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Advanced Glycation End Product Inhibitory Effect of Quercetin-3-O-Rutinoside, Isolated from *Teucrium polium*

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ABSTRACT

Background: Several mechanism including autoxidation of glucose, protein glycation and advanced glycation end products (AGEs) formation may to be involved in hyperglycemia mediated oxidative stress. Herein, in this study, the effects of rutin with high antioxidant activity isolated from Teucrium polium on structural changes of human serum albumin (HSA) were performed. ***Methods:*** The major compounds of T. polium methanol extract were isolated and their radical scavenging properties were established through various approaches including 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl radicals scavenging, and reducing power. The structural changes of HSA with glucose, in the presence of rutin were studied with far-UV CD spectroscopy. ***Results****:* Based on our results, rutin, a flavonol glycoside isolated from T. polium exhibited high antioxidant activity compared to the other isolated compounds from T. polium. Rutin inhibited high glucose induced oxidative damages to protein by decreasing protein carbonyl formation (PCO) and preventing thiols group from oxidation. In addition, evaluating the structural changes of HSA by glucose in the presence of rutin showed that rutin may be able to increase the helicity of the protein and prevent helix decrement in the secondary structure of HSA. ***Conclusion:*** We found that rutin with high antioxidant activity effectively inhibited the generation of amadori products and exhibited good inhibitory effects on the formations of α-dicarbonyl compounds and AGEs.

Introduction

Reactive oxygen species (ROS) have been shown to play a critical role in many diseases such as diabetes mellitus, cancer, Alzheimer, arteriosclerosis and other conditions.[1] Chronic glucose levels in blood or cells of diabetic patients are known to cause oxidative damages, followed by an increasing in ROS production and/or dysfunction of antioxidant defense system (enzyme or non-enzymatic) in biological systems.[2] Several mechanism including autoxidation of glucose, protein glycation and advanced glycation end products (AGEs) formation may to be involved in hyperglycemia mediated oxidative stress.[2] Non enzymatic protein glycation initiated by the reversible formation of a Schiff base which leads to formation of heterogeneous and toxic advanced glycation end products (AGEs).[3] The accumulation of the reaction products of protein glycation in living organisms leads to structural and functional modifications of tissue proteins.[4] Regarding the significance of oxidative stress to diabetic pathology, numerous natural and synthetic ROS scavengers and antioxidants have been evaluated to inhibit the process of protein modifications against the attack of ROS and/or to suppress the resultant damages.[5] In spite of the sufficient effects of aminoguanidine (AG) on diabetic complications, its consumption has been associated with some deleterious side effects such as drug resistance and hepatotoxicity.[6] Thus, consumption of complementary components (mainly from plant sources), with an antioxidant effect and low side effects, make them good candidates for the development of new functional drugs with potential protective and/or prevention properties against oxidative stress-related diseases such as diabetes and its complications.[7]

In an attempt to find new antioxidants agents from plant materials as therapeutic agents, we are interested in the development of antioxidants that have potent anti-glycation activity. *Teucrium polium* was used as a hypoglycemic agent in folk medicine in Iran. The biological activities of *T. polium* including anti-inflammatory, anti-nociceptive, anti-bacterial, anti-hypertensive, hypolipidemic, anti-rheumatoid, and hypoglycemic widely has been reportd. [8-10] Recently, Esmaeili and Yazdanparast,[11] showed the high insulinotropic and anti-hyperglycemic activity of *T. polium* crude extract using both animal and/or isolated rat pancreatic islets. There are also some data in literature about antioxidant activity of various crude extract of *T. polium*. [12, 13] Based on these data, the present study was hence, undertaken to isolate and

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structural elucidate the high antioxidant flavonoid compound(s) from *T. polium* on glycation and oxidation dependent damages to albumin induced by glucose.

Material and Methods

Chemicals

Butylated hydroxytoluene (BHT), Nitroblue tetrazolium (NBT), 5, 5′-dithiobisnitro benzoic acid (DTNB) and human serum albumin (HSA) were obtained from Sigma (St. Louis, MO, USA). Thioflavin T (ThT), 2, 4, 6-trinitrobenzene sulfonic acid (TNBSA) and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Fluka (Buchs, Switzerland). All other reagents were used in this study were of analytical grade.

Plant material and extraction

Arial parts of *Teucrium polium* L. were collected from Fars province, Iran in spring. A voucher herbarium specimen (No. 570) was deposited at the Herbarium of faculty of pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran. The aerial parts were air-dried and then powdered. The powder (250 g) was passed through a sieve in order to maintain particle size unity (300 ml) and was extracted by the method described by Sharififar and colleagues[13] via percolation with petroleum ether, chloroform, methanol and water, successively. The plant extract was concentrated under reduced pressure on a rotary evaporator and the methanol extract (32 g) was fractionated by column chromatography on silica gel 60 (70–230 mesh, Merck; Darmstadt, Germany), using petroleum ether and petroleum ether: chloroform (9:1, 8:2, 5:5, v/v), chloroform, and chloroform: ethyl-acetate (9:1, 8:2, 5:5, v/v), ethyl-acetate and ethyl-acetate: methanol (9:1, 8:2, 5:5, v/v) as eluents to afford nine fractions (TP1–TP9)[13]. All the fractions obtained, were tested for their antioxidant activity. Fraction TP-4 (2.4 g) which showed the most antioxidant activity was purified by TLC on silica gel 230–400 mesh and eluted with ethyl-acetate: water: formic acid: glacial acetic acid (100:26:11:11, v/v/v/v) to obtained four fractions (1–3, 4–8, 9–13, and 14–18). The combined fractions of 9-13 (0.85 g) and 14-18 (1.04 g) (the most antioxidant activity) were purified on Sephadex LH-20 with methanol to obtained four compounds. By comparing the obtained spectral data (UV, IR, ^{1}H, and ^{13}C NMR) with literature values[13], purified compounds were identified as rutin (1) (0.096 g), apigenin (2) (0.074 g), 3', 6 dimethoxy apigenin (3) (0.039 g) and 4',7 dimethoxy apigenin (4) (0.036). In further investigation rutin with powerful antioxidant activity was used for in *in vitro* glycation studies.

Rutin (Quercetin-3-O-rutinoside) (Figure 1) (1): yellow-green amorphous powder. m.p. 208-212 ^{0}C; UV–vis (nm): λ_{max} = 257, 351 nm. IR (KBr, cm^{-1}): 3600-3200 (OH), 2920-2700 (CH), 1648 (C=O), 1608-1550 (aromatic rings), 1435, 1321, 1294, 1265. ^{1}H NMR (300.1 MHZ, CD_3OD): 6.21(1H, d, J=2, C6-H), 6.40 (1H, d, J=2, C8-H),7.55 (1H, d, J=2.1,C2'-H),6.86 (1H, d J=9,C5'-H),7.56 (1H, dd, J=9,2.1, C6'-H),9.71 (1H, s, C4'-OH), 9.21 (1H, s, C3'-OH), 12.62 (1H, s, C5-OH), 10.86 (1H, s, C7-OH), 5.35 (1H, d, J=7.4, H1-G),5.12 (1H, d, J=1.9, H1-R), 1.00 (3H, d, J=6.1,CH3-R); ^{13}C NMR (75.47 MHZ, CD_3OD) 157.3 (C-2),134.1 (C-3), 178.2 (C-4), 157.5 (C-5), 99.5 (C-6), 164.9 (C-7), 94.5 (C-8), 162.1 (C-9), 104.8 (C-10), 122.5 (C-1'), 116.1(C-2'), 145.6 (C-3'), 149.3 (C-4'), 117.1 (C-5'), 122.0 (C-6'),101.6 (C1-G), 74.9 (C2-G), 77.3 (C3-G), 72.7 (C4-G), 76.7 (C5-G), 67.9 (C6-G), 102.2 (C1-R), 70.8 (C2-R), 71.2 (C3-R), 71.4 (C4-R), 69.1(C5-rhamnose), 18.6 (C6-R).

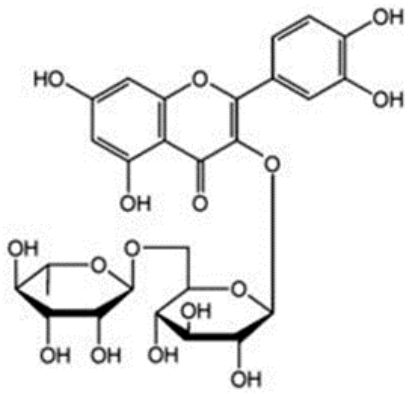

Figure 1. Chemical structure of quercetin-3-O-rutinoside (rutin)

DPPH free radicals scavenging activity assay

Radical scavenging capacity was determined according to the technique reported by Blois.[14] An aliquot of 1.5 ml of 0.25 mM DPPH solution in ethanol and 1.5 ml of rutin at various concentrations were mixed. The mixture was shaken vigorously and allowed to reach a steady state at room temperature for 30 min. Decolorization of DPPH was determined by measuring the absorbance at 517 nm with a Varian spectrophotometer. The DPPH radicals scavenging activity was calculated according to the following equation:

Scavenging activity = $[(A_0-A_1/A_0)\times 100]$

Where A_0 is the absorbance of the control (blank, without rutin) and A_1 is the absorbance in the presence of the rutin or standard sample.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of samples was measured by two different methods named site-specific assay and nonsite-specific assay.[15] For the nonsite-specific hydroxyl radical system, the reaction mixture, contained 2.8 mM deoxyribose, rutin and/or BHT as positive control, 0.1 mM ascorbic acid, 0.1 mM $FeCl_3$, 1 mM H_2O_2 and 0.1 mM EDTA in KH_2PO_4–KOH buffer (20 mM pH 7.4), was incubated at 37 °C for 1 h. For the site-specific hydroxyl radical system, EDTA was replaced by phosphate buffer. The extent of deoxyribose degradation was measured by

thiobarbituric acid (TBA) method. TBA (1 ml, 1% w/v) and trichloroacetic acid (TCA) (1 ml, 2% w/v) were added to the mixture and heated at 100 °C for 20 min. the absorbance of the colored developed was measured at 532 nm using a spectrophotometer. The hydroxyl radical scavenging ratio was calculated by following formula:

Inhibition (%)= [1-(A_0-A_t)], Where A_0 is the absorbance of the control (blank, with out sample) and A_t is the absorbance in the presence of the rutin or standard sample.

Reducing power assay

The reducing power of the purified compound (rutin) was determined according to the method of Oyaizu.[16] Briefly, 1 ml of rutin and BHT at concentration of 50 µg/ml was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of a potassium ferricyanide solution (30 mmol/l). After incubation of mixture at 50 °C for 20 min, 2.5 ml of a TCA solution (0.6 mol/l) was added to the mixture and then centrifuged at 3000×*g* for 10 min. the upper layer of solution was mixed with 2.5 ml of distilled water and 0.5 ml of a ferric chloride solution (6 mmol/l), and the absorbance was measured at 700 nm using a spectrophotometer.

Evaluation of various stages of protein glycation

According to a slightly modified method of Rahbar, [17] early stage of protein glycation was achieved. 200 µl of fresh human blood with 40 µl of δ-gluconolactone (50 mM) were incubated in the presence of 10 µl of rutin in a final concentration 50 µg/ml at 37 ^{0}C for 16 h and the percentage of glycated hemoglobin was determined using commercially available methods. PBS buffer was used as appropriate blank and AG as a final concentration 10 mM was used as positive control. The % inhibition of HbA_{1c} formation was calculated using the following formula:

[(HbA_{1c} of δ-gluconolactone control - HbA_{1c} of test sample)/ (HbA_{1c} of δ-gluconolactone control - HbA_{1c} of blank)] ×100.

For investigation the effect of inhibitors on middle stage of protein glycation, the human serum albumin-methylglyoxal (HSA-MGO) assay was performed based on Lee method with minor modification.[18] After incubation HSA (50 mg/ml) with of 100 mM MGO in 0.1 M phosphate buffer (pH 7.4) under sterile conditions at 37 ^{0}C for 9 days in the presence of rutin as test and AG as positive control, fluorescence intensity of each samples were measured at the excitation and emission maxima of 330 and 410 nm, respectively using Varian-spectrofluorometer, model Cary Eclipse. N-acetyl-glycyl-lysine methyl ester (G.K.) peptide-Ribose assay determines the last glycation product. According to Nagaraj method, [19] 80 mg/ml G.K. peptide was incubated with 0.8 M ribose in 0.5 M sodium phosphate buffer (pH 7.4) in the presence of purified compound at 37 ^{0}C for 24 h. the fluorescence intensity of samples was recorded at the excitation and emission maxima of 340 and 420 nm, respectively. In another approach, HSA (50 mg/ml) was incubated with glucose (50 mM) and sodium azide (0.02%) with or without $CuSO_4$ (100 µM) in 0.2 M phosphate buffer (pH 7.4). [20] The rutin was added to the reaction mixture, and the reaction mixture was incubated for 21 d at 37 °C. AG (10 mM) was used as a positive control. Fluorescence intensity of all samples was measured at the excitation and emission maxima of 330 and 410 nm, respectively.

Evaluation the protein carbonyl and thiol groups level

For determination of protein carbonyl content, 1 ml of DNPH (10 mM) was added to samples and after incubation for 30 min at room temperature, 1 ml of cold TCA (10 %, w/v) was added to the samples and centrifuged at 3000 ×g for 10 min. The protein pellet was washed three times with 2 ml of ethanol/ethyl acetate (1:1, v/v) and dissolved in 1 ml of guanidine hydrochloride (6 M, pH 2.3). The absorbance of the sample was read at 370 nm. The data were expressed as nmol/mg protein. Thiol groups of native and glycated protein under effect of rutin were measured according to the Ellman's assay.[21]

Structural experiments

The secondary structural changes of glycated samples were evaluated by far-UV CD spectroscopy. Measurements were recorded over wavelength range of 200–250 nm using an AVIV spectropolarimeter, model 215 (USA) with a 0.1 cm path length sample cell. All CD measurements were carried out at 25 °C with the help of a thermostatically controlled cell holder. Spectra were collected at 1nm intervals, with a scan speed of 20 nm/min. Each spectrum was the average of two scans and the noise in the data was smoothed using the AVIV 215 software. For evaluation the tertiary structural changes of glycated samples, fluorescence experiments were performed using a Varian spectrofluorometer, model Cary Eclipse, at 25 °C, with a 10 mM path length sample cuvette. For recording the intrinsic protein fluorescence spectra of native and glycated samples, the excitation and emission wavelengths were set at 280 and 290–400 nm range, respectively. The fluorescence spectra were corrected with respect to the respective blanks, under all identical experimental conditions and with the excitation and emission slit widths of 5 nm. ANS fluorescence studies were performed to evaluate the accessible surface located hydrophobic pockets of the assigned protein samples using a Varian spectrofluorometer at the excitation and emission wavelengths of 350 nm and 450-600 nm range, respectively. The determination of the level of free lysine residues was carried out based on Sharma method.[22] The fibrillar state of the incubated HSA was determined via thioflavin T (Th T), a reagent used for detecting the β-sheet configuration in proteins.[23]

Statistical analysis

All results shown represent means ± SD from triplicate experiments performed in a parallel manner unless otherwise indicated. Statistical analyses were performed using an unpaired, two-tailed Student's t-test. All comparisons are made relative to untreated controls and significance of difference in means measured at $p< 0.05$.

Results and Discussion

Antioxidant activity of rutin using different in vitro anti-oxidant assay system DPPH scavenging

Based on many reports on anti-oxidant and free radical scavenging activity of *T. polium* in this study we aim to isolate and evaluate the high anti-glycation compound(s) from methanol extract of *T. polium*. Fractionation of the methanol extract yielded four major flavonoids. The isolated compounds were screened for their antioxidant and free radical scavenging activities using DPPH radical-scavenging method. A rapid, simple and inexpensive method to measure antioxidant capacity of food involves the use of the free radical, 2, 2-Diphenyl-1-picrylhydrazyl (DPPH). DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity of foods.[14] The IC_{50} values (defined as the concentration of test compound required to produce 50% inhibition) for DPPH scavenging by various isolated compound(s) from *T. polium* was shown in Table 1. According to our data, the highest scavenging activity was found for isolated compound 1(rutin) of (22.6 ± 1.05 µg /ml), followed by isolated compound 2 (apigenin) (26.14 ± 1.52 µg /ml) and compound 3 (3', 6 dimethoxy apigenin) (33.20 ± 2.01 µg /ml). Compound 4 (4',7 dimethoxy apigenin) exhibited the weakest antioxidant activity in this test system of which IC_{50} value is ~38.11 ± 2.90 µg /ml. Our data clearly established that the rutin isolated from *T. polium* possesses potent antioxidant properties. Rutin a natural derivative, was first isolated in buckwheat. It has been widely used in treating disease, and has several pharmacological activities including antiallergic, anti-inflammatory, antitumor, antibacterial and antiprotozoal.[24] The inhibitory effect of rutin at various concentrations versus BHT as standard radical scavenger on DPPH radical was also shown in Figure 2. As shown in Figure 2, rutin at the concentration of 50 µg /ml exhibited a high scavenging efficiency toward DPPH radicals, while BHT at the same concentration showed weak to moderate activity compared to rutin (80% and 60%, respectively). In general, antioxidant activity of flavonoids depends on the structure and substitution pattern of hydroxyl groups. The essential requirement for effective radical scavenging is the 3′, 4′-orthodihydroxy configuration in ring B and 4-carbonyl group in ring C. The presence of 3-OH group or 3- and 5-OH groups, giving a catechol-like structure in ring C, is also beneficial for the antioxidant activity of flavonoids and make them 10-fold more potent than the corresponding catechol and 3-OH free flavonoids. The presence of the C2–C3 double bond configured with a 4-keto arrangement is known to be responsible for electron delocalization from ring B and it increases the radical-scavenging activity. Rutin has a catechol structure in ring B, as well as a 2, 3-double bond in conjunction with a 4-carbonyl group in ring C, allowing for delocalization of the phenoxyl radical electron to the flavonoid nucleus. Flavonols have a hydroxyl group at position 3, which suggests a structurally important role of the 3-OH group of the chroman ring responsible for enhancement of antioxidant activity. The unsaturation in the C ring of flavonoids allows electron delocalisation across the molecule for stabilisation of the aryloxyl radical [24]. Theses results showed that isolated compound 1 (rutin) has a noticeable effect on scavenging free radicals in a dose-dependent manner, thus, this isolated compound from *T.polium* was used in further studies.

Table 1. Antioxidant activity of the various compounds isolated from *T. polium* using DPPH radical scavenging activity approach.

Sample	DPPH (IC_{50})
Methanolic extract	21.54 ± 1.62
Compound 1 (rutin)	17.8 ± 1.05
Compound 2 (apigenin)	26.14 ± 1.52
Compound 3 (3', 6 dimethoxy apigenin)	33.20 ± 2.01
Compound 4(4',7 dimethoxy apigenin)	38.11 ± 2.90
BHT	18.42± 1.21

IC_{50} values were expressed as µg/ml. Each value represents the mean ± SD of triplicate measurements

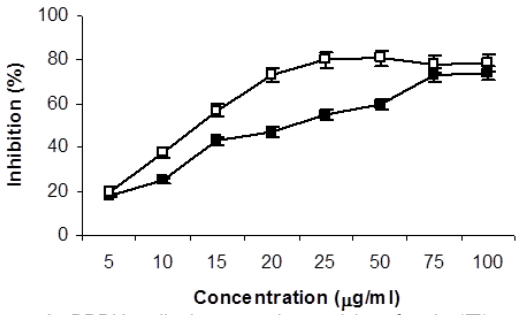

Figure 2. DPPH radical scavenging activity of rutin (□), and BHT (■) at various concentrations. For experimental details refer to Section 2. Each value represents the mean ± SD (n=3)

Hydroxyl radical scavenging activity

The hydroxyl radical passes easily through membranes at specific sites react with most biomolecules and furthermore cause tissue damage and cell death.[25] Thus, removing hydroxyl radical is a major factor for protection of living systems. Fig 3a and 3b showed the ability of rutin to inhibit hydroxyl radical-mediated

deoxyribose degradation in a reaction mixture with (nonsite-specific assay) or without EDTA (site-specific assay). The relative extents of inhibition of deoxyribose degradation will give an indication of hydroxyl radical-scavenger and/or iron-chelating activities. When hydroxyl radical, generated by the Fenton reaction, attacks to deoxyribose, it degrades into fragments that react with TBA on heating at low pH to form a pink color. Rutin neutralized nonsite-specific (in the presence of EDTA), hydroxyl-radical induced deoxyribose cleavage in a concentration-dependent manner (Figure 3a), with EC_{50} value of 25 µg/ml. Moreover, rutin exhibited a site-specific (in the absence of EDTA), hydroxyl radical scavenging activity in a dose-dependent manner (Figure 3b) with EC_{50} value of 38 µg/ml, though such activity was relatively weaker than that of nonsite-specific hydroxyl radical at the same concentrations. In reactions containing EDTA, hydroxyl radicals are generated by the Fe–EDTA complex and are subsequently released to free solution. Since EDTA is in stoichiometric excess of ferric ions, there will be very little interaction between ferric ions and deoxyribose. In this case, inhibition of deoxyribose degradation reflects the ability of a substance to directly compete with the reaction between deoxyribose and hydroxyl radical. Clearly, the rutin possesses hydroxyl radical-scavenger activity. When EDTA is omitted from the reaction, iron ions are free to bind to the deoxyribose molecule and hydroxyl radical generation becomes site-specific. Under these conditions, any inhibition of deoxyribose degradation upon addition of the rutin is likely to be due to iron ion-chelating effects that compete with the binding of the deoxyribose molecule. Under both conditions, rutin inhibited the generation of hydroxyl radical in a concentration-dependent manner.

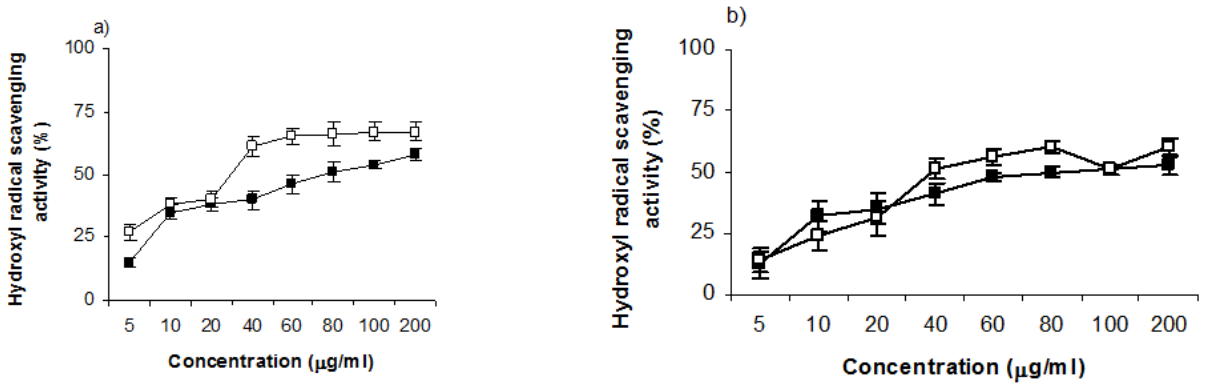

Figure 3. Hydroxyl radical scavenging (non site specific assay) (a) and (site-specific assay) (b) of rutin (□), and BHT (■) at various concentration. For experimental details refer to Section 2. Each value represents the mean ± SD (n=3)

Reducing power

The reducing capacity of compounds may be a significant marker of their antioxidant ability. Some evidence reveals that there is a direct correlation between antioxidant ability and reducing power of compounds from natural sources (especially plants).[26] To measure the reductive ability, we investigated the Fe^{3+}/Fe^{2+} transformation in the presence of rutin using the method of Oyaizu [16]. As shown in Figure 4 the reducing potential of rutin increased in a dose-dependent manner, with a high correlation index ($r^2 = 0.9954$). At all used concentrations, reductive capacity of rutin was similar to BHT. Regarding these results, it might be concluded that rutin as an electron donor capable to neutralize the free radicals. This would have the effect of converting free radicals to more stable products and thus terminating free radical initiated chain reactions. The results of this study clearly indicated that rutin had powerful antioxidant capacity against various antioxidant systems in vitro and the capacity was concentration dependent. Since glycation and oxidation induced biochemical changes are known to participate in the overall diabetic complications, the studies were also extended to determine the possibility of preventing the glucose-induced protein glycation by rutin isolated from *T. polium.* The present study was hence, undertaken to evaluate the inhibitory effect of rutin on glycation dependent damages to human serum albumin induced by glucose.

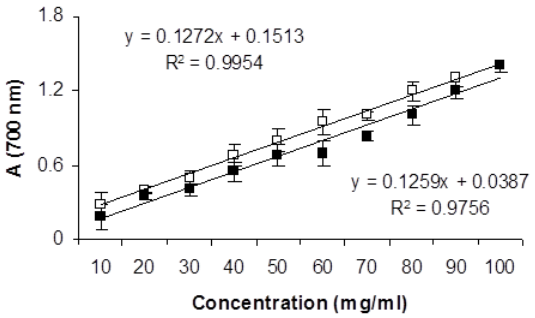

Figure 4. Reducing power activity of rutin (□) compared with BHT (■). For more details refer to Section 2.

The protein glycation products formation in various glycation stages

The AGEs formation process in a protein–glucose reaction system is subdivided simply into three main stages. In the early stage, reducing sugars such as glucose and ribose react with free amino groups of protein to form Schiff bases and the formed Schiff bases undergo rearrangement to form early, reversible stable ketoamine named Amadori products.[27] Regarding the link between glycation and protein oxidation, antioxidant flavonoids might possess anti-glycation properties.[28] Based on the ability of Amadori products to react NBT, monoformazan dye with a maximum absorption at 530 nm is produced.[29] Some evidences also have shown that reduction of NBT could occur by superoxide anions that formed by glucose autoxidation,[30] and/or by glycated protein.[31] In our experimental condition, during glucose autoxidation in the presence and/or absence of Cu^{+2} no NBT reduction was observed (data not shown). These finding are similar to Wu studies[32] doesn't confirm the acceleration of NBT reduction in the presence of his subject. However, regarding literature, generation Amadori products may be accelerated by the presence of Cu^{+2}.[33] Thus, we evaluated another approach to delaminate the effect of rutin on the early stage of protein glycation (Hemoglobin-δ-gluconolactone assay). As shown in Figure 5a, co incubation of human hemoglobin with δ-Glu for 16 hours in the presence of rutin and AG reduces the glucated hemoglobin content nearly 30 and 24 %, respectively. These results showed that rutin as a potent free radical scavenging agent was not able to inhibit the hemoglobin glycated more effective than AG. In the middle stage of protein glycation, the Amadori products degrade into α-carbonyl compounds such as glyoxal, methylglyoxal and deoxy glucosones. These compounds are more reactive to react with amino groups of proteins to produce AGEs. Methylglyoxal (MGO) readily reacts with protein Lysine and Arginine residues to form high molecular weight cross linked, products.[34] Thus, scavenging the RCS compounds is likely to be a sufficient strategy to inhibit the AGEs formation and prevent AGEs-mediated complications. Therefore, evaluating the effect of agents on scavenging of RCS should be another direction of controlling diabetic complications. To detect the inhibitory effect of flavonoids on MGO-mediated protein glycation, fluorescence intensity was performed. The results represented in Figure 5b indicated that in the presence of rutin significant inhibition about 75 % was achieved. However, AG as an anti-glycation agent (positive control) suppressed the protein glycation by almost 83 %. In the large stage on glycation process, α-dicarbonyl compounds again react with free amino groups and form advanced glycation end products. The accumulation of the reaction products of protein glycation (non-enzymatic reaction of proteins with glucose and other reducing sugars) in living organisms leads to structural and functional modifications of tissue proteins.[17] To evaluate the inhibitory effect of rutin against AGEs formation, the fluorescence studies were measured. As shown in Figure 5b, the rutin at the concentration of 50 µg/ml, exhibited 85 % of inhibitory activity. The ability of AG to inhibit AGE generation was about 90 %. These results indicated that rutin are effective in the prevention of high-glucose mediated protein glycation. In addition, incubation of G.K. peptide with ribose increased the late glycation products formation. In G.K. peptide–ribose system a lysine residue are able to generate peptides with advanced Maillard reaction product with dimerization through lysine-lysine cross linking[19] Thus, we expected that rutin exhibited anti-cross linking activity. The inhibition effect of rutin (63 %) was observed when incubation with G.K. peptide–ribose system for 24 hours (data not shown).

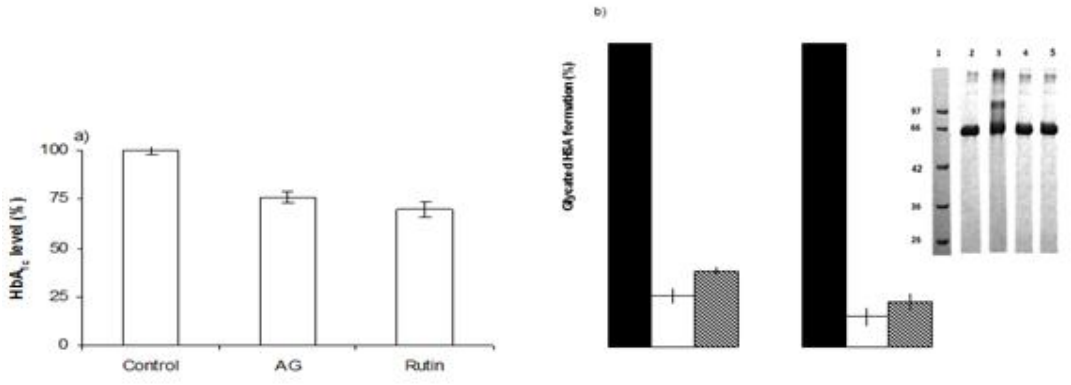

Figure 5. Inhibitory effect of rutin (▒) on early (a), middle and late (b) stages of protein glycation. AG (□) was used as a positive control at final concentration of 10 mM. (■), represents the glycated HSA with out any additive. The *inset* in *b* shows the SDS-PAGE profile of glycated protein under effect of AG and rutin. Lane 1, MW marker; lane 2, native HSA; lane 3, glycated HSA; lane 4, AG and Lane 5, rutin. For more details refer to Section 2. Results are mean SD for n=3. P <0.05 compared with blank

AGEs are a heterogeneous group of high molecular weight aggregates stabilized by non-reducible cross linking components with characteristic fluorescence spectra. In this study, SDS-PAGE was also performed to evaluate the formation of AGEs by incubation of HSA with glucose after three week. In the SDS-PAGE profile shown in Fig. 5b, a very clear band (lane 3) related to glycated HSA with molecular weight ~90 kDa larger than native HSA (lane 2) was observed. Based on our data, cross linking between proteins and binding sugars to protein has been existed during AGEs formation. Rutin suppressed the formation of glycated protein as well as AG. Thus, the polymerized protein bands in lanes 4 and 5 (AG and rutin, respectively) are very weak, which is similar to that of non-glucose-treated protein in lane2. There might be trimer or more polymerized protein present, but we failed to identify such polymerized protein clearly under our experimental condition. Our data obtained from various glycation processes indicated that rutin as high anti-oxidant activity agent isolated from *T. polium* are able to reduce the glycation products formation especially in middle and late stages of protein glycation process. In diabetic patients, the concentration of Schiff's base and AGEs is higher than those in normal human.[35] Thus; it could be of great interest to propose the usage of natural flavonoids for the prevention of protein glycation. For example, Daidzein (an isoflavonoid) has been shown to interfere with AGEs mediated oxidative DNA damage in hypertensive rats.[36] In addition, flavones glycosides isolated from Zea mays, showed the glycation inhibitory activity like to that of AG.[36] In conclusion, it may possible that anti-glycation activity of rutin mainly due to an inhibition of other stages of protein glycation reactions, namely the free radical mediated conversion of Amadori products to AGE. Some researches indicated that ginseng,[37] ethyl-acetate extract of *T. polium*,[13] and Luobuma tea [38] was able to inhibition the AGE formation by their antioxidant properties. Thus, it has been suggested that no oxidation process is involved in the Amadori rearrangement products formation, whereas oxidation play a sufficient role in the fluorescence intensity spectra and the molecular cross-linking, which are characteristic features of AGEs.[19] In addition, the present study demonstrated that exposure of HSA to high-glucose level could enhance protein oxidation levels in terms of protein carbonyl (PCO) formation and loss of protein thiols. Rutin inhibited high glucose-induced oxidative damages to protein by decreasing PCO formation and preserving protein thiols from oxidation (Table 2). This indicates that rutin by decreasing oxidative stress may be effective in preventing oxidative protein damages which are believed to occur under the glycoxidation processes. The determination of free thiol groups in HSA was performed according to Ellman's method [21] (Table 2). The results showed a significant decrease in the number of free thiol groups when HSA is incubate with glucose. The incubation of HSA solution (pH 7.4) at 37 °C for a period of 3 weeks is sufficient to decrease the number of reduced thiol groups in albumin by 61% in the presence of glucose. However, rutin in the presence of glucose significantly inhibited the oxidation of these thiol groups (Table 2). In this study, AG, as a positive standard, moderately inhibited PCO formation and the loss of thiol groups.

Table 2. Effect of rutin isolated from *T. polium* and AG on protein carbonyl (PCO) formation and thiol groups content of glucose-glycated HAS.

Sample	PCO (nmol/mg protein)	Thiol group (pmol/mg protein)
Control [a]	0.40 ± 0.05	11.14 ± 1.01
Control [b]	2.98 ± 0.13	4.45 ± 0.78
AG	1.18 ± 0.07*	7.12 ± 0.46*
Rutin	0.87 ± 0.04*	9.71 ± 0.21*

Each value represents the mean ± SD (n = 3). Significantly different from control contained glucose (*$p < 0.001$).
[a] Reaction mixture with out glucose
[b] Reaction mixture in the presence of glucose

Structural evaluation of the glycated HSA under effect of rutin

There are two major questions regarding the protein conformational changes that may be associated with disease. First, it is important to know the possible effects of the change of conformation so that better structural insights into the pathogenesis of the disease are obtained. For example, the high loss of heme from glycated hemoglobin known to affect diabetes patients may imply that the structural integrity of the assembly of α- and β-chains in hemoglobin is impaired, as heme helps to maintain such integrity.[39] For non-heme proteins, glycation-induced overall changes in conformation have already been reported.[40] For serum

albumin, glycation results in a higher propensity for formation of β-sheet. Based on literatures β-sheet generation is accompanied by loss of alpha helix in a compensatory way.[39] The structural changes of HSA occurring after incubation with glucose were evaluated in the presence of rutin. HSA is a single-chain protein, which is largely α-helical, and consists of three structurally homologous domains, reflected by the presence of negative CD band around 208 and 222 nm. The principle site of glycation of HSA is Lysa-525, but the lysine residues in position 199, 281, and 439 are also susceptible to glycation. As shown in Figure 6a, glycation of the protein results in a reduction the negative ellipticity value at 208 and 222 nm, indicating of a reduction in secondary structural content. The α-helicity of glycated protein decreased dramatically from 61.29 % to 54.75 % after three weeks of incubation, while β-conformation increased from 7.67 % to 9.81 % (Table 3). However, in the presence of rutin the CD data of glycated protein showed more negative ellipticity value relative to glycated protein. In addition, inhibitory effect of rutin on α→β transfer during the glycation of human serum albumin was observed (Table 3). Therefore, rutin may stabilize the native protein structure. HSA has a single tryptophal residue at position 214. In order to obtain more details information concerning the environment of this residue under effect of rutin, tryptophan emission spectra were obtained after excitation at 295 nm. Figure 6b showed that the relative fluorescence intensity of glycated HSA reduced to 37 % compared to native HAS. In addition, λ_{max} was slightly blue-shifted, namely from 336 to 334 nm for the glycated HAS. In contrast, the spectra obtained for the glycated HAS in the presence of rutin was very similar to that of native HSA. It may be concluded that the observed changes of tryptophan fluorescence could result from changed location of the tryptophan ring, possible interaction with other amino acid residues or overall protein structure changes on glycation process. The ANS fluorescence was also indicated that the blue shift of λ_{max} (inset to Figure 6b) from 530 nm to 528 nm can be explained by a decrease in the polarity of the environment of ANS which indicated that the AGEs of serum albumin shield the ANS probe more effectively from the water than the non-or less glycated form of HSA. However, in the presence of rutin protein hydrophobicity does not increase significantly compared to the glycates samples. The formation of protein cross-links requires spatial proximity of lysine and arginine residues, which may be limited by steric constraints of the tertiary or quaternary protein structure. This factor may limit propagation of damage, especially in extracellular matrix proteins that are organized in relatively structurally rigid networks. On the other hand, ROS can be generated from either glucose or the Amadori intermediate within a short distance from susceptible amino acid residues and diffuse rapidly to their targets. Free lysine residues of HSA in glycation processes were also measured by TNBSA. The free lysine residues in glycated protein decreased by approximately 45 % compared to the control sample, whereas in the presence of rutin, the percent of free lysine residues is nearly similar to the control sample (data not shown). It seems that in the presence of rutin in protein/glucose system, glucose may be unable to react with the lysine residues of the protein, there by preventing AGEs formation.

Table 3. The percentage of secondary structure of native HSA and glycate HSA in the absence and/or presence of rutin after three week incubation at 37 ^{0}C

Sample	α-helix (%)	β-sheet (%)	Random coil (%)
Native HSA	61.29 ± 0.3	6.67 ± 0.3	32.04 ± 0.4
Glycated HSA	54.75 ± 0.4	9.81 ± 0.4	35.44 ± 0.3
Glycated HSA + Rutin	58.13 ± 0.3	7.74 ± 0.2	34.13 ± 0.3

Amyloidosis comprises a group of systemic and localized diseases with varied clinical presentations. In these diseases, amyloid forms when proteins with a largely α-helical structure lose their original conformation and are converted into a predominantly β-sheet form, thereby increasing their propensity to form highly insoluble and fibrillar aggregates. Most soluble amyloid precursor proteins have substantial β-pleated sheet secondary structure, and extensive β-pleated sheet structure occurs in all of the deposited fibrils. The aberrant deposition of proteins as cellular inclusions or plaques in the form of amyloid fibrils is a characteristic hallmark of all amyloid diseases (or amyloidoses) and of the so-called conformational diseases. Currently, Obrenovich and Monnier,[41] showed that glycation causes albumin, a globular protein with a largely α helical structure, to adopt a β-pleated sheet structure and the quaternary structural element known as the cross-β conformation. ThT is a dye that interacts with the fibrillar structure of proteins, upon which interaction its fluorescence intensifies, while in its free form is only weakly fluorescent. This quality has been employed in the detection of amyloid fibril structures in proteins.[23] The results obtained from ThT fluorescence revealed that glycation protein induced the formation of the fibrilar state in protein. However, there is no major difference between intensity of ThT fluorescence for HSA incubated in the presence of rutin and/or AG compared to that in native protein (data not shown). These data indicated that rutin may be inhibited AGEs formation after developing the β-conformations.

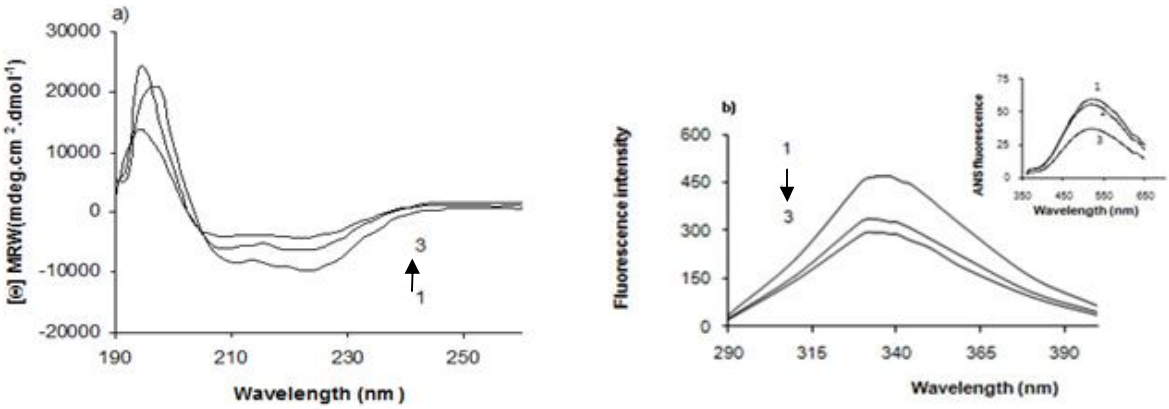

Figure 6. a) Far-UV CD (a) and fluorescence spectra (b) of native (curve 1), and glycated HSA in the presence of rutin (curve2) in the glucose-protein system after three week incubation at 37 ^{0}C. Curve 3, represents the spectra of glycated HSA with out any additive. The *inset* in Figure *b*, represents the ANS fluorescence spectra of various samples (Curve 1, native HSA; Curve 2, glycated HSA in the presence of rutin and Curve 3, glycated HSA). The experimental conditions are described in Section 2.

Conclusion

In this study, we found that rutin with high antioxidant activity isolated from *Teucrium polium* effectively inhibited protein glycation and its activity was nearly similar to that of aminoguanidine, a synthetic antiglycation agent. Rutin showed slight inhibitory effects on the generation of Amadori products and exhibited good inhibitory effects on the formations of α-dicarbonyl compounds and AGEs. It displayed a significant effect on the cross-linking of protein from the SDS-PAGE results. In addition, rutin inhibited the consequent formation of fluorescence AGEs, masking the amino groups in lysine residues of protein. Structural studies revealed that rutin may be preventing helix decrement in the secondary structure of protein in the presence of sugar. Therefore, the interaction of rutin with protein may provide protective effects toward oxidation of protein. However, the mechanism of antiglycation by this flavonoid requires more investigation. Regarding our findings with previous data in literature, it was suggested that daily consumption of *T. polium* may be beneficial for preventing complications in diabetic patients. However, further investigations in *in vivo* condition from diabetic patients are required.

Competing interests

The author declares that he has no competing interests.

Authors' contributions

The author contributed completely to this work and approved the final manuscript.

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