JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Low-Density Lipoprotein, Collagen, and Thrombin Models Reveal that *Rosemarinus officinalis* L. Exhibits Potent Antiglycative Effects

CHIU-LAN HSIEH,[†,‡] CHIUNG-HUEI PENG,[§] CHARNG-CHERNG CHYAU,[‡] YUH-CHARN LIN,[‡] HUI-ER WANG,[†] AND ROBERT Y. PENG*[,‡]

Department of Food and Nutrition, Research Institute of Biotechnology, and Division of Basic Medical Sciences, Hung-Kuang University, No. 34 Chung-Chie Road, Shalu County, Taichung Hsien, Taiwan 43302

Using the low-density lipoprotein (LDL), collagen, and thrombin models, we report here that the rosemary extracts (REs), either the aqueous (RE_w) or the acetonic (RE_A), all possessed many antiglycation-related features, and the effective concentrations required were as follows: 0.1 mg/mL for suppressing the relative electrophoretic mobility, 1.3 μg/mL for anticonjugated diene induction, 0.5 mg/mL for inhibition of thiobarbituric acid reactive substances production, 0.1 mg/mL for AGEs (advanced glycation end products) formation, 0.1 mg/mL to block glucose incorporation, and 0.05 mg/mL as an effective anti-antithrombin III. Using high-performance liquid chromatography/mass spectrometry, we identified five major constituents among eight major peaks, including rosmarinic acid, carnosol, 12-methoxycarnosic acid, carnosic acid, and methyl carnosate. In the LDL model, RE_A was proven to be more efficient than RE_w; yet, the reverse is true for the collagen and the thrombin III models, the reason of which was ascribed to the higher lipid-soluble antioxidant content (such as rosmarinic acid, carnosol, carnosic acid, 12-methoxycarnosic acid and methyl carnosate) in RE_A than in RE_w and the different surface lipid characteristics between LDL and collagen; although to act as anti-AGEs, both extracts were comparable. To assist the evidence, a larger 2,2-diphenyl-1-picrylhydrazyl radical scavenging capability with less total polyphenolic content was found in RE_A. We conclude that rosemary is an excellent multifunctional therapeutic herb; by looking at its potential potent antiglycative bioactivity, it may become a good adjuvant medicine for the prevention and treatment of diabetic, cardiovascular, and other neurodegenerative diseases.

KEYWORDS: Rosemary; anti-thrombin; collagen; low-density lipoprotein; glycation

INTRODUCTION

To date, 6% of the global population is affiliated with diabetes mellitus (DM). A five-fold increase could even be expected within the coming decade (*1*). DM has climbed to the top four in mortality since 2004, with a mortality rate of 39.26 per 100000, most of which are closely related with cardiovascular diseases (*2*). As is well-known, hyperglycemia is a pertinent initiating factor for atherosclerosis, vascular basal membrane atrophy, and the elevation of blood pressure, resulting in a reduction of blood circulation and eventually severe damage to the cardiovascular system pathogenetically (*3*). Glycation has often been considered to be associated with vascular damage; its relevant factors are as follows: (i) the polymerization of collagen inhibiting the proteolytic attacking enzyme and the atrophy of vascular basal membranes (*4*), (ii) the glycation of antithrombin III and plasminogen resulting in thrombosis (*5*), and (iii) the oxidative glycative modification of the low-density lipoprotein (LDL) acting as the intiative factor of atherosclerosis (*6*).

As frequently cited, the unsaturated fatty acids contained in LDL are extremely susceptible to oxidative stress, and the peroxidation of fatty acids may trigger the formation of foam cells (*7*), fatty steaks, facilitating the deposition of calcium fibrin complexes inside the blood vessels and resulting in artherosclerosis. Alternatively, the hardened fibrin caps are apt to rupture, causing bleeding, forming thrombosis and artherosclerosis, or finally sudden death. Currently, extensive research has extended its interests in the preventive protocols against aging and DM and their complications and has come to the conclusion that glycation is pertinently relevant to these pathogentic processes, which suggests that glycation has indeed become an important problem that needs to be solved both clinically and nutraceutically. Well-known chemical compounds, such as

* To whom correspondence should be addressed. Tel: +886-2-2758-5767. Fax: +886-2-27585767. E-mail: ypeng@seed.net.tw.
† Department of Food and Nutrition.
‡ Research Institute of Biotechnology.
§ Division of Basic Medical Sciences.

10.1021/jf0631833 CCC: $37.00
Published on Web 03/27/2007

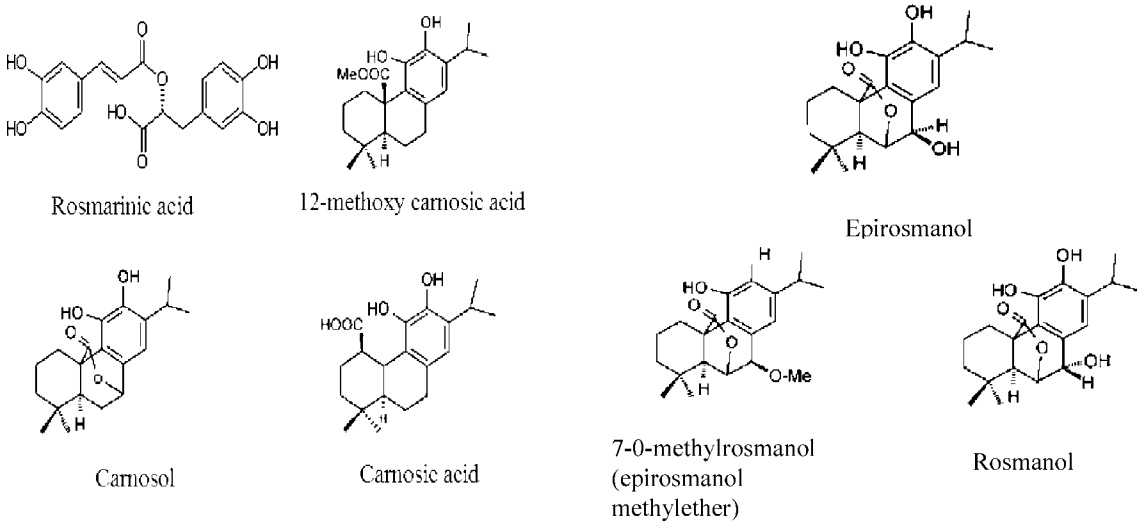

Figure 1. Major compositions usually found in *R. officinalis* L.

aminoguanidine (AG) and aspirin, although effective against in vivo glycative reactions, are worth great concern because of their side effects. Consequently, natural herbs and animal origins are promising sources for supplying new antiglycatives, and fortunately, some have been well-developed accordingly (*8*). Extracts of rosemary and tea are promising examples (*9*, *10*). Concomitantly, Hipkiss et al. (*11*) indicated the inhibitory effect of the peptidic carnosine on protein glycation. From the herbs of South America, an active antiglycative constituent, achyrofuran, has been isolated (*12*). Literature elsewhere has also documented that vitamin C and α-tocopherol are very potent antioxidants; they are as effective as an antiglycative, they are powerful advanced glycation end product (AGE) inhibitory agents (*13*), much more prominently effective than AG in vitro (*14*). Technically, rosemary extract (RE) has been widely used as an antioxidant in food industries. The main constituents are carnosol and carnosic acid (*15*) (**Figure 1**).

In addition, glycation is well-associated with oxidative stress (*16*, *17*). In view of the excellent antioxidant capability of rosemary and the speculation that rosemary may play an important role in antiglycative therapy, we performed several experiments to investigate its suppressing capabilitites on the formations of conjugated diene (CD), thiobarbituric acid reactive substances (TBARs), and AGEs and its antithrombotic activity using LDL, collagen, and thrombin models. Our aim was to discover the potent protective effects and therapeutic uses of herbal rosemary in reducing the risks of cardiovascular and neurodegenerative diseases, which might be complicated by DM and aging processes.

MATERIALS AND METHODS

Materials and Chemicals. *Rosemarinus officinalis* L. was purchased from the local Taichung Transform Farm and identified by The Research Institute of Medical Herbs (Taichung, Taiwan). Glucose (G), glyoxal (GO), methylglyoxal (MGO), AG, bovine serum albumin (BSA), sodium azide, butylated hydroxytoluene (BHT), phosphotungstic acid, thiobarbituric acid (TBA), sodium dodeyl sulfate (SDS), disodium ethylenediamine tetraacetate (EDTA) dehydrate, potassium bromide, and sodium carbonate were purchased from Sigma Chemical Co. (St. Louis, MO). Ethanol, *n*-butanol, and methanol were obtained from E. Merck (Darmstadt, Germany). All reagents used were of analytical grade. Paragon Lipo Gel was obtained from Beckmann (California). A Bio-Rad kit and SAR-PRO-ARG-pNA were obtained from Bio-Rad (California). Authentic carnosic acid, 12-methoxycarnosic acid, carnosol, rosemarinic acid, and gallic acid were also products of Sigma Chemical Co.

Sample Preparation. Fresh sample leaves of *R. officinalis* L. were immediately debranched when harvested and brought back to the laboratory from the local Taichung Transforming Farm.

Preparation of the Pulverized Rosemary Leaves. Leaves of *R. officinalis* L. were pulverized using a centrifugal pulverizer (Ultra Centrafugal Mill Retsch, Germary) and screened through a #20 mesh; the powder obtained was stored for further treatment.

REs. Aqueous Extract of Rosemary (RE_w). A pulverized leaf sample of *R. officinalis* L. (20 g) was sequentially extracted thrice with boiling water (each 200 mL) for 30 min. The extracts were filtered through Whatman #2 filter paper. The filtrates were combined, lyophilized, and pulverized. The % yield (w/w, on dry basis) of the aqueous extracts was 9.05.

Acetonic Extract of Rosemary (RE_A). Leaves of *R. officinalis* L., after they were rinsed with distilled water, were lyophilized to remove all water contents. An accurately weighed lyophilized leaf sample (150 g) was soaked in a 10-fold volume (1500 mL) of acetone for 1 h, macerated, collected into a serum bottle, and finally ultrasonicated for 10 min. The extraction was repeated three times. The combined extracts were filtered, and the filtrate was concentrated under reduced pressure with a vacuum evaporator to almost dryness to remove the acetone, then lyophilized, and pulverized to obtain RE_A. The product was stored at −80 °C for use. The % yield (w/w, on dry basis) of RE_A was 13.77.

LDL Preparation. Plasma used for LDL isolation was collected from normal, healthy, fasting volunteers. The patients were venipunctured and bled, and the blood was collected in tubes previously containing 1 mg/mL of EDTA in each tube. LDL (d = 1.019−1.063 g/mL) was isolated by sequential ultracentrifugation using a Hitachi Ultracentrifuge (Himac CS 150GXL, Hitachi) according to the method previously described by Yamanaka et al. (*18*) with slight modification. The LDL solution flushed with N_2 was stored at 4 °C and used within 1 week after preparation. The protein content was measured using a Bio-Rad kit against a BSA standard. To carry out glycation and oxidation experiments, LDL was previously dialyzed three times against 1 L (1000-fold volume) of phosphate-buffered saline (PBS, containing 0.01 M phosphate buffer and 0.15 M NaCl, pH 7.4) in the dark at 4 °C for 24 h.

Relative Electrophoresis Mobility (REM). REM was measured according to Miura (*19*). LDL (0.3 mg protein/mL) was diluted with 10 mM PBS and incubated with Cu^{2+} (10 μM) plus G (400 mM), GO (2.5 mM), or MGO (2.5 mM) at 37 °C for 24 h in the presence and the absence of REs (0.1 mg/mL). Electrophoresis of LDL was performed on Paragon Lipo Gel buffered at pH 8.6 with Paragon Lipo buffer. The former was previously stained with Sudan B black. Results were expressed in REM against the native LDL (REM was arbitrarily assigned as 1.0).

CD. LDL (0.1 mg protein/mL) was diluted in 10 mM PBS and incubated with Cu^{2+} (2.4 μM) and G (30 mM) at 37 °C in the presence and the absence of 1.3 μg/mL RE_w and RE_A, respectively. The amount

of CD formed was measured by determining the increased absorbance at 234 nm at 5 min intervals for a course span of 600 min using a Hitachi U-3000 spectrophotometer. Results were expressed in relative absorbance at 234 nm. The duration of the lag phase was determined from the extrapolation of the propagation phase.

TBARS. LDL (0.3 mg protein/mL) was diluted with 10 mM PBS and incubated with Cu^{2+} (50 μM) and G (400 mM) at 37 °C in the presence (0.5 mg/mL) and the absence of RE_w and RE_A for 1, 3, 5, and 20 h, respectively. AG (4 mM) was used as the positive control. The TBARS were measured according to Yagi (*20*). An aliquot of LDL solution (100 μL, 100 μg protein/mL) was mixed with 50 μL of BHT (4% w/v), 0.5 mL of SDS (0.3% w/v), 2.0 mL of HCl (0.1 N) solution, 0.3 mL of PTA (10% w/v), and 1.0 mL of aqueous solution of TBA (0.8% w/v). The mixture was incubated in a water bath at 100 °C for 45 min while loosely capped. After the mixture was cooled in an ice bath, 3.0 mL of *n*-butanol was added to each tube, and the mixtures were vigorously shaken for 30 s and centrifuged at 3000 rpm for 10 min; the absorbance of the organic layers was read at 532 nm using a Hitachi U-2000 spectrophotometer.

AGEs. LDL (0.3 mg protein/mL) or collagen (2 mg/mL) was diluted with 10 mM PBS and incubated with G (800 mM; 1 M for collagen), GO (2.5 mM), or MGO (2.5 mM) at 37 °C in the presence (0.1 mg/mL) and the absence of RE_w and RE_A, respectively, for 9 days (for collagen, 15 days). AGE formation was measured by determining the fluorescence (excitation at 355 nm and emission at 460 nm; for collagen, excitation at 370 nm and emission at 440 nm) (*21*) using a Hitachi Fluo-Polar star. Data were presented as means ± standard deviations (SDs). The percent inhibition for AGEs formation (PIAF) in LDL was calculated according to the following equation:

$$\mathrm{PIAF} = [(1 - F_s)/F_c] \times 100 \quad (1)$$

where F_s is the intensity of fluorescence of the sample with excitation at 355 nm and emission at 460 nm (for collagen, excitation at 370 nm and emission at 440 nm) and F_c is the intensity of fluorescence of the control with excitation at 355 nm and emission at 460 nm (for collagen, excitation at 370 nm and emission at 440 nm).

Glycation of Collagen. The early glycation reaction of collagen was assessed by the modified phenol−sulfuric acid method (*22*). Collagen (5 mg/mL) was used as the collagen blank or added with G (1 M) and incubated at 37 °C for 24 h. In the other treated groups, 0.1 mg/mL of either RE_A or RE_w was added to compare with a positive control AG (4 mM). Immediately after the incubation period, the tendons were thoroughly washed in PBS to remove unbound materials and dialyzed against 0.2 M phosphate buffer (pH 7.4) for 24 h. To 1 mL of deionized water containing about 5.0 mg of collagen, 3.0 mL of concentrated sulfuric acid was added and vortexed. The solution was then cooled in an ice bath, then added to 0.05 mL of phenol (80%), and incubated at 37 °C for 30 min. The absorbance was taken at 485 nm against a G standard using an LKB Pharmacia spectrophotometer (England).

Determination of Antithrombin III Activity. The antithrombin III activity was measured by following the chromogenic method (*5*, *23*). SAR-PRO-ARG-pNA was used as a specific thrombin substrate, and the reciprocal of the residual thrombin activity was taken. Briefly, the collected whole blood was centrifuged at 3000 rpm for 10 min, and the supernatant plasma was decanted and diluted with four-fold diluent PBS (10 mM, pH 7.4). On addition of G (80 mM) and 0.05 mL of RE, the mixture was incubated at 37 °C for 48 h. An aliquot of 80 μL of the reacted plasma was added with 90 μL of thrombin solution (30 NIH U/mL) and 6 μL of heparin (1 U/μL) and incubated at 37 °C for 20 min. Thereafter, 20 μL of SAR-PRO-ARG-pNA (3.1 mM) was added and left to stand at ambient temperature for 2 min. Finally, the absorbance was read at 385 nm against the control. AG (1 mM) was used as the positive control.

Determination of Polyphenolic Contents. The total polyphenolic contents in REs were determined by means of the Folin−Ciocalteau colorimetric method (*24*). The sample of extract (each 0.2 mL) was mixed with 0.8 mL of Folin−Ciocalteau reagent (Kanto Chemicals, Tokyo, Japan) and 0.5 mL of Na_2CO_3 (10%), the mixture was allowed to stand for 1 h at ambient temperature to facilitate the reaction, and the absorbance was measured at 760 nm. A calibration curve was established using gallic acid as the reference standard. The total polyphenolic content was expressed as mg gallic acid equivalents (GAE) per gram of extract (GAE/g).

Table 1. Programmed Gradient Elution Performed in HPLC Analysis

time	eluent A	eluent B
0.00	5.0	95.0
5.00	5.0	95.0
35.00	60.0	
40.0		
55.00	95.0	5.0
60.00	95.0	5.0
65.00	5.0	95.0

Free Radical Scavenging Activity on 2,2-Diphenyl-1-picrylhydrazyl (DPPH). The scavenging capability against DPPH radicals was measured according to the method described by Hatano et al. (*25*). Briefly, 0.3 mL of extract (either dissolved in methanol or distilled water) was added to Tris-HCl buffer (pH 7.9; 0.1 mL of 1 M) and DPPH methanolic solution (0.6 mL; 100 mM in methanol). The mixture was mixed thoroughly for 20 min at room temperature. During the whole course of the experiment, the reaction mixture was kept away from light exposure. Finally, the absorbance was read at 517 nm against the blank (using either deionized water or methanol). The percent scavenging capability (PSC) for DPPH was calculated according to eq 2:

$$\mathrm{PSC} = [(A_b - A_s)/A_b] \times 100\% \quad (2)$$

where A_b is the absorbance for the blank at 517 nm and A_s is the absorbance for the sample at 517 nm. The IC_{50} concentration required for 50% scavenge was calculated thereof from eq 2.

Liquid Chromatography/Mass Spectrometry (LC/MS) Analysis. The high-performance liquid chromatography (HPLC) analyses were carried out using a Finnigan Surveyor Modular HPLC system (Thermo Electron Co., United States). In chromatographic separations, an analytical column Luna 3 μg C18(2) 150 mm × 2.0 mm and a guard column were used. A Security Guard C18 (ODS) 4 mm × 3.0 mm i.d. (Phenomenex, Inc., Torrance, CA) at a flow rate of 0.2 mL/min was adopted. The mobile phases A and B were run according to the programmed protocol listed in **Table 1**.

Briefly, from 0 to 5 min, the elution was run with 95% of B; from 5 to 35 min, 95−40% of B was used; from 35 to 55 min, eluted with 40 to 5% of B in a linear gradient manner; within 55−60 min, 5% of B was adopted, while from 60 to 65 min, isocratic 95% B was used. The photodiode array detector was operated at wavelengths between 220 and 400 nm. This system was coupled to a Finnigan LCQ Advantage MAX ion trap mass spectrometer, which was operated in an electrospray ionization (ESI) mode. An aliquot of sample extracts (20 μL) was directly introduced into the column through the Rheodyne (model 7725i) injection valve. ESI source and negative ionization mode were used with different fragment voltages. Nitrogen was used as the nebulizing and drying gas. The typical operating parameters adopted were as follows: the spary needle voltage, 5 kV; the ion transfer capillary temperature, 300 °C; the nitogen sheath gas, 40; and the auxiliary gas, 5 (arbitrary units). The ion trap containing helium damping gas was introduced in accordance with the manufacturer's recommendations. The mass spectra were acquried in an *m*/*z* range of 100−1000 with five microscans and a maximum ion injection time of 200 ms. The selected ion monitoring analysis was a narrow scan event that monitored the *m*/*z* value of the selected ion within a range of 1.0 Th centered on the peak for the molecular ions. This relevant function was used for the analyses of the flavonoid or diterpenoid molecular ions by MS/MS in negative ELS modes. The MS/MS fragment spectra were produced using normalized collision energies with an increment of 5% within 10−30% and also with wideband activtion "off".

Statistical Analysis. Statistical analyses (analyses of variance) were performed according to SAS User's Guide (*26*). All analyses were run in triplicate and averaged. Results were calculated and expressed as

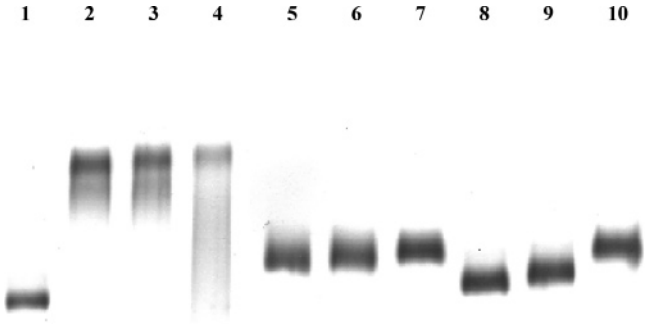

Figure 2. Shifts of eletrophoretic patterns of human LDL incubated with Cu^{2+} plus different initiators in the presence and the absence of *R. officinalis* L. extracts. LDL (0.3 mg protein/mL) was induced with Cu^{2+} (10 μM) and G (400 mM), GO (2.5 mM), or MGO (2.5 mM) at 37 °C in the presence and the absence of aqueous extract (RE_W) and acetone extract (RE_A) of *R. officinalis* L. at 0.1 mg/mL, respectively, for 24 h. Lane 1, naive LDL; lane 2, LDL plus Cu^{2+} and G; lane 3, LDL plus Cu^{2+} and GO; lane 4, LDL plus Cu^{2+} and MGO; lane 5, LDL plus Cu^{2+}, G with RE_W; lane 6, LDL plus Cu^{2+}, GO with RE_W; lane 7, LDL plus Cu^{2+}, MGO with RE_W; lane 8, LDL plus Cu^{2+}, G with RE_A; lane 9, LDL plus Cu^{2+}, GO with RE_A; and lane 10, LDL plus Cu^{2+}, MGO with RE_A.

means ± SD. Significance ($p < 0.05$) of mean differences was determined by Duncan's multiple range tests.

RESULTS

REs Can Effectively Suppress CD Formation Induced by Different Initiators. Taking the naive LDL (without any modification) as the control (arbitrarily REM = 1; lane 1 in **Figure 2**), the REM shifts were compared among three systems: system 1, Cu^{2+} (10 μM) plus G (400 mM); system 2, Cu^{2+} (10 μM) plus GO (2.5 mM); and system 3, Cu^{2+} (10 μM) plus MGO (2.5 mM). The REMs obtained were found to be 10.4, 10.8, and 11.0 for systems 1, 2, and 3, respectively (lanes 2−4 in **Figure 2**). On addition of REs (0.1 mg/mL), the REMs in the above three systems were shifted to 4.4, 4.8, and 5.2 (lanes 5−7 in **Figure 2**) by RE_w and 2.8, 3.2, and 5.2 (lanes 8−10 in **Figure 2**) by RE_A, respectively. Apparently, better results were favored by RE_A in cases of G and GO (lanes 8 and 9), whereas in the case with MGO, results were only comparable between RE_w (lane 7) and RE_A (lane 10) (**Figure 2**).

REs Can Effectively Inhibit LDL Glycation Induced by G. In the presence of Cu^{2+} (2.4 μM) and G (30 mM), the lag time for CD appearance was seen to be only 45 min in the control (without REs) as compared to 150 min in the presence of RE_w (1.3 μg/mL) and to 600 min as effectively relapsed by RE_A (1.3 μg/mL) (**Figure 3A**).

As well-known, the oxidatively cleaved product malondialdehyde species can be measured as TBARs. In our study, the TBARS attained a maximum concentration at the fifth hour postinduction by G without any REs treatment (**Figure 3B**) and was effectively suppressed by the addition of REs (0.5 mg/mL) in the order of effectiveness: 74.29 ± 1.51, 71.22 ± 0.95, and 68.01 ± 0.19% by RE_A, RE_w, and AG (4 mM), respectively. Thus, the two REs at a concentration of 0.5 mg/mL were shown to have effectively suppressed the LDL glycation when compared with the positive control AG.

The AGE inhibition assay performed by the fluorescence determination revealed REs (0.1 mg/mL) to be stronger inhibitors than AG (1 mM) in this regard. The inhibitory effect of the two REs (0.1 mg/mL) against G-induced AGE formation

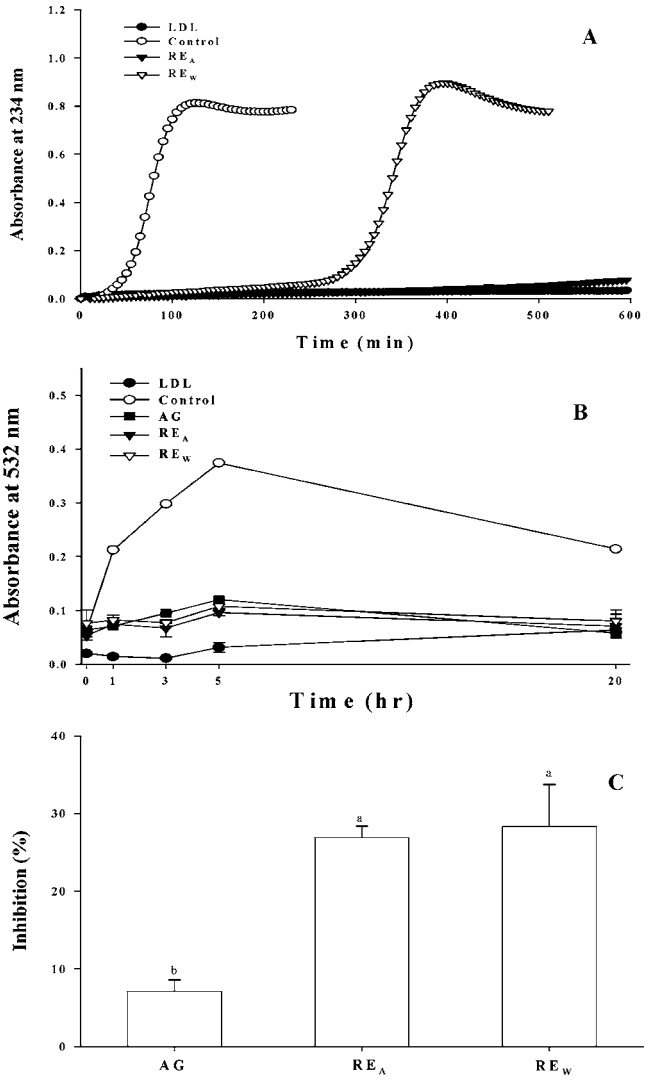

Figure 3. Effect of *R. officinalis* L. extracts on LDL glycation induced by G. Conjugated diene production profile (**A**): LDL (0.1 mg protein/mL) was incubated with Cu^{2+} (2.4 μM) and G (30 mM) at 37 °C in the presence and the absence of the aqueous extract (RE_w) or the acetone extract (RE_A) of *R. officinalis* L. at 1.3 μg/mL, respectively. The amount of conjugated dienes was measured at 234 nm at 5 min intervals for a total period of 600 min. TBARS formation profile (**B**): LDL (0.3 mg protein/mL) was incubated with Cu^{2+} (50 μM) and G (400 mM) at 37 °C for 1, 3, 5, and 20 h, respectively, in the presence and absence of *R. officinalis* L. extracts RE_w or RE_A at 0.5 mg/mL or AG (4 mM), respectively. Amount of AGEs formed (**C**) as follows: LDL (0.3 mg protein/mL) was induced with G (800 mM) at 37 °C in the absence or presence of *R. officinalis* L. extracts RE_w or RE_A at 0.1 mg/mL or AG (1 mM), respectively, for 9 days. The AGEs formation was measured by determining the fluorescence with excitation at 355 nm and emission at 460 nm. Data are expressed as means ± SD from triplicates. Different letters between each bar denote significant differences ($p < 0.05$).

almost had reached 30% in the order of effectiveness: RE_w (28.33%) ≥ RE_A (26.92%) > AG (7.14%) (**Figure 3C**).

REs Effectively Inhibited AGEs Formation In Collagen Induced by Different Initiators. The glycation on collagen induced by different initiators was most prominently exhibited by MGO with GO next, and the induction period required for AGEs formation was relatively long (15 days) (**Figure 4**) as compared to that (9 days) of LDL (**Figure 3**). As is well-known, among all inducers, MGO induces the most prominent AGE formation in collagen; hence, the antiglycative capability of REs

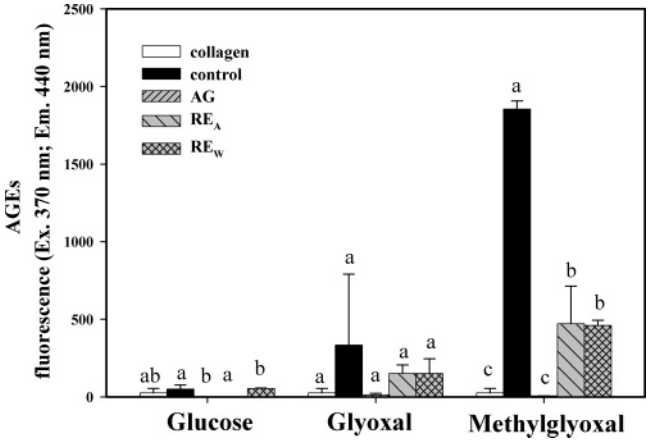

Figure 4. Effect of *R. officinalis* L. extracts on AGEs formation in collagen induced by G, GO, or MGO. Collagen (2 mg/mL) was induced with G (1 M), GO (2.5 mM), or MGO (2.5 mM) at 37 °C in the presence and absence of aqueous extracts of *R. officinalis* L. extracts RE_w or RE_A at 0.1 mg/mL or AG (4 mM), respectively, for 15 days. AGEs formation was measured by determining the fluorescence excited at 370 nm with emission at 440 nm. Data are expressed as means ± SD from triplicates. Different letters between each bar denote significant differences ($p < 0.05$) vs the control of each group.

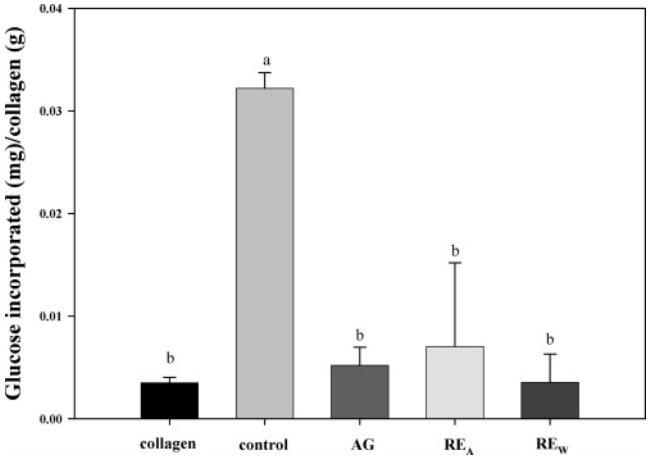

Figure 5. Effect of *R. officinalis* L. extracts on glycation in collagen induced by G. Glycation of collagen (**A**): The glycation of collagen was measured by the phenol–sulfuric acid method. Lane 1, naive collagen. Positive controls (lanes 2–5) using collagen (5 mg/mL) were incubated at 37 °C. Lane 2, with G (1 M) alone; lane 3, with G (1 M) and AG (4 mM); lane 4, with G (1 M) and 0.1 mg/mL RE_A; lane 5, with G (1 M) and 0.1 mg/mL RE_w, respectively, for 24 h. Data are expressed as means ± SD from triplicates. Different letters between each bar denote significant differences ($p < 0.05$).

was most apparently conceivable in the presence of MGO, whereby REs at 0.1 mg/mL were evidenced to have effectively suppressed the glycation of collagens initiated by G (1 mM), GO (2.5 mM), and MGO (2.5 mM), respectively. The order of suppressive capability in the case by G was AG > $RE_A \geq RE_w$; however, for RE_A and RE_W with GO and MGO (**Figure 4**), results were rather comparable. They seemed somehow different in capabilities between G and GO, yet statistically insignificant (**Figure 4**).

G Uptake into Collagen Can Be Inhibited by RE_s. The normal G incorporation into collagen was estimated to be 0.032 mg/g collagen after 1 day of incubation of collagen (5 mg/mL) with G (1 M) (control bar in **Figure 5**). Such a mobilization can be inhibited by antiglycative agents. On treatment with RE_w, RE_A (0.1 mg/mL), and AG (4 mM), values were significantly reduced to 0.0035, 0.0070, and 0.0055 mg/g collagen, respectively. Interestingly, in this respect, RE_w prevailed much more than those of RE_A and AG, exhibiting a more effective antiglycative efficiency (**Figure 5**).

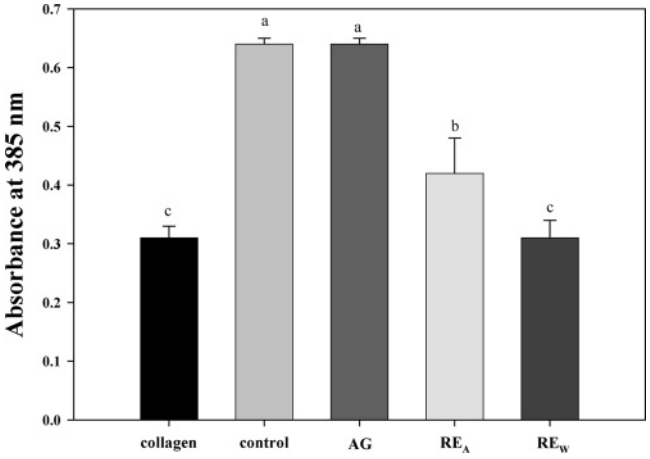

Figure 6. Effect of *R. officinalis* L. extracts on antithrombin III activity induced by G. Antithrombin III activity: Plasma was incubated with G (80 mM) control at 37 °C in a CO_2 (5%) incubator in the presence and the absence of *R. officinalis* L. aqueous (RE_w) or acetonic (RE_A) extracts at 0.05 mg/mL and 1 mM AG, respectively, for 48 h. Data are expressed as means ± SD from triplicates. Different letters between each bar denote significant differences ($p < 0.05$).

Table 2. Total Polyphenolics and DPPH Radical Scavenging Capability of Different *R. officinalis* L. Extracts

extract	DPPH radical scavenging capability[a] (%)	total polyphenolics (GAE)[b] (mg/g)
RE_w	57.94 ± 1.25	155.87 ± 7.18
RE_A	80.67 ± 2.24	63.03 ± 5.16

[a] Free radical scavenging capability of *R. officinalis* L. aqueous (RE_w) or acetone extracts (RE_A) at 0.1 mg/mL, respectively, on DPPH radicals. The scavenging capability (%) was calculated by the equation described in the text. [b] The amount of total polyphenols was expressed as GAE. Data are expressed as means ± SD from triplicates.

REs Are Able To Moderately Reduce the Anti-Thrombin III Activity. A study on the antithrombin III activity showed that RE_w was more efficient than both the RE_A and the positive control AG. As evidenced in the G (80 mM)-induced system at 37 °C, the inhibitory effect exerted by RE_A and RE_w (0.05 mg/mL) reached 34 and 51%, respectively, which was a comparatively rather large difference of 17%. As a prominent contrast, AG at 1 mM was not as effective as expected (**Figure 6**).

RE_w Has More Polyphenolic Content, while RE_A Is a Better Radical Scavenger. Astonishingly, although the total phenolics were more abundant in RE_w (155.87 GAE/g) than RE_A (63.03GAE/g), in regard to the free radical scavenging capability for DPPH radicals, the extracts RE_w and RE_A at 0.1 mg/mL reached 57.4 and 80.67%, respectively (**Table 2**).

LC/MS Reveals Some Major Bioactive Components. LC/MS analyses revealed the summed-up area of the eight main peaks to have a total of 50.64%, among which the five major constituents, rosmarinic acid, carnosol, 12-methoxycarnosic acid, carnosic acid, and methyl carnosate, as identified by us, occupied a subtotal of 20.82% (**Figure 7** and **Table 3**). Alternatively, in **Figure 7**, we found that there were still three other major peaks (Un1−Un3) that remain to us a huge space for further investigation.

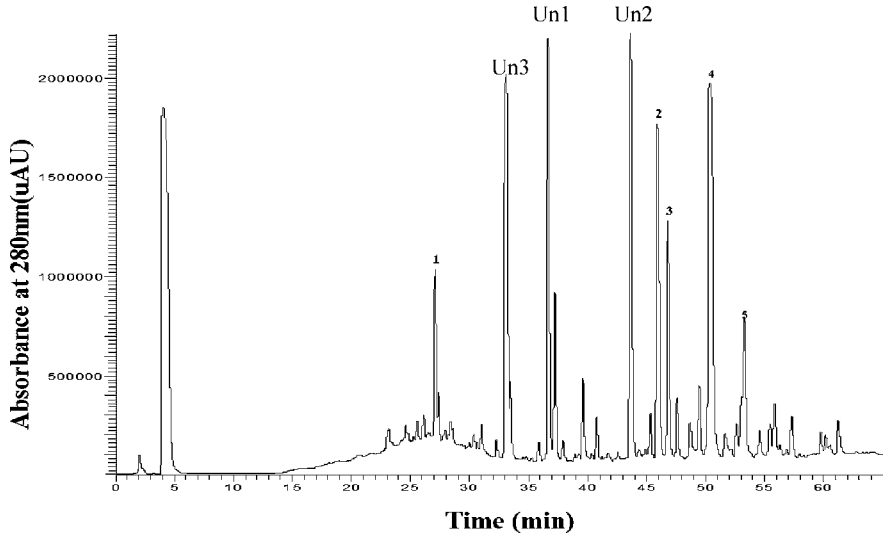

Figure 7. Characteristic HPLC chromatogram of the acetonic extract from *R. officinalis* leaves. Peaks: 1, rosmarinic acid; 2, carnosol; 3, 12-methoxycarnosic acid; 4, carnosic acid; and 5, methyl carnosate. Other unknown constituents Un1 to Un3 are indicated. The molecular mass of peak 5 has been identified; yet, its structure still remains unclear.

Table 3. Characteristic Parameters for LC/MS Analysis of the Compounds in the Extracts of *R. officinalis* L.

peak no.	retention time (min)	assigned identity	UV λ_{max} (nm)	$[M - H]^-$ *m/z*	LC/ESI-MS^{2a} *m/z*
1	27.11	Rosmarinic acid	237, 328	359.1	
Un1	36.65	unknown 1	237, 274, 330	345.3	
Un2	43.64	unknown 2	238, 275, 326	345.1	
2	45.91	carnosol	248, 270	329.2	329.1, 301.3
3	46.82	12-methoxycarnosic acid	242, 266	361.2	
4	50.36	carnosic acid	281, 329	331.1	331.1, 287.2
5	53.30	methyl carnosate	281, 327	345.1	

[a] MS^2 run with 30% collision energy; Un, unknown constituent.

DISCUSSION

Glycation has been cited to be relevantly associated with oxidative stress (*16*, *17*). When oxidized, the surface of LDL becomes more negatively charged and more intense with the extent of oxidation (*27*−*29*). The farther REM can mean more oxidized specimens (lanes 2−4 in **Figure 2**). In this regard, the acetonic extract RE_A (lanes 8 and 9 in **Figure 2**) showed a more efficient suppressing effect than the aqueous extract RE_w (lanes 5 and 6 in **Figure 2**) for both G and GO initiators; however, this was seemingly not the case with MGO (lane 10 vs lane 7 in **Figure 2**), implicating that MGO acted through a mechanism somehow different from the other two initiators. On glycation of LDL, conjugated dienes always appear first, then come up with the TBARS, and finally the AGEs (*21*, *30*, *31*) (**Figure 3**). The formation of AGEs is usually known to be an evitable process involved in LDL glycation induced by hyperglycemia. **Figure 3** revealed the more potent acetonic extract RE_A in anti-CD formation than the aqueous extract RE_w; the time lag relapsed approximately four-fold (600 min) as compared to that (150 min) of the aqueous extract (**Figure 3A**); however, both extracts were comparable in anti-TBARs formation (**Figure 3B**). Such a better outcome of RE_A than RE_w obviously was not due to the polyphenoilc content (**Table 2**) but could possibly be ascribed to the higher content of some key lipophilic antioxidants (such as carnosic acid, rosmarinic acid, 12-methoxycarnosic acid, rosmarinic acid, and carnosol, etc.; **Table 3**) in the fraction of RE_A, which could readily penetrate the LDL lipid moiety at the very initial phase. Accordingly, both the initiators G and GO are speculated to be actively and instantaneously effective in the initial phase contributing to the early stage of CD formation, whereas MGO may induce glycation only at a stage slightly later than G and GO (**Figure 2**).

At least 12 diterpene phenols have been isolated from rosemary (*32*). Several of them, such as carnosol, carnosic acid, rosmanol, rosmaridiphenol, rosmadial, and miltirone, all possess antioxidant activities and are known as major antioxidants in rosemary (*15*, *33*).

Because LC/MS analyses reveled the summed-up area of the eight main peaks (**Figure 7**) occupying a total of 50.64%, in which the combined area contributed by rosmarinic acid, carnosol, 12-methoxycarnosic acid, carnosic acid, and methylcarnosate was 20.82%, whether the antiglycative and antioxidative capabilities of REs can be attributed to these compounds, we carried out the CD inhibitory activities using the actual contents of carnosic acid and rosmarinic acid already found in this experiment, and we were excited to recognize that the lag times of carnosic acid and rosmarinic acid all surpassed 600 min (data not shown), which was comparable to the corresponding data revealed by the acetonic extract RE_A (**Figure 3A**). As cited, carnosic acid can be readily oxidied to form carnosol, implicitly indicating the major contribution of carnosol, carnosic acid, and rosmarinic acid in the role of antiglycation.

The aging process has been found to be largely associated with nonenzymatic glycation as well as the AGEs formation (*34*). Hence, currently, AGEs have been considered as markers of glycation and oxidative stress caused by glycation, but its link is extended to aging processes, such as atherosclerosis, diabetes, neurodegenerations, and other nephropathies (*35*, *36*). Our study indicated that the REs, despite RE_A or RE_w, were all

very effective against AGEs formation (**Figures 3C** and **4**), an implication of a potential protective capability against cardiovascular endothelial damage caused by hyperglycemia. Pathologically, hyperglycemia and aging may induce more extensive oxidative stresses, hence with greater probabilities to produce more severe damage involving the intra- and intermolecular cross-linking between many biomolecules between the proteins, enzymes, and DNA (*37*), prominently occurring in atherosclerosis (*6*) and late stage blindness caused by DM or senile cataracts (*38*). In this respect, G uptake can also be a major role to accelerate the glycative reactions; yet, the G uptake by collagen was greatly suppressed by RE_w and RE_A (**Figure 5**). As mentioned in above, in **Figure 5**, the effect of RE_w was seen to prevail over that of RE_A and the positive control AG, apparently contrary to the anti-CD effects found in LDL (**Figure 3A**); such a discrepancy may be ascribed to the stronger surface hydrophilicity possessed by collagen than the LDL.

Moreover, oxidation of unsaturated fatty acid can injure endothelial cells, inducing the proliferation of smooth cells and forming thrombus or blood clot and deposition of fibrin−calcium complexes (*7*). Polymerization of collagen and glycation of thrombin and fibrinogen is closely correlated with reactive oxygen species (*5*, *39*). In the past decade, many herbal constituents have been cited with rather satisfactory outcomes. Morimitsu et al. (*9*) and Nakagawa et al. (*10*) demonstrated that rosemary and tea extracts are effective in the inhibition of protein glycation. In addition, rosemary has also been found to be an excellent anti-inflammatory as well as antiviral agent other than an antioxidant (*40*).

Polyphenolics are very important plant constituents inherently having potent free radical scavenging capabilities due to their hydroxyl groups. Obviously, there was no positive correlation of the total phenolic content with the DPPH scavenging capability (**Table 2**) nor with the antiglycative capability (**Figure 3A−C**).

Antithrombin III is an inhibitor for the proteolytic enzyme of a 53 kDa protein. By inhibiting the thrombin and other blood coagulation factors, it can control the blood-clotting rate. Our study showed that the RE_w at 0.05 mg/mL inhibited 51% of the antithormbin III activity; in contrast, RE_A showed only 31% inhibition (**Figure 6**). Interestingly, AG was totally ineffective, implicating that their action mechanisms may be quite different in this respect.

In summary, in the LDL model, the acetone extract of rosemary RE_A has more effective antiglycative potential than the aqueous extract RE_w, while in the collagen and thrombin III models, the reverse is true. We attributed such results to the higher lipophilc diterpenoid polyphenolic content in RE_A (such as rosmarinic acid, carnosic acid, and carnosol) and the different surface lipid characteristics between LDL and collagen, although for anti-AGEs both extracts were comparable. To assist the evidence, a larger DPPH radical scavenging capability with less total polyphenolic content was indicated in RE_A.

In conclusion, on the basis of the results obtained from the LDL, collagen, and thrombin models, we conclude that rosemary is an excellent multifunctional therapeutic herb; its extracts are very effective antioxidants, antiglycatives, and rather potent antithrombin III agents, which can be attributed to the profound contents of rosmarinic acid, carnosic acid, and carnosol. By looking at its potential potent antiglycative bioactivity, it may be used as a good adjuvant medicine for the prevention and treatment of diabetic, cardiovascular, and other neurodegenerative diseases.

LITERATURE CITED

(1) Guillausseau, P. J. Classification and diagnostic criteria of diabetes: propositions of ADA and WHO. *Diabetes Metab.* **1997**, *23*, 454−455.

(2) Department of Health, Executive Yuan, ROC. http:/www.doh.gov.tw/statistic/data/CausesofMortality/2004/table1.xls.

(3) Givgliano, D.; Ceriello, A.; Paolisso, G. Oxidative stress and diabetic vascular complications. *Diabetes Care* **1996**, *19*, 257−267.

(4) Monnier, V. M.; Glomb, M.; Sell, D. R. The mechanism of collagen cross-linking in diabetes: A puzzle nearing resolution. *Diabetes* **1996**, *45* (Suppl. 3), S67−S72.

(5) Gugliucci, A.; Menini, T. The botanical extracts of Achyrocline satureoides and Ilex paraguariensis prevent methylglyoxal-induced inhibition of plasminogen and antithrombin III. *Life Sci.* **2002**, *72*, 279−292.

(6) Steinberg, D.; Parthasarathy, S.; Carew, T. E.; Khoo, J. C.; Witztum, J. L. Beyond cholesterol: Modifications of low-density lipoprotein that increases its atherogenicity. *N. Engl. J. Med.* **1989**, *320*, 915−919.

(7) Rosenfeld, M. E.; Tsukuda, T.; Grown, A. M.; Ross, R. Fatty streak initiation in Watanabe Heritable hyperlipemic and comparably hypercholesterolemic fat-fed rabbits. *Atherosclerosis* **1987**, *7*, 9−23.

(8) Craig, W. J. Health-promoting properties of common herbs. *Am. J. Clin. Nutr.* **1999**, *70*, S491−S499.

(9) Morimitsu, Y.; Yoshida, K.; Esaki, S.; Hirota, A. Protein glycation inhibitors from thyme (*Thymus vulgaris*). *Biosci., Biotechnol., Biochem.* **1995**, *59*, 2018−2021.

(10) Nakagawa, T.; Yokozawa, T.; Terasawa, K.; Shu, S.; Juneja, L. R. Protective activity of green tea against free radical-and glucose-mediated pritein damage. *J. Agric. Food Chem.* **2002**, *50*, 2418−2422.

(11) Hipkiss, A. R.; Brownson, C.; Bertani, M. F.; Ruiz, E.; Ferro, A. Reaction of carnosine with aged proteins: another protective process? *Ann. N. Y. Acad. Sci.* **2002**, *959*, 285−294.

(12) Carney, J. R.; Krenisky, J. M.; Williamson, R. T.; Luo, J. Achyrofuran, a new antihyperglycemic dibenzofuran from the South American medicinal plant Achyrocline satureioides. *J. Nat. Prod.* **2002**, *65*, 203−205.

(13) Vinson, J. A.; Howard, T. B., III. Inhibition of protein glycation and advanced glycation end products by ascorbic acid and other vitamins and nutrients. *J. Nutr. Biochem.* **1996**, *7*, 659−663.

(14) Booth, A. A.; Khalifah, R. G.; Hudson, B. G. Thiamine pyrophosphate and pyridoxamine inhibit the formation of antigenic advanced glycation end-products: comparison with aminoguanidine. *Biochem. Biophys. Res. Commun.* **1996**, *220*, 113−119.

(15) Frankel, E. K.; Huang, S.; Aeschbach, R. Evaluation of antioxidant activity of rosemary extracts, carnosol and carnosic acid in bulk vegetable oils and fish oil and their emulsions. *J. Sci. Food Agric.* **1996**, *72*, 201−208.

(16) Yim, H. S.; Kang, S. O.; Hah, Y. C.; Chock, P. B.; Yim, M. B. Free radicals generated during the glycation reaction of amino acids by methylglyoxal. A model study of protein-cross-linked free radicals. *J. Biol. Chem.* **1995**, *270*, 28228−28233.

(17) Bonnefont-Rousselot, D. Glucose and reactive oxygen species. *Curr. Opin. Clin. Nutr. Metab. Care* **2002**, *5*, 561−568.

(18) Yamanaka, N.; Oda, O.; Nagao, S. Prooxidant activity of caffeic acid, dirtary non-flavonoid phenolic acid, on Cu^{2+}-induced low density lipoprotein oxidation. *FEBS Lett.* **1997**, *405*, 186−190.

(19) Miura, S.; Watanabe, J.; Tomita, T.; Sano, M.; Tomita, I. The inhibitory of tea polyphenols (flavan-3-ol derivatives) on Cu^{2+} mediated oxidative modification of low density lipoproteion. *Biol. Pharmacol. Bull.* **1994**, *17*, 1567−1572.

(20) Yagi, K. A simple fluorometric assay for lipid peroxides in blood serum or plasma. *CRC Band Book of Free Radicals and Antioxidants in Biomedicine*; CRC Press Inc.: Boca Raton, FL, 1989; Vol. III, p 215.

(21) Galle, J.; Schneider, R.; Winner, B.; Lehmann-Bodem, C.; Schinzel, R.; Munch, G.; Conzelmann, E.; Wanner, C. Glycoxidized LDL impair endothelial function more potently than oxidized LDL: Role of enhanced oxidative stress. *Atherosclerosis* **1998**, *138*, 65−77.
(22) Rao, P.; Pattabiraman, T. N. Reevaluation of the phenol-sulfuric acid reaction for the estimation of hexoses and pentoses. *Anal. Biochem.* **1989**, *181*, 18−22.
(23) Gugliucci, A. A practical method to study functional impairment of proteins by glycation and effects of inhibitors using current coagulation/ fibrinolysis reagent kits. *Clin. Biochem.* **2003**, *36*, 155−158.
(24) Taga, M. S.; Miller, E. E.; Pratt, D. E. Chia seeds as a source of natural lipid antioxidants. *Am. J. Oil Chem. Soc.* **1984**, *61*, 928−931.
(25) Hatano, T.; Kagawa, H.; Yasuhara, T.; Okuda, T. Two new flavonoids and other constituents in licorice root: their relative astringency and radical scavenging effects. *Chem. Pharm. Bull. (Tokyo)* **1988**, *36*, 2090−2097.
(26) SAS Institute Inc. *SAS User's Guide: Statistics*; SAS Institute Inc.: Cary, NC, 1985.
(27) Yen, G. C.; Hsieh, C. L. Tnhibitory effects of Du-zhong (*Eucommia ulmoides* Oliv.) against low-density lipoprotein oxidative modification. *Food Chem.* **2002**, *77*, 449−456.
(28) Sobal, G.; Menzel, E. J.; Sinzinger, H. Troglitazone inhibits long-term glycation and oxidation of low-density lipoprotein. *J. Cardiovasc. Pharmacol.* **2005**, *46*, 672−680.
(29) Hsieh, C. L.; Lin, Y. C.; Ko, W. S.; Peng, C. H.; Huang, C. N.; Peng, R. Y. Inhibitory effect of some selected nutraceutic herbs on LDL glycation induced by glucose and glyoxal. *J. Ethnopharmacol.* **2005**, *102*, 357−363.
(30) Otero, P.; Herrera, E.; Bonet, B. Dual effect of glucose on LDL oxidation: Dependence on vitamin E. *Free Radical Med.* **2002**, *33*, 1133−1140.
(31) Mowri, H. O.; Frei, B.; Keaney, J. F. Glucose enhancement of LDL oxidation is strictly metal ion dependent. *Free Radical Med.* **2000**, *29*, 814−824.
(32) Ho, C. T.; Chen, J.; Lu, G.; Huang, M. T.; Shao, Y.; Chin, C. K. Antioxidative and antimutagenic properties of rosemary. In *Functional Foods for Disease Prevention II: Medicinal Plants and Other Foods*; Osawa, T., Shibamoto, T., Terao, J., Eds.; ACS Symposium Series 702; American Chemical Society: Washington, DC, 1998; pp 153−161.
(33) Wei, G. J.; Ho, C. T. A stable quinone identified in the reaction of carnosol, a major antioxidant in rosemary, with 2,2-diphenyl-1-picrylhydrazyl radical. *Food Chem.* **2006**, *96*, 471−476.
(34) Wautier, J. L.; Guillausseau, P. J. Advanced glycation end products, their receptor and diabetic angiopathy. *Diabetes Metab.* **2001**, *27*, 535−542.
(35) Brownlee, M. Advanced protein glycosylation in diabetes and aging. *Annu. Rev. Med.* **1995**, *46*, 223−234.
(36) Heidland, A.; Sebekova, K.; Schinzel, R. Advanced glycation end products and the progressive course of renal disease. *Am. J. Kidney Dis.* **2001**, *38*, S100−S106.
(37) Gugliucci, A. Glycation as the glucose link to diabetic complications. *J. Am. Osteopath. Assoc.* **2000**, *100*, 621−334.
(38) Ahmed, M. U.; Brinkmann, E.; Degenhardt, T. P.; Thorpe, S. R.; Baynes, J. W. N-epsilon-(carboxyethyl) lysine, a product of the chemical modification of proteins by methylglyoxal, increases with age in human lens proteins. *Biochem. J.* **1997**, *324*, 565−570.
(39) Sajithlal, G. B.; Chithra, P.; Chandrakasan, G. Effect of curcumin on the advanced glycation and cross-link of collagen in diabetic rats. *Biochem. Pharmacol.* **1998**, *56*, 1607−1614.
(40) Aruoma, O. I.; Spencer, P. E.; Rossi, R.; Butler, J.; Halliwell, B. An evaluation of the antioxidant and antiviral action of extracts of rosemary and provencal herbs. *Food Chem. Toxicol*. **1996**, *34*, 449−456.

Received for review November 5, 2006. Revised manuscript received February 23, 2007. Accepted February 28, 2007.

JF0631833