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RESEARCH ARTICLE

Rutin inhibits the *in vitro* formation of advanced glycation products and protein oxidation more efficiently than quercetin

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Abstract

Introduction: The exacerbated generation of advanced glycation end products (AGEs) triggers the onset of diabetic complications associated with hyperglycemia. The search for natural bioactive compounds that can inhibit AGE formation has gained immense interest. Quercetin and its glycoside derivative, rutin, are powerful antioxidants. They have been studied due to their potential to mitigate the disturbances observed in diabetes; however, studies comparing their antiglycation effects are limited. The aim of the present study was to compare the *in vitro* antiglycation potentials of quercetin and rutin. **Methods:** The *in vitro* model system of protein glycation was applied using bovine serum albumin (10 mg/mL) incubated with glucose (0.5 M) in the absence or presence of aminoguanidine (1 mM, prototype anti-AGE agent), metformin (1 mM), quercetin (100, 50, or 12.5 μM), or rutin (100, 50, or 12.5 μM). Before initiating incubations (day 0) and after 10, 20, and 30 days, aliquots were assayed for fluorescent AGEs. Markers of amino acid oxidation (dityrosine, N'-formylkynurenine, kynurenine), protein carbonyl groups (PCO), and protein crosslink formation were assessed after 30 days. **Results:** Both quercetin and rutin inhibited the formation of AGEs and decreased the PCO levels in a concentration-dependent manner, and moreover, the effect of rutin was more prominent than that of quercetin. Quercetin and rutin also decreased the formation of amino acid oxidation products and protein crosslinks; the best effects were observed in incubations with rutin. **Conclusion:** Rutin exhibited the most potent antiglycation and antioxidant activities, which may be attributed to the minor occurrence of interactions between albumin and rutin, making rutinnoside more available to exert its effects.

Keywords: Protein Glycation. Diabetes Mellitus. *in vitro* Model Systems. Rutin. Quercetin. Protein Crosslinking.

How to cite

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1. INTRODUCTION

Advanced glycation end products (AGEs) are a set of heterogeneous molecules generated by various mechanisms and precursors; protein glycation is the main process that leads to AGE formation. The first step in protein glycation is nonenzymatic condensation between a primary amino group of the protein (mainly in lysine and arginine residues) and a carbonyl group of the reducing sugar (Maillard reaction), leading to the formation of an imine (Schiff base), which undergoes rearrangement to form a ketoamine (Amadori product), a more stable product. Amadori products can undergo oxidative fission or retro-aldol fragmentation, yielding highly reactive dicarbonyl compounds, mainly 3-deoxyglucosone, glyoxal, and methylglyoxal. Moreover, the degradation of triose phosphate intermediates from glycolysis can produce dicarbonyl compounds. These compounds react with amino acid residues in proteins, mostly arginine, lysine, and cysteine residues, leading to the formation of AGEs[1].

Under conditions of prolonged hyperglycemia during diabetes mellitus (DM), the formation of glycated proteins and AGEs is accelerated. The generation of AGEs is strongly involved in the onset and maintenance of diabetic complications[2]. Some detrimental effects of AGEs are related to the extensive loss of function in proteins, mainly through crosslink formation[1]. Furthermore, AGEs also cause tissue injuries via activation of the receptor of AGE (RAGE); AGE/RAGE interaction enhances inflammation and oxidative stress[3].

Recently, the search for natural bioactive compounds with the ability to inhibit protein glycation and the formation of AGEs has gained immense attention. The antiglycation properties of natural compounds, in combination with their antioxidant properties, make them interesting candidates for complementary therapies attempting to mitigate the diabetic complications resulting from glycoxidative stress[4]. Quercetin (3,3',4',5,7-pentahydroxyflavone) (Figure 1A) is one of the most important bioflavonoids and is ubiquitously present in vegetables and fruits. Quercetin has been extensively studied in relation to its antioxidant properties, as well as its potential for treating metabolic disorders[5], including diabetes[6]. Some hydroxyl groups of quercetin (aglycone) can be glycosylated to produce quercetin glycosides, including rutin (3,3',4',5,7-pentahydroxyflavone-3-rhamnoglucoside) (Figure 1B), a quercetin derivative that contains the disaccharide rutinose[7]. Multiple pharmacological activities are related to rutin[8,9]. Rutin is known to attenuate several changes occurring in DM, and inhibits various intracellular pathways responsible for diabetic complications, including the formation of AGEs and reactive oxygen species (ROS)[10].

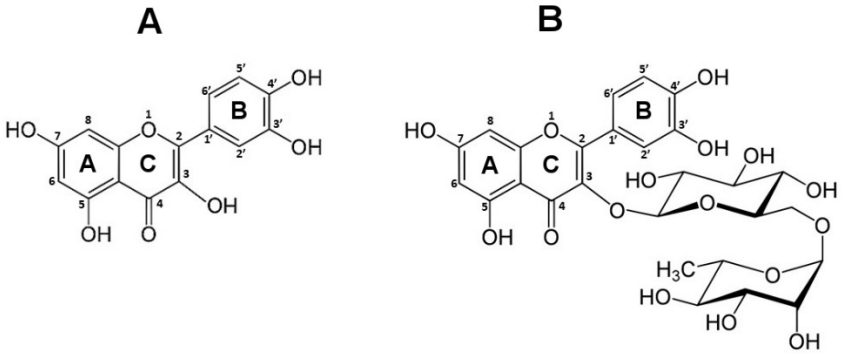

Figure 1: Structures of quercetin (A) and rutin (B).

Considering the potential of quercetin and rutin to mitigate the disturbances observed in DM, as well as the antioxidant activity of these bioactive compounds, the objective of the present study was to compare the antiglycation potentials of quercetin and rutin, using an *in vitro* model system of protein glycation, and to monitor markers of advanced glycation (fluorescent AGEs and protein crosslink formation) and protein oxidative damage (protein carbonyl groups and markers of amino acid oxidation).

2. MATERIAL AND METHODS

2.1. *In vitro* model system of protein glycation

The *in vitro* model system of protein glycation was applied according to dos Santos et al.[11] and Motta et al.[12] Bovine serum albumin (BSA, 10 mg/mL; ≥96%, A6003, Sigma-Aldrich, USA) was incubated with 0.5 M glucose (≥97%, G5250, Sigma-Aldrich, USA) in 0.1 M phosphate buffer (pH 7.4) containing 0.02% sodium azide, at 37°C for 30 days. Moreover, BSA (without glucose) and BSA + glucose were incubated in the absence or presence of aminoguanidine (1 mM, prototype therapeutic agent with anti-AGE activity; 97%, 109266, Sigma-Aldrich, USA) or metformin (1 mM; 99.56% purity, Gemini Indústria de Insumos Farmacêuticos Ltda, Brazil), and in the absence or presence of quercetin (100 µM, 50 µM, 12.5 µM; ≥ 95%, Q4951, Sigma-Aldrich, USA) or rutin (100 µM, 50 µM, 12.5 µM; ≥ 94%, R5143, Sigma-Aldrich, USA). Dimethyl sulfoxide (5% in the incubation) was used as the solvent for quercetin and rutin. Immediately after mixing the reagents and before initiating incubations at 37°C (day 0) and after 10, 20, and 30 days, aliquots were assayed for fluorescent AGEs. Furthermore, the markers of amino acid oxidation, levels of protein carbonyl groups, and protein crosslinking formation were analyzed in incubation samples after 30 days.

2.2. Fluorescent AGEs and markers of amino acid oxidation

The formation of fluorescent AGEs throughout the experimental period was assessed spectrofluorometrically, with excitation and emission wavelengths of 355 and 430 nm, respectively. Markers of amino acid oxidation were also assessed spectrofluorometrically, with the respective excitation and emission wavelengths: dityrosine (330/415 nm) (product of tyrosine oxidation), N'-formylkynurenine (325/434 nm), and kynurenine (365/480 nm) (products of tryptophan oxidation). The fluorescence values relative to AGEs and markers of amino acid oxidation were obtained after arithmetic subtraction of the fluorescence of incubations of flavonoids with buffer from those of flavonoids incubated with BSA or BSA + glucose. The results are expressed in terms of arbitrary units (AUs) of fluorescence. The fluorescence intensities were measured using a microplate multimode reader, with a split set at 16 nm (Synergy TM H1, BioTek Instruments Inc., USA).

2.3. Protein carbonyl groups

The levels of protein carbonyl groups (PCO) were measured as described by Levine et al.[13] and Meeprom et al.[14] PCO reacts with 2,4-dinitrophenylhydrazine and generates 2,4-dinitrophenylhydrazone, which is monitored at 370 nm. PCO levels were estimated using the molar extinction coefficient of the hydrazone (22,000 $M^{-1}.cm^{-1}$), and the results are expressed in terms of µmol/L.

2.4. Protein crosslink formation

Protein crosslinking was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as described by Santos et al.[11] and Motta et al.[12] Reducing buffer (62.5 mM Tris-HCl, 10% glycerol, 2% SDS, 33.2 mM dithiothreitol, 0.01% bromophenol blue; pH 6.8) was added to the incubation samples. Samples containing 5 µg of protein were subjected to SDS-PAGE in 10% polyacrylamide gels. Electrophoresis was performed at a constant potential of 60 V (first 30 min) and then at 120 V for 90 min. After electrophoretic separation, protein bands were visualized by staining the gels with Coomassie blue solution (0.01% Coomassie Brilliant Blue, 10% (v/v) methanol, and 10% (v/v) glacial acetic acid).

2.5. Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). One-way analysis of variance followed by the Student-Newman-Keuls test was used to compare the differences among groups. Data were considered statistically significant at $p < 0.05$. Statistical analyses were performed using GraphPad Prism 5.01 (GraphPad Software, USA).

3. RESULTS

3.1. Effects of quercetin and rutin on the levels of fluorescent AGEs

The fluorescence intensities relative to AGEs in the incubations of BSA in the presence of metformin, aminoguanidine, quercetin, or rutin (without glucose) were markedly low and similar to those observed in the incubations with BSA alone (Figure $2A_I$ and B_I). These findings indicated that these compounds do not emit fluorescence at the same wavelength used to monitor the fluorescent AGEs; therefore, they potentially do not interfere with the results of fluorescent AGEs when incubated with BSA + glucose.

When BSA was incubated with glucose for 30 days, a progressive increase in the fluorescence intensity relative to AGEs was observed, when compared with the fluorescence values of BSA incubated alone. After 10, 20, and 30 days, the levels of fluorescent AGEs after incubation with BSA + glucose were increased by 5-fold, 6-fold, and 5.8-fold, respectively, when compared with the corresponding values of BSA alone (Figure 2). This finding indicates that this *in vitro* model system of protein glycation leads to AGE formation.

Incubation of BSA + glucose with metformin did not prevent the formation of fluorescent AGEs (Figure $2A_{II}$ and B_{II}). Conversely, incubation of BSA + glucose with aminoguanidine, an anti-AGE agent, had low AGE levels throughout the experimental period, when compared with that of BSA + glucose (Figure $2A_{II}$ and B_{II}).

By monitoring the fluorescence relative to AGEs after 10, 20, and 30 days of incubation, it was observed that quercetin and rutin reduced the formation of AGEs during incubation with BSA + glucose, in a concentration-dependent manner, and during the entire study period (Figure $2A_{II}$ and B_{II}). After 30 days, the values of fluorescent AGEs were decreased by 45% and 10% during incubations of BSA + glucose with 100 and 50 μM quercetin, respectively, when compared with the fluorescence of BSA + glucose, whereas 12.5 μM quercetin did not reduce the formation of AGEs. After 30 days, 100 μM quercetin exhibited a profile similar to that of aminoguanidine in its ability to reduce AGE levels (Figure $2A_{II}$).

Rutin also reduced the fluorescence relative to AGEs after incubation with BSA + glucose, an effect that was more prominent than that of quercetin. After 30 days, the fluorescence relative to AGEs decreased by 80%, 61%, and 10% during incubations of BSA + glucose with 100, 50, and 12.5 μM rutin, respectively, in comparison with values of BSA + glucose. After 30 days, the levels of AGEs during incubations with 50 and 100 μM rutin were lower than those in incubations with aminoguanidine (Figure $2B_{II}$).

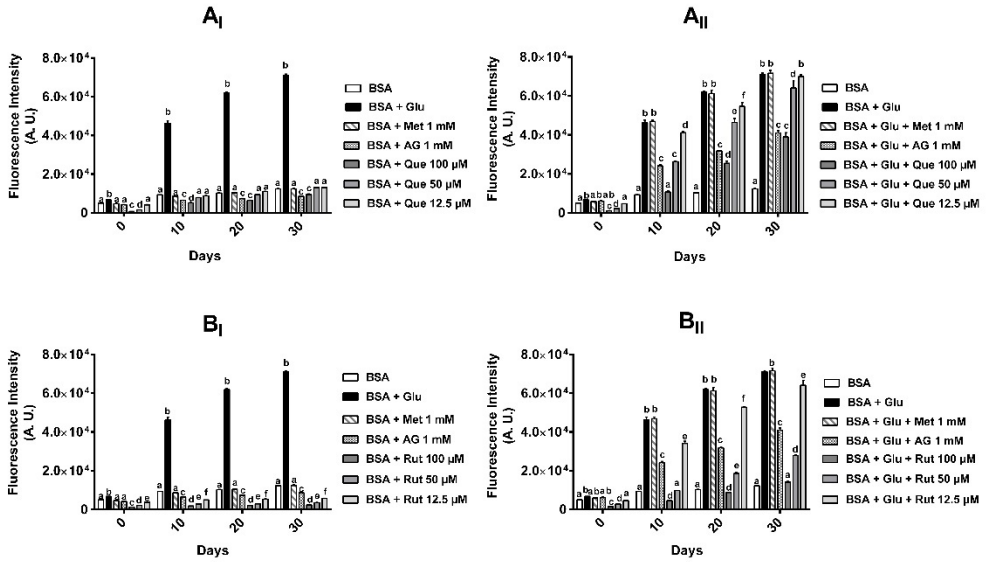

Figure 2: Effects of quercetin (A_I and A_{II}) and rutin (B_I and B_{II}) on the formation of fluorescent AGEs in an *in vitro* model system of protein glycation. A_I and B_I: incubations with BSA; A_{II} and B_{II}: incubations with BSA + glucose. Values are expressed as the mean ± SEM. Differences between groups were considered significant if $p < 0.05$, and were analyzed using one-way ANOVA followed by the Student-Newman-Keuls test. Means not sharing a common letter indicate significant differences.

3.2. Effects of quercetin and rutin on the levels of amino acid oxidation markers

After 30 days, the fluorescence intensities of dityrosine (product of tyrosine oxidation), N'-formylkynurenine and kynurenine (products of tryptophan oxidation) were increased by 5-fold, 4.4-fold and 4.2-fold in incubations of BSA + glucose, respectively, in comparison with the corresponding values of BSA alone (Figure 3).

Incubation of BSA + glucose with metformin resulted in marginal decreases in the levels of dityrosine (Figure 3A_I and B_I) and kynurenine (Figure 3A_{III} and B_{III}); however, there were no changes in N'-formylkynurenine (Figure 3A_{II} and B_{II}), when compared with BSA + glucose. In contrast, incubation of BSA + glucose with aminoguanidine significantly reduced the formation of all amino acid oxidation markers by 49% (dityrosine), 35% (N'-formylkynurenine) and 46% (kynurenine) (Figure 3).

The effects of quercetin and rutin on markers of oxidative changes in amino acid residues were distinct. Incubation of BSA + glucose with 100 μM quercetin significantly decreased dityrosine (Figure 3A_I), N'-formylkynurenine (Figure 3A_{II}), and kynurenine (Figure 3A_{III}), whose levels were similar to the corresponding oxidation product values in incubations of BSA + glucose with aminoguanidine. Conversely, during incubations of BSA + glucose, quercetin at concentrations of 50 and 12.5 μM did not decrease the levels of tyrosine and tryptophan oxidation markers (Figure 3A).

The best effects on decreasing the formation of tyrosine and tryptophan oxidation products were achieved in incubations with rutin. Incubation of BSA + glucose with rutin significantly reduced the fluorescence relative to dityrosine (Figure 3BI), N'-formylkynurenine (Figure 3B_{II}), and kynurenine (Figure 3B_{III}), in a concentration-dependent manner, attaining values that were lesser (100 μM rutin) or similar (50 μM rutin) than the corresponding oxidation product values after incubation of BSA + glucose with aminoguanidine.

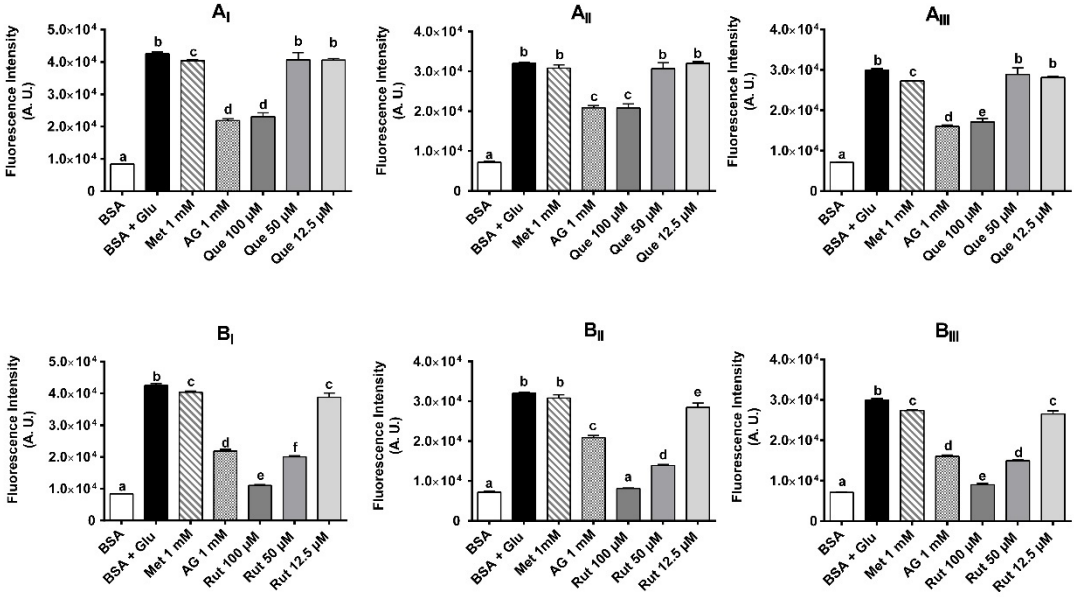

Figure 3: Effects of quercetin (A) and rutin (B) on the formation of dityrosine (A_I and B_I), N'-formylkynurenine (A_{II} and B_{II}), and kynurenine (A_{III} and B_{III}) in an *in vitro* model system of protein glycation. Values are expressed as the mean ± SEM. Differences between groups were considered significant if $p < 0.05$, and were analyzed using one-way ANOVA followed by the Student-Newman-Keuls test. Means not sharing a common letter indicate significant differences.

3.3. Effects of quercetin and rutin on the levels of protein carbonyl groups

In accordance with the changes observed in the *in vitro* formation of the amino acid oxidation products, the levels of PCO were increased significantly (9.8-fold) in BSA + glucose

samples after 30 days of incubation (Figure 4). Collectively, considering the increased levels of PCO and markers of tyrosine and tryptophan oxidation, these findings support the concept that oxidative damage occurs as a consequence of protein glycation processes.

In accordance with the findings about the levels of fluorescent AGEs and markers of tyrosine and tryptophan oxidation, incubation of BSA + glucose with metformin did not prevent the formation of PCO (Figure 4). The addition of aminoguanidine to BSA + glucose caused a marginal decrease (14%) in the PCO levels compared to those in BSA + glucose (Figure 4).

The response profiles of quercetin and rutin on PCO levels were consistent to those observed in AGE levels, that is, the inhibition of PCO formation occurred in a concentration-dependent manner, and the best effects were observed during incubation with rutin. Incubation of BSA + glucose with 100 μM quercetin resulted in a 17% decrease in the PCO levels compared to BSA + glucose, attaining values that were similar to the corresponding values during incubations of BSA + glucose with aminoguanidine. In contrast, the PCO levels remained increased in incubations of BSA + glucose in the presence of 50 or 12.5 μM quercetin (Figure 4A).

Rutin had the best protective effect against *in vitro* oxidative changes after incubation with BSA + glucose. Incubations with 100 μM and 50 μM rutin reduced the levels of PCO by 51% and 34%, respectively, in comparison with the corresponding values in BSA + glucose, reaching values lower than those observed in BSA + glucose incubated with aminoguanidine. Rutin at 12.5 μM did not reduce the PCO, whose levels remained increased and were similar to the values from BSA + glucose (Figure 4B).

Collectively, considering the decrease in the levels of tyrosine and tryptophan oxidation products and PCO, these findings reinforce the antioxidant effects of quercetin and rutin, mainly at concentrations of 100 μM, and indicate their potential to mitigate oxidative damage in biomolecules due to glycoxidative stress.

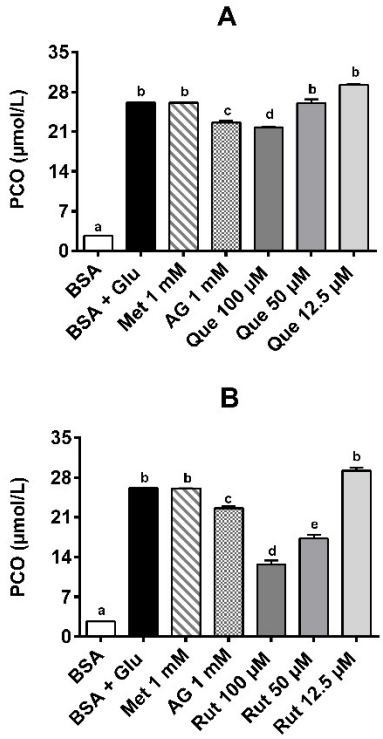

Figure 4: Effects of quercetin (A) and rutin (B) on PCO levels in an *in vitro* model system of protein glycation. Values are expressed as the mean ± SEM. Differences between groups were considered significant if $p < 0.05$, and were analyzed using one-way ANOVA followed by the Student-Newman-Keuls test. Means not sharing a common letter indicate significant differences.

3.4. Effects of quercetin and rutin on protein crosslinking

After 30 days, protein crosslinking appeared in incubations of BSA + glucose as a consequence of the structural changes that occur in the late stages of protein glycation. In BSA + glucose incubations, protein crosslinking can be visualized in samples subjected to electrophoresis as a protein band of a larger size, slightly "lagging" in relation to the BSA band, often observed as a "shadow" in an upper position relative to BSA, accompanied by a spreading BSA band (Figure 5A and B, line 2), compared to the single band of BSA at approximately 66 kDa in samples containing this protein incubated alone (Figure 5A and B, line 1).

Metformin (Figure 5A and B, line 3) or aminoguanidine (Figure 5A and B, line 5) did not cause changes in the electrophoretic pattern of BSA when compared with BSA incubated alone. After incubation with BSA + glucose, metformin did not prevent crosslinking and spread of BSA (Figure 5A and B, line 4), whereas aminoguanidine promoted a marginal reduction in the spread of BSA (Figure 5A and B, line 6).

Notably, incubation of BSA with quercetin, without glucose, led to the formation of protein crosslinks in a concentration-dependent manner (Figure 5A, lines 7, 9, 11), although this change occurred to a lesser extent than that observed in BSA + glucose incubations. In the incubation with BSA + glucose, 100 µM quercetin slightly inhibited both protein crosslink formation and the spread of BSA (Figure 5A, line 8). Quercetin at concentrations of 50 µM (Figure 5A, line 10) and 12.5 µM (Figure 5A, line 12) did not prevent the formation of crosslinking or the spread of BSA in incubation of BSA + glucose.

In the incubation of BSA with 100 µM rutin, without glucose, a discrete formation of protein crosslink was observed (Figure 5B, line 7), similarly to the effect caused by incubations of BSA with 12.5 µM quercetin (Figure 5A, line 11); however, incubations of BSA with rutin at concentrations of 50 µM (Figure 5B, line 9) and 12.5 µM (Figure 5B, line 11) did not lead to protein crosslink formation. During incubation with BSA + glucose, rutin significantly inhibited protein crosslinking and the spread of BSA in a concentration-dependent manner, mainly during incubation with rutin at 100 µM (Figure 5B, line 8) and 50 µM (Figure 5B, line 10).

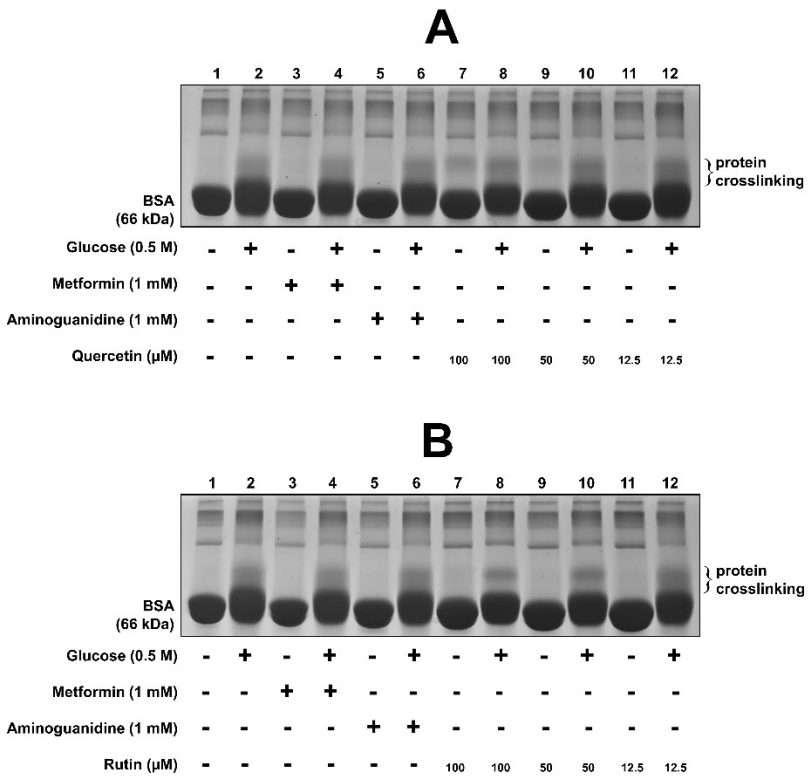

Figure 5: Effects of quercetin (A) and rutin (B) on the formation of protein crosslinking in an *in vitro* model system of protein glycation. Lines: (1) BSA; (2) BSA + glucose; (3) BSA + metformin; (4) BSA + glucose + metformin; (5) BSA + aminoguanidine; (6) BSA + glucose + aminoguanidine; (7) BSA + 100 µM flavonoid; (8) BSA + glucose + 100 µM flavonoid; (9) BSA + 50 µM flavonoid; (10) BSA + glucose + 50 µM flavonoid; (11) BSA + 12.5 µM flavonoid; (12) BSA + glucose + 12.5 µM flavonoid.

4. DISCUSSION

According to our results, the *in vitro* model system of protein glycation based on the incubation of 10 mg/mL BSA with high glucose concentration (0.5 M) for 30 days resulted in significant changes in markers related to glycoxidation of BSA: (*i*) progressive increase in fluorescent AGEs (markers of advanced glycation); (*ii*) increased levels of dityrosine (product of tyrosine oxidation), N'-formylkynurenine, and kynurenine (products of tryptophan oxidation); (*iii*) increased levels of PCO (marker of protein oxidative damage); and (*iv*) formation of protein crosslinks (representing structural changes in the late stages of protein glycation). Moreover, this *in vitro* model system of protein glycation is suitable for screening of bioactive compounds with antiglycation activity, since aminoguanidine, a prototype therapeutic agent with anti-AGE activity, effectively reduced the levels of the aforementioned markers of glycoxidative stress during incubation of BSA + glucose, mainly the fluorescent AGEs and the products of amino acid oxidation.

Conversely, although there is previous evidence that metformin can reduce fluorescent AGEs in *in vitro* protein glycation model systems[15,16], this drug did not reveal satisfactory *in vitro* anti-AGE effects, at least under the conditions used in this study. Metformin caused marginal reductions in the levels of dityrosine and kynurenine after incubation with BSA + glucose, without changes in other biomarkers. Using an *in vitro* model system of protein glycation similar to that used in our study, Sadowska-Bartosz et al.[17] observed that metformin did not protect proteins against glycoxidation. In an interesting study by Mehta et al.[18], aminoguanidine and metformin were used as carbonyl scavenging drugs to prevent glyoxal toxicity in isolated rat hepatocytes. The authors found that metformin, at all tested concentrations (1, 3, and 5 mM), failed to prevent glyoxal-induced protein carbonylation, although the glyoxal-induced ROS formation decreased, whereas aminoguanidine decreased both ROS formation (1 and 5 mM) and protein carbonylation (5 mM). The authors suggested that metformin may act as an ROS scavenger. These observations may help explain, at least partially, our findings on the effects of metformin on reducing the levels of products related to amino acid oxidation, without causing decreases in PCO levels.

According to our results, the flavonoids quercetin and rutin had inhibitory effects on AGE formation in an *in vitro* model system of protein glycation. Our findings are in accordance with those of previous studies that observed the *in vitro* anti-AGE effects of quercetin[19,20] and rutin[21,22]. Ashraf et al.[23] studied the *in vitro* antiglycation potential of quercetin using various *in vitro* model systems of early, intermediate, and advanced glycation. The authors found that, in all *in vitro* model systems, quercetin (mainly at concentrations of 200 and 500 μM) exhibited the ability to protect against protein glycation to a similar extent as that of 10 mM aminoguanidine, suggesting that quercetin is a more potent antiglycating agent than aminoguanidine at all stages of glycation. These findings are in accordance with our results; after 30 days of incubation with BSA + glucose, 100 μM quercetin inhibited the formation of AGEs to a similar extent as that of 1 mM aminoguanidine; however, the rutin effects were more prominent, since incubations of BSA + glucose with 100 and 50 μM rutin caused decreases in the formation of AGEs that were even greater than those promoted by aminoguanidine.

Although previous evidence suggests the inhibitory effects of quercetin and rutin on the formation of AGEs *in vitro*, studies comparing the effects of these two flavonoids are limited. Therefore, one main advantage of our study was the possibility of comparing the *in vitro* anti-AGE properties of rutin and quercetin. In this sense, we observed that rutin has a greater capacity to inhibit the formation of AGEs *in vitro* than quercetin. The findings of our study are contradictory with those of the few studies that investigated the anti-AGE effects of quercetin and rutin concurrently. Wu and Yen[24] found the same response profile as observed in our study, that is, the inhibitory potential against AGE formation was greater for rutin than that of quercetin. Conversely, Bhuiyan et al.[25] observed that quercetin had a greater anti-AGE effect than that of rutin, and that rutin was not able to inhibit the formation of AGEs *in vitro*; thus, the authors concluded that the C-3 hydroxyl group in quercetin may be crucial for its inhibitory

potential on AGE formation. On the other hand, Li et al.[19] found that C-6 and C-8 of quercetin are involved in trapping methylglyoxal, a dicarbonyl compound that acts as a precursor of AGEs. Cervantes-Laurean et al.[26] and Pashikanti et al.[27] suggested that the vicinal hydroxyl groups in the structures of rutin and its metabolites (including quercetin) are important for the *in vitro* potential of these flavonoids in suppressing the formation of AGEs. Collectively, these studies indicated that other functional groups besides the C-3 hydroxyl group in quercetin may participate in the *in vitro* anti-AGE effects of these flavonoids, supporting our and other findings regarding the capacity of rutin to inhibit the formation of AGEs.

Furthermore, in the present study, we obtained the absorption spectra of incubation samples containing the flavonoids with BSA (Figure 1S; Supplementary Material) and flavonoids with BSA + glucose (Figure 2S; Supplementary Material). Based on the analysis of the absorption spectra, incubations with quercetin or rutin had high absorption capacities between 300 and 390 nm, which encompasses the wavelength used to excite the fluorescent AGEs (355 nm). Therefore, the minor fluorescence intensities relative to AGEs observed in incubations of BSA + glucose + quercetin or BSA + glucose + rutin must be interpreted with caution, and this analytical interference must be considered. Nevertheless, this interference does not explain the differences observed in the anti-AGE potential between quercetin and rutin. For example, at 350 nm (a wavelength close to that used to excite AGEs), both 100 μM quercetin (Figure 1S-A; Supplementary Material) and 100 μM rutin (Figure 1S-B; Supplementary Material) presented exactly similar absorbance values (0.8285) during incubations with BSA.

In addition to inhibiting the formation of AGEs, quercetin and rutin exhibited *in vitro* antioxidant activities, as they reduced the levels of markers related to protein oxidation, including PCO and products of tyrosine and tryptophan oxidation. Protein damage due to oxidative and carbonyl stress is often observed as a consequence of glycation processes[17,28], which explains the increased fluorescence intensities of dityrosine, N'-formylkynurenine and kynurenine, and the increased levels of PCO after incubation with BSA + glucose for 30 days. Quercetin and rutin are powerful antioxidants that decrease the levels of these standard indices of protein oxidation; however, considering that (*i*) rutin had a more potent anti-AGE effect than quercetin, and (*ii*) the inhibitory effects on the formation of PCO and markers of tyrosine and tryptophan oxidation were more evident for rutin, it can be suggested that the anti-AGE effects of rutin play a pivotal role in mitigating protein oxidation *in vitro*. Using different glycating agents (glucose, fructose, ribose) during incubation with BSA, Sadowska-Bartosz et al.[17] found that rutin inhibited the formation of AGEs and, in parallel, caused significant reductions in the formation of dityrosine, N'-formylkynurenine, and kynurenine.

When analyzing protein crosslink formation in incubations of BSA and BSA + glucose with flavonoids, we propose a reasonable explanation for the lower effectiveness of quercetin in inhibiting AGE formation *in vitro*, as well as its minor antioxidant effects, in relation to the effects of rutin on the same parameters. Incubations of BSA + quercetin, without glucose, led to the formation of protein crosslinks in a concentration-dependent manner, although this change occurred to a lesser extent than that observed with BSA + glucose. The formation of protein crosslinking during incubations of BSA + rutin, without glucose, was practically nonexistent. Thus, the possibility that quercetin interacts with BSA cannot be ruled out, leading to crosslink formation. Notably, quercetin interacts with both HSA and BSA[29]. Although no previous evidence reveals that the interaction of quercetin with BSA causes crosslink formation, studies have suggested that phenolic compounds (flavonoids, phenolic acids, tannins) can interact with proteins, leading to changes in the electrophoretic profile[30] and crosslink formation[31]. Therefore, presumably, the interaction between BSA and quercetin made the flavonoid less available to exert its anti-AGE and antioxidant activities compared to rutin, which does not appear to have significantly interacted with BSA, thus being able to inhibit the formation of AGEs and the oxidation of BSA. This hypothesis is corroborated by the fact that, during incubations of BSA + glucose with quercetin *versus* BSA + glucose with rutin, rutin was able to decrease crosslink formation, whereas quercetin apparently had no protective effect.

Although no studies have revealed the possibility of BSA + quercetin interaction negatively interfering with its anti-AGE potential, evidence suggests that the antioxidant capacity of quercetin is significantly decreased in the BSA + quercetin condition, compared to free quercetin[32,33], which is in accordance with our findings on the decreased *in vitro* antioxidant activity of quercetin compared to rutin. Eventually, the BSA + quercetin interaction may decrease the *in vitro* anti-AGE potential of this flavonoid. Lastly, in studies that evaluated the ability of quercetin and rutin to interact with BSA[34] or HSA[35], it was observed that the binding affinity was lower for rutin than quercetin, which corroborates our findings of virtually no crosslink formation after incubation with BSA + rutin. Moreover, as an aglycone, quercetin is more hydrophobic and has a stronger affinity toward albumin than rutin. Furthermore, since rutin is a quercetin glucoside, the presence of rutinose renders rutin less hydrophobic than quercetin, and the large size of the rutinnoside may result in steric hindrance of its penetration into the hydrophobic pocket of albumin[34,35].

5. CONCLUSION

The present study reveals the differences in the anti-AGE and antioxidant activities of rutin and quercetin in an *in vitro* model system of protein glycation. Rutin has the most potent inhibitory effects on AGE formation and protein oxidation *in vitro*, which may be partly attributed to the minor occurrence of interactions between BSA and rutin, making rutinnoside more available to exert its anti-AGE and antioxidant activities. This study sheds light on the protective effects of quercetin and rutin against the consequences of protein glycoxidation, making these flavonoids interesting candidates for future studies attempting to explore their efficacies as complementary therapeutic options against diabetic complications resulting from glycoxidative stress.

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Authors' contributions

DTMD: Conceptualization, Methodology, Data Curation, Formal analysis, Investigation, Visualization, Writing - Original Draft; KRP: Methodology, Investigation, Visualization; BPM: Conceptualization, Methodology, Data Curation, Formal analysis, Investigation, Visualization, Writing - Original Draft; AKK: Conceptualization, Methodology, Data Curation, Formal analysis, Investigation, Visualization, Writing - Original Draft; TFOL: Methodology, Data Curation, Formal analysis, Investigation, Visualization, Writing - Original Draft; ILB: Conceptualization, Methodology, Resources; AMB: Conceptualization, Methodology, Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

SUPPLEMENTARY MATERIAL

Supplementary Material is available in OSF (public repository) at http://doi.org/10.17605/OSF.IO/WF6Y2.