J. Korean Soc. Appl. Biol. Chem. 52(5), 448-455 (2009)

Article

Flavonoids from the Leaves of *Thuja orientalis* Inhibit the Aldose Reductase and the Formation of Advanced Glycation Endproducts

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Received May 11, 2009; Accepted June 18, 2009

The purpose of this study is to evaluate the active properties of *Thuja orientalis* leaves for the treatment of diabetic complications. The ethyl acetate fraction showed a significantly higher inhibition both of recombinant human aldose reductase (rhALR2) inhibitory activity and advanced glycation endproducts (AGEs). The detected antioxidants (compounds 1-4) by online-HPLC-ABTS$^+$ method and other three compounds were isolated using preparative RP-HPLC and Sephadex LH-20 column chromatography. Among the seven compounds, compound 4 (quercitrin) which was isolated from ethyl acetate fraction, was found to show inhibition for both forms of rhALR2 and AGEs. This compound also inhibited oxidative stress which was measured by Photochem® apparatus. In conclusion, the ethyl acetate fraction from *T. orientalis* demonstrated antioxidant activity as well as inhibitory effects on rhALR2 and AGEs. Quercitrin was shown to be the active compound and hence could be offered as an active material of standardization for the development of natural products for food or medicines.

Key words: *advanced glycation endproducts, aldose reductase, online-HPLC-ABTS$^+$ method, quercitrin,* Thuja orientalis

There are four main hypothesis regarding the mechanisms of diabetic complications such as increased ALR2 activity, increased AGEs formation, activation of protein kinase C isoforms and increased hexosamine pathway flux [Brownlee, 2001].

It has been suggested that ROS play a key role in the pathogenesis of late diabetic complications because of their ability to directly oxidize and damage DNA, protein, and lipid [Nishikawa *et al.,* 2000; Rosen *et al.,* 2001]. In addition to their ability to directly inflict macromolecular damage, ROS can function as signaling molecules to activate a number of cellular stress-sensitive pathways that cause cellular damage, and are ultimately responsible for the late complications of diabetes. In the previous review paper, the four main molecular mechanisms of diabetic complications mentioned above, reflect a single hyperglycemia-induced process of overproduction of superoxide by the mitochondrial electron transport chain [Brownlee, 2001].

Recently, on-line high performance liquid chromatography (HPLC) free radical decoloration assays aimed at the rapid identification of antioxidants in natural products extracts have been used [Niederlander *et al.,* 2008]. The most widely used assays are the assays based on the stable free radicals DPPH (2,2'-diphenyl-l-picrylhydrazyl) [Koleva *et al.,* 2000] or ABTS$^+$ (2,2'-azinobis-(3-ethylbenzothiazolidine-6-sulfonate)) [Koleva *et al.,* 2001].

ALR2 (alditol/NADP$^+$ oxidoreductase, E.C.1.1.1.21) is the first enzyme of the polyol pathway which reduces excess D-glucose into D-sorbitol with concomitant conversion of NADPH to NADP$^+$ [Carper *et al.,* 1989; Tomlinson *et al.,* 1994], which has been demonstrated to play important roles not only in the cataract formation in the lens [Frank *et al.,* 1983] but also in the pathogenesis of diabetic complications such as neuropathy [Young *et al.,* 1983], nephropathy [Dunlop, 2000] and retinopathy [Robison *et al.,* 1989].

Prolonged hyperglycemia can also result in the

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Abbreviations: ABTS$^+$, 2,2'-azinobis-(3-ethylbenzothiazolidine-6-sulfonate; AGEs, advanced glycation endproducts; BSA, bovine serum albumin; DAD, diode array detection; HPLC, high performance liquid chromatography; rhALR2, recombinant human aldose reductase; ROS, reactive oxygen species

doi:10.3839/jksabc.2009.078

formation of AGEs in body tissues. The complex, fluorescent AGE molecules formed during the Maillard reaction can lead to protein cross-linking and contribute to the development and progression of several diabetic complications such as cataract, atherosclerosis, nephropathy and neuropathy [Ahmed, 2005].

Through the discovery that ALR2 and AGEs may play an important role in the pathogenesis of diabetic complications, a large variety of structurally diverse synthetic and naturally occurring compounds have been studied for their in vitro and in vivo activities. Several studies of naturally occurring flavonoids on the inhibition of ALR2 and AGEs, and their structure-activity relationship have also been reported [Matsuda *et al.,* 2002; Matsuda *et al.,* 2003; Fernandez *et al.,* 2005]. It would therefore seem desirable that ALR2 inhibitors would also possess AGEs inhibitory as well as antioxidant properties.

Thuja orientalis is an evergreen shrub belonging to the family Cupressaceae and native to Korea and China. It also has been used as a haemostatic agent in Korea and its essential oil is used as aromatic essence, insecticides and materials for making perfume. Several studies on the chemistry for the *T. orientalis* have been performed so far, [Pelter *et al.*, 1970; Chizzola *et al.,* 2004], but no information on the effect of diabetic complications is available.

In the present study, we investigated the antioxidant compounds of the leaves of *T. orientalis* by online-HPLC-$ABTS^+$ method. Furthermore, we tested to see whether the compounds can inhibit ALR2 and AGEs for treatment for diabetic complications. This investigation led to isolation and identification of the compounds that inhibit the activity of ALR2 and AGEs.

Materials and Methods

Chemicals. First grade solvents were used for extraction, fractionation and column chromatography. Kiesel gel 60 (70-230 mesh, Art. 7734, Merck, Whitehouse Station, NJ, U.S.A.) was used as the column packing material. Kiesel 60 F_{254} (precoated plate, Art. 5559, Merck, NJ, U.S.A) was used for thin layer chromatography (TLC). Iodine vapor and 10% H_2SO_4 were used for TLC detection. DL-Glyceraldehyde, D-glucose, bovine serum albumin (BSA), DTT, $ABTS^+$ and NADPH were purchased from Sigma Chemical (St. Louis, MO). Ampicillin, IPTG and imidazole were purchased from USB Corporation (Cleveland, OH). Human ALR2 cDNA clone was purchased from 21C Frontier Human Gene Bank (Daejeon, Korea). All other chemicals were of analytical grade.

Plant materials. The leaves of *T. orientalis* were purchased in the Kyungdong market, Korea and the voucher specimen (D-602) was deposited at the Herbarium of KIST Gangneung institute, Korea.

On-line detection of radical scavenging activity. The radical scavenging activity of the leaves of *T. orientalis* was determined using the online-HPLC-$ABTS^+$ assay according to the method of Stewart *et al*. [2005] with some modification.

A 2 mM $ABTS^+$ stock solution containing 3.5 mM potassium persulphate was prepared and incubated overnight in the dark at room temperature, to allow for stabilization of the radical. The $ABTS^+$ reagent was prepared by diluting the stock 8 fold in methanol. The ethyl acetate fraction of *T. orientalis* (10 mg/mL) was injected and separated using an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA) equipped with binary pumps, a diode array detection (DAD), a ultra violet (UV)/Vis detector, and an additional reagent pump. The analytical column was a reversed-phase Hydrosphere C_{18} (4.6×250 mm and 5 μm particle size; YMC, kyoto, Japan). The mobile phase consisted of acetonitrile (solvent A) and water with trifluoroacetic acid (TFA) (0.1 %, v/v) (solvent B). The gradient consisted of 90-10% B for 45 min. DAD was performed in the 200-800 nm range and the chromatographic profile was recorded at 254 nm. The sample injection volume was 10 μL and the flow rate was 1.0 mL/min. The analyses were performed at 35°C. The HPLC eluent from the DAD arrived at a "T" piece, where the $ABTS^+$ was added. The $ABTS^+$ flow rate was 0.5 mL/min delivered by an additional Agilent 1200 Pump. After mixing through a 1 mL loop maintained at 40°C, the absorbance was measured at 734 nm by a UV/Vis detector. Finally, the data were analyzed using ChemStation software (Agilent Technologies, Santa Clara, CA).

Isolation and identification. The leaves *T. orientalis* (1.5 kg) was extracted four times with hot EtOH (7.0 L) for 4 h. This residue was evaporated *in vacuo* to yield the total extract (320.0 g). This extract was then suspended in distilled water and partitioned sequentially with *n*-hexane (49.7 g), methylene chloride (21.0 g), and ethyl acetate (31.2 g). The ethyl acetate fraction (3.5 g) was subjected to preparative RP-HPLC (20×250 mm and 4 μm particle size; KYA TECH HiQ-Sil, Tokyo, Japan) using ACN-H_2O gradient system (10:90→90:10) to provide 4 fractions (fractions 1-4). From fraction 2 (1.2 g), compounds **1** (27.3 mg), **2** (5.7 mg), **3** (23.0 mg), **4** (30.2 mg), and **5** (30.5 mg) were isolated using preparative RP-HPLC (20×250 mm and 4 μm particle size; KYA TECH HiQ-Sil, ACN-H_2O=25%→45%) and then purified by Sephadex LH-20 column chromatography (3.5×45 cm, MeOH). Compounds **6** (11.0 mg) and **7** (39.0 mg) were purified from fraction 3 (0.9 g) using a Silicagel column

chromatography (3.0×50 cm, CH_2Cl_2:MeOH=9:1→7:1).

Preparation of recombinant human ALR2 (rhALR2). Open reading frame of human ALR2 was amplified by PCR and inserted into *E. coli* expression vector pET-23b (Merck, Darmstadt, Germany). Then the recombinant expression plasmid was transformed into *E. coli* BL21 (DE3) host strain. The transformant was grown in LB broth containing 50 μg/mL of ampicillin at 37°C, 200 rpm. When the absorbance at 600 nm reached 0.8, the expression of recombinant ALR2 was induced by IPTG and the culture continued at 30°C. After 4 h of additional culture, bacterial cells were harvested and stored at −20°C until further use. Further procedures were all performed at 4°C, except the lysis of bacterial cells, which was performed at room temperature. Harvested cells were lysed by adding 5 mL of BugBuster master mix lysis solution (Merck, Darmstadt, Germany) per 1 mg of wet cell. After adding about 3 volumes of binding buffer (20 mM Tris-Cl, pH 8.0, 0.5 M NaCl, 5 mM imidazole, and 1 mM DTT), cell lysates were centrifuged to remove cell debris. The supernatant was mixed with 1 mL of Ni-NTA resin (Merck, Darmstadt, Germany) on a twist shaker for 1 h.

Then, protein bound resin was collected by weak centrifugation and loaded onto a column. After washing with 10 column volumes of washing buffer (20 mM Tris-Cl, pH 8.0, 0.5 M NaCl, 20 mM imidazole, and 1 mM DTT), bound proteins were eluted with 4 mL of elution buffer (100 mM sodium phosphate, pH 6.2, 0.5 M NaCl, 250 mM imidazole, and 1 mM DTT). The concentration of the protein solution was determined by using Bradford dye (BioRad, Hercules, CA), and adjusted to a final concentration of 40 μM recombinant human ALR2. Small aliquots of enzyme solution were frozen with liquid nitrogen and stored at −80°C until further use.

Measurement of rhALR2 activity. rhALR2 activities were assayed spectrophotometrically by measuring the decrease in absorption of NADPH at 340 nm over a 5 min period with DL-glyceraldehyde as a substrate [Sato and Kador, 1990]. Each 1.0 mL cuvette contained equal units of enzyme, 0.10 M sodium phosphate buffer (pH 6.2), 0.3 mM NADPH with or without 10 mM substrate and inhibitor. The concentration of inhibitors giving 50% inhibition of enzyme activity (IC_{50}) was calculated from the least-squares regression line of the logarithmic concentrations plotted against the residual activity.

Determination of inhibition-type of rhALR2 by the compounds. Reaction mixtures consisted of 0.1 M potassium phosphate (pH 7.0), 0.16 mM NADPH, 2 μM of rhALR2 with varied concentrations of substrate DL-glyceraldehyde and ALR2 inhibitors, epalrestat or quercitrin in a total volume of 200 μL.

Concentrations were ranged from 0.02 mM to 0.2 mM for DL-glyceraldehyde, from 0.5 μM to 1.5 μM for epalrestat, and from 1.0 μM to 2.0 μM for quercitrin. rhALR2 activity was assayed spectrophotometrically by measuring the decrease in absorption of NADPH at 340 nm after substrate addition using BioTek Power Wave XS spectrophotometer (BioTek Instruments, Winooski, VT).

Effects on AGEs formation. The AGEs formation was assessed by characteristic fluorescence reported by Morimitsu *et al*. [1995] with slight modifications. Briefly, the reaction mixture of 100 mg D-glucose, 10 mg BSA in 1 mL sodium phosphate buffer (100 mM, pH 7.4) was incubated at 60°C for 2 days with or without the test compound. The reaction solution (0.2 mL) was diluted with water (2 mL), and the intensity of fluorescence was measured using a fluorophotometer (Luminescence Spectrometer LS50B, Perkin-Elmer Ltd, Massachusetts) at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The reaction mixture without D-glucose was used as a blank solution.

Antioxidative capacity determination. For the determination of the integral antioxidative capacity of lipid soluble substances in *T. orientalis*, the method of photochemiluminescence was used. ACL (Analitik Jena AG, Jena, Germany), where the luminol plays a double role as photosensitizer and also as oxygen radical detection reagent was used according to the manufacturer's instruction [Jung *et al.*, 2007]. Results were calculated in Trolox equivalents.

Results and Discussion

Here we report on the properties of the leaves extract of *T. orientalis* on the inhibition of rhALR2 and AGEs activities that would be useful for the treatment of diabetic complications. It is clearly shown in the present studies that *T. orientalis* is a potent inhibitor of both ALR2 and AGEs.

In order to identify the active compounds from *T. orientalis*, biological activity guided fractionation of the extract were performed. Five systematic fractions were then tested for ALR2 inhibitory activity using DL-glyceraldehyde as a substrate and inhibitory effect on BSA glycation by a fluorescence method. Among them, ethyl acetate fraction showed a significantly higher inhibition which was concentration dependent (Table 1).

Reactive oxygen species (ROS) can directly oxidize DNA, protein, lipid, and carbohydrate, which are believed to play a key role in the pathogenesis of diabetic complications. Recently, a hyperglycemia-induced process of overproduction of the superoxide anion radical ($^{\cdot}O_2$) by the mitochondrial electron transport chain was reported to

Table 1. Inhibitory effect of the leaves of *T. orientalis* on rhALR2 and AGEs

Samples	rhAL2 Activity			AGEs Activity		
	Concentration (μg/mL)	Inhibition (%)	IC_{50} (μg/mL)	Concentration (μg/mL)	Inhibition (%)	IC_{50} (μg/mL)
Epalrestat[1)]	2.5 1.3 0.63 0.31	99.9±4.5 89.0±3.2 42.2±7.1 16.5±5.8	0.68			
Aminoguanidine[2)]				2500.0 1250.0 625.0	61.5±0.1 37.3±2.1 20.7±1.3	1736.9
n-Hexane fr.	20.0	-	20.0 >	200.0	-	200 >
Methylene chloride fr.	20.0	13.7±2.4	20.0 >	200.0	16.9±0.6	200 >
Ethyl acetate fr.	20.0 2.5 0.63 0.31	98.1±3.5 63.3±7.1 29.4±4.5 16.1±11.7	1.43	200.0 100.0 50.0	87.8±2.3 58.7±0.8 24.7±4.7	85.6
n-Butanol fr.	20.0	-	20.0 >	200.0	5.5±3.8	200 >
Water fr.	20.0	-	20.0 >	200.0	-	200 >

Inhibition rate was calculated as percentage with respect to the control value and expressed as mean±standard deviation of triplicate experiments. The concentration of each test sample giving rise to 50% inhibition of activity (IC_{50}) was estimated from the least-squares regression line of the logarithmic concentration plotted against inhibitory activity.
[1)]Epalrestat and [2)]Aminoguanidine were used as positive control.

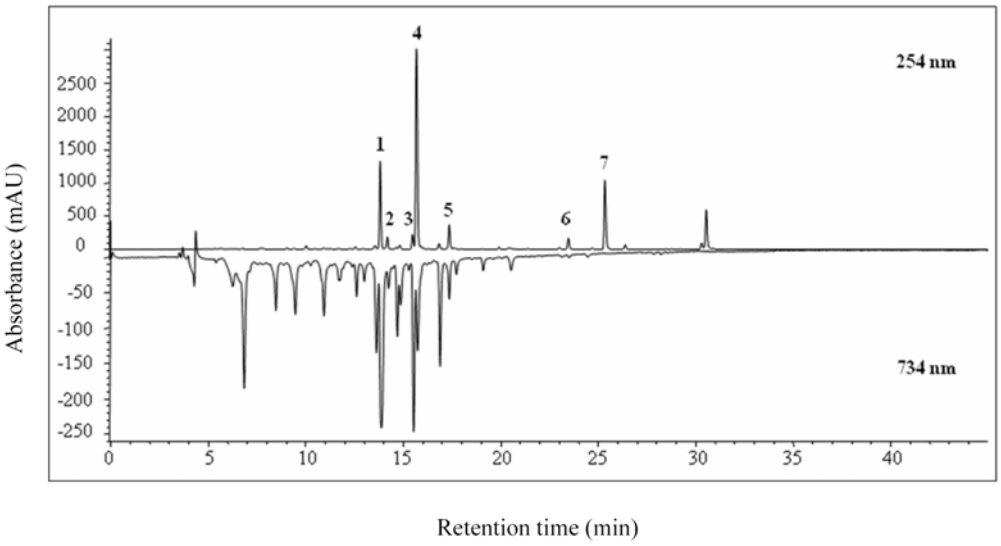
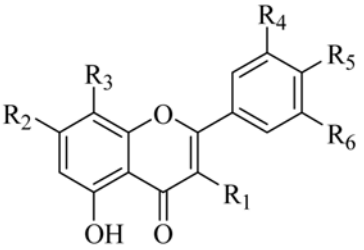


Fig. 1. On-line HPLC-ABTS+ analysis of ethyl acetate fraction of the leaves of *T. orientalis*.

partially inhibit the glycolytic enzyme glyceraldehyde phosphatase dehydrogenase, thereby diverting upstream metabolites from glycolysis into the polyol pathway and AGEs formation, etc [Brownlee, 2001].

For the reason, it is important for drugs used to treat diabetic complications to possess antioxidant properties as well as ALR2 and AGEs inhibitory activities.

In this study, for a rapid assessment of pure antioxidant compounds in ethyl acetate fraction of *T. orientalis*, online-HPLC-$ABTS^+$ method was used. Following HPLC separation, the HPLC eluate was mixed with a stabilized solution of $ABTS^+$ radicals, and the solution was directed to a UV/Vis detector monitoring absorbance at 734 nm. The radical solution had a deep blue color, and any quenching of the $ABTS^+$ radical resulted in a loss of color, which was indicated by a negative peak on the absorbance profile. The more rapidly the absorbance decreases, the more potent the antioxidant activity of the



1. R_1= *O*-Rhamnose, R_2= OH , R_3=H, R_4=OH, R_5=OH, R_6=OH
2. R_1= *O*-Glucose, R_2= OH , R_3=H, R_4=OH, R_5=OH, R_6=H
3. R_1= H, R_2= *O*-Xylose, R_3=OH, R_4=OH, R_5=OH, R_6=H
4. R_1= *O*-Rhamnose, R_2=OH, R_3=H, R_4=OH, R_5=OH, R_6=H
5. R_1= *O*-Rhamnose, R_2=OH, R_3=H, R_4=H, R_5=OH, R_6=H
6. R_1= OH, R_2=OH, R_3=H, R_4=H, R_5=OH, R_6=H

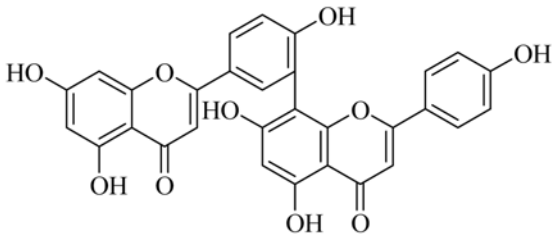


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Fig. 2. Chemical structures of phenolic compounds isolated from the leaves of *T. orientalis*.

compound in terms of hydrogen-donating ability [Wu *et al.,* 2008].

Figure 1 shows the online-HPLC-ABTS$^+$ analysis of the ethyl acetate fraction of *T. orientalis*. From the combined UV (positive signals) and ABTS$^+$ quenching (negative signals) chromatograms, compounds **1-4** showed free radical scavenging activity.

In order to isolate pure compounds, ethyl acetate fraction was subjected to RP-HPLC separation to yield four sub-fractions. Further chromatography of fraction 2 using RP-HPLC and Sephadex LH-20 gave five compounds, and two compounds were purified from fraction 3 by Silica gel column chromatography.

Their chemical structures were elucidated by spectral analysis and direct comparison with authentic compounds, their structures were identified as myricitrin (**1**), isoquercitrin (**2**), hypoletin-7-*O*-β-D-xylopyranoside (**3**), quercitrin (**4**), kaempferin (**5**), kaempferol (**6**), and amentoflavone (**7**). Their structures were shown in Fig. 2.

ALR2 was also shown to be dose-dependently inhibited by hypoletin-7-*O*-β-D-xylopyranoside (**3**) and quercitrin (**4**) with IC_{50} value of 1.19 and 1.29 μM which was 2 times less active as for epalrestat (Table 2).

In our previous study, we postulated the possible relationships of structure to the inhibitory activities of flavonoids: 1) chalcones having 4'-hydroxyl at the A ring show stronger activity; 2) chalcones having catechol moiety at the B ring (the 3,4-ortho-dihydroxyl moiety) show stronger activity; 3) chalcone shows a stronger activity than dihydrochalcone, in which 3,4,2',4'-tetrahydroxychalcone was the most potent inhibitor for ALR2; 4) ring-closure form of 3,4,2',4'-tetrahydroxychalcone exhibit stronger activities than other flavonoids [Matsuda *et al.,* 2002; Jung *et al.,* 2004]. Moreover, flavonol having 3-*O*-rhamnoside (quercitrin) was the strongest inhibitory potency among the 30 flavonoids [Jung *et al.,* 2004].

This study not only confirms that quercitrin has potent ALR2 inhibitory property but also suggests that hypoletin-7-*O*-β-D-xylopyranoside could be offered as a leading compound for further study as an ALR2 inhibitor. Moreover, these data are consistent with structure activity relationship studies reported by Matsuda *et al*. [2002] where it was shown that catechol moiety of flavone was important for ALR2 inhibition.

We adopted BSA-glucose model in vitro as the phenomenon of protein glycation. Glucose is used as glycated agent, which is commonly adopted in Maillard reaction studies, and BSA could serve as targets for glycated agent [Matsuda *et al.,* 2003]. Among the compounds isolated from *T. orientalis*, quercitrin (**4**) and amentoflavone (**7**) showed the strongest AGEs inhibition, and the inhibitory potencies as expressed by IC_{50} values, were 82.8 and 97.7 μM, respectively (Table 2).

It has been reported that the hydroxyl groups of flavones at the 3-, 4-, 5-, and 7-positions increased the AGEs inhibitory activities and the methylation or glycosilation of the 3- or 4-hydroxyl group reduced the activity [Lou *et al.,* 2001; Matsuda *et al.,* 2003]. In agreement with this report, 3'-, 4'-, 5-, and 7-tetrahydroxylated flavone, quercitrin, and 4'-, 5-, and 7-trihydroxylated biflavone, amentoflavone, increased the activity. In each case the biologically important factor, ALR2 and AGEs for diabetic complications was inhibited significantly by quercitrin.

From the overall results, quercitrin has antioxidative as well as ALR2 and AGEs inhibitory effects. Kinetic analysis of the inhibitory activity of quercitrin (**4**) against rhALR2 using Lineweaver-Burk plots of 1/velocity and 1/concentration of substrate was shown in Fig. 3. According to the result, inhibition of rhALR2 by epalrestat and quercitrin (**4**) was uncompetitive, meaning that both compounds can neither bind to substrate binding region

Table 2. Inhibitory effects of the compounds isolated from the leaves of *T. orientalis* on rhALR2 and AGEs

Samples	rhAL2 Activity			AGEs Activity		
	Concentration (μM)	Inhibition (%)	IC_{50} (μM)	Concentration (μM)	Inhibition (%)	IC_{50} (μM)
Epalrestat[1)]	2.5	99.9±4.5	0.68			
	1.3	89.0±3.2				
	0.63	42.2±7.1				
	0.31	16.5±5.8				
Aminoguanidine[2)]				2500.0	60.4±1.9	1780.3
				1250.0	38.1±0.7	
				625.0	22.5±0.3	
1	5	42.9±3.2	5 >	200.0	9.2±2.3	200 >
2	5	39.9±5.2	5 >	200.0	27.5±1.4	200 >
3	5.0	88.1±3.2	1.19	200.0	11.3±0.5	200 >
	2.5	72.0±3.9				
	1.3	50.0±6.5				
	0.63	33.0±3.2				
4	5.0	90.8±2.0	1.29	200.0	83.9±1.7	82.8
	2.5	76.6±2.6		100.0	57.6±1.5	
	1.3	46.8±0.7		50.0	30.4±4.5	
	0.63	26.2±11.7				
5	5.0	73.4±9.1	1.69	200.0	38.4±0.1	200 >
	2.5	59.6±2.0				
	1.3	43.1±2.0				
	0.63	28.0±1.3				
6	5	43.5±3.2	5 >	200.0	36.7±1.6	200 >
7	5.0	59.2±9.1	1.63	200.0	85.2±2.5	97.7
	2.5	53.7±6.5		100.0	52.0±0.2	
	1.3	49.5±3.2		50.0	16.3±0.6	
	0.63	40.4±3.2				

Inhibition rate was calculated as percentage with respect to the control value and expressed as mean±standard deviation of triplicate experiments. The concentration of each test sample giving rise to 50% inhibition of activity (IC_{50}) was estimated from the least-squares regression line of the logarithmic concentration plotted against inhibitory activity.
[1)]Epalrestat and [2)]Aminoguanidine were used as positive control.

nor the nucleotide binding region of rhALR2. From the result, we can conclude that inhibitory mechanisms of both epalrestat and quercitrin might be some what similar to each other.

For the quantitative measurement of antioxidative activity of ethyl acetate fraction, and quercitrin, Photochem® apparatus were used. Free radicals were generated by the instrument by means of a photosensitizer. The free radicals generated were detected by their reaction with a chemiluminogenic substance luminal. In this way luminal can act both as a photosensitizer as well as the detecting reagent [Apati *et al.*, 2003].

If antioxidants are added to this measuring system, the intensity of the photochemiluminescence signal is attenuated dependent on concentration. In this way, the total antioxidative capacity of the samples under investigation can be quantified. The antioxidative capacity is given in equivalent concentration units of Trolox for lipid-soluble substances.

The antioxidative capacity of ethyl acetate fraction of this plant was measured as 1.66, 0.49 and 0.46 nmol equivalents of Trolox at 1000.0, 100.0 and 10.0 ng/mL, respectively. Quercitrin, isolated from this plant have antioxidative capacity as 4.53, 1.91 and 0.46 nmol equivalents of Trolox at 625.0, 125.0 and 62.5 ng/mL, respectively (Table 3).

Overall findings suggest, therefore, the ethyl acetate fraction from *T. orientalis* had antioxidant activity as well as ALR2 and AGEs inhibitory effects. Especially, quercitrin isolated from ethyl acetate fraction of this plant was possibly acting as active compounds. Further studies on *T. orientalis* should be carried as a new natural product source for the treatment of diabetic complications. Also quercitrin could be offered as an active material of

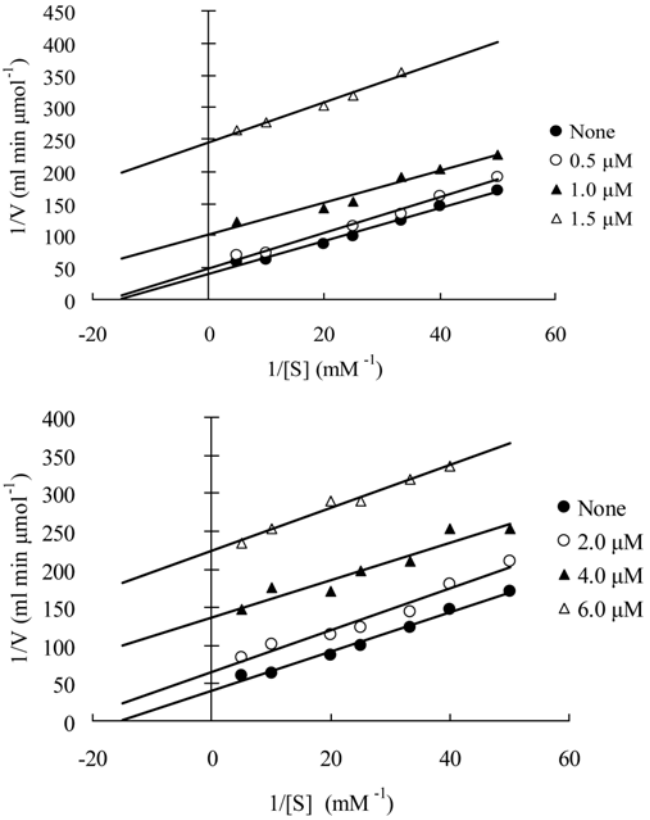

Fig. 3. Lineweaver-Burk plots from the kinetic studies of rhALR2 in the presence of ALR2 inhibitor epalrestat (A) and quercitrin (B). Results are the representative of three independent experiments.

Table 3. Lipid soluble antioxidative capacity (ACL) of ethyl acetate fraction, and quercitrin isolated from the leaves of *T. orientalis*

Samples	Photochem		
	Concentration[1)]	Inhibition (%)	Trolox equivalent (nmol)
Ethyl acetate fr.	1000.0	70.0	1.66
	100.0	38.5	0.49
	10.0	37.1	0.46
Quercitrin	625.0	74.1	4.53
	125.0	49.1	1.91
	62.5	18.3	0.46

[1)]The concentration unit of ethyl acetate fr. and quercitrin was ng/mL and nM, respectively.
The sample and luminol mixtures were run for 3 min in the Photochem, and results were obtained from a workstation employing PCL software. ACL was assayed as the area under the curve and expressed as Trolox equivalent after dilution with methanol, reagent kits, and tested samples. The 'Signal (V)' means arbitrary signal unit. The tests were carried out in duplicate.

standardization for the development of natural products for food or medicines.

Acknowledgment. This work was supported by grant No.RTI05-01-02 from the Regional Technology Innovation Program of the Ministry of Knowledge Economy (MKE).

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