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Research article

Antioxidant activity and protective role on protein glycation of synthetic aminocoumarins

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ABSTRACT

Background: Synthesized aminocoumarins are heterocyclic compounds possessing potential for the treatment of insulin-dependent diabetes mellitus with unexplored anti-glycative action.
Results: In this study 4-aminocoumarin derivatives (4-ACDs) were evaluated in vitro for antiglycation (AG) activities by using the human serum albumin (HSA)/glucose system, for 8 weeks of incubation. The glycation and conformational alteration of HSA in the presence of the tested compounds were evaluated by Congo red assay, fluorescence and circular dichroism spectroscopy. The antioxidant (AO) capacity were also tested by four different assays including: DPPH (2,2′-diphenyl-1-picrylhydrazyl radical), ABTS (2,2-azinobis (3-ethylbenzothiazoline-6-sulphonate) diammonium salt), FRAP (ferric reducing antioxidant power) and β-carotene-linoleic acid assay. The tested compounds showed AG and AO effects. The intensity of the accomplished AO potential is related to the type of the used assay. Significant alterations in the secondary (monitored by CD spectropolarimetry) and tertiary structure (assessed by spectrofluorimetry) of HSA upon glycation were mitigated by the 4-ACDs, suggesting their suppressive role in the late stage (post-Amadori) of the HSA glycation.
Conclusions: By the analogues, in vitro ascertained AO and AG properties of 4-ACD may be recognized as rationale for their protective role against oxidative changes of proteins, thereby precluding diabetic complications in humans.

1. Introduction

Coumarins are heterocyclic compounds, naturally occurring in green plants, fungi, bacteria and some fruits. An antioxidant and anti-inflammatory properties of coumarins and their derivatives have recognized to reduce the risk of cancer, diabetes, cardiovascular and brain diseases [1]. Moreover, *in vitro* inhibitory properties of 4-aminocoumarin derivatives (4-ACD) against human platelet aggregation, antioxidant, anticancer, antimicrobial and anti-mycobacterial activity have been described [2,3]. Likewise, cyclic 4-aminocoumarin derivatives have been reported to act on the viability of HepG2 cells through antioxidant activity [3]. Recently, antioxidant effect of coumarins was recognized as their novel mechanisms of action [4].

Increased content of free radicals (FRs) in living organisms occurs due to their increased production or insufficient sequestration by the innate antioxidative defense system (AODS). Free radicals initiate oxidative stress (OS), *i.e.* oxidative injury of all classes of biomolecules (proteins, lipids, DNA). This pathophysiological mechanism has been documented in major ailments such as diabetes, carcinogenesis, atherogenesis, aging, etc. [5,6]. Protein glycation occurs spontaneously, but increasingly in the presence of oxidizing agents such as FRs. Glycated proteins are involved in long term complications of diabetes [7,8]. Reduced antioxidant status coexistence with hyperglycemia results in formation of heterogeneous molecules complexes known as advanced glycation end products (AGEs) [9]. Cytotoxicity of AGE adducts have been hypothesized to be tightly intertwined with OS [10,11]. Metal-catalyzed oxidation reactions were also found to

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increase the rate of AGEs production. Accordingly, compounds with both antioxidant and antiglycation (anti-glyoxidants) properties would be ideal candidates to suppress harmful effects caused by FRs in biological systems, as well as to obstruct the AGE-formation-based mechanism — pathways in diabetic patients. Recently, we reported that anti-AGE activity of balm extract was associated with its antioxidant properties [12]. Madhu and Devi demonstrated that OS was diminished by vitamins C and E intake in diabetic rats [13]. Moreover, it has been found that the hemoglobin glycation decreases with the supplement of vitamin C in the diabetic patient [14].

Current efforts have being made to synthetize coumarin modified analogs with better antioxidant properties and reduced adverse effects. In a previous paper, 4-hydroxycoumarin-3-carboxamide derivatives have been synthesized as potential drugs for the treatment of insulin-dependent diabetes mellitus [6]. The objective of this study was to evaluate if synthesized 4-ACDs exhibit antioxidant properties and if it is associated with antiglycemic activity.

2. Materials and methods

2.1. Chemicals

2,2-Azinobis(3-ethylbenzothiazoline-6-sulphonate) diammonium salt (ABTS), ascorbic acid, β-carotene, 2-deoxy-D-ribose, 2,2′-diphenyl-1-picrylhydrazyl (DPPH), disodium salt of ethylenediamine tetraacetic acid (EDTA), ferrozine, Folin–Ciocalteu reagent, gallic acid, hypoxanthine, iron(III) chloride, iron(II) chloride, linoleic acid, potassium hexacyanoferrate, trichloroacetic acid (TCA), Trolox, human serum albumin (HSA) and glucose were from Sigma Chemical Company (Germany).

2.2. Synthesized/tested compounds: analogues of 4-aminocoumarin

Three 4-ACDs were synthesized and diluted in relation to their water solubility [3 mg/mL], according to Ivanov et al. [15]: N-{2-[(2-oxo-2H-chromen-4-yl)amino]ethyl}acetamide or aminoethylacetamide (1), 4-[2-hydroxypropyl)amino]-2H-chromen-2-one or aminoalcohol (2) and N-{2-chromen-4-yl)ami-no]propyl}acetamide or aminopropylacetamide (3) [15] (Scheme 1).

2.3. In vitro antioxidant potential measurements

2.3.1. DPPH free-radical scavenging activity

The DPPH assay is based on the ability of an antioxidant [16] to donate hydrogen to DPPH radical (DPPH•). The change in color of DPPH• (from purple to yellow) is the measure of free radical scavenging activity. The hydrogen-donating activity of the 4-ACDs was measured according to the method by Gyamfi et al. [17]. By accepting hydrogen (H+ and e-), purple-colored DPPH• is being converted into the non-radical form (DPPH-H), yellow-colored diphenylpicrylhydrazine. Briefly, 50 mL of dissolved 4-ACDs was mixed with 450 mL of Tris–HCl buffer (50 mmol/L, pH 7.4) and 1 mL of (0.1 mmol/L) DPPH• (dissolved in methanol). After 30 min, the absorbance was recorded at 517 nm (absorption max for DPPH•). The percentage of inhibition was calculated using [Equation 1] and the concentration of the compound at which it exhibits 50% inhibition (IC_{50}) value was estimated using a non-linear regression algorithm.

$$\text{Percentage inhibition} = \left[\left(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}\right)/\text{Abs}_{\text{control}}\right] \times 100 \quad \text{[Equation 1]}$$

2.3.2. $ABTS^{\bullet+}$ free-radical scavenging activity

The antioxidant capacity of the tested compounds was estimated by the method of Re et al. [18]. The blue/green colored $ABTS^{\bullet+}$ solution used for the measurement of 4-ACDs antioxidative activities was prepared by mixing ABTS (10 mL of 7 mmol/L) with oxidizing agent $K_2S_2O_8$ (5 mL of 2.45 mmol/L) for 12–16 h in the dark and subsequently diluted with ethanol (a dilution of between 1/50 and 1/400 was performed in order to obtain absorbance value of 0.700, at 734 nm). The reduction of the radical cation ($ABTS^{\bullet+}$) by 4-ACDs was determined as decolorization at 734 nm, *i.e.* the percentage inhibition of absorbance of the $ABTS^{\bullet+}$ solution (since 1 min upon mixing of 1.5 mL of the prepared $ABTS^{\bullet+}$ solution with 15 μL of 3 mg/mL4-ACDs samples, at 5 min intervals, for 40 min).

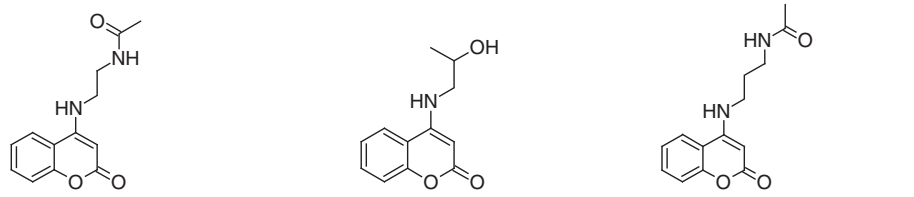
The results are expressed as the Trolox equivalent antioxidant capacity (TEAC, mmol/L Trolox) at different time intervals.

2.3.3. FRAP (ferric reducing antioxidant power) assay

The principle of this assay is based on one-electron reduction of Fe (III)/ferricyanide complex to the ferrous form Fe (II) [19]. In brief, 1 mL of 3 mg/mL compound was mixed with 2.5 mL of phosphate buffer (0.2 mol/L, pH 6.6) and 2.5 mL of a 10 g/L $K_3Fe(CN)_6$, and incubated at 50°C, for 30 min. After the incubation, 2.5 mL of a 100 g/L TCA solution was added to terminate the reaction and the mixture was centrifuged for 10 min (1800 rpm). Finally, 2.5 mL of supernatant was used to mix with 2.5 mL ultra-pure water and 0.5 mL of a 1 g/L $FeCl_3$. The absorbance was recorded at 700 nm and the data were presented as ascorbic acid equivalents (AscAE; mmol ascorbic acid/g sample).

2.3.4. β-Carotene-linoleic acid bleaching inhibition

The determination of antioxidant activity was evaluated by the ability of the compounds to inhibit the bleaching of the β-carotene by linoleic acid. Namely, during the incubation at 50°C linoleic acid produces peroxyl radical which becomes neutralized by the presence of antioxidants, at the same time the β-carotene oxidation is avoided (*i.e.* inhibition of β-carotene bleaching occurs, thus yellow color of β-carotene in the system persists in the presence of antioxidant) [20]. Briefly, 0.2 mg β-carotene dissolved in 1 mL chloroform, 20 mg of linoleic acid and 200 mg of Tween 20 were transferred into a round-bottom flask. Once the chloroform had been removed under



Scheme 1. Structures of tested compounds.

the nitrogen stream, 50 mL distilled H_2O was added and the resulting mixture was stirred for 30 min. Five mL aliquots of the emulsion were mixed with either 200 mL of dissolved compounds or 200 mL of positive controls (1 mg/mL) and measured at 470 nm twice, before and after the 2 h incubation in a water bath at 50°C for 2 h. The data were presented as a percentage of an antioxidant activity % (AA %), using [Equation 2]:

$$AA\% = \left[1-\left(Abs_{0sample}-Abs_{120\ sample}\right)/\left(Abs_{0control}-Abs_{120\ control}\right)\right] \times 100$$

[Equation 2]

2.4. Antiglycation potential of the tested compounds: in vitro measurements

2.4.1. Preparation of the glycated HSA

The stock solution of 4 mg HSA/mL [prepared in 50 mM phosphate buffer (pH 7.4)] was mixed with equal volume of 100 mM glucose solution (dissolved in the same buffer) to gain a final concentration of 2 mg/mL HSA and 50 mM glucose. Samples (50 mL of 2 mg/mL HSA as control and 50 mL of 2 mg/mL HSA with 50 mM glucose) were pre-incubated in the presence and absence of each of the synthetic compounds. All samples were sterile-filtered with a 0.22 μm millipore filter and incubated in capped vials at 37°C for up to 8 weeks. Unbound glucose molecules were removed from HSA by exhaustive dialysis against 50 mM phosphate buffer pH 7.4 and stored at 4°C prior to analysis.

2.4.2. Congo red binding assay

Congo red binding to amyloid cross-β structure of HSA was performed by Klunk et al. method [21]. The absorbance of the formed complex was measured at 530 nm. Briefly, 500 μL of each sample (HSA, glycated HSA with and without each compound) were incubated with 100 μL of Congo red (100 μM) in PBS with 10% (*v*/v) ethanol for 5 min at room temperature and afterwards, the absorbance of the reaction mixture was measured.

2.4.3. Intrinsic fluorescence measurements

Intrinsic fluorescence spectra of native and glycated-HSA, incubated with 50 mM glucose in the presence and absence of the coumarins were collected. Both, native and glycated-HSA were excited at 270 nm, and the spectra were corrected for buffer baseline fluorescence. The glycation of HSA was also confirmed by AGE-related auto-fluorescence assay. Fluorescence of relevant samples (0.15 mg/mL) was measured after exciting at 270 nm, and monitoring the emission at 295–450 nm using a Hitachi F-2500 spectrofluorometer. Correction for spectra was done with the appropriate protein and buffer blanks.

2.4.4. Circular dichroism (CD) spectropolarimetry

All far-UV CD spectra of the mixtures of the tested compounds with HSA-GA, and HSA were recorded in the far-ultraviolet region (190–250 nm) using solutions with a protein concentration of about 0.15–0.2 mg/mL. Experiments were performed on a 215 Aviv spectropolarimeter (USA) and measuring parameters were set as follows: bandwidth: 1 nm, response time: 2 s; scan speed: 20 nm/min; and resulted spectra is an average of four scans; baseline was corrected for the respective blanks. Results are expressed as molar ellipticity, $[\Theta]$ deg·cm^2 dmol^{-1}, based on a mean amino acid residue weight (MRW).

3. Statistical analysis

One-way ANOVA and Tukey's post hoc multiple test was used (software GraphPad version 5) for statistical data analysis. Values are presented as the means ± standard deviation. Correlation coefficients were calculated using the Spearman's test. Differences were considered statistically significant for $p < 0.05$ for all tests [31].

4. Results and discussion

Three 4-ACDs (compounds 1–3) were evaluated *in vitro* for the potential antioxidant properties. We attempted to clarify if the structurally different side chains of the 4-ACDs interfere with their antioxidative and/or antiglication activities.

Due to known limitations of the *in vitro* methods [22], the antioxidant capacity of the tested compounds was measured by four different assays including: DPPH and ABTS radical scavenging activities, iron (III) reduction power and inhibition of β-carotene-linoleic acid bleaching assay. Achieved antioxidant activities closely depend to the applied assays and is related decisively to the conditions and required red-ox mechanism patterns, specifically for the particular test. Thus, obtained values of the antioxidant capacity for the certain compound differs according to the applied test. The principles of these measurements are different and are based on: a) one or two electron(s) transfer between the particular substrate and tested compound (FRAP assay); b) hydrogen radical (H•) donating to certain substrate (ABTS• and DPPH• assays); or c) spearing already present antioxidant (for instance: β-carotene bleaching assay) [23]. Thus, documented AO activity for one substance measured by one test does not mean that it will be accomplished by using another test for the same substance.

The DPPH and ABTS assays were used to test the ability of the examined 4-ACDs to donate H• and thus neutralize corresponding reactive radicals (DPPH• and ABTS•$^+$). After H• abstraction from the enamine group at the position 4 of the coumarin ring, formed coumarin radical is partially stabilized by resonance and induces stability [24].

Based on the nitrogen-centered DPPH• assay, only compound-1 showed the free radical scavenging potential, i.e. antioxidative effect (Fig. 1a), although significantly lower than vitamin C ($p < 0.0001$) [25].

An alternative test that provides good estimation of the antioxidant ability, applicable for both lipophilic and hydrophilic antioxidants is ABTS•$^+$ decolourization assay which also enables spectral interferences [26]. In general, highly pigmented and hydrophilic antioxidants are better assessed for antioxidant activity using the ABTS assay compared to the DPPH assay [25]. The ABTS•$^+$ is a moderately stable nitrogen center radical [20] with higher solubility than DPPH•. Additional advantage of the ABTS assay is that it can be used in different pH ranges and therefore takes into consideration the effect of pH on the antioxidant activity of the tested sample [27]. All of the compounds [3 mg/mL] reached maximal activity (plateau) at 30 min and to the following intensities: compound-1 > compound-2 > compound-3 (Fig. 1b).

The results of the ABTS assay should be comparable to the results obtained by DPPH assay and may be considered as a confirmation of the DDPH assay, although the absolute values from the ABTS assay are generally higher [28]. Both radicals (DPPH•, ABTS•$^+$) show the same stoichiometry with water-soluble vitamin E analogue, Trolox (*e.g.*, two moles of ABTS•$^+$ [29] or two moles of DPPH radicals [30] are scavenged by one mole of Trolox).

One of the mechanisms by which antioxidants achieved their role is related to their reducing potency [26]. For this reason, FRAP assays were used to measure capability of the compounds to reduce Fe^{3+} into Fe^{2+} in acidic medium, in the presence of e-donors. The data were presented as ascorbic acid equivalent (AscE) values. All tested compounds [3 mg/mL] were capable of reducing Fe^{3+} and comprised similar reducing potential, around double lower than vitamin C (Fig. 1c). Having similar effect, we concluded that e^- donating properties of the tested compounds are addressed to the coumarin part of the molecule, independently of substituents on the position 4. Keto enol tautomerie might occur in coumarin heterocyclic part of the molecule and in the amide part. Lactone carbonyl group is coumarin heterocyclic part which is an electron acceptor. The tested 4-ACDs showed similar redox potential evaluated by FRAP assay, suggesting that differences in the structure of alkyl radicals in position

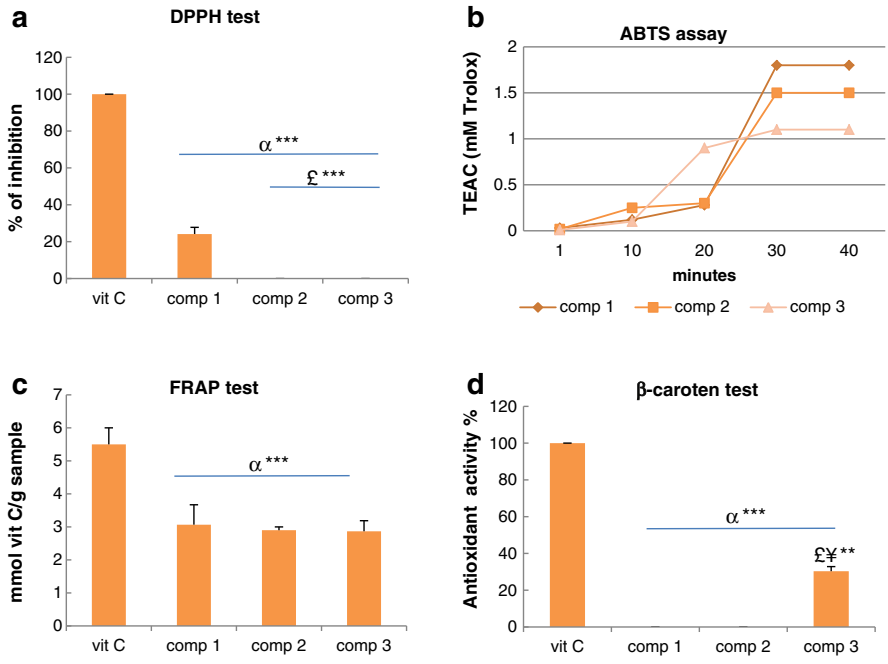


Fig. 1. Antioxidative capacity of 4-aminocumarin derivatives [3 mg/ml] measured by the following assays: (a) DPPH•; (B) ABTS•$^{+}$; (c) FRAP; (d) beta-carotene bleaching inhibition. (a): DPPH assay: the results are presented as a percentage of inhibition the absorbance at 517 nm (absorption max for DPPH•); (b) ABTS assay: the results are expressed as the Trolox equivalent antioxidant capacity (TEAC, mmol/L Trolox) at different time intervals; (c) FRAP assay: the data were presented as ascorbic acid equivalents (AscAE; mmol ascorbic acid/g sample); (d) β-Carotene bleaching: The data were presented as a percentage of an antioxidant activity % (AA %). Values are presented as means ± S.D. Statistical significance was considered at: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.0001$. Labels of statistical significance: α: significantly different from vit C; £: significantly different from compound-1; ¥: significantly different from compound-2.

4 have no influence on the redox potential of the tested compounds. The tested compounds [3 mg/mL] accomplished almost double lower AO activity compared to vitamin C ($p < 0.0001$).

What guided us to try β-carotene–linoleic bleaching inhibition assay was the finding that the side chain in the 4-ACDs was the reason for increased lipophilicity and lower reducing potential than hydrophilic vitamin C. Considering that lipo- or hydro-philicity of 4-ACDs may interfere with AO respond, we employed additionally a membrane-based lipid peroxidation model, using β-carotene-linoleic bleaching inhibition assay to test if the examined compounds [3 mg/mL] may prevent/inhibit lipid peroxidation [28]. A membrane-based lipid peroxidation model, using β-carotene-linoleic bleaching inhibition assay offers a good biologically relevant matrix compared to DPPH and ABTS free-radical scavenging assays. Briefly, in this system, linoleic acid in an oil–water emulsion phase undergoes thermally induced oxidation, thereby producing free radicals which react with the β-carotene's chromophore, resulting in a bleaching effect [20]. Only compound-3 showed AO activity, by using the β-carotene bleaching test, what is in accordance with side chain length, although only 30% of vitamin C activity ($p < 0.0001$) (Fig. 1d). It is known that lipophilicity increases with the CH length increase for approximately 0.5 (log p = [comp] org/[comp] H_2O) [25].

Diabetes is an oxidative stress-associated disease, thus the long-term complications in diabetes may partially be reduced by taking antioxidants. To examine if the 4-ACDs prevent glycation of proteins *in vitro*, Congo red binding assay in the HSA/glucose system [2 mg/mL HSA and 50 mM glucose] was applied. Recently, we have reported that secondary structure alteration of albumin occurs if exposed to glucose *in vitro* [8]. The amount of the secondary structure alterations due to glycation can be followed by measuring the absorbance at 530 nm, which reflects the intensity of the binding of the dye to the hydrophobic clefts between anti-parallel β-strands [21]. All compounds [3 mg/ml] significantly inhibited the secondary structure alterations of HSA, compared to glycated HSA (G-HSA) (for compounds1–3: $p < 0.05$ and for compound-2: $p < 0.001$) (Fig. 2). We showed that 4-ACDs have potential to prevent glycation of HSA hindering the transition of α to β conformer of HSA, therefore they may have a suppressive role in the late stage (post-Amadori) of HSA glycation. Slightly higher suppressive effect of compound 2 compared with compounds 1 and 3 on glucose-induced HSA damage may be prescribed to the hydroxyl group of the side chain i.e. the absence of acetamide group.

Furthermore, due to the possibility of the tested compounds to prevent secondary structure alteration of HSA, the HSA-AGE structure was analyzed in greater detail with CD spectropolarimetry. As illustrated in Fig. 3, a large alteration in the secondary structure of

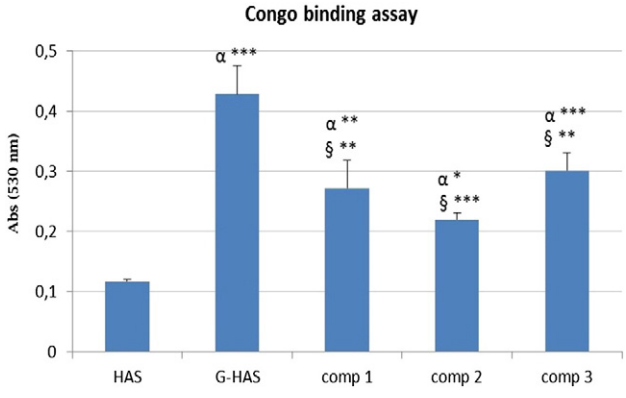


Fig. 2. Time evolution of the secondary structure alteration of HSA [2 mg/mL HSA and 50 mM glucose] by Congo red assay. Values are presented as means ± S.D. Statistical significance was considered at: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.0001$.Labels of statistical significance: α: significantly different from HSA; §: Significantly different from G-HSA. The values are expressed as average ± STDEV and represented plateau (reached 6–10 weeks).

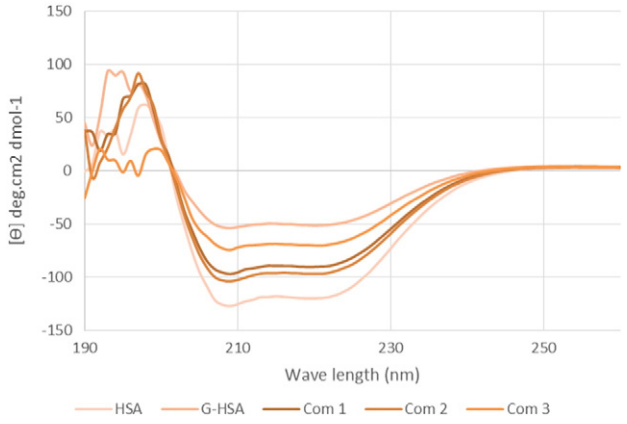


Fig. 3. Far-UV CD spectra of native HSA and glycated HSA [2 mg/mL HSA and 50 mM glucose] in the presence and/or absence of the 4-aminocumarin compounds [3 mg/mL]. CD data were expressed as molar ellipticity (deg cm^2 $dmol^{-1}$).

HSA occurred if not treated with the synthesized compounds. Evidence from AGE formation studies suggests the appearance of cross-β structures of proteins as a unique recognition signal for globular protein structures changes upon glycation [29]. We showed that the aminocoumarins profoundly prevent the structural changes caused by D-glucose, keeping the protein molecule close to its native polar conformation. The tested compounds [3 mg/mL] have potential to keep proteins in α-conform by concealing the glycation sites and lowering the extent of the solvent-accessible surface area, thereby producing barriers for cross β-structure formation.

Oxidative stress in diabetes is coupled with the formation of AGEs within non-enzymatic glycation reactions [27]. In order to evaluate the ability of used compounds [3 mg/mL] to prevent AGE formation, autofluorescence measurements were performed. The glycophore fluorescence (λ_{ex}, 280 nm) spectra of albumin-AGE preparations (0.15 mg/ml) were recorded and compared with those in the presence of the synthesized coumarins [3 mg/mL]. Fluorescence intensity at 295–445 nm was decreased in the glycated sample (Fig. 4) and retained to be reduced upon the treatment with the tested compound-2. The two other compounds failed to protect HSA conformational alteration under the glycation. The molecular basis for this effect appears to be linked with hydroxyl group of compound-2 hindering the incorporation of albumin with C1′–OH of glucose, thereby interfering with Schiff base formation and the subsequent Amadori reaction.

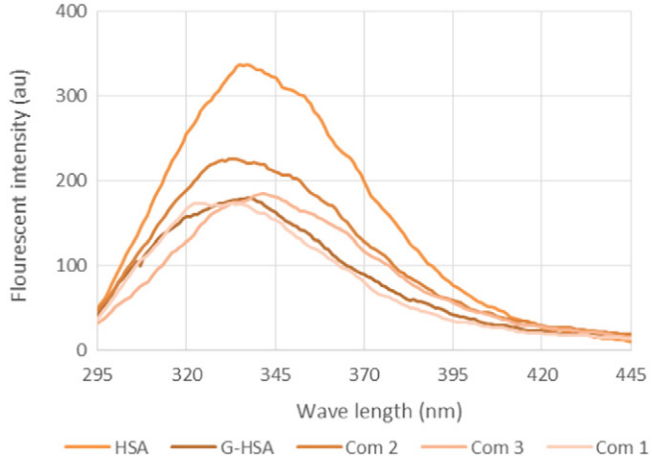


Fig. 4. Fluorescence spectra of glycated HSA (0.15 mg/mL). Spectra were obtained in the wavelength range of 295–445 nm after excitation at 270 nm in the presence and/or absence of the compounds [3 mg/mL].

5. Conclusion

In summary, we showed that *in vitro* tested 4-ACDs achieved moderate antioxidant effect (comparing with vitamin C) and antiglycant activity, indicating their possible protective role against oxidative biomolecule damage *in vivo*. We showed that antioxidant properties of the tested compounds correlate inversely with antiglycant activity. Compound-2 accomplished stronger antiglycant activity than compounds 1–3, but weaker antioxidative effect and *vice versa*. We demonstrated that substituents on the position 4 of the aromatic ring of the tested coumarins influence antiglycant activity. Observed moderate antioxidant effects of the 4-ACDs along with antiglycant properties should be considered as rationale for further *in vivo* investigation related to their potentially preventive/protective role in diabetes.

Conflict of interest

The authors declare that they have no conflict of interest.

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