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Evaluation of in vitro effects of natural substances of plant origin using a model of protein glycoxidation

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Abstract

In an in vitro model with purified porcine aspartate aminotransferase (AST, EC 2.6.1.1) as the protein, the effects of phenolic antioxidants of plant origin (arbutin, methylarbutin, ferulic and isoferulic acids, *o*-coumaric and *p*-coumaric acids, quinic acid), flavonoids (baicalin and baicalein), and of hydroxycitric acid (HCA) at 0.5–50 mM concentration on the enzyme activities and on its glycation by 50mM D-fructose as the glycating agent were studied. During incubation with AST at 37 °C up to 24 days, fructose alone decreased AST activities as a result of protein glycation. In the absence of fructose, 50 mM phenolic compounds gradually decreased AST activity, while no or a weak effect of individual compounds was found at 3 mM concentration. A direct negative effect on AST was pronounced with ferulic acid. On the other hand, beneficial influences of phenolic compounds on glycation of AST by fructose were found mostly at 3 mM concentration. Effects on glycation were vague at 50 mM concentration, probably due to a combination of direct negative influences and antiglycation effects of individual compounds. No effect, neither positive nor negative, on AST activity and protein glycation, was found with quinic acid. The flavonoid baicalin and its aglycon baicalein rapidly decreased the in vitro activity of the enzyme in all concentrations used (0.5–3 mM), and no beneficial effects of the compounds on glycation of the enzyme by fructose were found. The influence of HCA on glycation was concentration-depended, ranging from beneficial inhibition of glycation at 2.5 mM concentration to a strong decrease in AST activity at 10 mM HCA. Both the beneficial and undesirable effects of natural antioxidants should be considered in case they are used as antiglycation factors. The results obtained can contribute to the evaluation of quality of various generally recommended antioxidants.

Keywords: Aspartate aminotransferase; Protein glycation; Phenolic antioxidants; Hydroxycitric acid; Flavonoids; Enzyme activity

1. Introduction

Glycation, a non-enzymatic reaction between free amino groups of proteins and reducing sugars, is closely associated with the pathogenesis of age- and diabetes-related complications like neuropathy, angiopathy and nephropathy [1]. This process represents a common posttranslational modification of proteins, which can impair their functions in living organisms. The oxidative steps are also involved and the process can be therefore called glycoxidation [2]. Free radicals, products of the autooxidation of the glycating sugar, and a heterogeneous group of substances called advanced glycation end products (AGEs) are formed in the course of glycoxidation [3].

Aspartate aminotransferase (AST, EC 2.6.1.1) is a very useful enzyme in clinical laboratory diagnostics. The AST

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molecule is composed of two identical, non-covalently bound subunits, with one molecule of the coenzyme pyridoxal-5′-phosphate bound to each subunit. Regardless of its metabolic role, the enzyme represents a suitable model for glycation studies [4], because the molecule contains 15–20 lysine residues (according to animal species) participating in the course of glycation. Glycation of AST by reactive monosaccharides (e.g. glucose, fructose) is accompanied by a decrease in its catalytic activity in dependence on the concentration and activity of the glycating agent [4,5].

With regard to the presence of free radicals and oxidative steps in the glycoxidation process, compounds with antioxidative effects have been tested in order to slow down or to stop glycoxidation [6–8]. The list include compounds like α-lipoic acid, α-tocopherol, ascorbic acid, β-carotene, aminoguanidine, pyridoxamine, which showed different antiglycation potential from no effect (α-tocopherol) to the effect at the dose 600 mg/day (α-lipoic acid). Description of antioxidant properties and their explanations are ambiguous.

Antioxidants may be divided into several groups according to several aspects (e.g. solubility, origin, and mechanism of action). Primary antioxidants inhibit the formation of free radicals (e.g. inhibitors of xanthinoxidase and NADPH oxidase, ion chelators); secondary antioxidants are able to scavenge the already formed free radicals (e.g. superoxid dismutase, substances with reducing activity); tertiary antioxidants repair or eliminate molecules damaged by free radicals [9]. The present study deals with secondary antioxidants of plant origin possessing reducing activity.

Baicalin and its aglycon baicalein are natural polyphenolic compounds belonging to the group of flavones, which occur in the plant *Scutellaria baicalensis Georgi*. These flavonoids have proven the following effects: antioxidative, immunomodulative, antiflogistic, antibacterial, antiviral, sedative, and partially cytostatic. Both substances are able to scavenge hydroxyl- and alkyl-radicals, and baicalein also traps superoxide anion radicals [10].

Hydroxycinnamic acids (e.g. caffeic, ferulic, coumaric) are widely distributed compounds in the plant kingdom, which usually exist as simple esters with quinic acid or glucose. Only carboxyl groups of hydroxycinnamic acids are included in the formation of these esters [11]. Hydroxycinnamic acids form highly resonance-stabilized radicals (phenoxy radical) after a reaction with reactive radicals, since the unpaired electron may be delocalised across the entire molecule. These phenoxy radicals are not able to initiate or propagate a radical chain reaction and their most probable fate is a collision and condensation with another radical [12].

Arbutin, a glucoside of hydroquinone, is found in the leaves of some medicinal plants (*Arctostaphylos uva-ursi*). Hydroquinone and its derivatives act as antioxidants by scavenging free radicals, and therefore, arbutin also seems to possess an antioxidative activity towards lipid peroxidation [13].

Hydroxycitric acid is a major metabolite in the fruit rinds of certain species of the plant *Garcinia* (*G. cambogia*, *G. indica*, *G. atroviridis*). This substance is at the centre of interest for its unique regulatory effect on fatty acid synthesis, lipogenesis, appetite, and weight loss [14]. Properties of hydroxycitric acid have been intensively studied in our laboratories using the tests of antiaggregation, cytotoxicity, and acute toxicity (results not yet published).

The aim of the present study was to evaluate a possible antiglycation effect of a group of the above-mentioned natural antioxidants of plant origin using our in vitro model of glycation.

2. Experimental

2.1. Chemicals

Aspartate aminotransferase, a cytosolic enzyme from the porcine heart suspended in saturated ammonium sulphate (291 U/mg), was obtained from Serva Electrophoresis GmbH, Germany. Baicalein and (−)-threo-hydroxycitric acid were purchased from Fluka Chemie, GmbH, Switzerland. Baicalin hydrate was obtained from Aldrich Chem. Co., Milwaukee, USA. Arbutin and methylarbutin were isolated from the leaves of *Arctostaphylos uva-ursi* at the Department of Pharmaceutical Botany and Ecology. The following compounds were products of Sigma Chemicals Co. (St. Louis, USA): ferulic acid, isoferulic acid, *o*-coumaric acid, *p*-coumaric acid, quinic acid, and D-(−)-fructose. Sodium azide was obtained from Lachema, Brno, Czech Republic. All chemicals were of analytical grade. Chemical structures of the compounds tested are presented in Fig. 1.

2.2. Sample preparation and incubation

The enzyme suspension was centrifuged at 5000 rpm at 4 °C for 20 min, the supernatant was removed, and the protein pellet was reconstituted in 0.1 M sodium phosphate buffer (pH 7.4, 0.05% sodium azide) to yield a stock solution of 2.66 mg/ml. After that, the enzyme solution was used for the preparation of four different types of incubation mixtures: (a) with buffer only (control samples), (b) with D-fructose (Frc) in a final concentration of 50 mmol/l (i.e. “protein glycation” samples), (c) with individual antioxidants in a final concentration of 0.5–50 mmol/l (i.e. “direct protein–antioxidant interaction” samples), (d) with individual compounds in a final concentration of 0.5–50 mmol/l and D-fructose in a final concentration of 50 mmol/l (i.e. “antiglycation” samples). The final concentration of the enzyme protein was 1.33 mg/ml. Incubation mixtures were incubated in the dark at 37 °C for up to 24 days.

2.3. Enzyme assay

The enzyme was assayed using the IFCC-recommended kinetic UV-method (Roche Diagnostics, Mannheim, Germany, an automatic analyzer Hitachi 917) [15]. Sampling

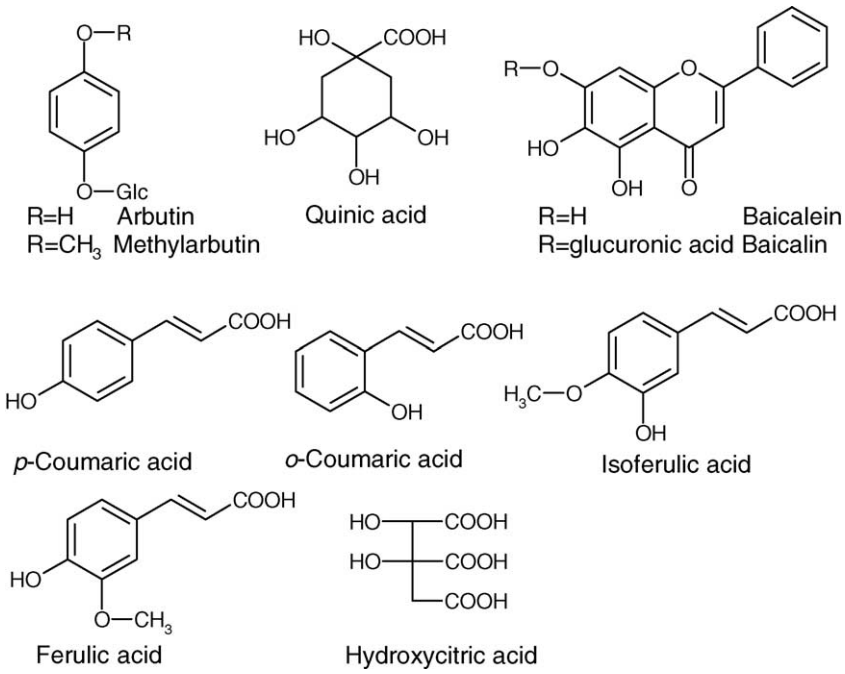

Fig. 1. Chemical structures of tested antioxidants.

and measuring was carried out on days 0–24, individual intervals were adopted according to the schedules of the clinical laboratory. Sample aliquots were diluted 1:2000 with 0.1 M sodium phosphate buffer (pH 7.4, 0.05% sodium azide) to obtain enzyme activities in the analytical range of the methods used. All experiments were carried out in triplicates. AST catalytic activity was calibrated by measurement of the standards Precinorm U and Precipath U (Roche Diagnostics, Germany) before and after each assay. The between-run coefficients of variation of the both assays for Precinorm U and Precipath U were lower than 2.0%.

2.4. Statistical analysis

Absolute values of enzyme activities (μkat/l) are given as the mean ± standard deviation (S.D.). Some presented data are expressed as the percentage of the respective control ± S.D. in order to avoid some day-to-day fading of enzyme activities and fluctuations caused by daily recalibrations of the analyzer. Statistical significance was determined by the use of Student's *t*-test and the differences were regarded as significant when $p < 0.05$.

3. Results and discussion

Most of tested compound had a more or less pronounced negative direct effect on enzyme activity, which was probably due to a direct interaction of the molecule of the antioxidant with the molecule of the enzyme. In addition, positive (indirect, antiglycation) effects were observed with some compounds.

A negative direct influence on enzyme activity was mostly pronounced in the case of baicalin (see Fig. 2).

This substance at 0.5 mM concentration caused a statistically significant decrease in enzyme activity already after five days of incubation. The direct effect of baicalin was dependent upon concentration (day 5: control sample 5.36 ± 0.04 μkat/l; baicalin 0.5 mM 3.97 ± 0.47 μkat/l; 1.0 mM 2.89 ± 0.05 μkat/l; 1.5 mM 2.42 ± 0.27 μkat/l;

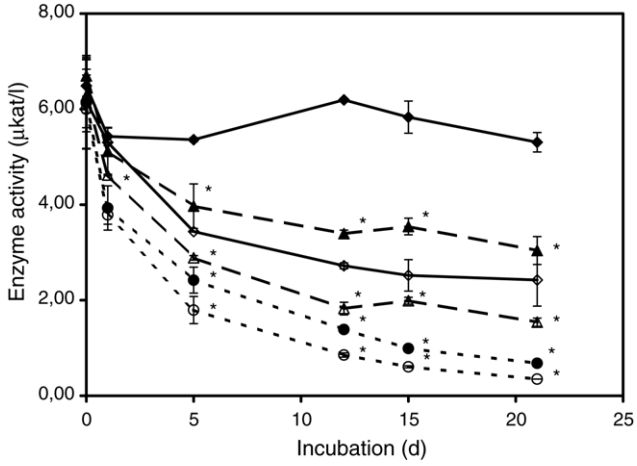

Fig. 2. Direct effect of baicalin on the AST activity in vitro. Concentration of AST in incubation mixtures was 1.33 mg/ml in 0.1 M sodium phosphate buffer (pH 7.4; 0.05% sodium azide). Incubation at 37 °C. The samples were diluted before the assay to fit to the analytical range of the method. Values are expressed as mean ± S.D. of six (control and AST + Frc 50 mM) or three (with baicalin) independent samples ($^{*}p < 0.05$). (♦): Control sample (AST), (◊): AST + Frc 50 mM, (▲): baicalin 0.5 mM + AST, (△): baicalin 1.0 mM + AST, (●): baicalin 1.5 mM + AST, (○): baicalin 3.0 mM + AST.

3.0 mM 1.79 ± 0.28 μkat/l). Similar results were obtained in the case of baicalein (results are not presented).

Activities of AST samples containing baicalin or baicalein and fructose were compared with those of the samples of AST and fructose 50 mM, whose activity was taken as 100%. Both baicalin and baicalein deepened a deteriorating influence of sugar on enzyme activity (day 5: control sample 5.36 ± 0.04 μkat/l; baicalin 0.5 mM + AST + Frc 50 mM 3.09 ± 0.08 μkat/l) and had no beneficial effects on the glycation of enzyme by fructose (day 5: 3.44 ± 0.01 μkat/l). Concentration dependence was determined in both cases. The results are summarized in Table 1. Obviously, no antiglycation effect of baicalin and baicalein was found.

Flavonoids with the greatest antioxidant activity fulfil the following structural arrangements: the 2,3-double bond in combination with both the 4-oxo function and the 3-hydroxyl group in the C ring; the *meta*-5,7-dihydroxy arrangements in the A ring; the *ortho*-3′,4′-dihydroxy moiety in the B ring [16]. Baicalin and baicalein, according to above-mentioned structure–activity relantionships, would react as week antioxidants.

Fig. 3 shows direct influences of hydroxycitric acid on AST activity. The enzyme activities of samples with fructose demonstrate a remarkable decrease caused by sugar in comparison with a minor direct effect of hydroxycitric acid. The direct effect of the compound on AST was negligible in all tested concentrations (2.5 mM, 5.0 mM, 7.5 mM, and 10.0 mM). A statistically significant decrease in enzyme activity was observed only in a few individual samples (once at any tested concentration) and could be omitted.

Hydroxycitric acid at 2.5 mM concentration showed a statistically significant positive antiglycation effect on days 5–15 (see Fig. 4). The same antiglycation effect was not observed at higher concentrations (5.0 mM, 7.5 mM, and 10.0 mM). For example, samples with fructose and 5.0 mM hydroxycitric acid showed a nearly identical enzyme activity as AST samples with fructose alone. It seems that hydroxycitric acid at higher concentrations supports the negative influence of fructose on AST activity. In general, the positive effects of hydroxycitric acid are prevailing at lower concentration, contributing thus to several other positive effects of the compound, which are described in the Introduction.

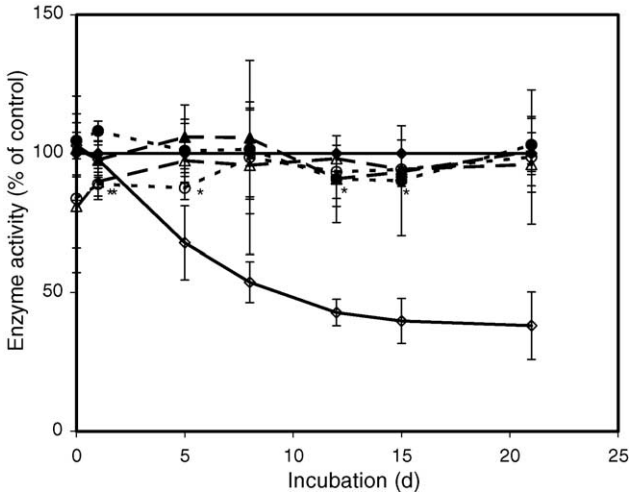

Fig. 3. Direct influence of hydroxycitric acid (HCA) on the AST activity in vitro. Values are expressed as a percentage of the activity of respective control (AST) ± S.D. (%) of six independent samples ($^{*}p < 0.05$). (♦): Control sample (AST), (◊): AST + Frc 50 mM, (▲): HCA 2.5 mM + AST, (△): HCA 5.0 mM + AST, (●): HCA 7.5 mM + AST, (○): HCA 10.0 mM + AST. For other details see Fig. 2.

Phenolic antioxidants were tested at two different concentrations (3 mM and 50 mM). Table 2 summarizes direct effects of the group of phenolic antioxidants at 50 mM concentration on AST activity. Results obtained at 3.0 mM concentration are not presented, because the activity declined maximally by 8.3 ± 3.7% (ferulic acid) or apparently increased by 15.0 ± 5.1% (*p*-coumaric acid) in comparison with the control sample (100%), respectively. Quinic acid, *o*-coumaric acid, and isoferulic acid at 50 mM concentration had no effect on the AST activity and an apparent increase in enzyme activity was also observed. There is no exact explanation for

Table 1
Evaluation of antiglycation effect of baicalin and baicalein in enzyme model

Concentration	Incubation sample[a]	Enzyme activity (% of control)[c]		
		Day 5	Day 12	Day 21
	AST + Frc[b]	100.0 ± 0.3	100.0 ± 2.3	100.0 ± 13.1
0.5 mM	Baicalin + AST + Frc	89.9 ± 2.3	76.4 ± 1.8	41.7 ± 3.1
	Baicalein + AST + Frc	103.5 ± 2.6	61.4 ± 1.3	23.9 ± 3.2
1.0 mM	Baicalin + AST + Frc	74.7 ± 3.1	40.2 ± 4.1	15.3 ± 0.6
	Baicalein + AST + Frc	134.4 ± 55.2	52.5 ± 0.4	19.4 ± 0.9
1.5 mM	Baicalin + AST + Frc	61.4 ± 32.8	38.5 ± 10.1	11.6 ± 3.9
	Baicalein + AST + Frc	100.7 ± 3.4	74.0 ± 7.9	19.5 ± 1.3
3.0 mM	Baicalin + AST + Frc	62.3 ± 17.2	33.2 ± 5.6	9.1 ± 1.7
	Baicalein + AST + Frc	95.6 ± 21.1	47.3 ± 5.3	12.1 ± 1.3

[a] Protein concentration: 1.33 mg/ml in sodium phosphate buffer (0.1 M; pH 7.4) with 0.05% sodium azide. Incubation at 37 °C.
[b] Final concentration: 50 mM in all samples.
[c] Results are expressed as % of enzyme activity of the respective control (AST + Frc) ± S.D. Each value represents six (AST + Frc) or three (with baicalin or baicalein) individually prepared samples.

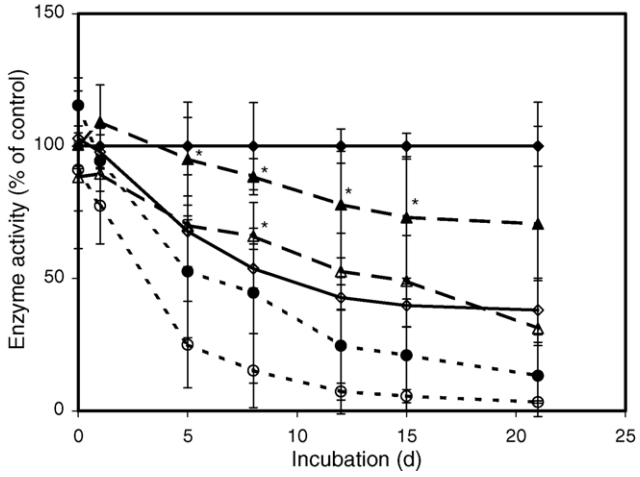

Fig. 4. The effect of hydroxycitric acid (HCA) on the glycation of AST by fructose in vitro. Values are expressed as a percentage of the activity of respective control (AST) ± S.D. of six independent samples ($^{*}p < 0.05$). (♦): Control sample (AST), (◊): AST + Frc 50 mM, (▲): HCA 2.5 mM + AST + Frc, (△): HCA 5.0 mM + AST + Frc, (●): HCA 7.5 mM + AST + Frc, (○): HCA 10.0 mM + AST + Frc. For other details see Figs. 1 and 2.

this increase. A statistically significant decrease in AST activity since the fourth day was observed in the case of arbutin, methylarbutin, *p*-coumaric acid, and ferulic acid at 50 mM concentration. The enzyme activity in the presence of the latter compound was the lowest among the phenolic antioxidants (see Table 2).

Antiglycation influences of the group of phenolic antioxidants at 3.0 mM and 50 mM concentrations are shown in Table 3.

Table 2
Direct effects of a group of phenolic antioxidants on AST activity

Incubation sample	Enzyme activity (% of control)[a]		
	Day 0	Day 4	Day 13
Control sample (AST)	100.0 ± 10.9	100.0 ± 8.2	100.0 ± 7.0
Arbutin 50 mM + AST	108.9 ± 5.4	79.1 ± 1.0*	79.9 ± 2.7*
Methylarbutin 50 mM + AST	104.2 ± 23.7	85.9 ± 1.8*	75.1 ± 7.7*
Quinic acid 50 mM + AST	99.5 ± 1.5	100.4 ± 8.7	97.1 ± 2.2
p-Coumaric acid 50 mM + AST	100.5 ± 2.7	93.6 ± 0.9*	50.5 ± 4.2*
o-Coumaric acid 50 mM + AST	103.7 ± 1.1	107.6 ± 2.3	110.0 ± 4.9
Ferulic acid 50 mM + AST	119.0 ± 2.5	47.4 ± 2.2*	10.5 ± 0.2*
Isoferulic acid 50 mM + AST	123.9 ± 3.0	112.8 ± 1.8	122.3 ± 10.0

[a] Results are expressed as % of enzyme activity of respective control (AST) ± S.D. (%). Enzyme activities of marked data (*) were significantly decreased in comparison with enzyme activity of control sample ($p < 0.05$). Each value represents six individually prepared samples. For other conditions see Table 1.

Table 3
Evaluation of antiglycation effect of a group of phenolic antioxidants in enzyme model

Incubation sample	Enzyme activity (% of control)		
	Day 0	Day 4	Day 13
AST + Frc 50 mM	100.0 ± 17.0	100.0 ± 3.2	100.0 ± 15.9
Arbutin 3 mM + AST + Frc	104.7 ± 0.5	123.8 ± 18.3*	115.4 ± 13.8
Arbutin 50 mM + AST + Frc	101.2 ± 1.9	139.0 ± 6.1*	121.9 ± 5.8*
Methylarbutin 3 mM + AST + Frc	95.1 ± 9.1	106.2 ± 2.4	102.5 ± 26.7
Methylarbutin 50 mM + AST + Frc	95.6 ± 0.9	91.9 ± 3.7	74.3 ± 10.5
Quinic acid 3 mM + AST + Frc	94.6 ± 7.8	100.5 ± 15.3	91.9 ± 46.5
Quinic acid 50 mM + AST + Frc	95.5 ± 1.4	72.9 ± 11.5	69.6 ± 31.9
p-Coumaric acid 3 mM + AST + Frc	96.0 ± 9.5	144.8 ± 29.6*	163.1 ± 28.7*
p-Coumaric acid 50 mM + AST + Frc	97.6 ± 1.7	180.4 ± 2.7*	81.1 ± 3.5
o-Coumaric acid 3 mM + AST + Frc	95.3 ± 13.0	142.9 ± 15.3*	140.0 ± 7.3*
o-Coumaric acid 50 mM + AST + Frc	106.6 ± 6.0	202.2 ± 9.1*	174.9 ± 4.3*
Ferulic acid 3 mM + AST + Frc	100.3 ± 14.9	113.6 ± 43.8	112.2 ± 1.1
Ferulic acid 50 mM + AST + Frc	112.9 ± 5.0	84.4 ± 5.2	23.4 ± 0.3
Isoferulic acid 3 mM + AST + Frc	101.3 ± 1.1	125.8 ± 36.1	161.9 ± 5.3*
Isoferulic acid 50 mM + AST + Frc	115.5 ± 4.9	108.2 ± 8.1	138.3 ± 1.6*

For conditions see Tables 1 and 2.

Quinic acid had no antiglycation effect at either concentration tested. Isoferulic acid at both concentrations showed a statistically significant influence on the glycation of enzyme only on the 13th day. These two above-mentioned compounds had neither a negative direct nor a positive indirect (antiglycation) effect and they may be considered as neutral. Since no beneficial effects were observed in the case of methylarbutin and ferulic acid and the compounds had negative direct influences, they can be regarded as negatively acting compounds. The only substance with a fully positive effect seems to be *o*-coumaric acid, which showed both no negative direct effect and a remarkable support of enzyme activity at both concentrations since the first day of the glycation experiment. Beneficial antiglycation effects outweighed the negative direct influences of arbutin (at both concentrations and throughout the experiment) and of *p*-coumaric acid (at 3 mM concentration between the 1st and 13th day, and at 50 mM concentration on days 1 and 4).

Antioxidant activity of hydroxycinnamic acids is mainly due to *para*-hydroxyl group, while there is little or no effect when OH group is present in the *meta* or *ortho* position. The presence of methoxy group (ferulic acid) enhances the electron donating properties in the *para*-position in comparison with monophenolics (*p*-coumaric acid). The expected or-

der of antioxidant activities of hydroxycinnamic acids is ferulic > *p*-coumaric ≫ *o*-coumaric [12,16,17]. This order was confirmed only in the case of negative direct effects on the enzyme activity, which suggests at least that the structural features of compounds, providing them antioxidant activities, are closely connected with their reactivity with molecules that should be in the same time protected (proteins).

4. Conclusions

The influences of antioxidants of natural origin on the catalytic activity of AST and on the enzyme glycation by fructose were evaluated using two different enzyme assays. Both beneficial and possible undesirable effects of antioxidants were found. The authors believed that these equivocal effects should be considered in view of various conditions (e.g. concentration, presence of other compounds) before the use of the compounds is generally recommended. The compounds with beneficial effect may join the list of antiglycation compounds described so far.

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