J Appl Biol Chem (2017) 60(1), 29–33
https://doi.org/10.3839/jabc.2017.006

Online ISSN 2234-7941
Print ISSN 1976-0442

Article: Bioactive Materials

Aldose reductase inhibitory activity of quercetin from the stems of *Rhododendron mucronulatum* for. *albiflorum*

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Received: 19 October 2016 / Accepted: 1 November 2016 / Published Online: 31 March 2017

Abstract The methanol extract of *Rhododendron mucronulatum* for. *albiflorum* (RMFA) stems inhibited aldose reductase (AR) activity. The RMFA fractions obtained by stepwise extraction with solvents of different polarity were tested for AR inhibition *in vitro* using the lens of a rat. Among them, the ethyl acetate (EtOAc) fraction inhibited AR more than the other fractions. Quercetin (**1**) from the EtOAc fraction showed a high AR inhibition with IC_{50} of 2.11 μM. The stems of RMFA contained the highest amount (5.12 mg/g extract) of quercetin. Our results suggest that RMFA, which contained quercetin, could be a useful material for the development of supplementary functional foods.

Keywords Aldose reductase inhibition · High-performance liquid chromatography · Quercetin · *Rhododendron mucronulatum* for. *albiflorum*

Introduction

Aldose reductase (AR) is a member of the aldo-keto superfamily and accelerates the reduction of glucose to sorbitol. Accumulation of excessive sorbitol influences the development of disproportionate ratios of NADPH/$NADP^+$ and NAD^+/NADH cofactors and facilitates cell transformation (Kao et al. 1999). Thereby, AR promotes the generation of osmotic and oxidative stress. Among them, oxidative stress can cause diseases, including diabetes-related complication and disorders, including retinopathy, neuropathy, and nephropathy (Enomoto et al. 2004; Jung et al. 2007; Ha et al. 2009; Jung et al. 2011). The AR accumulation can cause numerous disorders and, therefore, the discovery of AR inhibitors is crucial.

Rhododendron mucronulatum (RM) is a vascular plant that is distributed widely worldwide, especially in the northern hemisphere. An ancient source reported that RM can cause toxic honey poisoning (Gunduz et al. 2007). Despite this observation, RM has been used as a folk medicine (Gunduz et al. 2008). e.g., as a tonic, diuretic, for stomach disorders, and gonorrhoea while Koreans have used RM in cakes, wine, and as juice (Lee et al. 2007; Guleria et al. 2011). Among these products, the wine produced from the flowers exhibits significant antioxidant activity (An et al. 2005). *R. mucronulatum* for. *albiflorum* (RMFA) is a sub-species of RM, which is shrub with white flowers, and is endemic in Korea. RMFA is a rare plant, that has been endangered by indiscriminate uprooting and cutting (Lee et al. 1991). Previous studies have reported that the flowers of RMFA contain flavonoids (Mok and Lee 2012; Mok et al. 2013). However, there are limited studies on RMFA and, therefore, additional investigations of this plant are needed.

Therefore, the aim of this investigation was to evaluate the AR inhibition of RMFA on the rat lens as well as compound isolation from the stems.

Materials and Methods

Plant materials

The RMFA and RM samples used in this study were collected from Chilgap Mountain, 2013, Chungnam, Republic of Korea. These voucher specimens of RMFA and RM were deposited at our Department.

Apparatus and chemicals

Nuclear magnetic resonance (NMR) and electron ionization-mass

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spectrometry (EI-MS) were recorded by using a Bruker AVANCE 500 NMR spectrometer (Rheinstetten, Germany) and a JEOL JMS-600W mass spectrometer (Tokyo, Japan), respectively. The reagents and solvents including 3,3-tetramethyleneglutaric acid (TMG), β-NADPH, DL-glyceraldehyde, potassium phosphate buffer, and sodium phosphate buffer were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Extraction, fractionation, and isolation of a flavonoid from RMFA stems

The dried, finely powdered RMFA stems (3.4 kg) were extracted with methanol (MeOH) for 3 h (8 L×4) under reflux (65-75 °C). After removal of solvent *in vacuo*, the extract (186.4 g) distilled in water was partitioned successively with *n*-hexane (40.0 g), CH_2Cl_2 (25.8 g), EtOAc (48.0 g), and *n*-BuOH (25.1 g). A part of the EtOAc fraction (20 g) from the RMFA sample was chromatographed using a silica gel column (6×80 cm, No. 7734) by a stepwise gradient of $CHCl_3$ and MeOH solvent systems to obtain 5 fractions. Compound **1** was isolated from sub-fraction 4 ($CHCl_3$:MeOH=9:1). Among them, sub-fraction 4 yielded compound **1** by recrystallization using MeOH. Then, compound **1** was subsequently isolated.

Measurement of AR activity

The rat lenses were harvested from Sprague-Dawley rats (weighing 250-280 g) and kept frozen before they were used. The homogenized lenses were centrifuged at 10,000 rpm (4 °C, 20 min) and the supernatant was used as the enzyme source for the AR activity testing. The AR (EC 1.1.1.21) activity was spectrophotometrically determined by measuring the decrease in absorption of β-NADPH at 340 nm over a 4 min period at room temperature with DL-glyceraldehyde as a substrate. The assay mixture contained 0.1 M potassium phosphate buffer (pH 7.0), 0.1 M sodium phosphate buffer (pH 6.2), 1.6 mM NADPH, and the test extract sample (in dimethyl sulfoxide) with 0.025 M DL-glyceraldehyde as the substrate (Mok and Lee 2012; Mok et al. 2012).

Sample preparation for high-performance liquid chromatography (HPLC)

To analyze the components of compound **1** from the various parts of the RMFA and RM plants, the extracts were dissolved in MeOH, filtered by a Whatman 0.45-μm syringe filter (Cat No. 6779, Piscataway, NJ, USA), and then, the solution was analyzed by using HPLC.

Quantitative analysis of parts of RMAF and RM

The HPLC analysis of compound **1** from the different parts of the RMFA and RM was performed. A Waters Spherisorb® INNO C18 (4.6×250 mm, 5 μm) reverse phase column was used for the determination. The mobile phase was 0.5 % acetic acid and MeOH (regeants A and B, respectively). The aliquot volume was 10 μL. The flow rate was at 1 mL/min. The gradient system was run as follows: initially, reagent A and B at 80:20, the linear gradient was increased to 0:100 for 35 min and maintained for 5 min, and then finally the gradient was increased to 80:20 for 5 min again and maintained for 5 min for a total run time of 55 min. The UV wavelength absorbance was detected at 330 nm for quantification of the flavonoids.

Limit of detection and quantification (LOD and LOQ) of compound 1 from RMFA and RM

The validation of compound **1** as a standard was conducted by using LOD and LOQ. The LOD and LOQ were calculated based on a linear regression equation, and the values were determined separately at a signal to noise ratios of 3 and 10, respectively.

Calibration curve

The stock solution (1 mg/1 mL) of compound **1** was dissolved in MeOH and mixed while the same solvent was used repeatedly to compare the integrated peak area of the individual compound. The calibration function of compound **1** were calculated by peak area (Y), concentration (X, mg/mL) and mean values (n=5).

Result and Discussion

The extracts and fractions of RMFA were analyzed for their AR inhibitory effects, and the results are shown in Table 1. The EtOAc fraction exhibited a significant higher inhibition of the AR than the other fractions and extracts did. In a previous study, the MeOH extracts of white-colored natural products including RMFA were shown to inhibit AR activity (Mok et al. 2012). There are few literature reports on the various biological activities of RMFA, and these results demonstrated that the EtOAc fractions showed AR inhibitory effects on the rat lens (Mok and Lee 2012).

Table 1 IC_{50} of the extract and fractions from RMFA against rat lens AR

Fraction	Concentration (μg/mL)	AR inhibition[a] (%)	IC_{50}[b] (μg/mL)
MeOH ext.	10	45.83	-
n-Hexane	10	28.88	-
CH_2Cl_2	10	58.38	-
EtOAc	10	68.56	6.50
	5	24.75	
	1	24.75	
n-BuOH	10	37.88	-
TMG[c]	10	83.28	0.29
	1	62.21	
	0.1	40.13	

[a]Inhibition rate was calculated as a percentage of the control value
[b]IC_{50} calculated from least-squares regression line of logarithmic concentrations plotted against residual activity
[c]TMG was used as a positive control

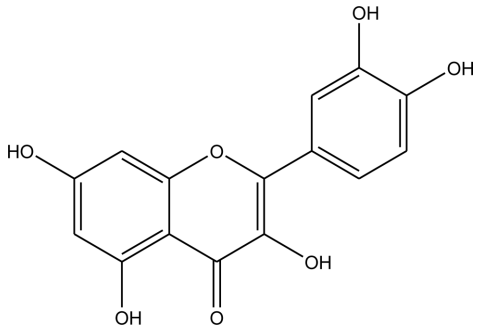

Fig. 1 Structure of quercetin

The EtOAc fraction of RMFA was repeatedly separated using silica gel and Sephadex LH-20 chromatography and led to the isolation of compound **1**. The structure of compound **1** was confirmed by a combination of ^{1}H-NMR and EI-MS. In the ^{1}H-NMR spectra, the typical flavonoid signals of compound **1** were observed, and its molecular weight was at *m/z* 302 $[M]^+$. The presence of singlet signals at δ 12.49 showed a 5-OH of an A-ring in the structure while H-6 and -8 signals are observed at δ 6.18 (d, *J*=2.0 Hz, H-6) and δ 6.40 (d, *J*=2.0 Hz, H-8). Furthermore, δ 6.88–7.67 showed the ABX pattern of the B-ring: δ 7.67 (1H, d, *J*=2.0 Hz, H-2'), 6.88 (1H, d, *J*=8.5 Hz, H-5'), and 7.54 (1H, dd, *J*=2.0, 8.5 Hz, H-6'). From the spectroscopic comparison with values in the literature (Sato and Kador 1990), the chemical structure of purified compound **1** was elucidated as quercetin (Fig. 1). Numerous quercetin (**1**) derivatives have been isolated from RM sp. (Jung et al. 1996; Hong et al. 2007).

Table 2 IC_{50} of compound **1** from RMFA against rat lens AR

Compound	Concentration (μg/mL)	AR inhibition (%)	IC_{50} (μM)
1	10	74.25	2.11
	1	18.06	
	0.1	3.01	
TMG	10	83.28	1.52
	1	62.20	
	0.1	40.13	

Same as in Table 1

Table 3 Linearity of standard curves of compound **1**

Compound	t_R	Calibration equation[a]	Correlation factor, $r^{2\,b}$
1	21.68	Y=030223X–715.45	1

[a]Y=peak area, X=concentration of standards (mg/mL)
[b]r^2=correlation coefficient for 3 data points in calibration curves (n=5)

Table 4 Quantities of compound **1** in each plant part of RMFA and RM

Sample	Content (mg/g extract)
Flower of RMFA	3.51±0.07
Stem of RMFA	5.12±0.07
Flowers of RM	2.22±0.00
Stem of RM	3.29±0.02
Root of RM	tr.

Data are mean ± SD (n=3) in μg/g of dried samples
tr., trace

Quercetin (**1**) from the EtOAc fraction of RMFA was evaluated for AR inhibitory activity (Table 2). Quercetin (**1**) exhibited

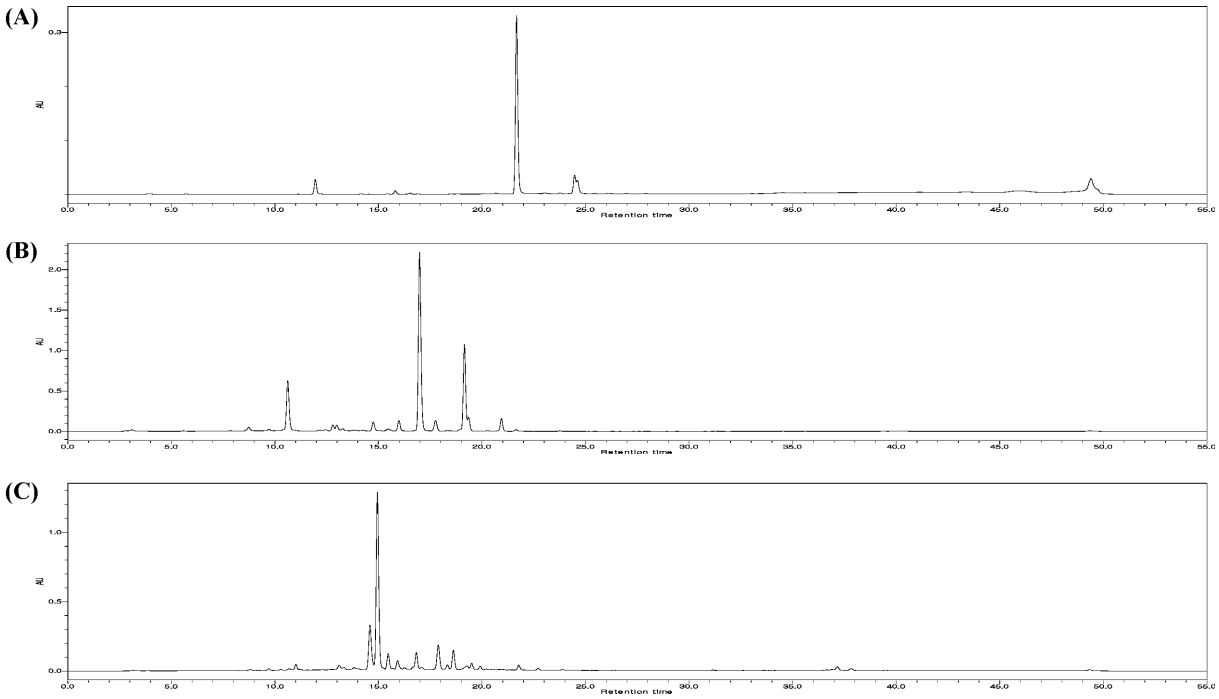

Fig. 2 HPLC chromatograms of quercetin (**A**), flowers of RMFA (**B**), and stems of RMFA (**C**)

Table 5 LOD and LOQ values of compound **1**

Compound	Calibration equation[a]	r^2 [b]	Linear range (mg/mL)	LOD (mg/mL)	LOQ (mg/mL)
1	Y=33335X–177.23	1	0.1–1.000	0.012	0.029

[a]Y=peak area, X=concentration of standard (mg/mL)
[b]r^2=correlation coefficient for 3 data points in calibration curve

significant AR inhibitory activity (IC_{50}, 2.11 μM) with TMG, as a positive control. There have been numerous reports of flavonoids and phenol constituents with significant AR inhibitory activity (Kawanishi et al. 2003; Jung et al. 2004; Lee et al. 2008). In addition, previous studies have demonstrated that flavonoids have various pharmaceutical activities including anti-ulcer, anti-viral, anti-inflammatory, and vasodilatory actions (Proestos and Komaitis 2006). Our study demonstrated that RMFA exhibits AR inhibitory effects. Recently, quercetin reduces manic-like behavior and brain oxidative stress (Kanazawa et al. 2016). Also it affects glutathione levels and redox in human aortic endothelial cells (Li et al. 2016).

The content analysis was performed to determine the concentration of quercetin (**1**) in the various parts of the RMFA and RM plants by using HPLC/UV analysis. The linear calibration equation of quercetin (**1**) was Y =30223X–715.45. The correlation coefficient (r^2) was 1 and shown in Table 3. The retention time of quercetin (**1**) was 21.68 min. The flowers and stems of RMFA contained high amounts of quercetin (**1**) at 5.12 and 3.51 mg/g extract, respectively, which was more than the other parts of the RM. The roots of RM showed a very low concentration of quercetin (**1**). The quercetin (**1**) content of the various parts of the RM and RMFA plants was quantified by using a calibration curve (Table 4). RMFA had more active than RM in a previous paper (Mok et al. 2012). We think that different concentrations of quercetin in RMFA and RM is main key for AR inhibition. The LOD and LOQ of compound **1** were 0.012 and 0.029 mg/mL, respectively (Table 5).

In conclusion, our study revealed that RMFA contains higher amount of quercetin (**1**) than RM. Furthermore, our results demonstrated that RMFA has the potential to be used as an AR inhibitory agent against diabetic complications.

Acknowledgment This work was supported by a Grant from Pyeongtaek High School, Pyeongtaek, Korea. We would like to thank the National Center for Inter-University Research Facilities (Seoul National University, Republic of Korea) for the NMR and MS measurements.

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