|---------------------------|----------------|----------------------|-------------|-------------|
| 1        | \(Y = 278511X + 348.97\) | 1              | 0.01–100             | 0.000       | 0.003       |
| 2        | \(Y = 394029X + 419.36\) | 1              | 0.01–100             | 0.000       | 0.002       |
| 3        | \(Y = 191896X + 96.42\)  | 1              | 0.01–100             | 0.001       | 0.005       |

\(a\) \(Y\) = peak area, \(X\) = concentration of standard (mg/mL)

\(b\) \(r^2\) = correlation coefficient for three data points in the calibration curve
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