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Influence of Flavonols As *in vitro* on Low Density Lipoprotein Glycation

Mohammad Ali Ghaffari* and Samad Mojab

Dept. of Clinical Biochemistry, School of Medical, Ahwaz Jundishapour University of Medical Sciences, Ahwaz, Iran

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ABSTRACT

Introduction. The non-enzymatic glycation of Low density lipoprotein (LDL) is a naturally occurring chemical modification of apolipoprotein B as a result of condensation between lysine residues and glucose. Glycated LDL is poorly recognized by LDL receptors and initiates different processes that can be considered proatherogenic. Thus, LDL glycation may contribute in the increased atherosclerotic risk of patients with diabetes. The objective of this study was to investigate the effect of naturally occurring flavonols on LDL glycation *in vitro*. **Methods.** In this study, LDL was isolated from EDTA-plasma by ultracentrifugation using a single step discontinuous gradient. Then, glucose was added to LDL and LDL glycation level was estimated in absence and presence of flavonols by sodium periodate assay. **Results.** This study was showed that five flavonols: quercetin, myricetin, kaempferol, rutin and morin decreased LDL glycation in a dose-dependent manner. Also, it was demonstrated this nutrients decreased electrophoretic mobility of glycated LDL. **Conclusion.** The results of this investigation show that flavonols probably with their antioxidant properties inhibited LDL glycation and thus may have a role in ameliorating atherosclerotic risk of patients with diabetes mellitus. *Iran. Biomed. J. 11 (3): 185-191, 2007*

Keywords: Low density lipoprotein (LDL), Glycation, Flavonols

INTRODUCTION

Low density lipoprotein (LDL) is a well-known risk factor for the premature development of atherosclerosis [1]. There is evidence that LDL gains or increases its atherogenic potential after chemical modification, including glycation [2]. Like other serum proteins, lipoproteins are non-enzymatically glycated in the presence of glucose. Non-enzymatic glycation of proteins known as Millard reaction which reduces sugars are covalently attached to free amino groups and ultimately forms advanced glycosylation end products (AGE) which has been found to occur during normal aging and at accelerated rate in diabetes mellitus. It has been suggested that glycated LDL provides the missing link between diabetes mellitus and hypercholesterolemia [3]. Thus, an increased rate of LDL glycation in diabetes could indeed contribute to the increased rate of cardiovascular morbidity in these patients [3].

Apolipoprotein B_{100} (apo-B_{100}) of LDL, usually has some of its lysine residues modified by non-enzymatic glycation [4]. The functional consequences of glycation are manifested in impaired LDL receptor recognition of glycated LDL in fibroblasts and human monocyte derived macrophages (HMDM) and increased cholesterol ester synthesis in HMDM, possibly via a high capacity, low affinity, non-LDL receptor pathway. Glycation of apo-B_{100} also slow the catabolism of LDL [4, 5].

The flavonols belong to a large group of compounds called flavonoids, which are diverse in their chemical structure and characteristics. Fruits, vegetables and beverages such as tea and red wine are major sources of flavonols in the human diet [6]. Flavonols are antioxidant phenolic compounds, which are known to be effective scavengers of free radicals and chelators of transitional metals (Fe, Cu) that catalyze free radical production. They also reduce macrophage mediated LDL oxidation [7].

*Corresponding Author; E-mail: ghaffarima@yahoo.com

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Consumption of flavonoids, particularly the quercetin (as a flavonol) has been associated with a reduced incidence of heart disease in diabetes mellitus [8]. Therefore, in present study, we investigated the role of five flavonols (quercetin, myricetin, kaempferol, rutin and morin) as *in vitro* on glycation LDL.

MATERIALS AND METHODS

Chemicals. The flavonols (quercetin, myricetin, kaempferol, rutin and morin) were purchased from Sigma (St. Louis, Mo, U.S.A.) and used without further purification. EDTA, DMSO, potassium bromide, bovine serum albumin, agarose, glucose, fructose and sodium periodate were obtained from Merck (Darmstadt, Germany). Sodium borohydride was obtained from Riedel-deHaen (Germany). Solutions were freshly prepared with double deionized water.

LDL isolation. Blood from normolipidemic overnight fasting volunteers (n = 25, age 30 ± 5 y, men, non- smokers, non-diabetics, not taking any drug since at least 2 weeks) was collected into syringes containing EDTA (1 mg EDTA/ml blood). LDL was isolated by ultracentrifugation using a single step discontinuous gradient according to the method of Gieseg *et al.* [9]. Briefly, anticoagulated (EDTA) plasma was obtained by centrifugation (3,000 ×g, 10 min). EDTA solution (4.5 ml at 1 mg/ml, pH 7.4) was placed in 10 ml centrifuge tubes. Using a long needle, this solution was under laid with dense plasma solution. The dense plasma solution was prepared by dissolving 0.632 g of solid potassium bromide in 2 ml of defrosted EDTA-plasma. The centrifuge tubes were ultracentrifuged at 40,000 ×g for 2 hours in a Damon B-60 ultracentrifuge. LDL fraction as the orange band at the middle of the tubes was carefully withdrawn. EDTA and potassium bromide were separated from LDL by dialysis tubing (10 mm flat width) with a molecular weight cutoff 12-4 kDa at 4°C for 24 hours against PBS, pH 7.4. The LDL protein content was determined by Bradford method [10], using bovine serum albumin as standard. The cholesterol, triglyceride, LDL-cholesterol and HDL-cholesterol were determined using commercially available Kits (Pars Azmon kits, Iran). The purified LDL was examined by electrophoresis carried out at pH 8.6 in 0.05M barbital buffer on 0.8% agarose gel. Gels were stained with Sudan Black B stain [11].

LDL was sterilized by filtration (0.45 µm Millipore filter). Then stored in darkness under nitrogen gas at 4°C and was used within 4 weeks.

LDL glycation. We prepared glycated LDL by incubation of LDL (0.2 mg protein/ml) with different concentrations of glucose (0-200 mM) and in different time intervals (1-11 days) at 37°C. The degree of LDL glycation was measured by periodate method [12]. Briefly, 500 µl LDL (0.2 mg protein/ml) was incubated with 100 µl of fresh prepared sodium borohydride (200 mM) in ice-cold NaOH (0.01 mM). A volume of 100 µl of NaOH (0.01 mM) was used as control. The reaction was stopped by adding 100 µl of HCl (0.2 mM). Then, 100 µl of sodium periodate (0.1 mM) was added and incubated at room temperature for 30 minutes. The samples were put on ice for 10 minutes and then 200 µl of ice-cold NaOH (0.7 mM) and 200 µl of zinc sulfate (15%) were added to each sample. The samples were centrifuged at 13,000 ×g for 10 minutes and then supernatants were removed and centrifuged again. Final supernatant (600 µl) of each sample was mixed with 300 µl color reagent. Color reagent was prepared by adding 46 µl of acetylacetone to 5 ml of ammonium acetate (6.6 mM). The samples were incubated at 37°C for 1 hour and then absorbance of each sample was measured at 450 nm.

We used fructose solutions (0-0.9 mM) to prepare a calibration curve for the periodate assay. Both fructose and amadori products (in the initial stages of glycation a labile Schiff base is formed, which rearranges to form a stable sugar adduct known as an amadori products) exist predominantly in a ring structure, and after periodate oxidation, both produce 1 mol of formaldehyde per mole sugar moiety [12]. The number of moles of amadori products per mole of protein can be calculated from the number of moles of formaldehyde released, because 1 mol of formaldehyde is released per mole of amadori products. Thus, in this study, degree of glycation of LDL expressed as mmol of formaldehyde released per mg LDL protein.

Effect of flavonols on LDL glycation. The effects of five flavonols: quercetin, myricetin, kaempferol, rutin and morin on glycation of LDL were examined by incubation of LDL (0.2 mg protein/ml) with glucose (120 mM) and varying concentrations (0-200 mM) in PBS, pH 7.4 at 37°C for 7 days. The flavonols were dissolved in 10% DMSO in PBS, pH 7.4. Degree of LDL glycation was determined by

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sodium periodate assay [12]. The electrophoretic mobility of native LDL and glycated LDL in absence and/or presence of 200 mM flavonols was compared by 5% polyacrylamide gel. The gels were stained with Coomassie blue stain [13].

Statistical analysis. The results are expressed as mean values ± SD. Glycated modification of LDL in absence (as control) or presence of flavonols was compared using ANOVA test. Differences were considered significant at $P \leq 0.05$.

Table 1. The comparison of lipid concentrations in plasma pool and LDL fraction.

Lipid	Plasma pool (mg/dl)	LDL fraction (mg/dl)
Total Cholesterol	167 ± 2.0	480 ± 2.5
Triglyceride	127 ± 1.5	60 ± 0.5
LDL-Cholesterol	104 ± 1.3	430 ± 1.8
HDL-Cholesterol	38 ± 0.9	22 ± 0.4

Values are mean ± SD of triplicated determinations on plasma and separated LDL. LDL, low density lipoprotein; HDL, high density lipoprotein.

RESULTS

Isolation of LDL was assessment by measurement of lipid concentration (Table 1). As shown in the Table, cholesterol and LDL-cholesterol amounts were increased in LDL fraction approximately by 65% and 76%, respectively. This measurement can confirm the isolation of LDL from plasma. This isolation was also confirmed by agarose gel electrophoresis (Fig. 1). This Figure shows the separated fractions (Fig.1, Lane2 and Lane3) according to LDL and very low density lipoprotein (VLDL) bands in plasma, as control (Fig.1, Lane1).

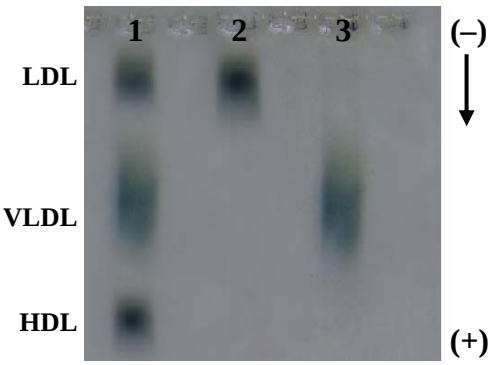


Fig. 1. Electrophoresis analysis of plasma (Lane 1)**,** LDL fraction (Lane 2) and VLDL fraction (Lane 3) on 0.8% agarose gel. LDL, low density lipoprotein; VLDL, very low density lipoprotein; HDL, high density lipoprotein.

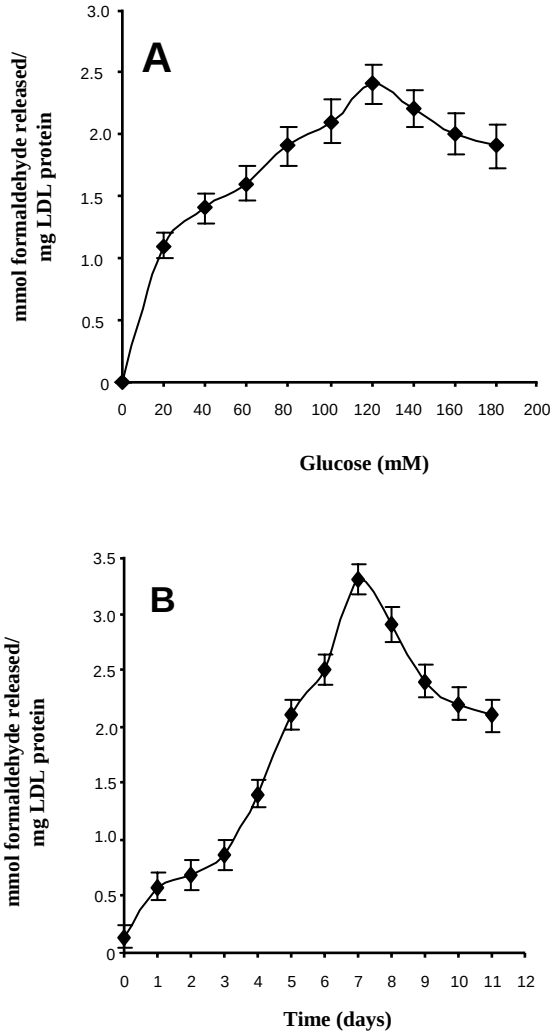


Fig. 2. The effect of glucose concentration **(A)** and incubation time **(B)** on glycation of LDL. Values represent the means ± SD of triplicate determinations.

The best concentration of glucose for glucose incorporation with LDL was investigated by incubation of varying concentrations of glucose (0-180 mM) with LDL (0.2 mg protein/ml) in PBS containing 1mM EDTA, pH 7.4 at 37°C for 7 days under sterile conditions (Fig. 2A). The best time of incubation for glucose incorporation with LDL was also investigated by incubating 0.2 mg protein/ml of LDL with 120 mM glucose for 1-11 days at 37°C in PBS and 1 mM EDTA, pH 7.4, under sterile condition (Fig.2B). As shown in Figure 2, optimum glucose concentration and incubation time were obtained for LDL glycation 120 mM and 7 days, respectively.

A series of experiments was performed to examine the influence of flavonols on LDL glycation process.

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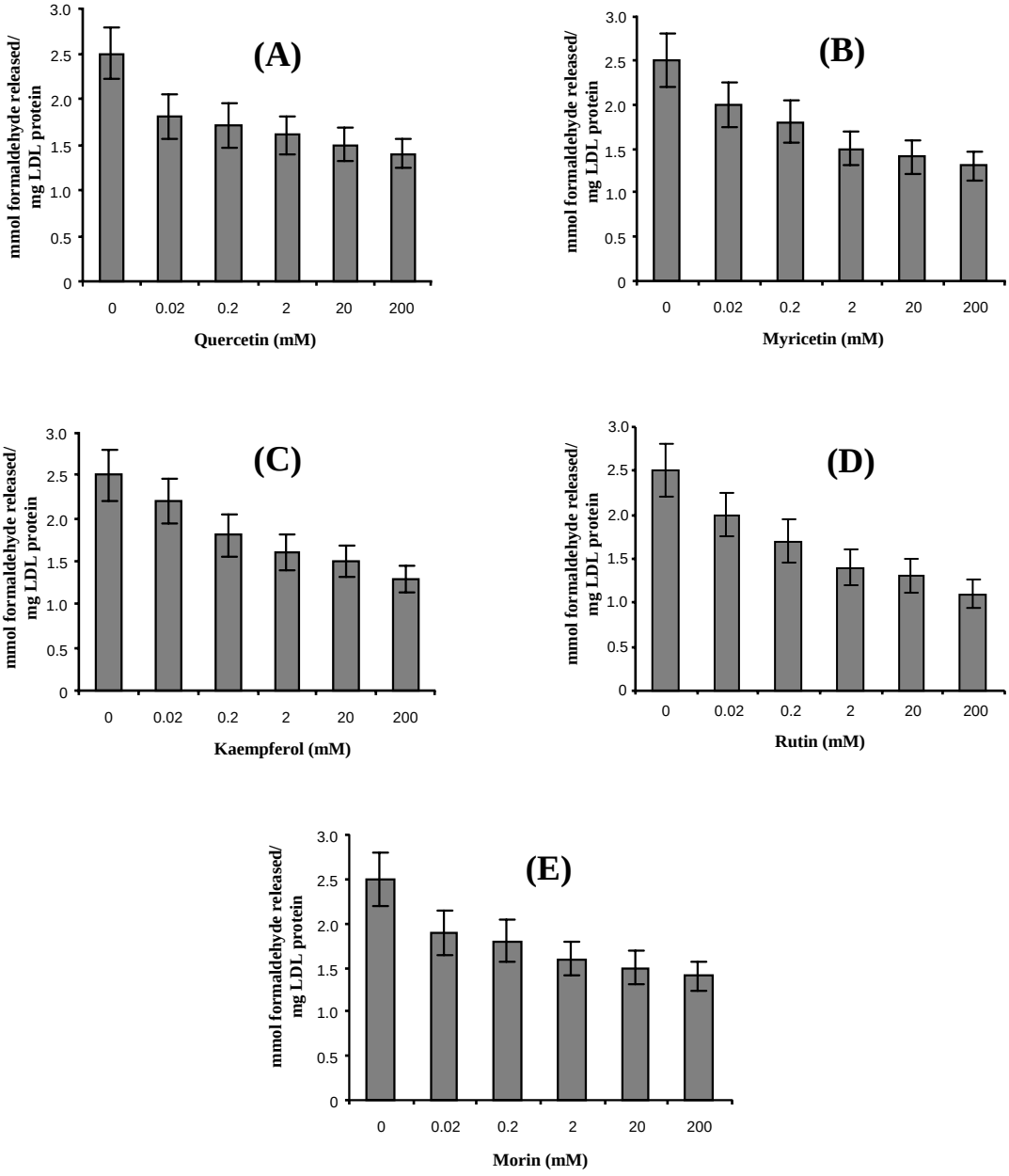


Fig. 3. The effect of 0.02-200 mM concentrations of quercetin **(A)**, myricetin **(B)**, kaempferol **(C)**, rutin **(D)** and morin **(E)** on glycation of LDL (0.2 mg protein/ml) by glucose (120mM). Values represent the means ± SD of triplicate determinations.

Quercetin, one of the flavonols tested, was incubated at concentration of 0 to 200 mM with LDL (0.2 mg protein/ml) and glucose (120 mM) at 37°C for 7 days. The extent of LDL glycation at absence (as control) and/or presence of quercetin were estimated from formaldehyde released as shown in Figure 3A. Glycation of LDL decreased in presence of 0.02 mM, 0.2 mM, 2 mM, 20 mM and 200 mM of quercetin concentrations. As shown in Figure 3A, this reduction was dependent on quercetin concentration. Same procedure was repeated to investigate the effect of myricetin (Fig. 3B), kaempferol (Fig. 3C), rutin (Fig. 3D) and morin (Fig. 3E) on LDL glycation. The results shown that these flavonols, decrease LDL glycation in a dose-dependent manner (Fig. 3B-3E). In this study, all

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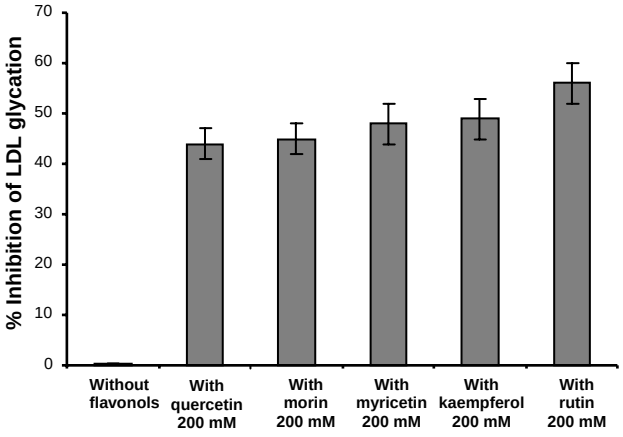


Fig. 4. The comparison of inhibition percent of LDL glycation in absence and presence of 200mM concentration of flavonols. This Figure was obtained by Figure 3 data.

flavonols in comparison to the control (without flavonols) were shown a significant inhibition of LDL glycation by the ANOVA test, $P<0.05$. According to this study 200 mM concentration of quercetin, morin, myricetin, kaempferol and rutin are able to reduce LDL glycation approximately by 44%, 45%, 48%, 49% and 56%, respectively (Fig. 4). We also investigated electrophoretic mobility of 200 mM flavonols treated LDL on polyacrylamide gel (Fig. 5A). Figure 5A shows that glycation increased anodic migration of LDL when compared to native LDL and flavonols (200 mM) decreased electrophoretic mobility LDL glycation. The comparison of sample rate of flow on gel electrophoresis was also showed in presence of flavonols decreased rate of flow of glycated LDL (Fig. 5B). These observations suggest that quercetin, myricetin, kaempferol, rutin and morin can decrease LDL glycation in presence of glucose.

DISCUSSION

In this study, the phenomenon of LDL glycation was demonstrated in the reaction mixtures of LDL with sugar by model system *in vitro*. Glucose was used as glycated agent, which is commonly adopted in many Millard reaction studies [14]. Apo-B_{100} of LDL, the amine source could serve as target for glycated agents. Considering that glucose adducts mainly with the ε-amino group of lysine residues, of which about 90% are contained in apo-B_{100} [4, 15]. In present study, experiments were performed with various concentrations of glucose (0-180 mM) on LDL glycation. The glycation of LDL was increased in the presence of 20-120 mM of glucose concentrations, however, in high glucose concentrations (140-180 mM) glycation was decreased this phenomenon. Our results showed that glucose with concentration of 120 mM has most effect on glycation of 0.2 mg protein per ml of LDL concentration. In the protein glycation, glucose reacts with an amine group to form a labile Schiff base that rearranges to amadori product. The Schiff base is prone to oxidation and free radical generation, which leads to the formation of reactive carbonyl species such as glyoxal [16]. Given the link mentioned above between glycation and oxidation, we hypothesized that antioxidant flavonols might

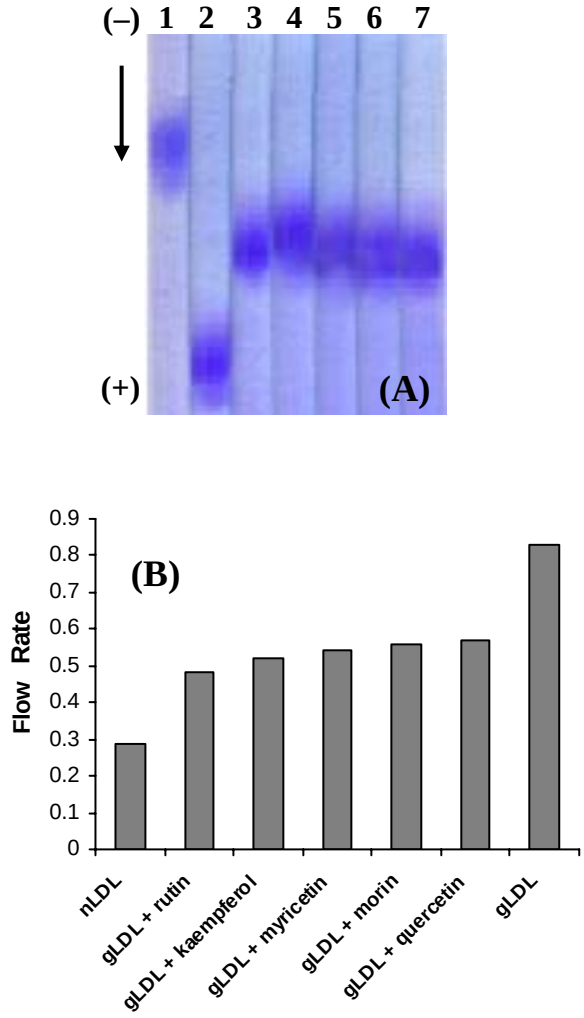


Fig. 5. (A) Electrophoresis analysis of native LDL (Lane 1) and glycated LDL in absence (Lane 2) and presence of 200 mM concentration of rutin (Lane 3), kaempferol (Lane 4), myricetin (Lane 5), morin (Lane 6) and quercetin (Lane 7) on 5% polyacrylamide gel; **(B)** The comparison of rate of flow (RF) of native LDL (nLDL) with glycated LDL (gLDL) in absence and presence of flavonols (200 mM) on 5% polyacrylamide gel.

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possess antiglycoxidative activities. Flavonols was chosen in this study as it rather universally found in plant foods [6]. Five flavonols were investigated including quercetin, myricetin, kaempferol, rutin and morin. They belong to class of flavonoids are distinguished by their structural feature. Our results revealed that five flavonols with concentrations of 0.02-200 mM reduced significantly (n = 3, ANOVA test, $P<0.05$) susceptibility of LDL to glycation. A marked reduction of glycation was observed in rutin (200 mM) treatment. Strong evidence supported the view that glycated albumin is a potential target of therapy in the treatment of diabetic complications [17]. For human serum albumin in blood plasma, the concentration ranges of Schiff's base adduct and AGE products were 1-5% and 6-15%, respectively [16]. This proportion typically increased between 2-3 fold in hyperglycemia [18]. Having available clinical and experimental information, it could thus be of great interest to propose that administrations of naturally occurring flavonoids are beneficial for the prevention of protein glycation [19]. Attention has been focused on preventing protein glycation by antioxidants [20]. The inhibition of free radicals generation derived from glycation process and subsequent inhibition of the protein modification was considered to be one of the mechanisms of antiglycation effect. Many data have shown that typical antioxidants/nutrients such as vitamin B_1, B_6, C, niacinamide, carnosine and sodium selenite inhibited the *in vivo* and *in vitro* AGE formation [21]. The present study also showed that flavonols suppressed LDL glycation approximately in the following order (56%)> kaempferol (49%)≥ myricetin (48%)> morin (45%)≥ quercetin (44%). It was found that when LDL was subjected to sugar-mediated modification, the addition of flavonols decreases mmol level of formaldehyde released per mg of LDL protein that this effect was concentration dependent. Rutin, with rhamnose-glucose group at C-3 position in the C ring and with hydroxyl groups at the C-5, 7 and 3', 4' positions in the B ring [22] was the most effective compound of LDL glycation in our study. Other four flavonols lack the C-3 glycosyl group, thus it may indicate that glucoside group at C-3 position in C ring would be the necessary functional group for inhibition. Others also suggested that presence of the hydroxyl groups in the B and C rings of flavonols affected their inhibitory activity against glycoxidative reaction [22]. These results were in agreement with other study that has suggested the possible contribution of the single hydroxyl group at position 5' of the B ring to the inhibition of glycation [23].

Our study also showed that glycated LDL had increased in mobility on polyacrylamide gel, which probably resulted from the increased negative charge caused by the modification of lysine amino groups. The increased electrophoretic mobility is in agreement with the finding of Witztum *et al.* [24]. The presence of flavonols decreased rate of flow of glycated LDL approximately followed the order of rutin (42%)> kaempferol (37%)> myricetin (35%)> morin (32%)≥ quercetin (32%). Thus, these results support the suggestion that rutin, kaempferol, myricetin, morin and quercetin play an important role in prevention of LDL glycation by glucose. The mechanism by which flavonols suppress glycation of LDL is still not known. Yim *et al.* [25] indicated that glycation of proteins generated some active centers for catalyzing one-electron oxidation-reduction reactions, which mimics the characteristics of the metal catalyzed oxidation system [25]. In addition, glycated proteins accumulated *in vivo* may provide stable active sites for catalyzing the formation of free radicals. Results from Jiang *et al.* [26] also demonstrated that reactive oxygen species (ROS) such as hydrogen peroxide and superoxide anion were generated during glycation process. Thus, we could suggest that antiglycation activity of flavonols was possibly correlated with their abilities to scavenge radicals.

In conclusion, the results obtained in the present study show that flavonols, especially of rutin, have inhibitory effects on LDL glycation. The scavenging of free radicals derived from glycation may play an important role in this phenomenon. This mechanism may help to provide a protective effect against hyperglycemia mediated LDL damage.

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