British Journal of Nutrition (2012), **107**, 941–949
doi:10.1017/S0007114511004077
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https://doi.org/10.1017/S0007114511004077 Published online by Cambridge University Press

British Journal of Nutrition

# Inhibition of advanced glycation end-product formation on eye lens protein by rutin

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*(Submitted 9 March 2011 – Final revision received 7 June 2011 – Accepted 27 June 2011 – First published online 25 August 2011)*

## Abstract

Formation of advanced glycation end products (AGE) plays a key role in the several pathophysiologies associated with ageing and diabetes, such as arthritis, atherosclerosis, chronic renal insufficiency, Alzheimer's disease, nephropathy, neuropathy and cataract. This raises the possibility of inhibition of AGE formation as one of the approaches to prevent or arrest the progression of diabetic complications. Previously, we have reported that some common dietary sources such as fruits, vegetables, herbs and spices have the potential to inhibit AGE formation. Flavonoids are abundantly found in fruits, vegetables, herbs and spices, and rutin is one of the commonly found dietary flavonols. In the present study, we have demonstrated the antiglycating potential and mechanism of action of rutin using goat eye lens proteins as model proteins. Under *in vitro* conditions, rutin inhibited glycation as assessed by SDS-PAGE, AGE-fluorescence, boronate affinity chromatography and immunodetection of specific AGE. Further, we provided insight into the mechanism of inhibition of protein glycation that rutin not only scavenges free-radicals directly but also chelates the metal ions by forming complexes with them and thereby partly inhibiting post-Amadori formation. These findings indicate the potential of rutin to prevent and/or inhibit protein glycation and the prospects for controlling AGE-mediated diabetic pathological conditions *in vivo*.

**Key words: Advanced glycation end products: Advanced glycation end products-fluorescence: Amadori product: Lens proteins: Metal chelation: Protein glycation: Rutin**

Non-enzymatic glycation is a complex cascade of reactions, initiated by the condensation of reducing sugars with free amino groups of protein to form reversible Schiff's base, which undergoes rearrangement to form a relatively stable Amadori product. Amadori products, over a period of time, undergo a series of reactions involving multiple dehydration, fragmentation and oxidative modifications through highly reactive dicarbonyl intermediates to form stable, heterogeneous adducts called advanced glycation end products (AGE)[1–3]. A wide variety of chemical structures of AGE such as carboxymethyllysine (CML), pentosidine, glyoxal-lysine dimer and methylglyoxal (MGO)-lysine dimer are reported[1–3]. Though, inside the cell, the impact of glycation is countered by high turnover and short half-life of many cellular proteins, the major consequence of glycation is the age-dependent chemical modifications of long-lived proteins, like eye lens proteins, skin collagen and basement membrane proteins[4–6].

Although AGE formation takes place during the normal ageing process, it is accelerated in hyperglycaemic conditions. An overwhelming body of evidence indicates that non-enzymatic glycation of proteins is implicated in a number of biochemical abnormalities associated with ageing and diabetes[4–6]. It has been shown that the formation of AGE *in vivo* contributes to several pathophysiologies associated with ageing and diabetes mellitus, such as arthritis, atherosclerosis, chronic renal insufficiency, Alzheimer's disease, nephropathy, neuropathy and cataract[4–9]. Glycation of eye lens protein has been considered to be one of the mechanisms responsible for both age-related and diabetic cataract, which is the leading cause of blindness[5,8,9]. It has been shown that the formation of AGE *in vivo* contributes to the cataract, by altering the surface charge of the protein, leading to conformational change that in turn may affect protein–protein and protein–water interactions and ultimately lead to decreased transparency of the eye lens[8–10]. The rate of AGE accumulation is related to the severity of diabetic cataract. Since considerable evidences have shown the contribution of AGE in the development of diabetic complications, inhibition of AGE is considered to be one promising approach for the prevention and treatment of diabetic complications. A wide variety of agents like pyridoxamine, carnosine, OPB-9195 (2-isopropylidenehydrazono-4-oxo-thiazolidin-5-ylacetanilide), phenyl thiazolium

**Abbreviations:** AGE, advanced glycation end products; ALR2, aldose reductase; BSA, bovine serum albumin; CML, carboxymethyllysine; HMW, high molecular weight; KLH, keyhole limpet haemocyanin; MGO, methylglyoxal; RNase, ribonuclease; TSP, total soluble protein.

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bromides, aspirin and lipoic acid have been investigated in several *in vitro* and *in vivo* studies for their potential against various pathologies including cataract[1,11–14]. However, except pyridoxamine, none have passed all the stages of clinical trials. While the extensively investigated hydrazine compound, aminoguanidine, has shown promising results *in vitro* and in animal models in terms of inhibition of AGE-formation and entered into phase 3 clinical trials[14–16], the trial was terminated due to various safety concerns[15]. It is known that glycation and AGE-induced toxicity are associated with increased free-radical activity. Recent studies have demonstrated the benefits of using compounds with combined antiglycation and antioxidant properties[17,18]. Such compounds not only prevent AGE formation, but also reduce free-radical-mediated toxicity. Hence, efforts are being made in identifying the natural sources of antiglycating agents that can be tested for their therapeutic value against AGE-mediated pathologies.

In the course of identifying and testing new antiglycating agents, we have evaluated a number of traditional and very common dietary sources and found that some spice principles, fruits and vegetables have the potential to inhibit AGE formation under *in vitro* conditions[19,20] and in animal models[21,22,23]. Flavonoids are abundantly found in fruits, vegetables, herbs and spices, and some of the flavonoids have been tested for their antiglycating activity[17,22–24]. Rutin is one of the commonly found dietary flavonols. Although the antiglycating activity of rutin and its metabolites has been reported[25,26], its potential against non-enzymatic glycation of eye lens protein has not been tested. Therefore, in the present study, we investigated the effect of rutin against glycation-induced alterations of the lens protein. We employed a set of complementary methods; spectroscopic, electrophoretic, chromatographic and immunochemical, to evaluate the antiglycating potential of rutin as well as its mechanism of action.

## Materials and methods

### Materials

Fructose, bovine serum albumin (BSA), Chelex-100, sorbitol, rutin, ascorbic acid, D-ribose, keyhole limpet haemocyanin (KLH), ribonuclease (RNase), MGO, Freund's complete and incomplete adjuvant, *m*-aminophenylboronic acid and horseradish peroxidase-conjugated goat anti-rabbit antibody were obtained from Sigma-Aldrich (St Louis, MO, USA). Glyoxylic acid and sodium cyanoborohydride were purchased from ICN Biochemicals (Aurora, OH, USA). All other chemicals and solvents used were of analytical grade.

### Preparation of advanced glycation end products antigens

AGE-RNase was prepared as described previously[10,19,21]. Briefly, RNase (25 mg/ml) was incubated with 1 M-glucose in 0·2 M-phosphate buffer (pH 7·4) containing 0·05 % sodium azide at 37°C for 90 d. KLH (50 mg/ml) was incubated with 0·045 M-glyoxylic acid and 0·15 M-sodium cyanoborohydride in 0·2 M-sodium phosphate buffer (pH 7·8) for 24 h at 37°C for the preparation of CML-KLH. For MGO-BSA, BSA (50 mg/ml) was incubated with 0·5 M-MGO in 100 mM-sodium phosphate buffer (pH 7·5) at 37°C in the dark for 3 d. Low molecular weight reactants and unbound sugars were removed by extensive dialysis.

### Production of polyclonal anti-advanced glycation end products antibodies

Antibodies were produced against AGE-RNase, CML-KLH and MGO-BSA by immunising 3-month-old female New Zealand white rabbits as described previously[10,20,21]. Rabbits were immunised by subcutaneous administration of a 1 ml solution containing 1 mg/ml AGE-protein antigen in Freund's complete adjuvant at multiple sites on the back of the rabbits and subsequently three boosters were given at 3-week intervals in Freund's incomplete adjuvant. For testing the titres, the rabbits were bled intermittently from the ear vein and the titre was checked by dot-blot analysis. The rabbits were bled after the last booster, and the serum was collected by centrifugation. Antiserum was partially purified by ammonium sulphate fractionation followed by diethylaminoethyl-sepharose anion exchange chromatography to obtain an IgG-rich fraction.

### Animal care

Institutional and national guidelines for the care and use of animals were followed and all experimental procedures involving animals were approved by the Institutional Animal Ethical Committee of National Institute of Nutrition. We adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

### In vitro glycation of proteins

Eye lens soluble proteins were obtained from 6-month-old goat lenses as described previously[27]. A 10 % homogenate of goat lenses was prepared in phosphate buffer saline, pH 7·4 and centrifuged at 10 000 ***g*** for 30 min at 4°C. The supernatant (total lens soluble protein referred to as total soluble protein (TSP), henceforth) was used for *in vitro* glycation. Each 1 ml reaction mixture contained 10 mg of TSP, 0·2 M-phosphate buffer, pH 7·4, 0·1 M-fructose, 50 μg of penicillin and streptomycin and 0·01 % sodium azide. Reaction tubes were incubated in the dark at 37°C for 3 weeks. At the end of the incubation, unbound sugars were removed by dialysis against the same buffer. Protein concentration was determined by the Lowry method using BSA as standard. Stock solutions of all the reaction contents were filtered through 0·20 μm syringe filters. The rationale behind using the lens proteins as a model protein was their longevity and their susceptibility to extensive accumulation of AGE, which is accelerated in diabetes-associated pathologies[10,21,27].

### Inhibition studies with rutin and aminoguanidine

For inhibition studies, concentrated stocks of rutin were prepared in dimethyl sulphoxide. Various concentrations of



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rutin (10–1000 μM) and aminoguanidine (10 and 100 mM) were added to *in vitro* protein glycation assay mixture and incubated in the dark at 37°C for 3 weeks as described earlier. At the end of the incubation, unbound reactants were removed by dialysis and protein concentration was determined as described previously. The extent of protein glycation in the absence and presence of rutin and aminoguanidine was evaluated by monitoring protein cross-linking on SDS-PAGE, AGE-related non-tryptophan fluorescence, protein carbonyl content, phenyl boronate affinity chromatography and immunodetection methods as described next. The percentage of inhibition with rutin and aminoguanidine was calculated considering the extent of glycation in their absence as 100 %.

### Fluorescence measurements

Non-tryptophan AGE fluorescence was monitored using 0·15 mg/ml protein in 20 mM-sodium phosphate buffer, pH 7·4, by exciting at 370 nm and recording the emission between 400 and 500 nm using a spectrofluorometer (Jasco FP-6500; JASCO Analytical Instruments, Tokyo, Japan).

### SDS-PAGE

Formation of high molecular weight (HMW) aggregates and protein cross-links in TSP as a result of protein glycation was monitored by SDS-PAGE under reducing conditions using 12 % gels.

### Glyco-oxidative damage

The effect of rutin and aminoguanidine on non-enzymatic glycation-mediated glyco-oxidative damage of TSP was monitored by estimating total protein carbonyls according to the method of Uchida *et al.*[28].

### Immunodetection of advanced glycation end products

Formation of specific AGE was detected by immunoblotting using anti-MGO-BSA, anti-CML-KLH and anti-AGE-RNase antibodies. Glycated proteins were resolved on a 12 % SDS-PAGE and transferred onto nitrocellulose membrane. The membrane was incubated for 2 h in blocking buffer containing 5 % skimmed milk powder. Subsequently, it was incubated with the respective primary antibodies (CML-KLH, 1:2000, MGO-BSA, 1:2000 and AGE-RNase, 1:1000) separately. The membrane was then incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody (1:5000) and detection was performed using the substrate buffer containing diaminobenzidine and $H_2O_2$.

### Affinity chromatography

The extent of glycation of TSP in the absence and presence of rutin was performed using phenyl boronate affinity chromatography[21,27]. In the present study, 10 mg of glycated TSP was passed through phenyl boronate affinity column (8 × 1 cm) equilibrated with 0·25 M-ammonium acetate buffer, pH 8·5, containing 0·05 M-$MgCl_2$. The unbound fraction containing non-glycated protein was washed with the earlier-mentioned buffer, while the bound glycated protein was eluted using 0·1 M-Tris-Cl, pH 7·5, and containing 0·2 M-sorbitol.

### Metal chelation

Metal-chelating activity was assessed by determining the metal-induced oxidation of ascorbic acid to dihydroascorbic acid using the HPLC method as described previously[29]. Copper chloride (1 μM) was preincubated with and without rutin in Chelex-100 treated Milli-Q element free water for 5 min, and then the ascorbate (0·01 mg/ml) was added to the reaction mixture and transferred to auto-injector vials to initiate the metal-catalysed oxidation reaction. Metal-catalysed oxidation of ascorbic acid was analysed using an RP-C18 column (3·9 × 300 mm) on a Shimadzu HPLC-(10AT VP) equipped with an SIL-10AD VP auto-injector and an SPD-10AV VP UV/Vis detector. The mobile phase was 70 % methanol and detection was at 265 nm. The peak area was integrated to estimate the percentage of ascorbic acid unoxidised with time in the absence and presence of rutin.

### Spectral absorbance shift

The shift in the absorbance spectrum of rutin due to complex formation with $CuCl_2$ was recorded[30]. The absorbance of rutin in the absence and presence of 100 μM-$CuCl_2$ in Chelex-100 treated 50 mM-sodium phosphate buffer (pH 7·4), was scanned from 200 to 700 nm in spectrophotometer (Spectramax-384; Molecular Devices, Sunnyvale, CA, USA).

### Post-Amadori inhibition

BSA (10 mg/ml) was incubated with ribose (0·4 M) at 37°C in 0·4 M phosphate buffer, pH 7·4, for 24 h. Glycation was then interrupted to remove excess ribose and also the reversible Schiff base by extensive dialysis against 20 mM-sodium phosphate buffer at 4°C. The glycated BSA intermediate containing maximal amount of Amadori product was then incubated at 37°C in the absence or presence of rutin, aminoguanidine and a combination of both for 5 d. This initiated conversion of Amadori intermediates to AGE products and the extent of conversion of Amadori to AGE[31] were measured by non-tryptophan AGE fluorescence as described previously.

### Statistical analysis

Results were expressed as means with their standard errors. Data were analysed using SPSS version 15.0 software (SPSS Inc., Chicago, IL, USA). Mean values were compared by one-way ANOVA with *post hoc* tests of least significant difference method. Heterogeneity of variance was tested by non-parametric Mann–Whitney test. Differences between comparisons of groups were considered to be significant at $P < 0.05$.

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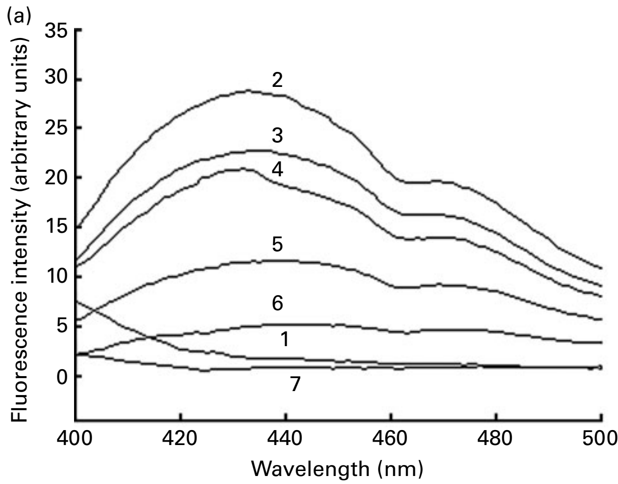


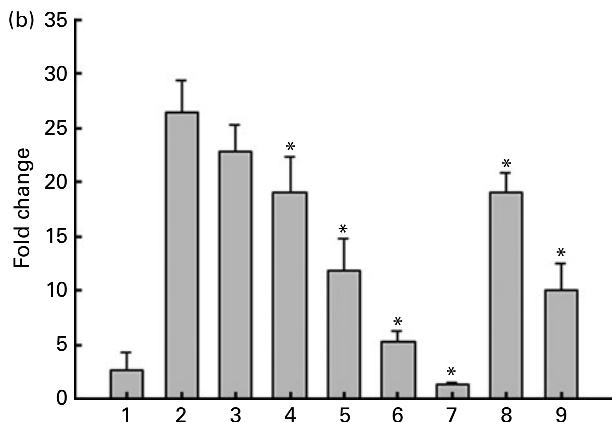
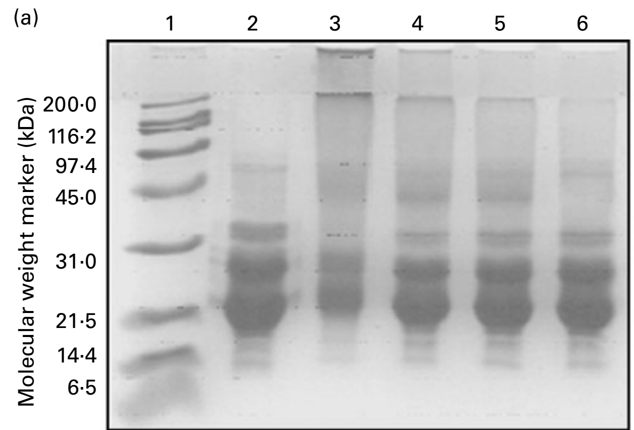




**Fig. 1.** Inhibition of advanced glycation end products (AGE) formation by rutin. (a) Representative non-tryptophan AGE-related fluorescence of total soluble protein upon *in vitro* glycation in the absence and presence of rutin. Trace 1, protein alone (P); trace 2, P + 100 mM fructose (F); trace 3, P + F + 10 μM-rutin; trace 4, P + F + 50 μM-rutin; trace 5, P + F + 100 μM-rutin; trace 6, P + F + 200 μM-rutin; trace 7, P + 200 μM-rutin. (b) Fold change in non-tryptophan AGE fluorescence was calculated considering the emission intensity (at 440 nm) of P as one fold. Bars 1–7 of (b) correspond to traces 1–7 of (a) and bars 8 and 9 correspond to P + F + 10 mM and 100 mM aminoguanidine, respectively. Values are means, with their standard errors represented by vertical bars of three independent experiments. * Mean values were significantly different from bar 2 ($P < 0.05$).

## Results

The antiglycation effect of rutin was evaluated by incubating it with TSP of goat lens and fructose for 21 d. AGE-related non-tryptophan fluorescence, which represents cumulative AGE fluorescence in a non-specific manner, was monitored to assess the effect of rutin (Fig. 1). Rutin inhibited AGE-related fluorescence in a dose-dependent manner with 90 % of reduction in AGE-fluorescence at 200 μM as opposed to aminoguanidine, which reduced 60 % AGE-fluorescence at 100 mM concentration (Fig. 1). Despite their heterogeneity, the propensity to form covalent cross-links is the common consequence of AGE which leads to the formation of HMW aggregates on proteins and ultimately lens opacification. Hence, the effect of rutin on the formation of AGE-mediated protein cross-links and HMW aggregates was investigated. Incubation of lens protein with fructose led to the appearance of non-disulphide dimers with a molecular weight of approximately 45 kDa and a large amount of HMW aggregates above 200 kDa that did not enter the stacking gel was observed on the SDS-PAGE (Fig. 2), similar to the modifications observed in the soluble portion of the lens protein from streptozotocin-induced diabetic cataract[32]. While rutin at 100 μM was found to reduce the formation of both cross-link and HMW aggregates (Fig. 2), 100 mM-aminoguanidine was required for comparable results (data not shown).

A wide variety of structurally diverse sugar-derived AGE have been demonstrated, such as pentosidine, argypyrimidine, carboxyethyllysine and CML[33–36]. Having shown the effective reduction of fructose-induced modification over the lens protein by rutin, next we evaluated the immunoreactivity of MGO-, CML- and glucose-derived AGE using antibodies raised against MGO-BSA, CML-KLH and AGE-RNase. Immunodetection with anti-AGE antibodies demonstrated the presence



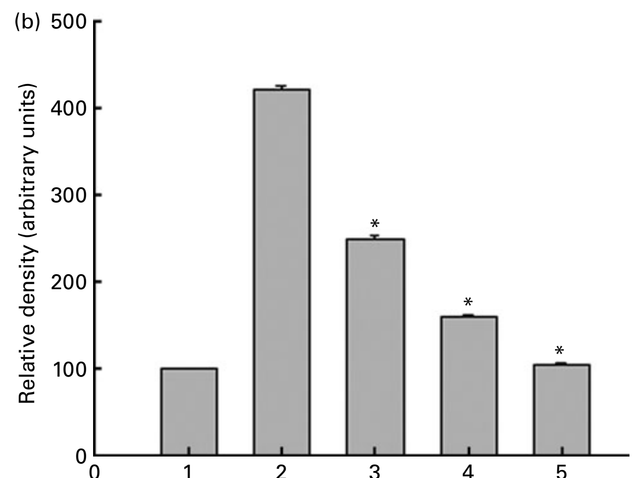




**Fig. 2.** Inhibition of advanced glycation end products-mediated protein cross-links by rutin. (a) Representative SDS-PAGE profile of total soluble protein upon *in vitro* glycation in the absence and presence of rutin. Lane 1, molecular weight markers; lane 2, protein alone (P); lane 3, P + 100 mM-fructose (F); lane 4, P + F + 10 μM-rutin; lane 5, P + F + 50 μM-rutin; lane 6, P + F + 100 μM-rutin. (b) Densitometry analysis of cross-linked and aggregated proteins. Intensity of protein bands above 31 kDa was quantified considering the intensity of lane 2 (a) as 100 %. Bars 1–5 of (b) correspond to lanes 2–6 of (a). Values are means, with their standard errors represented by vertical bars of three independent experiments. * Mean values were significantly different from bar 2 ($P < 0.05$).

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of diverse antigenic determinants over the protein (Fig. 3(a)). Anti-MGO-BSA detected cross-linked species of 45 and 26 kDa along with HMW aggregates >118 kDa. Anti-CML-KLH detected the HMW aggregates >118 kDa along with intermediate species of 45 and 26 kDa and anti-AGE-RNase detected the intermediate cross-linked species of 85 kDa. Densitometry analysis (Fig. 3(b)) indicates that CML- and MGO-derived AGE were prominent than glucose-derived AGE. Rutin showed a dose–response inhibition against all AGE. Densitometry analysis showed that rutin at 50 μM could reduce CML-derived AGE by approximately 90 %, glucose-derived AGE by 60 and 90 % reduction for the MGO-derived modification, respectively. Furthermore, boronate affinity chromatography shows that rutin could reduce the fraction of glycated protein in a dose-dependent manner (Fig. 4). Since boronate affinity allows the direct separation of glycated protein from the nonglycated one, these results corroborate the antiglycating potential of the rutin.

The reactive intermediates of the Maillard reaction, MGO and glyoxal, which can originate from all the stages of glycation (degradation of Schiff's base, auto-oxidation of sugar and from Amadori product), are a major source for AGE formation[35–37]. Trapping the reactive carbonyl compounds may be a valuable strategy for inhibiting or delaying the progressive glycation reactions. Hence, total protein carbonyls were estimated in the absence and presence of rutin. Increased carbonyl content (four-fold) of lens proteins upon fructose modification is an indication of glyco-oxidative damage (Fig. 5). While rutin was effective in lowering the carbonyl content in a dose-dependent manner, aminoguanidine – a known carbonyl scavenger – could not reduce glycation-induced carbonyl content in lens proteins on par with rutin even at 100 mM (Fig. 5). Metal-catalysed auto-oxidation of sugars or ascorbate is known to contribute to AGE formation[38,39]. Therefore, potent chelating activity of the compounds might increase the antiglycating action of AGE inhibition. For example, nucleophilic compounds, which are designed to trap reactive carbonyl or dicarbonyl intermediates as AGE inhibitors, have also been shown to have chelating activity[29,30]. We have also demonstrated the metal chelating activity of rutin by quantifying $CuCl_2$-catalysed ascorbic acid oxidation. Reduction of $CuCl_2$-catalysed oxidation of ascorbic

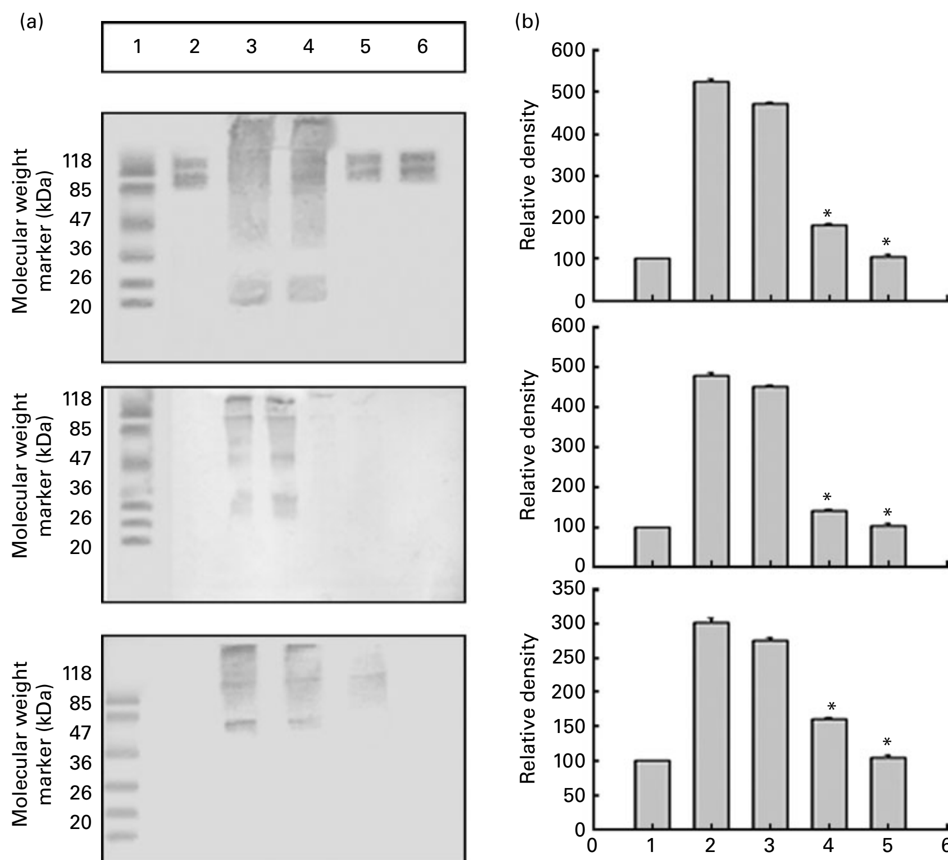


**Fig. 3.** Immunodetection of advanced glycation end products (AGE) in soluble lens protein. (a) Representative Western blot profile of total soluble protein upon *in vitro* glycation in the absence and presence of rutin. Blots were probed with anti-methylglyoxal-bovine serum albumin (top), anti-carboxy methyl lysine-keyhole limpet haemocyanin (middle) and anti-AGE-ribonuclease antibodies (bottom). Lane 1, molecular weight markers; lane 2, protein alone (P); lane 3, P + 100 mM-fructose (F); lane 4, P + F + 10 μM-rutin; lane 5, P + F + 50 μM-rutin and lane 6, P + F + 100 μM-rutin. (b) Densitometry analysis of AGE. Intensity of AGE signals was quantified considering the intensity of lane 2 (a) as 100 %. Bars 1–5 in (b) correspond to lanes 2–6 of (a). Values are means, with their standard errors represented by vertical bars of three independent experiments. * Mean values were significantly different from bar 2 ($P < 0·05$).

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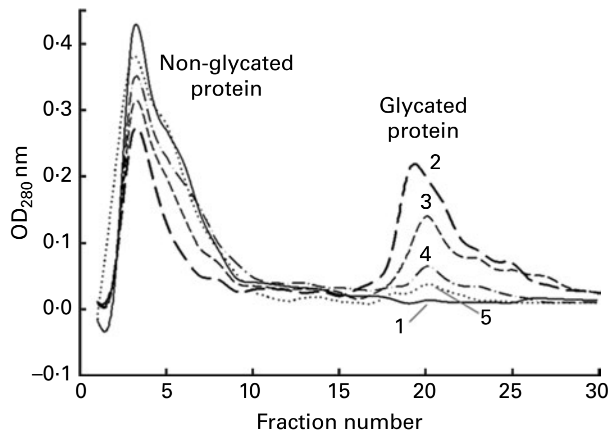


**Fig. 4.** The effect of rutin on the amount of glycated protein in total soluble protein upon *in vitro* glycation as analysed by phenyl boronate affinity chromatography. Trace 1, protein alone (P); trace 2, P + 100 mM-fructose (F); trace 3, P + F + 10 μM-rutin; trace 4, P + F + 50 μM-rutin; trace 5, P + F + 100 μM-rutin. OD, optical density.

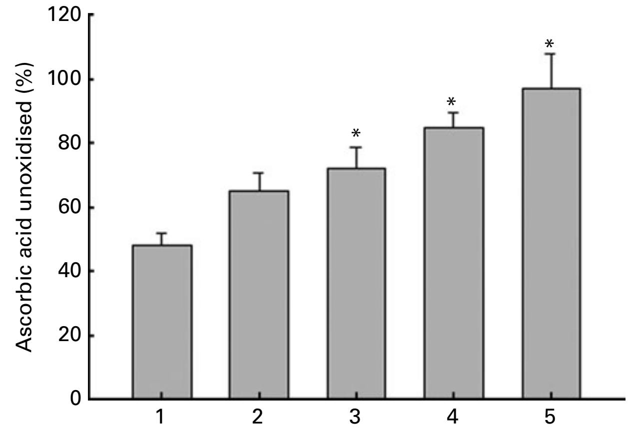


**Fig. 6.** Chelation of metals by rutin. Percentage ascorbic acid unoxidised due to metal catalysed reaction in the absence and presence of rutin. Bar 1, ascorbic acid (AA) + $CuCl_2$; bar 2, AA + $CuCl_2$ + 50 μM-rutin; bar 3, AA + $CuCl_2$ + 100 μM-rutin; bar 4, AA + $CuCl_2$ + 500 μM-rutin; bar 5, AA + $CuCl_2$ + 1000 μM-rutin. Values are means, with their standard errors represented by vertical bars of three independent experiments. * Mean values were significantly different from bar 1 ($P<0·05$).

acid by rutin in a dose-dependent manner (Fig. 6) indicates that inhibition of AGE by rutin could be due to its metal chelation ability. Further, a shift in the absorbance spectrum of rutin in the presence of $CuCl_2$ confirms the formation of rutin–metal complex (Fig. 7) and supports the finding that rutin may have metal chelation property. Finally, we have also assessed the potential of rutin to inhibit post-Amadori reaction as Amadori product after several rearrangement leads to the formation of stable and heterogeneous AGE. Rutin partly (30 %) inhibited the post-Amadori compound formation at 100 μM concentration, which is comparable to that of aminoguanidine at 10 mM (Fig. 8(a)). However, the effect of a combination of both rutin (100 and 200 μM) and aminoguanidine (10 mM) was not significantly different when compared with their individual potential alone (Fig. 8(b)).

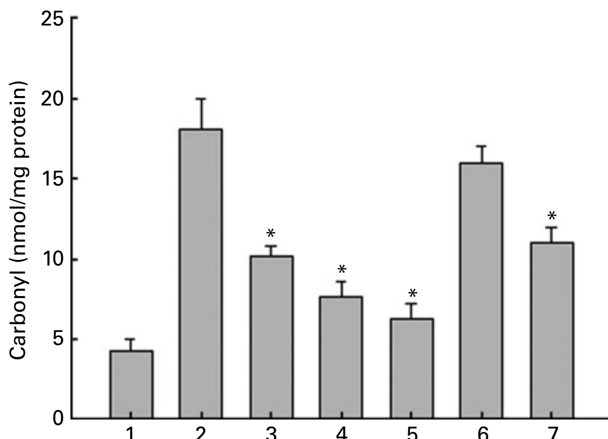


**Fig. 5.** Protein carbonyl content of total soluble protein upon *in vitro* glycation in the absence and presence of rutin. Bar 1, protein alone (P); bar 2, P + 100 mM-fructose (F); bar 3, P + F + 10 μM-rutin; bar 4, P + F + 50 μM-rutin; bar 5, P + F + 100 μM-rutin; bar 6, P + F + 10 mM-aminoguanidine; bar 7, P + F + 100 mM-aminoguanidine. Values are means, with their standard errors represented by vertical bars of three independent experiments. * Mean values were significantly different from bar 2 ($P<0·05$).

## Discussion

Numerous studies indicate that the world, in particular South-East Asia, is facing a growing diabetes epidemic, making it a major threat to public health[40,41]. Prolonged diabetes can lead to various short-term and long-term secondary complications, which represent the main cause of morbidity and mortality in diabetic patients. Hence, the long-term complications of diabetes, such as blindness due to cataract and retinopathy, remain serious problems to be dealt with. Hence, agents which can prevent diabetic complications have been studied for the management of secondary complications. Previously, we have identified some natural sources that include fruits, vegetables and spices for their potential to inhibit protein glycation and AGE formation, with the ultimate goal to prevent or treat diabetic complications[19–22]. Rutin is one of the commonly found flavonoids in these dietary sources. In the present study, we demonstrated the antiglycating effect of rutin and its mechanism of action using total soluble eye lens protein complement as a model protein system so as to translate these *in vitro* effects to an *in vivo* system in experimental models of diabetic complications.

A study examined the antioxidant activity of rutin and related it to its efficacy to inhibit glycation in some tissue protein extracts; and suggested that rutin and the rutin analogues exhibited significant antioxidant activity which corresponds to the ability to suppress the formation of the Maillard reaction intermediates in tissue protein sources[24]. Previously, it was also shown that dietary G-rutin suppresses the accumulation of glycation products in serum and kidney proteins as well as the oxidative modification of lipids and proteins in streptozotocin-induced diabetic rats[42]. However, the duration of diabetes (1 month) was too short for both – to study the extent of glycation and to evaluate the effect of rutin. Using a non-oxidative model of protein glycation, with histone H1, and glyoxal, MGO or ADP-ribose as the reducing sugar, a previous study tested the rutin metabolites as AGE inhibitors

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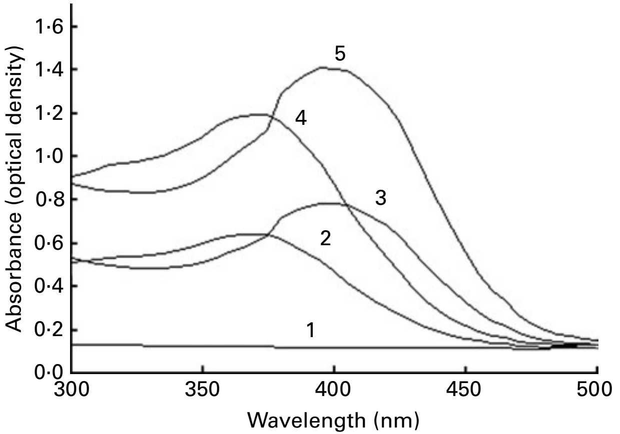


**Fig. 7.** Spectral shift of rutin in the presence of $CuCl_2$. Absorption spectrum of 50 μM-rutin in the absence (trace 2) and presence of 1 μM-$CuCl_2$ (trace 3), absorption spectrum of 100 μM-rutin in the absence (trace 4) and presence of 1 μM-$CuCl_2$ (trace 5). Absorption spectrum of 1 μM-$CuCl_2$ alone is also recorded (trace 1).

under non-oxidative conditions[26]. In the present study, we used glucose (fructose)-based protein glycation system because glucose-mediated protein glycation may occur under oxidative and non-oxidative conditions to form AGE protein adducts; and our results suggest that rutin could be effective under both oxidative and non-oxidative conditions. Rutin is metabolised by the gut microflora to a range of phenolic compounds such as quercetin and phenol derivatives such as 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxytoluene, 3-hydroxyphenylacetic acid and 4-hydroxy-3-methoxyphenylacetic acid (homovanillicacid)[26,43,44]. While a study showed that rutin and its circulating metabolites inhibit early glycation product formation induced by glucose glycation of collagen *in vitro*[43], the results of the present study indicate that rutin can inhibit both early and late glycation product formation.

Unlike the previous studies which focused on a specific aspect of AGE inhibition, in the present study we used a battery of methods to describe the antiglycating and AGE inhibitory potential of rutin. Particularly, the use of affinity chromatography (to quantify glycated proteins), immunodetection of specific but predominant AGE-antigens and post-Amadori inhibition systems helped us to provide information on the antiglycating property of rutin. However, it would be interesting to study the effect of rutin on late glycation product formation using glucose-mediated protein glycation; and efforts in this connection are already underway.

Rutin is a flavonol glycoside composed of quercetin and the disaccharide rutinose, i.e. quercetin-3-rutinoside. Structure–activity relationship study suggests the importance of vicinyl dihydroxyl groups in the B-ring of flavonoid for showing the property of AGE inhibition which correlates with the free-radical scavenging activity of flavonoids[45]. Probably, the presence of vicinyl dihydroxyl group in rutin contributes to its antiglycating activity. Being a free-radical scavenger, it may prevent the formation of dicarbonyls, the major source for AGE. In addition, the accumulation of intracellular sorbitol due to increased aldose reductase (ALR2 or AKR1B1) activity has been implicated in the development of various secondary complications of diabetes. Therefore, the inhibition of ALR2 is also one of the approaches to prevent or arrest the progression of diabetic complications. We have found that rutin also has the potential to inhibit ALR2 and suppress the formation of sorbitol, which has been shown as reported (G. B. R., unpublished results). Thus, these multiple properties of rutin support its utility for controlling AGE- and ALR2-mediated diabetic pathological conditions *in vivo*.

Although the beneficial impact of strict glycaemic control on the prevention of diabetic complications has been well established, most individuals with diabetes rarely achieve consistent euglycaemia. Hence, agents that can substantially delay or prevent the onset and development of diabetic complications, irrespective of glycaemic control, would offer many advantages. In principle, antiglycating agents and ALR2 inhibitors can be included in this category. Thus, intensive research continues to identify and test both synthetic as well as natural

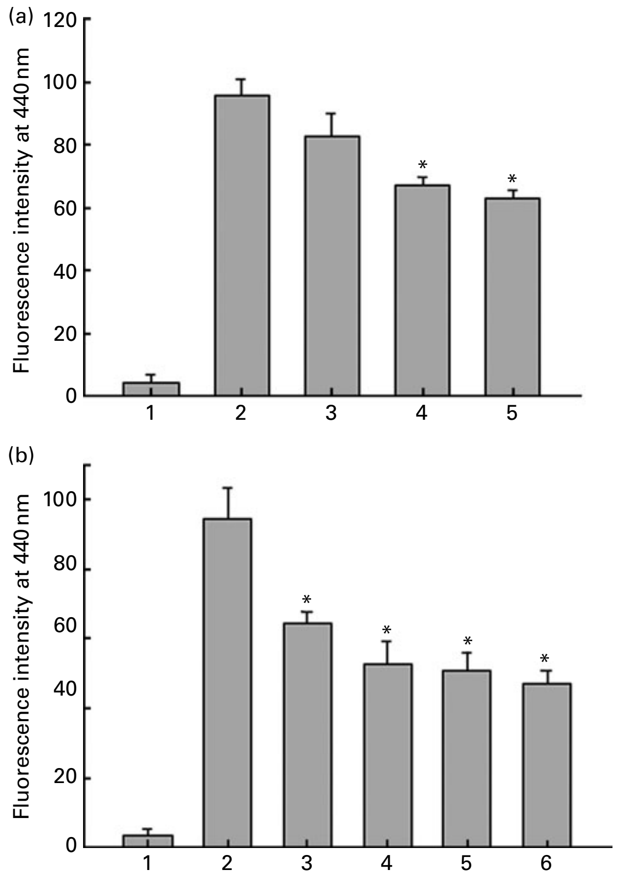


**Fig. 8.** Inhibition of post-Amadori product formation by rutin and aminoguanidine. Non-tryptophan advanced glycation end products fluorescence of bovine serum albumin (BSA) upon incubation with 0·4 M-ribose (R) in the absence and presence of rutin or aminoguanidine or both was recorded at 440 nm upon excitation at 370 nm. (a) Bars 1–5 correspond to BSA alone, BSA + R, BSA + R + 50 μM-rutin, BSA + R + 100 μM-rutin and BSA + R + 200 μM-rutin, respectively. (b) Bars 1–6 correspond to BSA alone, BSA + R, BSA + R + 100 μM-rutin, BSA + R + 100 mM-aminoguanidine, BSA + R + 100 μM-rutin + 10 mM-aminoguanidine and BSA + R + 100 μM-rutin + 100 mM-aminoguanidine, respectively. Values are means, with their standard errors represented by vertical bars, *n* 3. * Mean values were significantly different from bar 2 ($P<0·05$).

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https://doi.org/10.1017/S0007114511004077 Published online by Cambridge University Press

products for their therapeutic value to prevent the onset and/or delay the progression of diabetic complications. Studies are underway to investigate the potential of rutin against streptozotocin-induced diabetic cataract and other diabetic complications.

## Acknowledgements

The authors thank Dr. N. Balakrishna (National Institute of Nutrition) for his help in statistical analysis of the data. P. M., C. A. and M. S. were involved in data collection, data analysis, data interpretation, literature search and manuscript preparation. G. B. R. was involved in the study design, data analysis, data interpretation, literature search, manuscript preparation and review of the manuscript. P. M. received a research fellowship from University Grants Commission, Government of India; M. S. received a research fellowship from Indian Council of Medical Research, Government of India; G. B. R. received grants from the Department of Biotechnology under 7th FP of Indo-EU collaborative grant on functional foods (Grant agreement no. 245030) and Life Sciences Research Board of Defence Research and Development Organization, Government of India. The authors declare that they have no conflicts of interest.

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