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In Vitro Pro-Glycative Effects of Resveratrol and Caffeic Acid

Original research article/Review

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Abstract Resveratrol and caffeic acid belong to plant polyphenols and are known for their antioxidant effects. The aim of our research was to study their impact on Maillard reaction. This one occurs when the reducing saccharides react with amino groups of biomolecules including proteins, alter their protein conformation and transform to the variety of advanced glycation end products (AGEs). AGEs exhibit browning and generate fluorescence. There exist expectations that this oxidative protein glycosylation could be prevented by antioxidants. In this study, we incubated bovine serum albumin (BSA) with glucose for 7 days at 37°C and measured characteristic fluorescence and UV absorbance of the formed AGEs. Surprisingly, resveratrol and caffeic acid enhanced transformation of BSA to glycation products, which was confirmed either when cupric Cu(II) or ferric Fe(III) ions in nanomolar concentration were added to the system as pro-oxidant agent.

Keywords *Protein glycation – BSA – AGEs – caffeic acid – resveratrol*

INTRODUCTION

Polyphenols belong to a large and heterogeneous group of phytochemicals. They are present in food such as tea, coffee, wine, cereal grains, vegetables and fruits. The structural diversity of polyphenols extends from simple monophenolic substances (*e.g.*, *p*-hydroxycinnamic acid) to large polymeric macromolecules like proanthocyanidins and ellagitannins (Hanhineva, 2010). Recently, polyphenolic compounds have shown their biological activities linking to human health benefits, such as antioxidant, cardioprotective, anticancer, antiinflammatory, antiaging and antimicrobial properties (Xia et al., 2010, Pascual-Teresa et al., 2010, Kurin et al. 2012a). Resveratrol possesses some of these effects. There is growing evidence that resveratrol can prevent or delay the onset of cancer, heart disease, ischemic and chemically induced injuries, diabetes, pathological inflammation and viral infection (Baur & Sinclair, 2006, Kurin et al, 2013). Caffeic acid has been studied due to its antibacterial, antifungal, antiviral and antiproliferative properties (Matus, 2010). Both, resveratrol and caffeic acid are known as antioxidant agents (Wang et al., 1999, Kurin et al. 2012b).

Well-known antioxidant effect of both molecules is connected with the protection from reactive oxygen species (ROS). ROS are continuously produced during normal physiological events; there should be a balance between the generation and inactivation of ROS by the functional antioxidant system in an organism. ROS are overproduced under pathological conditions and the result is an oxidative stress, which leads to different oxidative modifications of cellular membranes or intracellular molecules (Gülçin, 2006). However, the same polyphenols can act as pro-oxidant under certain experimental conditions, for example, depending on the concentration, or source of free radicals (Alarcón De La Lastra & Villegas, 2007) and particularly in the presence of transition metal ions such as iron or copper (Bhat et al., 2007). Resveratrol can act as a pro-oxidant of DNA through reduction of ADP-Fe(III) (Miura et al., 2000) and also by switching to pro-oxidant in the presence of Cu(II) by the ROS generation (Alarcón De La Lastra & Villegas, 2007, Hadi et al., 2010). Similar effects were observed for caffeic acid as well (Bhat et al., 2007, Fan et al., 2009). Caffeic acid accelerated LDL oxidation rate in the propagation phase, which means that it exerts pro-oxidant activities in free radical chain reactions such as lipid peroxidation (Yamanaka et al., 1997).

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Oxidation of polyphenols produces $O_2^{\cdot}$, H_2O_2 and a complex mixture of semiquinones and quinones, which are potentially cytotoxic (Halliwell, 2008). Formation of pro-oxidant molecules has been observed in model systems during the early phases of Maillard browning (López-Galilea et al., 2006). Highly reactive radicals are formed in the early stages of the Maillard reaction just prior to the Amadori rearrangement and their disappearance is accompanied by a gradual development of browning (Nicoli, 1999). Reducing saccharides react with amino groups of biomolecules including proteins, lipids, and nucleic acids during Maillard reaction to form Schiff bases. These ones in turn undergo a transformation to the variety of AGEs (Fatima, 2008). Increasing protein glycation and the gradual build-up of AGEs in body tissues caused by hyperglycaemia play an important role in the pathogenesis of diabetic complications (Ahmed, 2005). In this study, we incubated bovine serum albumin (BSA) with glucose and measured characteristic fluorescence and UV/VIS absorbance of the created AGEs. Effects of resveratrol and caffeic acid were examined in this system with or without the presence of cupric or ferric ions as known pro-oxidant agents.

MATERIAL AND METHODS

Incubation of BSA with glucose

The reaction was carried out under the conditions as reported by Morimitsu et al. (1995) with some modifications. The reaction mixture was made of 250 mg D-(+)-glucose (ACS reagent, Sigma-Aldrich, China) and 25 mg bovine serum albumin (pH 7, ≥ 98%, Sigma-Aldrich, USA) in 2.5 ml sodium phosphate buffer (PBS, 67 mM, pH 7.2) containing $Na_2HPO_4 \times 12\ H_2O$ and $NaH_2PO_4 \times 2\ H_2O$ (p.a., Centralchem, Slovakia). Mixture was incubated at 37°C for 7 days with or without the tested compound, diluted with distilled water in 2.5 ml. Samples (100 μM): caffeic acid (CA, ≥ 98%, Sigma-Aldrich, USA) and resveratrol (Re, ≥ 99%, Sigma-Aldrich, USA) were used with aminoguanidin (AG, ≥ 98%, Sigma-Aldrich, USA) as a positive control. Cupric ions ($CuSO_4 \times 5\ H_2O$, Lachema, Czech Republic) or ferric ions ($FeSO_4 \times 7\ H_2O$, Lachema, Czech Republic) (3.906 nM) were added to another tested group and were incubated in the same way.

Fluorescence measurement

The formation of fluorescent AGEs was assessed by characteristic fluorescence of the glycated BSA and was measured at 370 nm excitation wavelength and 440 nm emission one according Wu et al. (2009) using Tecan Infinite M200 (Tecan AG, Austria) microplate reader and 96-well Nunc PP black (0.5 ml, round bottom) microplates. The value (%) of glycation inhibition by different concentrations of the tested polyphenols was calculated as follows: [1 - (fluorescence of the test group/fluorescence of the control group)] ´ 100. All measurements were done in quadruplicate.

UV absorbance measurement

Browning of the samples of different concentration were recorded by their absorbance in 96-well Greiner UV-Star microplates (Greiner-Bio One GmbH, Germany) with Tecan Infinite M200 microplate reader (Tecan AG, Austria) at 420 nm according to Morales & Jiménez-Pérez (2001). All the measurements were done in quadruplicate.

Statistical analysis

All the data were expressed as mean ± SD. Differences between the groups were examined for statistical significance using the Student's *t*-test. A *p*-value less than 0.05 was considered as significant.

RESULTS AND DISCUSSION

It is now well recognized that the reaction of reducing saccharides with proteins can cause marked alterations in protein conformation. Several investigators have shown that reaction of saccharides and dialdehydes with protein can also lead to the formation of structures showing strong emission between 400 and 500 nm, when excited at a wavelength of 370 nm (Fatima et al., 2008, Plaza et al. 2010). This method is different from the fluorescence spectroscopy of BSA excited at 295 nm and emission collected between 260 and 400 nm, where intrinsic fluorescence of albumin are observed (Dufour & Dangles, 2005).

Fig. 1 shows the fluorescence spectra of BSA solutions in the absence and presence of glucose in PBS at pH 7.2 and 370 nm excitation wavelength. We observed only a small increase in the BSA fluorescence intensity in the presence of glucose as compared to the untreated sample.

However, after 7 days of incubation with glucose, fluorescence intensity was measured at 370 nm excitation and 440 nm emission wavelengths. A significant increase of relative fluorescence (BSA fluorescence = 7 399.75 ± 850.43 *vs*. BSA fluorescence treated with glucose = 19 055.00 ± 1 135.85, $p < 0.001$) was observed. This can be caused by new fluorophore formation (Fatima et al., 2008). Protein glycation initiated by a nucleophilic addition reaction between a free amino group of a protein and a carbonyl group of a reducing saccharide forms a reversible Schiff base. This reaction can occur over a period of hours, and once formed, the labile Schiff base rearranges to a more stable ketoamine or Amadori product. It needs a period of days to be formed and then it is practically irreversible. Glycated proteins can undergo further reactions giving rise to AGEs. AGEs exhibit browning and generate fluorescence.

There exist expectations that whereas the protein glycosylation is an oxidative reaction, antioxidants should be able to prevent this reaction. Study of Asgary describes an inhibition of haemoglobin glycation by quercetin, rutin and kaempferol (Asgary et al., 1999). Urios measured inhibition

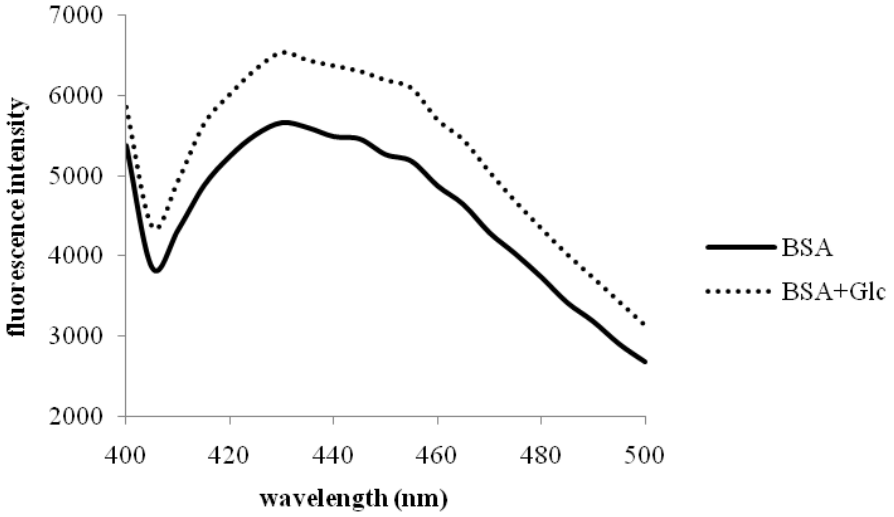

Figure 1. Fluorescence spectra of BSA solutions in the absence (—) and presence (·····) of glucose in PBS pH 7.2, without incubation, λ_{EXC} = 370 nm, λ_{EM} = 440 nm.

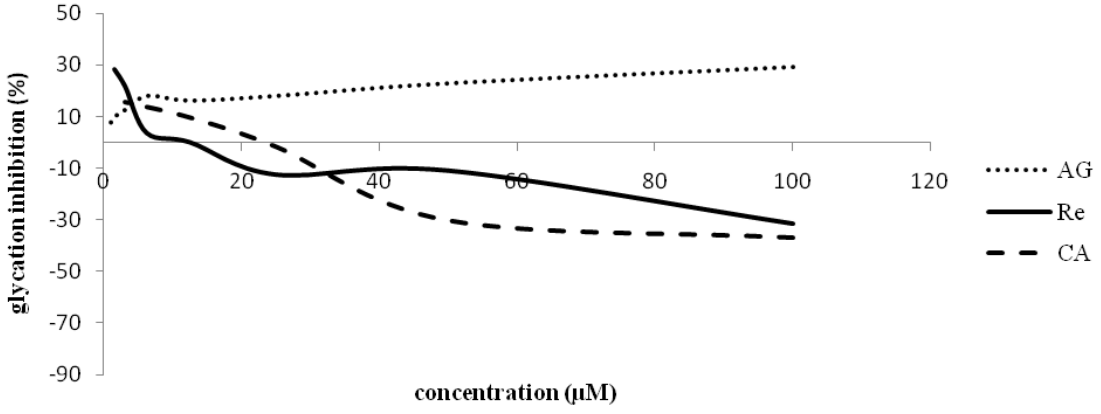

Figure 2. Glycation inhibition (%) of 100 μM samples (AG – aminoguanidin, CA – caffeic acid, Re – resveratrol) after 7 days incubation with BSA and glucose at 37 °C, λ_{EXC} = 370 nm, λ_{EM} = 440 nm.

effects of some monomeric and oligomeric flavonoids on pentosidine formation in collagen incubated with glucose (Urios et al., 2007). Morimitsu tested methanol extracts of 34 types of spices for the inhibitory activity of the AGEs formation, and even though most of these were inhibiting, some of them accelerated the formation of AGEs (i.e., mustard, tarragon, cinnamon, cardamom, cumin, coriander, celery) (Morimitsu et al., 1995).

Fig. 2 shows the results of 7 days of incubation of BSA with glucose in the presence of resveratrol, caffeic acid and aminoguanidine, respectively. Aminoguanidine, which acts as a nucleophilic scavenger by blocking the first step in the glycation (Lunceford & Gugliucci, 2005), was used as a positive control. Surprisingly, resveratrol and caffeic acid enhanced the transformation of BSA to glycation-like products in concentration dependent manner.

Many polyphenols (flavonoids, caffeic acid) possess the ability to reduce transition metal ions, and consequently, to act as pro-oxidants (Simić et al., 2007). It was also described that Cu(II)-induced pro-oxidant activity of phenolics proceeds *via* intra- and inter-molecular electron transfer reactions accompanying ROS formation, and a copper complexation followed by an oxidation of resveratrol analogues (e.g., 3,4-dihydroxystilbene) ended up with quinone production (Apak et al., 2007). As shown in the Fig. 3, the combination of polyphenols with metals leads to the concentration dependent formation of BSA glycated/transformed products. Only the combination of ferric ions with resveratrol results in a slight inhibition of the glycation. The increase in proglycative (= pro-oxidative) activity of phenolics in the presence of Fe(III) or Cu(II) is primarily associated with their ability to reduce metal ions. Subsequently, Fe(III) and Cu(II) can be re-oxidized in Fenton-type reactions leading to the production of hydroxyl radical and other ROS. The antioxidant/pro-oxidant activities of phenolics are determined by many factors: the concentration and nature of transition metal ion(s) and the concentration and pH of phenolics (Apak et al., 2007).

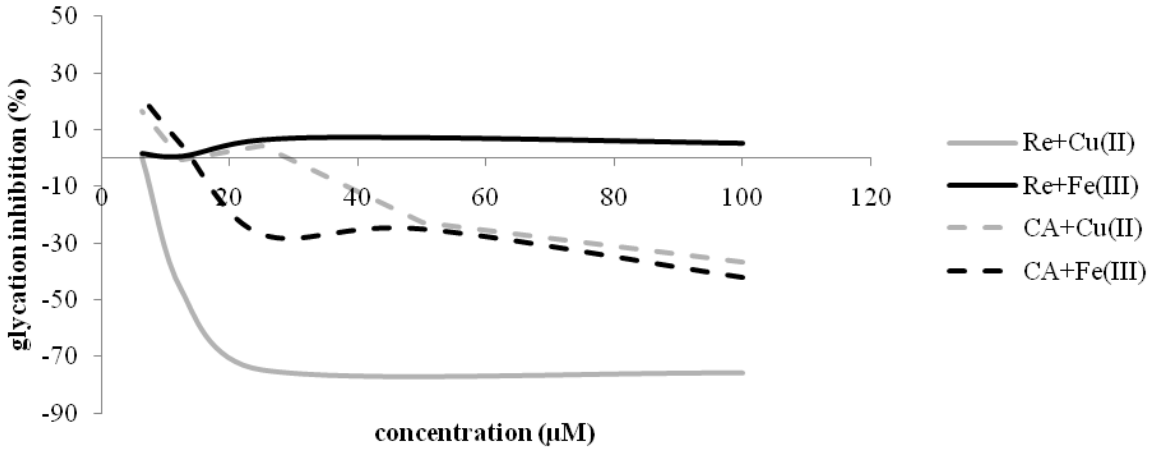

Figure 3. Glycation inhibition (%) of polyphenols at 100 µM (CA – caffeic acid, Re – resveratrol) with metal catalyst Cu(II) or Fe(III) (3.906 nM) after 7 days incubation with BSA and glucose at 37 °C, λ_{EXC} = 370 nm, λ_{EM} = 440 nm.

The Maillard reaction produces a variety of intermediate products and the final brown pigments (Lertittikul et al., 2007). Intensity of brown colour is often used as an indicator of the extent of the reaction. It symbolizes an advanced stage of the Maillard reaction (Plaza et al., 2010). Because of the variety and the complexity of Maillard reaction products, it is usually admitted to classify them into early (or precursors), advanced and final products. Following this classification, the intensity of non-enzymatic browning was generally based on the changes in absorbance at 294–297 nm, 320–350 nm and 420–450 nm, respectively.

Cu(II) or Fe(III) ions were used as a catalyst for pro-oxidant action of the polyphenols in the presence of protein and glucose. As Fig. 4 shows, we observed an absorbance increase of samples of BSA + glucose and resveratrol (100 µM) or caffeic acid (100 µM) when incubated with ferric ions. Only caffeic acid with cupric ions did not show any impressive changes in the *in vitro* glycation model. An increased absorbance in the 340–360 nm regions should correspond to the formation of heterocyclic derivatives and intermediate water-soluble compounds (reductones, amino-reductones or pre-melanoidins). Conversely, absorbance values at 420 nm should correspond to the formation of brown pigments or melanoidins (Billaud et al., 2004).

The absorbance increase at 420 nm is used as an indicator for the browning development in the final stage of the browning reaction. As Fig. 5 and Fig. 6 show, we observed an absorbance increase in the reaction mixture of BSA depending on the concentration of polyphenols and the presence of metals after 7 days of incubation. Fluorescent compounds – AGEs – are also possible precursors of brown pigments. The higher concentration of saccharide used, the higher the increase in browning was found (Billaud et al., 2005). We observed, at a stable concentration of glucose and varying concentration of polyphenols, a higher content of resveratrol or caffeic acid in the presence of pro-oxidant metal ions led to a higher increase in browning. Thus, we can postulate that the presence of brown pigments are the Maillard reaction products in the reaction mixtures.

Results of fluorescence and absorbance measurements indicated that the Maillard reaction had progressed to advanced stages in the amino acid–glucose reaction. However, coloured and fluorescent compounds need not be identical and fluorogens may be precursors of brown pigments showing a shorter induction period (Morales & Jiménez-Pérez, 2001).

In our work, using fluorescence measurement of AGEs, we observed that resveratrol and caffeic acid incubated with glucose and BSA accelerated formation of AGEs after 7 days at 37°C. The nanomolar presence of Cu(II) confirmed the pro-glycative effects of resveratrol and caffeic acid. Presence of Fe(III) with caffeic acid increased the formation of AGEs, but there was observed slight inhibition of the glycation with resveratrol. The observed pro-glycative effects of polyphenols can be based on the pro-oxidant activity of these. Resveratrol undergoes oxidation in the presence of Cu(II). The oxidative product of resveratrol is a dimer, which might be formed by dimerization of resveratrol phenoxyl radical as a result of the reductive activation of molecular oxygen (Alarcón De La Lastra & Villegas, 2007). Caffeic acid could dissociate to form a phenoxide, which chelates Cu(II) ions as bidentate ligand and undergoes intramolecular electron transfer to form an *o*-hydroxyphenoxyl radical (semiquinone radical). The radical intermediate was also proven by the formation of the caffeic acid dimer (furofuran bislacton) in the presence of Cu(II) ions (Fan et al., 2009). Pro-oxidant action of plant polyphenols may be an important mechanism of their anti-cancer or apoptosis-inducing properties (Hadi et al., 2010), and therefore, our results can be useful for the next research of their exact mechanism of action. However, in multicomponent mixtures, the risk of the pro-oxidative and pro-glycative effect is present, when phenolics are combined with even

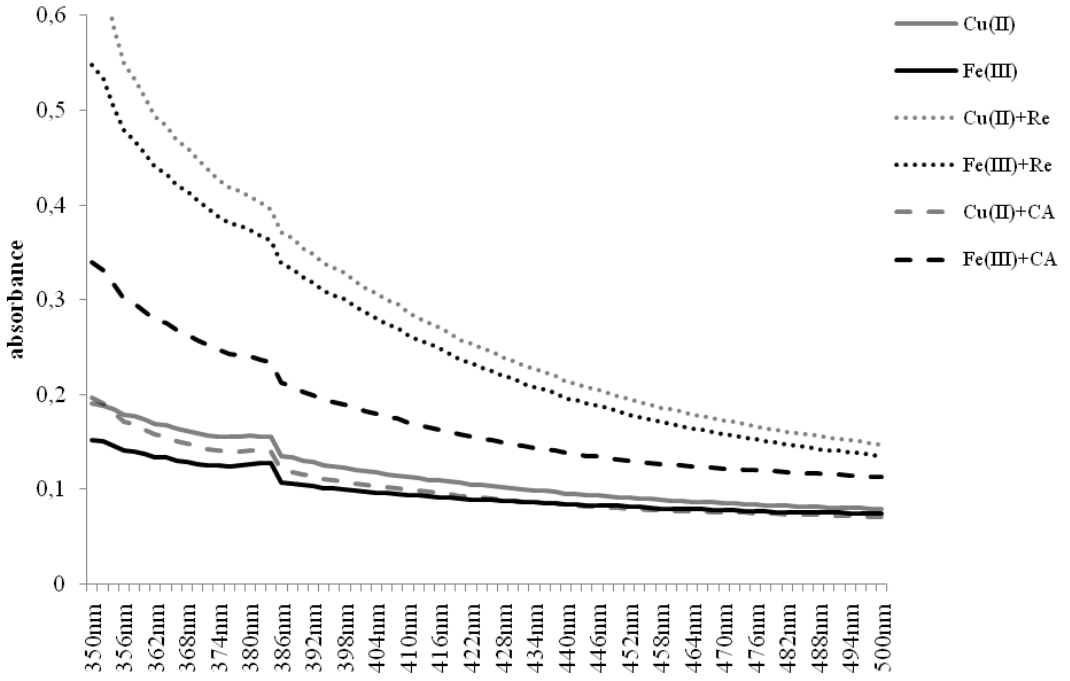

Figure 4. Absorbance of polyphenols (CA – caffeic acid, 100 μM, Re – resveratrol, 100 μM) with metal catalyst Cu(II) or Fe(III), always 3.906 nM, after 7 days incubation with BSA and glucose at 37 °C.

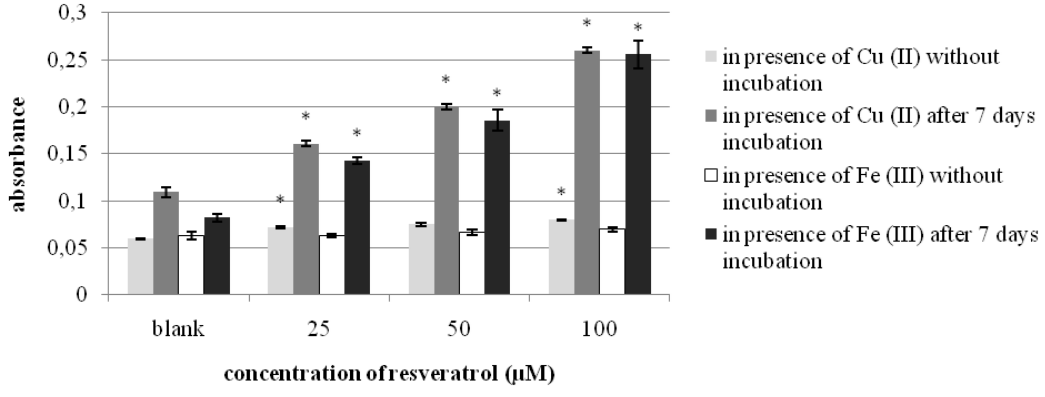

*Figure 5. Absorbance of resveratrol (100 μM) at 420 nm with metal catalyst Cu(II) or Fe(III), always 3.906 nM, without or after 7 days incubation with BSA and glucose at 37 °C. *p < 0.05 (error bars = mean ± SD).*

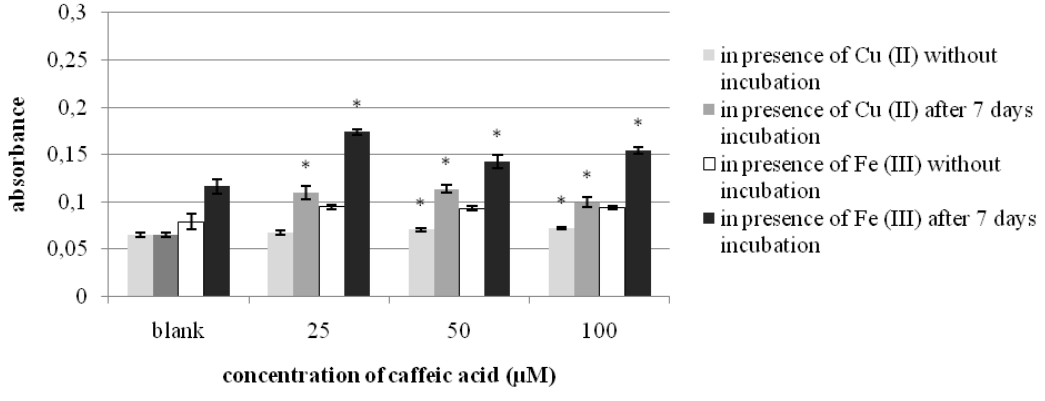

*Figure 6. Absorbance of caffeic acid (100 μM) at 420 nm with metal catalyst Cu(II) or Fe(III), always 3.906 nM, without or after 7 days incubation with BSA and glucose at 37 °C. *p < 0.05 (error bars = mean ± SD).*

nanomolar concentration of transition metal ions. This should be considered when multimineral food supplements with natural substances are made.

CONCLUSION

In conclusion, resveratrol and caffeic acid are well known antioxidants. However, in certain conditions, they can act as pro-oxidants and trigger pro-glycative action in the presence of glucose and amino acid. We found out that resveratrol and caffeic acid alone, as well as in the nanomolar presence of Cu(II) or Fe(III) ions, when incubated with glucose and BSA after 7 days, can initiate Maillard reaction and accelerate the formation of AGEs. We confirmed this by fluorescence measurements of AGEs, which act as a fluorophore, and by specific absorbance increase following the browning development. Whereas glycation can negatively alter protein activity, folding or degradation, it is important to research the conditions, which leads to the pathological formation of AGEs.

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