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Biological Activity and Inhibition of Non-Enzymatic Glycation by Methanolic Extract of *Rosa davurica* Pall. Roots

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Abstract

The methanolic extract of *Rosa davurica* Pall. roots exhibited strong antioxidant activity in a 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay and was found to be a dose-dependent inhibitor of non-enzymatic formation of advanced glycation end products (AGEs), which are relevant to diabetes complications. HPLC-diode array detector (DAD) analysis of the *R. davurica* Pall. root extract led to the identification of four compounds: hydrocaffeic acid, catechin, epicatechin, and ellagic acid. Catechin was present in the largest amount and exhibited high antiglycation activity. A CYP3A4 assay was used to investigate potential interactions between drugs and the extract, and results suggest that the *R. davurica* Pall. root extract had moderate potential for interfering with drug metabolism. The *R. davurica* Pall. extract did not display anti-inflammatory activity on the level of that for tumor necrosis factor-α (TNF-α) in a lipopolysaccharide (LPS)-stimulated macrophage assay; however, the extract did exhibit low to moderate immunostimulatory activity in a pro-inflammatory macrophage assay. Therefore, we conclude that *R. davurica* Pall. root is a promising anti-AGE agent with low to moderate risks of associated inflammation or drug interaction.

Key words: antioxidant, advanced glycation end products (AGEs), phenolic compounds, *Rosa davurica* Pall.

INTRODUCTION

Rosa davurica Pall. (Rosaceae) is a shrub that is widely distributed in the northeastern parts of Asia. Its fruits, roots, and flowers have been used in traditional Chinese medicine for the treatment of stomach problems and indigestion. The fruit and leaf of this plant, which are rich in vitamin C, are used in nutraceutical drinks (1, 2). The methanol extract from the *R. davurica* Pall. roots had high levels of antioxidants and free radical scavenging activities. *R. davurica* Pall. contains phenolics, and these may contribute to the antioxidant activity (3). Many phenolic compounds, including flavonoids, not only possess potent antioxidant activity but also inhibit the formation of advanced glycation end products (AGEs). AGEs form under high blood sugar conditions in diabetes and contribute to degenerative processes such as nephropathy, neuropathy, and other diabetic complications (4). The inhibition of AGE formation by silk extracts from different corn genotypes correlated strongly with the phenolic content of the silk (5,6). A study of the *in vitro* inhibitory effects of naturally occurring flavonoids demonstrated that quercetin and other flavonoids show potent inhibitory effects on protein glycation, and their scavenging of free radicals is proposed to play an important role in this reaction (7).

While inhibition of glycation may lead to new useful treatments for diabetic complications, candidate medicinal plants need to be evaluated for safety. Since diabetics often take a variety of drugs, one complication that can occur is undesirable drug interactions with the plant-derived medication. The most common mechanism is related to plant extract modulation of drug metabolizing enzymes such as cytochrome P450 (CYP). For example, the most common enzyme, CYP3A4, metabolizes statins, which are frequently prescribed for type 2 diabetics. Another complication is the role of inflammation in diabetes and the possible modulation of the immune system by plant extracts. In particular, an excessive stimulation of pro-inflammatory mediators such as tumor necrosis factor-α (TNF-α) can produce complications in both type 1 and type 2 diabetes, whereas anti-inflammatory activity may help reduce the pathology of these conditions (8-10).

Investigation of the inhibitory activity of *R. davurica* Pall. extract on AGE formation has not been reported.

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Therefore, the purpose of this study was to investigate any potential AGE-inhibitory activity of *R. davurica* Pall. using an extract for which the antioxidant activity and phenolic content have been well characterized. To provide some preliminary information on the safety of the use of this phytomedicine in diabetes, we investigated its potential to inhibit CYP3A4 and to modulate TNF-α in cell culture condition.

MATERIALS AND METHODS

Plant material

R. davurica Pall. roots were collected in the suburbs of Jeongseon-Gun, Gangwon-Do, in Korea, and roots were dried in an oven at 60°C. Roots were extracted three times in methanol (20 mL/g) at room temperature for 12 hr. All samples were filtered through a 0.2 μm PTFE non-sterile filter (Chromatographic Specialties Inc., Brockville, Ontario, Canada) prior to analysis.

Cell culture

THP-1 cells (www.ATCC.org; cell culture TIB-202), an immortal human macrophage line, were cultured in RPMI 1640 media (Invitrogen, Mississauga, Ontario, Canada) with 50 μM β-mercaptoethanol, 1% Penstrep (Invitrogen), 10% fetal bovine serum (Invitrogen) and 1% MEM sodium pyruvate, in a 37°C humidified environment with 5% CO_2.

Total phenolic estimation assay

A Folin reagent-based assay was conducted to estimate total phenolic content of the root extract. One milliliter of sample was mixed with 2 mL Folic reagent and 2 mL 35% Na_2CO_3. The mixture volume was brought to 10 mL and shaken vigorously. The absorbance (Elx 800, Biotec Instruments, Winooski, VT, USA) was measured at 765 nm after incubation at room temperature for 30 min. The result was expressed as mg of tannic acid equivalents per gram of extract.

1,1-Diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging activity

DPPH free radical scavenging activity was assessed as described by Hu et al. (11) with some modifications. Two milliliters of various concentrations of sample or water (control) were added to 2 mL DPPH solution (2 mM in MeOH). Blanks contained 2 mL each of distilled water and sample solution. The mixture was shaken immediately after adding DPPH and allowed to stand at room temperature in the dark; the decrease in absorbance at 517 nm was measured after 30 min. All the experiments were run in triplicate. The experiment was carried out in triplicate, and the results were averaged. The ability to scavenge DPPH radicals was calculated using the equation: $I\ (\%) = [1 \times (A_i - A_j)/A_c] \times 100\%$. In this equation A_c is the absorbance of the DPPH solution without sample; A_i is the absorbance of the test sample mixed with DPPH solution; and A_j is the absorbance of the sample without DPPH solution. α-Tocopherol was used as a positive control.

The inhibition of AGE formation assay

Stock solutions of glucose (200 mM)/fructose (200 mM) and bovine serum albumin (BSA; 2 mg/mL) were prepared in 100 mM sodium phosphate monohydrate buffer (pH 7.4). The final mixture contained glucose (100 mM)/fructose (100 mM); bovine serum albumin (BSA; 1 mg/mL); and vehicle alone (negative control), vehicle with extract (experimental), or positive control. The extract was tested at multiple concentrations (0.107, 0.214, 0.428, 0.856, 1.71, 3.43, 6.85, 13.7, 27.4, 54.8 μg/mL) with sugar and BSA to determine the effects on glycation and in the absence of BSA to control for fluorescent properties of the extract. The positive control was quercetin, a flavonoid with anti-glycation activity. Triplicate samples of 200 μL were transferred into wells of sterile opaque polystyrene 96-well clear bottom plates with a BSA control (no sugar) and negative and positive controls. The plate was sealed with parafilm and incubated for seven days at 37°C in darkness while shaking with a Series 25 Incubator Shaker (New Brunswick Scientific Co., Inc., Edison, NJ, USA). Following incubation, fluorescence was measured using a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA) at excitation and emission wavelengths of 355 nm and 460 nm, respectively. The average fluorescence reading of the BSA blank was subtracted from each fluorescence reading of the experimental ($F_{experimental}$) and negative control ($F_{negative}$) treatments. The average fluorescence reading of the phosphate buffer blank was subtracted from each reading of the extract blank treatment ($F_{extract\ blank}$). The average $F_{extract\ blank}$ was subtracted from each corresponding $F_{experimental}$ to determine the treatment fluorescence values ($F_{experimental\ corrected}$). Percent inhibition of AGE formation was determined according to the formula: $I\ (\%) = [(F_{negative\ control} - F_{experimental\ corrected})/ F_{negative\ control}] \times 100\%$.

HPLC analysis of terpenoid compounds in *R. davurica* Pall. roots

HPLC analyses were conducted on an Agilent (Palo Alto, CA, USA) 1100 LC system with an autosampler, quaternary pump, and diode array detector (DAD). Separations were performed using a Waters YMC ODS-AM column (100×2 mm i.d.; 3 μm particle size; Mississauga, Canada) maintained at 45°C at a flow rate of 0.3 mL min^{-1}. The mobile phase consisted of aqueous TFA

(0.05%) at pH 3.4 (solvent A) and acetonitrile (solvent B) with an initial ratio of 95:5 (A:B) maintained 0～2 min followed by two linear gradients of 5～25% B in 20 min and 25～100% B in 5 min. Next, the column was washed with 100% B for 2 min before returning to initial conditions (96:4) in 3 min. A 10 μL aliquot of each sample was injected, and the subsequent elution profiles were monitored on-line at 350 nm, 280 nm, 254 nm, and 210 nm (DAD). Identified metabolites were quantified on the basis of area under the peak of HPLC-DAD chromatograms at 280 nm using calibration curves produced using pure standards analyzed on the same day. Mean quantities were calculated from freshly dissolved triplicates of each extract (minimum n=3). An Agilent 1100 LC MSD VL APCI system with an online atmospheric pressure chemical ionization (APCI) mass detector with mass range of 50～15000 amu was used to identify terpenoid compounds known to occur in *R. davurica* Pall. root. MS detection was performed in positive ionization mode with optimized spray chamber conditions as follows: drying gas flow rate of 6.0 mL/min, nebulizer pressure of 60 psig, drying gas temperature of 300°C, vaporizer temperature of 400°C, capillary voltage of 3000 V, and corona current of 3.0 μA. The MS was operated in scan mode within 100 to 800 amu with ramping fragmentation voltages from 20 to 50 V.

CYP3A4 inhibition assay

The extract was diluted ten-fold with water prior to the assays. A volume of 10 μL per well of the sample was tested in triplicate. The fluorescence was measured using a Cytoflour 4000 Fluorescence Measurement System (Applied Biosystems, Foster City, CA, USA). The positive inhibitor used was 1.9 μM ketoconazole dissolved in MeOH. The sample, 1.5 nM CYP3A4, 1 μM dibenzylfluorescein (DBF) (dissolved in acetonitrile), and 0.6 mM NADPH were incubated in 0.19 M phosphate-buffered solution (pH 7.4). The final fluorescence was read after 20 min of reaction time at 37°C. Readings were performed at 485 nm excitation and 530 nm emission with a gain of 50 for CYP3A4. Initial fluorescence was subtracted from the respective final fluorescence for the calculations. The percent inhibition of each extract was calculated with respect to the CYP activity of the ethanol control.

Anti-inflammatory and pro-inflammatory assays

THP-1 cells (3×10^5) were added to the wells of a 96-well plate, followed by the addition of controls and *R. davurica* Pall. extract at 1, 10, or 100 μg/mL for a total volume of 300 μL. Final ethanol concentration in the wells was 0.5% with the ethanolic treatments. In anti-inflammatory assays, cells were incubated for 1 hr, stimulated with 1 μg/mL lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO, USA), and incubated for 24 hr; 10 μg/mL parthenolide (Sigma-Aldrich) was used as a positive control. In the pro-inflammatory assays, cells were incubated for 24 hr following the addition of treatment; *Echinacea angustifolia* ethanol extract, *Echinacea purpurea* water extract, and LPS were used as positive controls. Following the incubation period, cells were centrifuged at 2000 rpm for 10 min at 20 °C after incubation. Cell culture pellets and supernatants were separated and stored at -80°C for further analysis. ELISA kits (R and D Systems, Minneapolis, MN, USA) were used according to the manufacturer's protocol to analyze TNF-α level in anti-inflammatory and pro-inflammatory macrophage assay supernatants.

RESULTS

Total phenolic estimation and DPPH free radical scavenging activity

The yield of *R. davurica* Pall. root extract was 16.40 %, and the total phenolic content was 62.95±0.56 mg Tan equivalent/g (Table 1). The IC_{50} values for the DPPH radical scavenging activity was 46.91±0.29 μg/mL (Table 2). α-Tocopherol, a positive control, had an IC_{50} value of 16.45±0.89 μg/mL.

The inhibition of AGE formation assay

The extract was a highly active, dose-dependent anti-glycation agent (Fig. 1). The concentration of the *R. davurica* Pall. root extract required to reach 50% inhibition of fluorescent AGE formation (IC_{50}) was 32.60±2.65 μg/mL.

HPLC analysis of terpenoid compounds in *R. davurica* Pall. roots

Phytochemical characterization of the *R. davurica* Pall. root extract performed using HPLCDAD-APCI-MS led to the identification of six compounds (Fig. 2): (1) hydrocaffeic acid, (2) catechin, (3) epicatechin, (4) ella-

Table 1. The yield and total phenolic content of methanol extract of *R. davurica* Pall. roots

R. davurica Pall.	Yield (%)	Total phenolic content (Tan mg/g)[1)]
Root	16.40	62.95±0.56

[1)]Tan means tannic acid equivalent.

Table 2. DPPH radical scavenging activity of extract from *R. davurica* Pall. roots

Sample	IC_{50} (μg/mL)
R. davurica Pall. root extract	46.91±0.29
α-Tocopherol	16.45±0.89

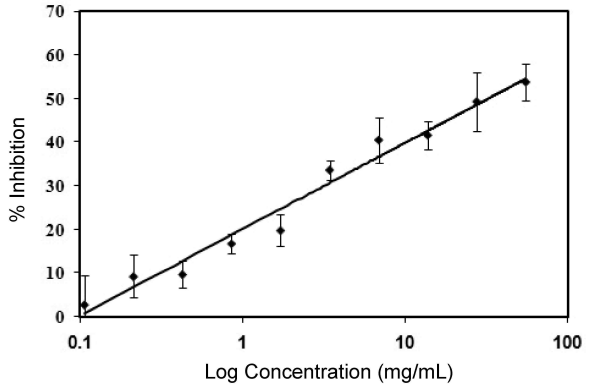

Fig. 1. Inhibition of the formation of fluorescent AGE by *R. davurica* Pall. root extract. The extract concentration ranged from 0.107 to 54.800 μg/mL in the assay. Results are expressed as the percent inhibition relative to negative control versus plant extract concentration (means ± SE; n=3). A logarithmic regression was fit to the data: y=8.59 ln(x)+20.1, R^2=0.976.

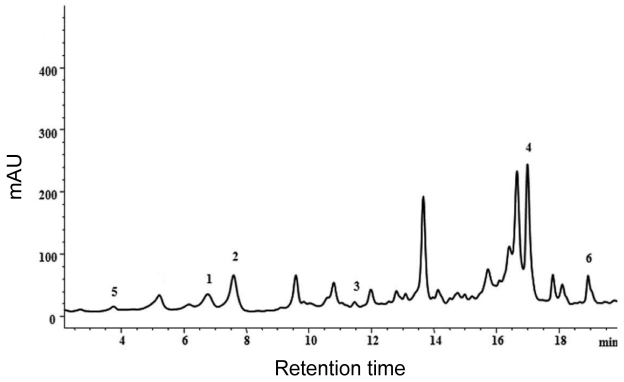

Fig. 2. The HPLC chromatogram of methanolic extract of *R. davurica* Pall. root. The mobile phase followed a linear gradient from acetonitrile: 5% aqueous TFA (0.05% v/v): 95% in 18 minutes with absorbance at 280 nm. The peaks were identified as (1) hydrocaffeic acid, (2) catechin, (3) epicatechin, (4) ellagic acid, (5) catechin gallate, and (6) gallotannin.

gic acid, and two tentatively identified gallotannins (5) and (6). Due to the lack of appropriate standards, (5) and (6) were not quantified. As catechin was the most abundant phenolic in the plant (Table 3), it was evaluated for antiglycation activity. It revealed an IC_{50} of 5.58 μg/mL (data not shown).

Table 3. Phytochemical constituents of methanolic extract of *R. davurica* Pall. roots

Peak number	Compound	Quantitation[1)] (mg compound/g dry material)
1	Hydrocaffeic acid	2.97 ± 0.05
2	Catechin	10.65 ± 0.20
3	Epicatechin	2.21 ± 0.04
4	Ellagic acid	3.65 ± 0.01

[1)]Quantification results based on calibration curves of authentic reference standards; data presented as mean ± SEM, n=3.

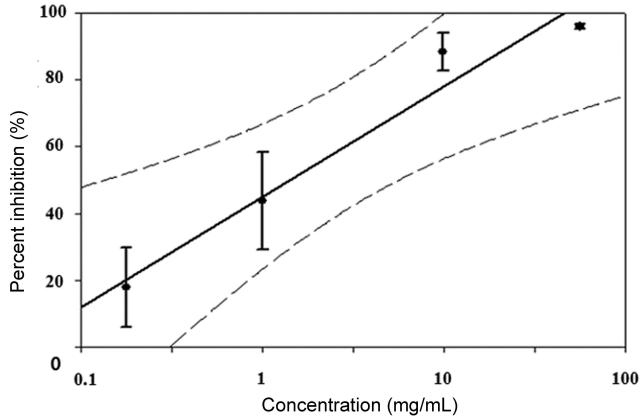

Fig. 3. The inhibitory concentrations (IC_{50}) for the *R. davurica* Pall. root extract by CYP3A4. Results are expressed as the percent inhibition relative to negative control versus plant extract concentration (means ± SE; n=3). A logarithmic regression was fit to the data: y=82.71 ln(x) − 37.68, R^2=0.961.

CYP3A4 inhibition assay

We investigated the inhibitory effects of *R. davurica* Pall. root extract on drug metabolism. CYP3A4 is a critical member of the CYP3A subfamily of cytochrome P450 that has been implicated in exhibiting clinically important, drug-drug interactions and toxicities related to CYP3A4 inhibition (12). The concentration of *R. davurica* Pall. extract required to reach 50% CYP3A4 inhibition (IC_{50}) of was 1.23 μg/mL (Fig. 3).

Anti-inflammatory and pro-inflammatory assays

We observed that *R. davurica* Pall. root extract had no significant effect on TNF-α levels in the anti-inflammatory assay at the three concentrations tested (Fig. 4). *R. davurica* Pall. root extract did exhibit moderate activity in the pro-inflammatory assay, comparable to that of the pro-inflammatory activity observed in the *E.*

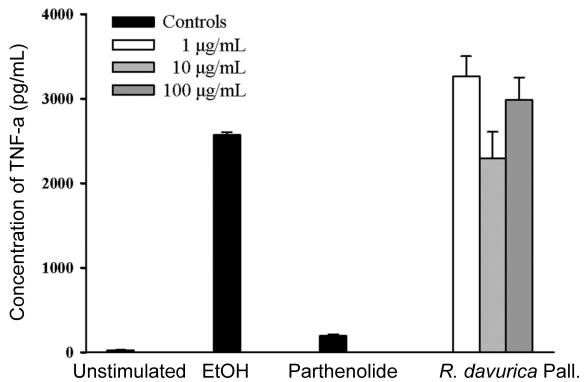

Fig. 4. Effects of *R. davurica* Pall. root extract on TNF-α production by THP-1 macrophages stimulated with LPS. Unstimulated controls contained no LPS, ethanol was the vehicle control, and 10 μg/mL parthenolide was used as a positive control. *R. davurica* Pall. root extracts were tested at 1, 10, and 100 μg/mL. Results are means ± SE, n=3.

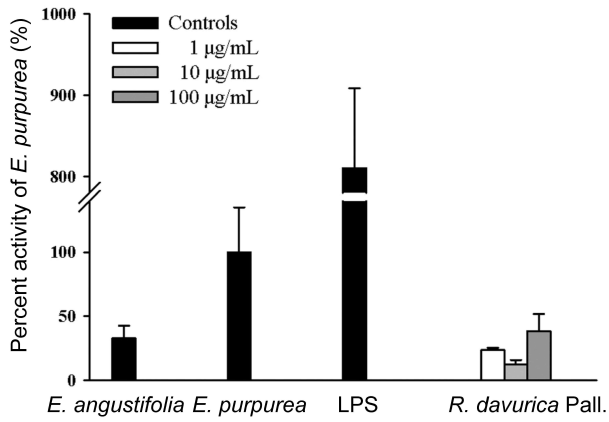

Fig. 5. Effects of *R. davurica* Pall. root extract on TNF-α production by THP-1 macrophages. Results are presented as percent activity of *Echinacea purpurea*, where the activity of the vehicle control is set to 0% and the activity of *E. purpurea* is set to 100%. Positive controls are 100 μg/mL ethanol extract of *E. angustifolia*, 100 μg/mL water extract of *E. purpurea*, and 1 μg/mL LPS. *R. davurica* Pall. root extract are tested at 1, 10, and 100 μg/mL. Results are means ± SE, n=3.

angustifolia ethanol extract although not as high as the pro-inflammatory activity of *E. purpurea* water extract (Fig. 5). Importantly, the levels of TNF-α production by *R. davurica* Pall. root extract were well below the toxic level of TNF-α production observed in the LPS treatment.

DISCUSSION

In this study, *R. davurica* Pall. root exhibited strong antiglycation and antioxidant activities. The IC_{50} for antiglycation by *R. davurica* Pall. root extract was 32.6 μg/mL, which is in the range of the most active maize silk extracts we tested previously (IC_{50}= 9.5～82 μg/mL) (5). This may be due in part to the higher phenolic content of the *R. davurica* Pall. root extract (63 mg/g) compared to that of maize silk extract (14～53 mg/g). Meanwhile, the DPPH free radical scavenging activity of *R. davurica* Pall. root extract also was high compared with positive control (α-tocopherol) at the same concentration. Phenolic antioxidants from dietary sources may have a number of beneficial effects in diabetic complications such as promotion of wound healing; they also serve as potent inhibitors of AGE formation through their radical-scavenging effects. The mechanism of flavonoids' action against glycation also is, in part, related to their antioxidant properties (13). Oxidative stress is elevated under hyperglycemic conditions, and free radicals (produced by oxidative stress) play an important role in the formation of AGEs (14). The phenolic oxy radicals are stabilized by resonance delocalization and may react with other radical species to form nonradical products (15). We quantified four compounds from *R. davurica* Pall. root extract (hydrocaffeic acid, catechin, epicatechin, ellagic acid). The main phytochemical constituent was catechin (Table 3). Catechin is quite active in the antiglycation assay (IC_{50} = 5.58 μg/mL), but accounts for only part of the inhibition of AGE formation. Bioassay guided isolation is required to identify all the active compounds.

CYP3A4 is largely responsible for first pass metabolism of drugs in the small intestine (16). The CYP3A4 inhibition activity of the extract was significant (Fig. 3), but it is not in the high range compared to many plant extracts we have tested; therefore, *R. davurica* Pall. root extract may not be responsible for any strong interaction with pharmaceutical drugs due to this P450. Although *R. davurica* Pall. root extract did not exhibit any concentration-dependant effects on TNF-α release in the anti-inflammatory assay, it did display moderate immunostimulatory production of TNF-α in the pro-inflammatory assay. However, the immunostimulatory activity of *R. davurica* Pall. root extract is well below the immunostimulatory activity observed in the LPS treatment, which suggests that it is at low risk for the potential deleterious effects of acute pro-inflammatory activity in diabetes. Overall, the *R. davurica* Pall. root possesses activities that suggest it may be a candidate for further investigation of complementary treatments of type 2 diabetes symptoms.

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