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Inhibitory Effects of Total Extract and Flavonols from Hardy Rubber Tree (*Eucommia ulmoides* Oliver) Leaves on the Glycation of Hemoglobin

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Abstract Our previous study demonstrated that aqueous ethanolic extract of hardy rubber tree (HRT; *Eucommia ulmoides* Oliver) leaves potently inhibited glycation, and that two known flavonols and one new flavonol were the active compounds in protein glycation *in vitro* using the model system of bovine serum albumin and fructose. The aim of this study was to examine the effectiveness of HRT extract and its flavonols against the glycation of hemoglobin (Hb) in primary cultured rat erythrocytes. The extract at 40-400 μg/mL and the flavonols at 0.2-20 μg/mL significantly inhibited Hb glycation in a concentration-dependent manner. They were more effective than aminoguanidine, a well-known inhibitor of glycation. Three flavonols seemed to be responsible for the inhibitory activity of the extract, furthermore, the extract of HRT leaves showed advantages over single isolated flavonols in the inhibition of Hb glycation.

Keywords: *Eucommia ulmoides*, flavonols, protein glycation, hemoglobin, erythrocytes

Introduction

Diabetes mellitus is a common endocrine disorder characterized by hyperglycemia and long-term complications affecting the eyes, nerves, blood vessels, skin, and kidneys. Increased glycation of proteins and the accumulation of advanced glycation end products have been implicated as one of main molecular mechanisms by which hyperglycemia causes diabetic complications (1). Nature offers an abundance of antidiabetic resources with lesser side effects, with most of these being found in medicinal plants (2). The leaves of hardy rubber tree (HRT; *Eucommia ulmoides* Oliver) have been reported to elicit beneficial pharmacological effects with regard to diabetes mellitus (3).

Our previous study demonstrated that aqueous ethanolic extract of the leaves of HRT inhibited protein glycation in an *in vitro* model system using bovine serum albumin (BSA) and fructose. A new flavonol glycoside, quercetin 3-*O*-α-L-arabinopyranosyl-(1→2)-β-D-glucopyranoside, and known flavonols, kaempferol 3-*O*-β-D-glucopyranoside (astragalin), and quercetin 3-*O*-β-D-glucopyranoside (isoquercitrin) were isolated as glycation inhibitors (4). The extract and flavonols from HRT leaves deserve more attention and should be investigated to see whether they can reduce the glycation of proteins in an organ culture system which retains at least in part the architectural characteristics of the tissue *in vivo*. Glycation-related damages are particularly pronounced in long-lived molecules and cells (1). The glycation of hemoglobin (Hb) is more harmful than that of BSA, in that the half-life of Hb is 28 days compared to 7 days for BSA.

The glycation-induced structural modification of Hb is associated with its functional modification leading to oxidative stress in diabetic patients (5). The glycation of Hb results in reduced deformability of erythrocytes and their increased adherence to endothelium (6). Therefore, Hb has been used as a model protein that has provided insights into the nonenzymatic glycation of other more complex tissue proteins (7). The primary cultures of rat erythrocytes have been shown to provide a valuable *in vitro* system for evaluating the glycation of Hb. Hemoglobin in erythrocytes is glycated at two sites: at the N terminal valine residues of the β chains, and at the ε amino group of several lysine residues on both the α and the β chains. Glycated hemoglobin (GHb), usually as HbA_1 or a subfraction of HbA_1 (e.g., HbA_{1C}), has wide acceptance as a long-term index of diabetic control (8). A rapid chromatographic method for separating GHb from non-glycated Hb, using a boronate-agarose affinity medium which selectively binds the *cis*-diol groups of GHb, was used to quantitate GHb.

The objective of this study was to investigate the inhibitory effect of the extract and flavonols from HRT leaves on Hb glycation in primary cultured erythrocytes, and to explain whether the whole extract offers advantages over each flavonol or vice versa.

Materials and Methods

Preparation of the extract HRT leaves were collected from Wonju (Gangwon, Korea) in July 2003. Voucher specimens have been deposited in the laboratory of the Korea Food Research Institute. They were air-dried in a sunless place and powdered with a grinder (Hanil Electronics, Seoul, Korea). The powder was extracted with 50%(v/v) ethanol at a ratio of 20 mL/g for 2 hr under reflux, followed by paper filtration (Toyo No. 2 and 4; Advantec, Tokyo, Japan). The extraction and filtration was repeated three times. The combined filtrate was concentrated in a rotary vacuum evaporator below 40°C. until all extracting solvent was completely removed so that

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solid residue was obtained. The residue was dissolved in dimethyl sulfoxide (DMSO) and used for subsequent bioassay. DMSO was found to have no effect on bioassay results at less than 1%(v/v).

Isolation of the flavonols The three flavonols were isolated by the method described previously (4), dissolved in DMSO, and used for subsequent bioassay.

Quantitative analysis of the flavonols in the extract The three flavonols of the extract were determined by high-performance liquid chromatography (HPLC) using a Jasco PU-980 liquid chromatograph (Jasco Corporation, Tokyo, Japan) fitted with a Waters Xterra™, reversed-phase C_{18} column (150×4.5 mm, i.d. 5 mm; Waters Corporation, Milford, MA, USA). The mobile phase consisted of water adjusted to pH 2.5 with trifluoroacetic acid (solvent A) and acetonitrile (solvent B). Elution was carried out as follows: 0-30 min, 80% A / 20% B at zero min, 50% A / 50% B at 30 min. The flow rate was 1 mL/min, and the peaks were detected at 365 nm using a UV-975 UV/VIS detector (Jasco Corporation).

Erythrocyte incubations Blood was collected from the abdominal aorta of male 10-week old Sprague-Dawley rats using heparinized tubes. After centrifugation at 800 g for 10 min at 4°C, the supernatant, buffy coat, and upper 15% of the packed erythrocytes were removed by aspiration. The remaining erythrocytes were washed with phosphate-buffered saline three times at 4°C. The final cell suspensions (20%, v/v) were then incubated in Krebs-Ringer bicarbonate buffer (pH 7.4) with 250 mM glucose and with or without HRT extract or various flavonols at 37°C in a water-jacketed humidified incubator with an atmosphere of 95% air and 5% CO_2 for 48 hr. Aminoguanidine (Sigma Chem. Co., St. Louis, MO, USA), a typical antiglycation agent, was dissolved in buffer and used as a positive control.

Analysis of glycated hemoglobin (GHb) content GHb levels were determined with the Glyc-Affin GHb micro-columns and the method described by the manufacturer was strictly followed (Wallac Inc., Akron, OH, USA). This method measured all GHb, not just HbA_1 or HbA_{1C}. Glycated forms of Hb variants such as HbS, HbC, HbD, etc., are detected as GHb. The level of GHb was calculated as the percentage of GHb compared to sum of GHb and nonglycated Hb. Percent of GHb was calculated at follows:

$$\%GHb = 100 \times G / (G + N)$$

where, G is the amount of the GHb fraction and N is the amount of the nonglycated Hb fraction. The % inhibition of GHb formation = 100% - (%GHb of a sample - %GHb of the blank of a sample)]/(%GHb of the control - %GHb of the blank of the control) × 100%. The blank was the incubation of erythrocytes and a sample without glucose.

Statistical analysis Experiments were performed in triplicate and replicated three times. All values were expressed as the mean and standard deviation (SD). Duncan's multiple range test in the SAS (SAS Institute,

Table 1. Inhibitory effect of the extract and flavonols from hardy rubber tree leaves on the formation of glycated hemoglobin in erythrocyte-glucose reactions *in vitro*[1)]

Samples	Conc. (μg/mL)	GHb (%)	Inhibition (%)	IC_{50}[3)]	
				μg/mL	μM
Control[2)]	-	14.65±0.34a[4)]	-	-	-
Quercetin 3-*O*-α-L-arabinopyranosyl-(1→2)-β-D-glucopyranoside (**1**)	0.2	12.34±1.81b	15.75±8.73	17.76 ± 2.25	29.8 ± 3.77
	2	11.77±0.88bc	19.66±4.23		
	20	6.68±1.22d	54.40±5.89		
Kaempferol 3-*O*-β-D-glucopyranoside (**2**)	0.2	13.58±1.15b	7.29±3.29	17.77 ± 1.64	39.67 ± 3.66
	2	9.43±0.19c	35.63±0.89		
	20	6.85±0.74d	53.24±3.58		
Quercetin 3-*O*-β-D-glucopyranoside (**3**)	0.2	13.33±1.05b	56.12±7.74	9.26 ± 4.25	19.93 ± 9.14
	2	10.50±1.67bc	66.41±5.45		
	20	5.33±1.05d	72.46±4.84		
Extract	40	13.73±1.10b	6.31±1.31	370.0 ± 1.8	
	200	8.97±1.34c	38.74±6.45		
	400	7.48±0.48cd	49.10±2.32		
Aminoguanidine	100	10.19±0.49b	28.95±2.39	6664 ± 57.2	60.07 ± 517.6
	1000	9.13±1.04c	36.31±5.12		
	10000	5.83±0.49d	59.37±2.41		

1)Mean ± SD, n = 9.
2)Control : incubation with erythrocytes (20%, v/v) with 250 mM glucose only.
3)Concentration of sample required to inhibit 50% of the control. Calculated from linear regression equation in semilogarithmic manner. Molar concentration was based on the molecular weights of each, 1 = 596, 2 = 448, 3 = 464, and aminoguanidine • HCl = 110.5.
4)Groups with different letters are significantly different (p<0.01).

Table 2. Flavonol and total flavonoid content in hardy rubber tree leaves

Contents	mg/kg in Dry weight of leaves	mg/kg in Extract
Quercetin 3-*O*-α-L-arabinopyranosyl-(1→2)-β-D-glucopyranoside (**1**)	1536.98±291.90	6295.33±27.85
Kaempferol 3-*O*-β-D-glucopyranoside (**2**)	234.67±11.47	831.29±40.61
Quercetin 3-*O*-β-D-glucopyranoside (**3**)	971.06±1.15	3439.81±4.08

Inc., Cary, NC, USA) was used for the statistical analysis. $p<0.01$ was assumed to be statistically significant.

Results and Discussion

Table 1 shows the inhibitory effect of HRT extract and three flavonols on Hb glycation. Three flavonols include quercetin 3-*O*-α-L-arabinopyranosyl-(1 → 2)-β-D-glucopyranoside (**1**), and the known flavonols, kaempferol 3-*O*-β-D-glucopyranoside (astragalin) (**2**), and quercetin 3-*O*-β-D-glucopyranoside (isoquercitrin) (**3**). Co-incubation of rat erythrocytes with 250 mM glucose for 48 hr increased glycated Hb 14.33-14.65% over the blank. The results shown in Table 1 indicate that the inhibitory effects of HRT extract and the flavonols were more effective than that of aminoguanidine (AG), a well-known inhibitor for advanced glycation endproducts. The IC_{50} value of the extract was 370.0 μg/mL compared with 6664 μg/mL for AG, indicating that the extract was 18 times more effective than AG on a by weight basis. The IC_{50} value of each flavonol was 29.80, 39.67, and 19.93 μM compared with that of AG, 60.07 μM, indicating that the flavonols were more effective than AG by 1.5-3 times on a molar basis. The order of inhibitory potency among the flavonols was **3** > **1** > **2**.

Within HRT extract at the concentration of 370.0 μg/mL (the IC_{50} value of the extract; Table 1), the three flavonols were present at 2.329 μg/mL for **1**, 1.273 μg/mL for **2**, and 0.3076 μg/mL for **3**. The calculation was based on the contents of the flavonols in the extract, i.e., 6295 mg/kg extract for **1**, 3440 mg/kg for **2**, and 831.3 mg/kg for **3** (Table 2). The IC_{50} value of each flavonol was 17.76, 17.77, and 9.26 μg/mL (Table 1). It is important to note that the concentration of each flavonol required to inhibit 50% of the control was equivalent to 9.26 to 17.77 μg/mL, whereas 50% inhibition was seen in the extract only with a concentration of 0.3076 to 2.329 μg/mL, i.e., 3 to 13% of the IC_{50} value of each flavonol. The extract of HRT leaves showed advantages over single isolated flavonols in the inhibition of Hb glycation.

In this study, the phenomenon of protein glycation was demonstrated in the reaction mixtures of Hb and glucose *in vitro*. Glucose, which is a common sugar in animal blood, was used as a glycating agent. The results reveal that HRT extract and three flavonols from HRT leaves are able to inhibit the glycation of Hb. Flavonoids have been reported to inhibit protein glycation (9). Antiglycating agents such as green tea extract (10) and tomato paste fraction (11) are similar in their flavonoid contents.

The present study shows that flavonols can suppress Hb glycation. In the present study, the quercetin glycosides (1 and 3) were more potent than the kaempferol glycoside (2). The presence of the hydroxyl group at the 3' position in the B ring of the flavonols affected their inhibitory activity against glycation. Between the two quercetin glycosides, the monoglycoside (3) was more potent than the diglycoside (1). Results from Wu and Yen (9) demonstrated that quercetin aglycone was slightly more effective than kaempferol aglycone, and that quercetin-3-rutinoside (rutin) was more effective than quercetin aglycone against glycation in several model systems using human Hb, bovine serum albumin, and N-acetyl-glycyl-lysine methyl ester as target proteins.

The whole extract from HRT leaves showed advantages over a single isolated flavonols with regard to inhibitory activity. The three flavonols seemed to be responsible for the inhibitory activity of the extract. However, it is possible that a number of active compounds are responsible for the activity, and synergistic interactions are documented for flavonols within HRT leaf extract as well.

In conclusion, the results of this study suggest that the extract of HRT might be used as an antiglycation agent against hyperglycemia-mediated protein damage. The present results also show that the three flavonols isolated from HRT leaves have potent inhibitory effects toward Hb glycation, and that the extract has more potent effects than that of the single flavonols.

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