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Isolation and Identification of Flavonoids with Aldose Reductase Inhibitory Activity from *Petasites japonicus*

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The purpose of this study was to evaluate the therapeutic potential of naturally occurring aldose reductase inhibitors extracted from *Petasites japonicus*. Methanol extract and stepwise polarity fractions of *P. japonicus* leaves were tested for aldose reductase inhibition on rat lenes *in vitro*. Of these, the ethyl acetate (EtOAc) fraction exhibited aldose reductase inhibitory activity (IC_{50} value, 0.26 μM). Chromatography of the active EtOAc fraction led to the further isolation of four flavonoids, identified as kaempferol-3-*O*-(6"-acetyl)-β-D-glucoside (**1**), quercetin-3-*O*-(6"-acetyl)-β-D-glucoside (**2**), kaempferol-3-*O*-β-D-glucoside (**3**) and quercetin-3-O-β-D-glucoside (**4**). Compounds **1-4** exhibited high aldose reductase inhibitory activity, with IC_{50} values of 6.08, 3.46, 8.55 and 2.21 μM, respectively. Compounds **1** and **2** were isolated for the first time from this plant and compound **4** showed the highest aldose reductase inhibitory activity. These results suggest that compound **4** extracted from *P. japonicus* is a potent aldose reductase inhibitor and could be a useful lead compound in the development of a novel aldose reductase inhibitory agent against diabetic complications.

Keywords: Aldose reductase inhibition, Diabetic complication, Flavonoid, *Petasites japonicus*.

INTRODUCTION

Petasites japonicus is a perennial herb belonging to the family Compositae and grows wild in mountain and field areas of Korea, Japan, China and other countries[1,2]. *P. japonicus* grows to a height of 30 cm, its leaf is kidney-shaped and it bears cylindrical white flowers from May to June[3]. It is a wild vegetable used widely as a side dish in Korea. Its leafstalk is edible and the flower stems are often used as a spice in food[1,2]. *P. japonicus* is commonly prescribed as a traditional treatment for cough, phlegm, stomach ache, swelling, diuresis and body ache[4-7]. In addition, the rhizomes are used in Chinese folk medicine to treat tonsillitis, contusions and poisonous snake bite[8]. The plant has beneficial effects on osteoblast cells and has been shown to prevent osteoporosis. *P. japonicus* is also thought to have antiallergic[4], antioxidant and anticancer effects[2,9]. This plant has shown other interesting properties, such as fatty diet effects[10] and anti-apoptotic activity[11].

Further studies on the rhizomes of this plant have reported isolation of eremophilane type sesquiterpene lactone compounds, eremopetasitenins A1, A2, B1, B2, C1, C2, C3, D1, D2 and D3, sulfoxide bearing lactone eremopetasinsulfoxide[12,13], secoeremopetasitolides A and B[14], eremopetasidione[15] and petasiphenone and petasiphenol[16]. Eremophile-nosides[17] and isopetasoside[18] were also isolated from the flower stalks and furofuran lignan[9], petasinophenol[19] and flavonoid glycosides[3,19] from the leaves. Some of these compounds, furofuran lignans, kaempferol-3-*O*-(6"-acetyl)-β-D-glucoside and quercetin-3-*O*-(6"-acetyl)-β-D-glucoside have been shown to be antioxidant and novel inhibitors of eukaryotic DNA polymerase α[3,9,19].

Aldose reductase is the first enzyme in the polyol pathway. These enzymes catalyze the reduction of various sugars to sugar alcohols, including glucose to sorbitol[20]. In diabetes, sufficient glucose can enter the tissues, but this pathway operates to produce sorbitol and fructose. These abnormal metabolic products have been reported as factors responsible for diabetic complications, including cataracts, retinopathy, neuropathy and nephropathy[21]. Aldose reductase inhibitors can prevent or reverse early stage diabetic complications. Nevertheless, no currently available aldose reductase inhibitors

have achieved worldwide use because of limited efficacy or undesirable side-effects. Evaluating natural sources of aldose reductase inhibitors may lead to the development of safer and more effective agents against diabetic complications[22].

The present study was undertaken to isolate and identify phytochemical constituents from *P. japonicus* leaf extract and determine their aldose reductase inhibitory capacity.

EXPERIMENTAL

P. japonicus leaves were supplied by Korea National Arboretum, Republic of Korea. A voucher specimen was deposited at the Herbarium of Department of Integrative Plant Science, Chung-Ang University, Republic of Korea.

Mass spectrometry (MS) was performed with a Jeol JMS-600W (Japan) mass spectrometer. ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were recorded with a Bruker Avance 500 NMR spectrometer (Rheinstetten, Germany). Chemical shifts were reported in parts per million (δ) and coupling constants (J) were expressed in Hertz. Evaporation was conducted using an Eyela rotary evaporator system (Japan) under reflux *in vacuo*. *n*-Hexane, chloroform, ethyl acetate and *n*-butanol were purchased from Sam Chun Pure Chemical Co. (Pyeongtaek, Korea). Recycling preparative HPLC was performed using a JAI LC-9014 system (Tokyo, Japan) equipped with an L-6050 pump and an UV-3702 UV/visible detector. Auto collector was measured using an FC-339 fraction collector. Thin layer chromatography (TLC) was conducted using Kiesel gel 60 F254 (Art. 5715; Merck Co., Germany) plates (silica gel, 0.25 mm layer thickness) and compounds were visualized by spraying with 10 % H_2SO_4 in MeOH. Fluorescence was measured with a Hitachi U-3210 spectrophotometer. Silica gel (No. 7734; Merck Co., Germany), Sephadex LH-20 (20-100 μm), DL-glyceraldehyde, β-NADPH, sodium phosphate buffer, potassium phosphate buffer, tetramethylene glutaric acid (TMG) and dimethyl sulfoxide (DMSO) were purchase from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Extraction and isolation: Dried and powdered *P. japonicus* leaves (2,950 g) were extracted with MeOH (8 L × 5) under reflux at 65-75 °C. Filtrate was concentrated until dry *in vacuo* to produce MeOH extract (541.9 g), suspended in H_2O and then partitioned using *n*-hexane, $CHCl_3$, EtOAc and *n*-BuOH, in that order. A portion of the EtOAc fraction (31.3 g) was chromatographed on a silica gel column (6 × 80 cm, No. 7734) using a stepwise gradient of the $CHCl_3$-MeOH solvent system to yield twenty nine subfractions. By recrystallization ($CHCl_3$-MeOH), subfractions 13, 17 and 18 yielded compounds **1**, **2** and **3**, respectively. Subfraction 19 (0.3 g) was processed with Sephadex LH-20 eluted with $CHCl_3$-MeOH to yield subfractions 19.1-19.10. Subfraction 19-6 yielded subfractions 19.6.1-19.6.17 upon Sephadex LH-20 rechromatography. Subfraction 19.6.14.24 also was processed with rechromatography and yielded compound **4** (subfraction 19.6.14.24.9.15).

Measurement of aldose reductase activity: Rat lenses were removed from Sprague-Dawley rats (weighing 250-280 g) and preserved by freezing until use. Lenses were homogenized and centrifuged at 10,000 rpm (4 °C, 20 min) and the supernatant was used as an enzyme source. Aldose reductase activity was spectrophotometrically determined by measuring the decrease in NADPH absorption at 340 nm for a 4 min period at room temperature with DL-glyceraldehydes as a substrate[23]. The assay mixture contained 0.1 M potassium phosphate buffer (pH 7), 0.1 M sodium phosphate buffer (pH 6.2), 1.6 mM NADPH and test extract sample (in DMSO) with 0.025 M DL-glyceraldehyde as substrate prepared in quartz celsl. The total volume of the test assay mixture was 1 mL. IC_{50} values (the concentration of inhibitors with 50 % inhibition of enzyme activity) were calculated from the least-squares regression line of the logarithmic concentrations plotted against residual activity. Tetramethylene glutaric acid, a known aldose reductase inhibitor, was used as a positive control. Each MeOH extract test sample and three fractions (each 1 mg) were dissolved in DMSO (1 mL).

RESULTS AND DISCUSSION

P. japonicus MeOH extract was tested for inhibitory effects on rat lens aldose reductase activity. Stepwise polarity fractions of the MeOH extract were evaluated for aldose reductase inhibitory activity with an *in vitro* evaluation system. *P. japonicus* leaf EtOAc fraction of exhibited significant rat lens aldose reductase inhibition, with an IC_{50} value of 0.26 μg/mL (Table-1). Its activity is more potent than tetramethylene glutaric acid (0.84 μg/mL), which is known to be a strong aldose reductase inhibitor in natural constituents.

TABLE-1
IC_{50} OF THE MeOH EXTRACT AND FRACTIONS OF *P. japonicus* ON ALDOSE REDUCTASE INHIBITION IN RAT LENSES

Sample	Concentration (μg/mL)	Aldose reductase inhibition (%)	IC_{50} (μg/mL)
MeOH extract	10	54.39	8.53
	5	37.19	
	0.1	4.56	
n-Hexane fraction	10	56.91	7.68
	5	42.11	
	1	29.93	
MC fraction	10	43.39	-
EtOAc fraction	10	92.00	0.26
	5	84.31	
	0.1	38.46	
n-BuOH fraction	10	87.84	1.33
	5	75.99	
	1	44.38	
TMG	10	87.62	0.84
	5	69.88	
	0.1	45.71	

Each sample concentration was 1 mg/mL DMSO.Inhibition rate was calculated as percentage with respect to the control value. IC_{50} value was calculated with the least-squares regression method and logarithmically plotted at three graded concentrations *vs*, % inhibition. TMG was used as a positive control

Chromatographic separation of the active *P. japonicus* leaf EtOAc fraction led to the isolation of compounds **1-4** (Fig. 1). Compound structures were identified through spectral analysis.

Spectral signal characteristic of flavonoids were observed upon NMR. Common kaempferol type flavonoids were seen in compounds **1** and **3** and quercetin type was seen in compounds **2** and **4** (Tables 2 and 3). Compound **1** was obtained

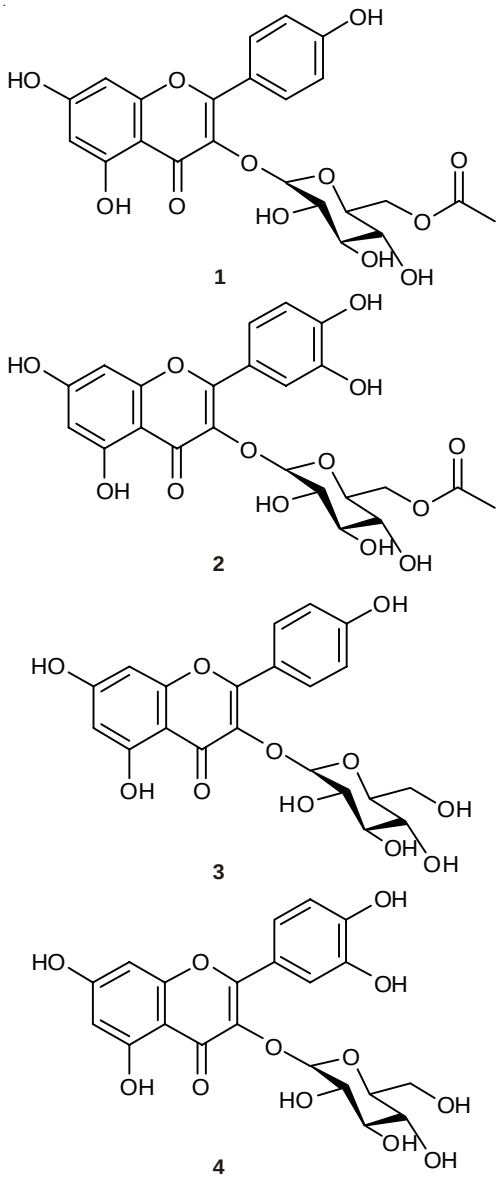

Fig. 1. Chemical structures of compounds **1-4**

TABLE-2
^{1}H NMR SPECTRAL DATA FOR COMPOUNDS **1-4** (*J* IN Hz) IN DMSO

No.	1	2	3	4
6	6.17 (d, 1.5)	6..22 (d, 2.0)	6.20 (d, 2.0)	6.19 (d, 2.0)
8	6.40 (d, 1.5)	6.42 (d, 2.0)	6.43 (d, 2.0)	6.39 (d, 2.0)
2′	7.99 (d, 8.5)	7.55 (d, 2.0)	8.04 (d, 8.5)	7.58 (d, 2.0)
3′	6.87 (d, 8.5)	-	6.88 (d, 8.5)	-
5′	6.87 (d, 8.5)	6.84 (d, 6.5)	6.88 (d, 8.5)	6.84 (d, 9.0)
6′	7.99 (d, 8.5)	7.54 (dd, 2.0, 6.5)	8.04 (d, 8.5)	7.58 (dd, 2.0, 8.7)
5-OH	12.54 (s)	12.61 (s)	12.61 (s)	12.49 (s)
Glc-1′	5.34 (d, 7.5)	5.39 (d, 7.5)	5.45 (d, 7.5)	5.46 (d, 7.5)
6″-Ha	4.10 (dd, 2.0, 11.5)	4.13 (dd, 2.0, 11.5)		
6″-Hb	3.95 (q, 6.0, 11.5)	3.96 (q, 6.0, 11.5)		
-COC<u>H</u>$_3$	1.74 (s)	1.74 (s)		

Chemical shifts are reported in parts per million (δ) and coupling constants (*J*) are expressed in Hertz

TABLE-3
^{13}C NMR SPECTRAL DATA FOR COMPOUNDS **1-4** IN DMSO

No.	1	2	3	4
2	155.9	156.0	155.7	158.5
3	132.6	132.6	132.7	135.6
4	176.9	176.9	176.9	179.5
5	160.7	160.7	160.7	163.1
6	98.2	98.2	98.2	99.8
7	163.8	169.4	163.7	166.1
8	93.2	93.0	93.1	94.6
9	156.1	155.8	155.9	158.9
10	103.4	103.4	103.4	105.4
1′	120.3	120.6	120.4	123.2
2′	130.3	114.6	130.4	116.4
3′	114.5	144.3	114.6	145.6
4′	159.5	148.0	159.4	149.4
5′	114.5	115.7	114.6	117.6
6′	130.3	121.0	130.4	123.4
Glc-1	100.6	100.5	100.3	104.3
Glc-2	73.4	69.3	73.7	75.7
Glc-3	73.6	73.5	75.9	78.9
Glc-4	69.3	62.3	69.4	71.4
Glc-5	75.6	75.7	77.0	78.0
Glc-6	62.2	59.3	60.3	62.3
-<u>C</u>OCH$_3$	169.3	169.4	-	-
-CO<u>C</u>H$_3$	19.6	19.6	-	-

as a yellow powder with a molecular ion peak at *m/z* 513 $[M+Na]^+$ in the FAS-MS and a molecular formula of $C_{23}H_{22}O_{12}$. The ^{1}H NMR spectrum of **1** exhibited a flavonoid skeleton. The presence of one singlet signal (5-OH) was observed at δ 12.54. A-ring proton signals of 6 and 8 were observed at δ 6.17 and 6.40 (1H, d, *J* = 1.5 Hz), respectively. Two doublet signals at δ 6.87 (1H, d, *J* = 8.5 Hz) and δ 7.99 (1H, d, *J* = 8.5 Hz) were confirmed protons of 3', 5' and 2', 6' in a B-ring. A doublet glucose anomeric signal was observed at δ 5.34 (1H, d, *J* = 7.5 Hz, H-1″). An acetyl group singlet was investigated at δ 1.74 (3H, s). The ^{13}C NMR-spectrum of compound **1** indicated 23 carbon resonances. Two carbon signals at δ 169.3 and 176.9 were each confirmed as an acetyl carbonyl group and a C-ring (C-4). One methyl carbon signal for the acetyl group was observed at δ 19.6. Like the ^{1}H NMR, C-3',-5' and C-2',-6' carbon signals (A_2B_2 system) appeared at δ 114.5 and 130.3, respectively. Significant HMBC correlations were observed between H-1" and C-3, confirming the location of the sugar group and between O=C- and H-6", confirming the location of the acetyl group. As shown in Tables 2 and 3, the ^{1}H- and ^{13}C NMR spectra of compound **1** were quite similar to those of kaempferol-3-*O*-β-D-glucoside (**3**)[19,24,25], except for the acetyl group. Accordingly, the structures of compounds **1** and **3** were identified as kaempferol-3-*O*-(6"-acethyl)-β-D-glucoside and kaempferol-3-O-β-D-glucoside (astragalin), respectively, after comparison with spectral data in the literature[24-27].

Compound **2** was obtained in the form of a yellow powder and showed a molecular ion peak at *m/z* 529 $[M+Na]^+$ in the FAB-MS, which corresponds to $C_{23}H_{22}O_{13}$ molecular formula. In the ^{1}H NMR spectrum, compound **2** showed ABX system (H-2', -5' and -6') with coupling constant signals at δ 6.84 (1H, d, *J* = 6.5 Hz, H-5'), 7.54 (1H, dd, *J* = 6.5, 2 Hz, H-6') and 7.55 (1H, d, *J* = 2 Hz, H-2') in a B-ring structure. The glucose anomeric signal showed a doublet at δ 5.34 (1H, d, *J* = 7.5 Hz, H-1″). The ^{1}H NMR spectrum for compound **2** also clearly showed that the following proton systems were implicated in the structure: two *meta*-coupled proton signals at H-6 and -8, which appeared at δ 6.22 and 6.42 (1H, d, *J* = 2 Hz), respectively. The other ^{1}H NMR spectrum signal for **2** was similar to data of compound **1**. The ^{13}C NMR also exhibited signals

suggesting 23 carbon atoms. One characteristic carbonyl carbon C-ring signal was observed at δ 176.9 and another carbonyl acetate group carbon was appeared at d 169.4. This means the chemical shift moved depending on electronegativity and shoul have a hydroxyl group. Additionally, the C-3 signal at δ 132.6 was seen to be connected to the glucose anomeric carbon. Expect for the acetyl group, the ^{1}H- and ^{13}C NMR spectra of compound **2** were quite similar to those of quercetin-3-*O*-β-D-glucoside (**4**)[19,25]. Accordingly, the structures of compounds **2** and **4** were identified as quercetin-3-*O*-(6"-acetyl)-β-D-glucoside and quercetin-3-O-β-D-glucoside (isoquercetin), respectively, upon comparison with the spectral data in the literature[25-27].

To evaluate aldose reductase inhibitory activity in rat lenses, inhibitory percentage and IC_{50} values were calculated for each compound. Results are presented in Table-4. IC_{50} values of kaempferol-3-*O*-(6"-acetyl)-β-D-glucoside (**1**), quercetin-3-*O*-(6"-acetyl)-β-D-glucoside (**2**), kaempferol-3-O-β-D-glucoside (**3**) and quercetin-3-O-β-D-glucoside (**4**) were shown to be 6.08, 3.46, 8.55 and 2.21 μM, respectively. Aldose reductase inhibition (2.21 μM) by quercetin-3-*O*-(6"-acetyl)-β-D-glucoside (3.46 μM) (**2**) and quercetin-3-*O*-β-D-glucoside (2.21 μM) (**4**) was more potent than TMG, a positive control (4.50 μM). Furthermore, aldose reductase inhibition of 3',4'-dihydroxyl groups of B-ring (compounds **2** and **4**) was stronger than that of the 4'-monohydroxyl group (compounds **1** and **3**).

TABLE-4
IC_{50} VALUES OF COMPOUNDS **1**-**4** FROM *P. japonicus* LEAF EXTRACT ON ALDOSE REDUCTASE INHIBITION IN RAT LENSES

Compound	Concentration (μg/mL)	Aldose reductase inhibition (%)	IC_{50} (μM)
1	10	72.49	6.08
	5	60.52	
	0.1	29.13	
2	10	83.02	3.46
	5	71.07	
	1	38.99	
3	10	69.67	8.55
	5	54.67	
	1	23.33	
4	10	85.89	2.21
	5	76.18	
	0.1	13.17	
TMG	10	87.62	4.50
	5	69.88	
	0.1	45.71	

Same as in Table-1

Conclusion

P. japonicus leaf EtOAc fraction demonstrated good inhibitory activity against aldose reductase in rat lenses based on *in vitro* data. It is postulated that quercetin-3-*O*-glucoside (**4**) from *P. japonicus* could be effective in preventing and/or retarding cataractogenic or diabetic complications through aldose reductase inhibition. Consequently, we suggest *P. japonicus* may provide a new natural resource for therapies targeting aldose reductase inhibition.

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