The Combined Effect of Pycnogenol® with Ascorbic Acid and Trolox on the Oxidation of Lipids and Proteins

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Abstract. Pycnogenol® (PYC), a procyanidin-rich extract of French maritime pine bark (*Pinus pinaster*) has strong antioxidant potential and promotes cellular health. The aim of this study was to investigate a possible cooperation of natural antioxidant PYC with synthetic antioxidants ascorbic acid and trolox in the model system of lipid peroxidation determined as conjugated dienes formation in liposomes and on the oxidation of proteins (in BSA and plasma proteins) determined as protein carbonyls. The present study shows that PYC and trolox significantly increased inhibition of lipid peroxidation initiated by copper acetate and *tert*-butylhydroperoxide in concentration and time dependence compared with untreated unilamellar liposomes. PYC and trolox added simultaneously to the oxidized liposomes exerted an additive preventive effect. PYC's inhibitory effect on formation of carbonyl compounds in BSA and plasma proteins, oxidized by two oxidative systems – $H_2O_2/FeSO_4$ and HOCl, were studied in co-operation with other synthetic antioxidants – ascorbic acid and trolox. We found the synergistic or additive effect of PYC with mentioned antioxidants.

Key words: Oxidative stress — Pycnogenol® — Conjugated dienes — Protein carbonyls — Synergism

Introduction

Free radicals and other oxygen-derived species are constantly generated *in vivo* and cause damage to DNA, lipids, proteins, and other biomolecules (de Zwart et

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al. 1999; Stadtman 2002). Antioxidant defense systems scavenge and minimize the formation of oxygen-derived species, but they are not absolutely effective. A large number of synthetic and natural exogenous antioxidants have been demonstrated to induce beneficial effects on human health and disease prevention. In recent years, a growing interest has been seen in the utilisation of flavonoids, which have gained considerable attention as beneficial antioxidant agents (Packer et al. 1999; Santos and Mira 2004).

Antioxidative properties of polyphenols arise from their high reactivity as hydrogen or electron donors, and from the ability of the polyphenol-derived radical to stabilize and delocalize the unpaired electron (chain-breaking function), and from their ability to chelate transition metal ions (Rice-Evans et al. 1997; Pietta 2000; Ozgová et al. 2003). The molecular mechanism of such a wide scale of biologically active group as flavonoids has not been examined yet. The antioxidant ability of flavonoids *in vivo* in comparison with other important antioxidant systems in the organism has been recently discussed (Grimm et al. 2004; Tesoriere et al. 2004). To study antioxidant properties of flavonoids, it is important to choose systems and substrates modelling the *in vivo* system as close as possible.

Pycnogenol® (PYC) is a proprietary mixture of bioflavonoids extracted from French maritime pine *Pinus pinaster* with a potent ability to scavenge free radicals (Packer et al. 1999; Rohdewald 2005). PYC displays important biological effect (Rohdewald 2005), its *in vitro* antioxidant activity is well known and it has strong free radical-scavenging *in vitro* activity against reactive oxygen and nitrogen species (van Acker et al. 1995; Nelson et al. 1998; Virgili et al. 1998). These properties result from structure of individual components of PYC. Aromatic rings bearing hydroxyl groups are potentially able to quench free radicals by forming resonance-stabilised phenoxyl radicals (Packer et al. 1999).

PYC can protect the chain of lipid bilayer against oxidative damage to fatty acids. During the process of lipoperoxidation, free radicals react with polyunsaturated fatty acids first resulting in the formation of conjugated dienes from which peroxyl radicals and hydrolipoperoxides are formed in aerobic conditions. These products, mainly in the presence of transition metal ions, initiate further free radical chain reactions (Deiana et al. 1999; Liptáková 1999; Liao and Yin 2000; Tang et al. 2000; Vasiljeva et al. 2000; Filípek et al. 2001). Rong et al. (1995) have reported that the pre-incubation of cultured normal endothelial cells with PYC at the concentrations from 20 to 80 μg/ml is associated with significant protection of these cells against lipid peroxidation and cell damage induced with *tert*-butylhydroperoxide. The generation of thiobarbituric acid-reactive substances (TBARS) is also significantly decreased, indicating that the protective effect of PYC is due to its antioxidant activity. Nelson and co-workers (1998) have shown that PYC has protective effect in preventing lipid peroxidation of low-density lipoproteins in human plasma.

PYC can bind to proteins, thus altering their structure and modulating the activity of key enzymes (Fitzpatrick et al. 1998). Oxygen radicals have been implicated in oxidative modification of proteins leading to their rapid degradation. Early marker of protein oxidation may be carbonyls (Reznick and Packer 1994; Lyras et

al. 1996; Chen et al. 2001). One of the ways to study carbonyl production is the enzymatic system of myeloperoxidase-H_2O_2-Cl^-, producing HOCl in the phagocytic vesicles. This system is working in phagocytic vesicles during phagocytosis as microbicidal system. HOCl reacts with susceptible fragments of protein molecules causing deamination and formation of carbonyl groups and oxidizing thiol groups in polypeptic chains (Naskalski 1994; Drozdz et al. 1995).

Because flavonoids are consumed with other antioxidants in the diet, their combination may prove to be more beneficial. It was shown that flavonoids coordinate their effects with other antioxidants. Cossins et al. (1998) detected the direct *in vitro* interaction and cooperation between flavonoids and ascorbate. Vinson and Jang (2001) confirmed *in vivo* cooperation of flavonoids with ascorbate, on the other hand, Silliman et al. (2003) did not confirm it.

The aim of our study was to examine oxidative damage to different substrates (lipids in the form of liposomes and proteins in the form of plasma proteins and bovine serum albumin (BSA) alone) evoked by different oxidants (Cu^{2+}/*tert*-butylhydroperoxide, Fe^{2+}/H_2O_2 and HOCl) and to determine *in vitro* protective effect of antioxidants such as PYC, trolox and ascorbic acid and their combination.

Materials and Methods

Chemicals

Chemicals of analytical grade were obtained from Sigma Company (USA); trolox, copper acetate (Aldrich Chem. Co., USA); BSA (albumin fraction V; MERCK, Germany); Pycnogenol® (Drug Research Institute, Modra, Slovakia). Antioxidant solutions were prepared freshly before experiment.

Unilamellar liposomes preparation

Egg yolk phosphatidylcholine (EYPC) was isolated from hen egg yolk as described by Singleton et al. (1965) at Faculty of Pharmacy, Comenius University (Bratislava, Slovakia). Its purity was checked by two-dimensional thin layer chromatography.

Natural phospholipids contain only non-conjugated double bonds, and therefore have a UV absorbance peak at a very short wavelength (200–205 nm). Removal of hydrogen atom from a methylene group located between two double bonds spreads the unsaturation over five carbon atoms and results in the formation of conjugated dienes (Babincová et al. 1999). As a result, second absorbance maximum at 234 nm appears.

The suspension of EYPC was dissolved in 0.15 mol/l NaCl, pH 7.4 (final concentration of EYPC was 50 mg/ml) and unilamellar liposomes were prepared by extruder (Liposo Fast^{tm} – Basic) with a membrane filter (pores size 50 nm). The number of squeezings through membrane filter was 21.

Standard curve for determination of unilamellar liposomes concentration was constructed from 50 mg EYPC dissolved in 1 ml of methanol (10–50 mg/ml). The absorbance of standard solutions and suspension of unilamellar liposomes was

measured at 234 nm. The suspension of unilamellar liposomes was sealed under nitrogen at −20 °C.

Lipid peroxidation of unilamellar liposomes

Reaction mixture contained 250 μl of unilamellar liposomes (final concentration 13.5 mg/ml), and 20 μl of tested antioxidants at final concentration: PYC 10–100 μg/ml, trolox 1–20 μmol/l and ascorbic acid 10–200 μmol/l diluted in methanol. Sample without an antioxidant (control) contained 20 μl of methanol.

This suspension was incubated for 30 min at 37 °C and the lipid oxidation was triggered with 30 μl of copper acetate (final concentration 50 μmol/l) and 7 μl of *tert*-butylhydroperoxide (final concentration 158 μmol/l). This suspension was incubated at 37 °C and an absorption spectrum was measured at time 0, 30, 60, 90, 120 min. The production of conjugated dienes was determined as an absorbance change at the wavelength 234 nm (ε = 29,500 $mol^{-1} \cdot cm^{-1} \cdot l$ for the EYPC). The final data were expressed as μmol of conjugated dienes/l of EYPC. The percentage of inhibition of lipid oxidation was calculated in relation to the samples without tested antioxidants.

Oxidative damage to proteins

The effect of PYC, trolox and ascorbic acid on the oxidative damage to proteins was examined in two different *in vitro* systems: Fe^{2+}/H_2O_2 and HOCl.

Plasma proteins oxidized by Fe^{2+}/H_2O_2 system

The plasma carbonyl content was measured in samples consisted of 30 μl of plasma, 20 μl H_2O_2 (final concentration 10^{-2} mol/l), 20 μl $FeSO_4$ (final concentration 80 μmol/l) and 30 μl of tested antioxidants at final concentrations: PYC (40 μg/ml), trolox (1, 10 μmol/l) and ascorbic acid (1, 10 μmol/l). Blank contained 30 μl of phosphate buffer (10 mmol/l, pH 7.4) instead of an antioxidant.

Plasma samples without an oxidative agent (H_2O_2 + $FeSO_4$) were pre-incubated with antioxidants or phosphate buffer at 37 °C for 30 min. To induce the oxidative damage to plasma proteins, H_2O_2 and $FeSO_4$ were added and incubated for 60 min. Carbonyl levels were determined according to the protocol by Reznik and Packer (1994). Briefly, plasma samples were divided into two portions (blank and sample). 80 μl of 10 mmol/l dinitrophenyl hydrazine (DNPH) dissolved in 2.5 mol/l HCl (320 μl) was added to the sample, while only 2.5 mol/l HCl (320 μl) was added to the blank. Both tubes were incubated for 45 min at room temperature. In order to precipitate proteins, we added 400 μl (20 % w/w) trichloroacetic acid and centrifuged both the sample and the blank for 10 min at 1000 × *g*. The sediment was washed four times with ethanol : ethyl acetate (1 : 1; 400 μl). After the final rinse, the samples and blank were centrifuged for 10 min at 2000 × *g* and the sediment was dissolved in 6 mol/l guanidine (600 μl) in 20 mmol/l KH_2PO_4, with final pH 2.3. The absorbance spectra of BSA-DNP conjugates were recorded from 280 to 450 nm. The peak of absorbance around 370–376 nm was used to quantitate

protein carbonyls. The final data were expressed as nmol of carbonyl groups/mg of proteins ($\varepsilon = 22{,}000\ \mathrm{mol}^{-1}\cdot\mathrm{cm}^{-1}\cdot\mathrm{l}$ for the DNPH derivates).

Total protein concentration in plasma was determined by measuring the absorption at 280 nm. The amount of proteins was calculated from a BSA standard curve (0.25–2 mg/ml).

BSA oxidized by Fe^{2+}/H_2O_2 system

BSA (4 mg/ml) was oxidized by Fe^{2+}/H_2O_2 system (in concentrations 1 and 10 mmol/l, respectively) in phosphate buffer (50 mmol/l, pH 7.4) at 37℃, for 15 min. The reaction was stopped by desferoxamine (15 mmol/l). Antioxidants were added before Fe^{2+}/H_2O_2 system. Antioxidants were studied in the following concentration ranges: trolox (10–500 μmol/l), PYC (2–150 μg/ml). Also combinations of PYC (10, 40 μg/ml) and trolox (100, 250, 500 μmol/l) were studied.

BSA oxidized by HOCl

BSA at different protein concentrations (1–4 mg/ml) was oxidized by HOCl at final concentration range of 50–200 μmol/l in phosphate buffer (10 mmol/l, pH 7.4), at 37℃, for 30 min. The concentrations of hypochlorite/hypochlorous acid in the diluted commercial NaOCl solution were determined spectrophotometrically ($\varepsilon_{290} = 350\ \mathrm{mol}^{-1}\cdot\mathrm{cm}^{-1}\cdot\mathrm{l}$). Oxidation was finished by addition of methionine (final concentration of 1 mmol/l). To study preventive effects of antioxidants, BSA (1 mg/ml) was oxidized by HOCl (200 μmol/l) in phosphate buffer (10 mmol/l, pH 7.4) at 37℃, 30 min. Antioxidants were added before HOCl. PYC (20–40 μg/ml) was tested in combination with trolox (50, 100, 500 μmol/l) or ascorbic acid (20, 50 μmol/l). PYC and ascorbic acid were diluted in water and trolox in phosphate buffer, pH 7.4. Carbonyl levels were determined according to the protocol described above (Reznik and Packer 1994).

Assay for HOCl scavenging

The compound 5-thio-2-nitrobenzoic acid (TNB) can easily be oxidized by HOCl. The inhibition of TNB oxidation by antioxidants was a measure of their HOCl scavenging activity. TNB (84 μmol/l) was incubated with or without a scavenger. The absorbance at 412 nm indicating the presence of TNB was measured before and after the addition of HOCl (55 μmol/l) as well as in the samples where antioxidant was added before HOCl.

Measurement of absorption spectra of BSA and PYC

Absorption spectra were measured on a Specord 40 PC spectrophotometer (Analytic Jena, AG, Germany) in the range of 200–400 nm. The spectra of PYC and BSA were measured individually and in their mixtures. Absorbance of individual PYC and BSA spectra were added (summarized) mathematically and compared with absorbance of mixtures measured experimentally. PYC and BSA individually and in their mixtures were incubated in phosphate buffer (pH 7.4) for 90 min.

Calculation of percentage of inhibition of conjugated dienes and carbonyl groups production

$$\text{Inhibition } (\%) = [100 - (a_{with}/a_{without})] \times 100\,\%$$

where a_{with} is activity of conjugated dienes and carbonyl groups production in the system with antioxidants; $a_{without}$ is activity of conjugated dienes and carbonyl groups production in the system without antioxidants.

Statistical analysis

Data presented as a mean ± SEM for the normally distributed parameters were evaluated by data analysis (Excel) using first two samples F-test for variances for the determination of equal or unequal variances. After that Student's paired *t*-test was used to compare investigated values.

Results

Lipid peroxidation of unilamellar liposomes

Unilamellar liposomes were incubated with copper acetate and *tert*-butylhydroperoxide in the absence (control) or presence of PYC, trolox, ascorbic acid and their mixture. The lipid peroxidation was determined spectrophotometrically by measuring conjugated dienes formation at 234 nm.

PYC significantly inhibited lipid peroxidation of liposomes in concentration dependence (from 10 to 80 μg/ml) during the different time of incubation (0–90 min) with copper acetate and *tert*-butylhydroperoxide, compared with untreated unilamellar liposomes with antioxidants ($p < 0.05$). The concentration of conjugated dienes did not apparently change with increasing concentration of PYC 100 μg/ml and time in incubation 120 min. Concentration of PYC 80 μg/ml was used for a time-dependent determination of inhibition of lipid peroxidation. At this PYC concentration, a time-dependent increase in the inhibition of lipid peroxidation was observed [0 min 0.25 ± 0.02 μmol of conjugated dienes/l (18.2 % inhibition); 90 min 0.57 ± 0.04 μmol of conjugated dienes/l (34 % inhibition)] ($r = 0.95$, $p < 0.01$).

The highest inhibition was reached at the incubation time of 90 min and this time was used in the next experiments. At this incubation time, PYC alone caused concentration-dependent inhibition of lipoperoxidation from 20.5 % (0.69 ± 0.10 μmol of conjugated dienes/l, concentration of PYC 10 μg/ml) to 34 % (0.57 ± 0.04 μmol of conjugated dienes/l, concentration of PYC 80 μg/ml) when compared with control sample without PYC (0 % inhibition; 0.87 ± 0.20 μmol of conjugated dienes/l) ($r = 0.98$, $p < 0.01$).

The concentration of PYC 80 μg/ml was tested for its possible synergistic effect with trolox in *in vitro* conditions. The measurement of conjugated dienes levels showed a link between an increase in trolox concentrations and the reduction of lipid peroxidation. Trolox alone at the concentration of 20 μmol/l inhibited the lipid peroxidation depending on time of incubation [0 min 0.22 ± 0.02 μmol

of conjugated dienes/l (8.6% inhibition); 90 min 0.64 ± 0.04 μmol of conjugated dienes/l (18.2% inhibition)] ($r = 0.98$, $p < 0.05$).

The cooperation of ascorbic acid with PYC was not examined due to the known pro-oxidative effect of ascorbate in this system.

We tested the effect of PYC (80 μg/ml) in the combination with trolox (20 μmol/l) at the incubation time 90 min. Mutual effect of PYC with trolox at these conditions had an additive character (0.32 ± 0.02 μmol of conjugated dienes/l; 51% inhibition) when compared to values obtained for individual antioxidants (PYC 0.45 ± 0.02 μmol of conjugated dienes/l, 36% inhibition; trolox 0.98 ± 0.03 μmol of conjugated dienes/l, 16.6% inhibition) and mathematically obtained values (0.31 ± 0.01 μmol of conjugated dienes/l, 52.6% inhibition).

Oxidative damage to proteins

Plasma proteins oxidized by Fe^{2+}/H_2O_2 system

The effect of PYC, trolox and ascorbic acid was investigated on the oxidative damage to plasma proteins. By oxidative system (H_2O_2 and $FeSO_4$), plasma proteins are oxidized and carbonyl compounds are formed. The extent of protein oxidation can be reduced by various antioxidants. In our experiments, PYC was tested alone and in combination with trolox and ascorbic acid. The inhibitory effect of PYC on the oxidative damage to proteins was concentration-depended and the highest effects were seen for PYC's concentration 40 μg/ml and did not change with the increasing concentration of this substance.

PYC alone (40 μg/ml) inhibited carbonyl production by 41% (from 7.59 ± 0.86 to 4.48 ± 0.53 nmol/mg proteins with absence and presence of PYC, respectively), trolox alone (1 μmol/l) by 40% (from 7.58 ± 0.11 to 4.55 ± 0.45 nmol/mg proteins with absence and presence of trolox, respectively) and trolox alone (10 μmol/l) by 46% (from 7.58 ± 0.11 to 4.09 ± 0.49 nmol/mg proteins with absence and presence of trolox, respectively). Combination of PYC (40 μg/ml) with trolox (10 μmol/l) had an additive effect, however, this effect was not observed at concentration 1 μmol/l of trolox (Fig. 1).

We investigated the effect of ascorbic acid alone (1, 10 μmol/l) on the oxidative damage to plasma proteins. Formation of carbonyls in plasma significantly declined with increasing concentration of ascorbic acid from 8.05 ± 0.21 to 5.39 ± 0.64 nmol/mg proteins with absence and presence of 1 μmol/l ascorbic acid, respectively (33% inhibition) or 10 μmol/l ascorbic acid by 60% inhibition (from 8.05 ± 0.21 to 3.22 ± 0.29 nmol/mg proteins with absence and presence of ascorbic acid, respectively). Combination of PYC (40 μg/ml) with ascorbic acid (1 μmol/l) had a synergistic effect ($p < 0.01$) when compared to the mathematically obtained value or an additive effect with 10 μmol/l ascorbic acid (Fig. 2).

When we used the constant concentration of ascorbic acid (1 μmol/l) and different concentrations of PYC (10, 40, 100 μg/ml), we confirmed a synergistic effect at the concentration of PYC 40 μg/ml as well as at the concentrations of PYC 10 and 100 μg/ml ($p < 0.01$). This effect was most obvious at PYC concentration 10 μg/ml (Fig. 3).

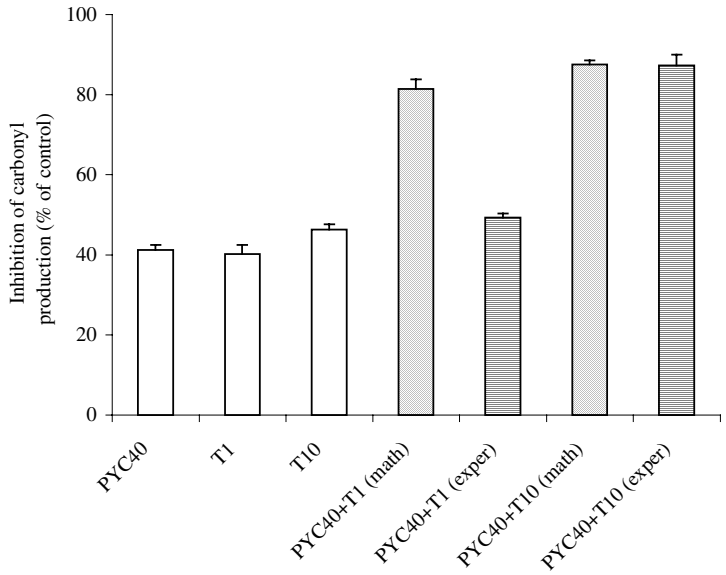

Figure 1. Effect of PYC, trolox and their combination on the oxidative damage to plasma proteins initiated by oxidative system $H_2O_2/FeSO_4$. Plasma samples were incubated with mentioned antioxidants or phosphate buffer (control) at 37°C for 30 min, then incubated with H_2O_2 (10^{-2} mol/l) and $FeSO_4$ (80 μmol/l) at 37°C for 60 min. PYC was used at the constant concentration of 40 μg/ml (PYC40) and trolox at different concentrations – 1 μmol/l (T1), 10 μmol/l (T10). Results are expressed as percentage inhibition of carbonyl production. Inhibition in the absence of antioxidants (control) is 0%. PYC40 + T1/T10 (exper) express the combination effect of PYC with T1 and T10 on carbonyl production obtained experimentally and PYC40 + T1/T10 (math) calculated mathematically as a sum of percentage inhibition of individuals agents. Values are mean ± SEM of eight independent experiments.

BSA oxidized by Fe^{2+}/H_2O_2 system

Control levels of protein carbonyls in BSA were in the range of 2 ± 0.12 to 3.45 ± 0.08 nmol/mg proteins, carbonyls in oxidized samples reached concentrations of 10.5 ± 0.3 to 16.3 ± 0.2 nmol/mg protein. Antioxidants in the concentration ranges of 10–500 μmol/l of trolox and 2–150 μg/ml of PYC only mildly inhibited protein carbonyl formation by concentration-dependent manner. Combinations of PYC (10, 40 μg/ml) and trolox (100, 250, 500 μmol/l) exerted neither an addive nor a synergistic preventive effect (not shown).

BSA oxidized by HOCl

Oxidation of BSA (1–4 mg/ml) by HOCl (50–200 μmol/l) in the time course of 5–50 min was studied. The most intensive HOCl concentration-dependent increase in protein carbonyls from 1.78 ± 0.03 to 6.47 ± 0.03 nmol/mg proteins was observed

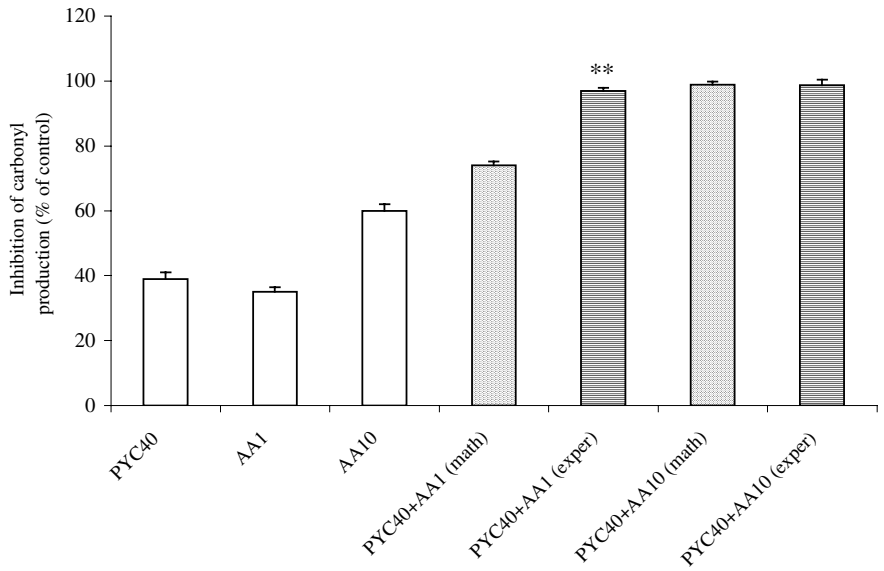

Figure 2. Inhibition of carbonyl production (initiated by oxidative system $H_2O_2/FeSO_4$) in plasma proteins by PYC, ascorbic acid and their combination. Plasma samples were incubated with mentioned antioxidants or phosphate buffer (control sample) at 37°C for 30 min, then incubated with H_2O_2 (10^{-2} mol/l) and $FeSO_4$ (80 μmol/l) at 37°C for 60 min. PYC was used at the constant concentration of 40 μg/ml (PYC40) and ascorbic acid at different concentrations – 1 μmol/l (AA1), 10 μmol/l (AA10). Results are expressed as percentage inhibition of carbonyl production. Inhibition in the absence of antioxidants (control) is 0%. PYC40 + AA1/AA10 (exper) express the combination effect of PYC with AA1 and AA10 on carbonyl production obtained experimentally and PYC40 + AA1/AA10 (math) calculated mathematically as a sum of percentage inhibition of individuals agents. Values are mean ± SEM of eight independent experiments. ** means significant differences between values obtained experimentally and mathematically ($p < 0.01$, for concentration of AA1).

after oxidation of 1 mg/ml protein of BSA. We studied the preventive effect of PYC (20 and 40 μg/ml) and ascorbic acid (20 and 50 μmol/l) and their combination on BSA oxidation by HOCl (200 μmol/l). PYC at the concentration of 20 μg/ml inhibited protein carbonyl production by 44.3%, and at the concentration of 40 μg/ml by 69.2%. Ascorbic acid (20 μmol/l) decreased protein carbonyl concentration by 7.9% and ascorbic acid of 50 μmol/l by 10.4%. The level of protein carbonyls in oxidized BSA in the absence of antioxidant was 6.04 ± 0.11 nmol/mg proteins. Combination of PYC (20 μg/ml or 40 μg/ml) with ascorbic acid 50 μmol/l induced a synergistic effect (Fig. 4). 20 μg/ml PYC exerted a higher synergism ($p < 0.01$) when compared with 40 μg/ml PYC in combination with ascorbic acid ($p < 0.05$). Combination of PYC with 20 μmol/l of ascorbic acid had only an additive effect (not shown).

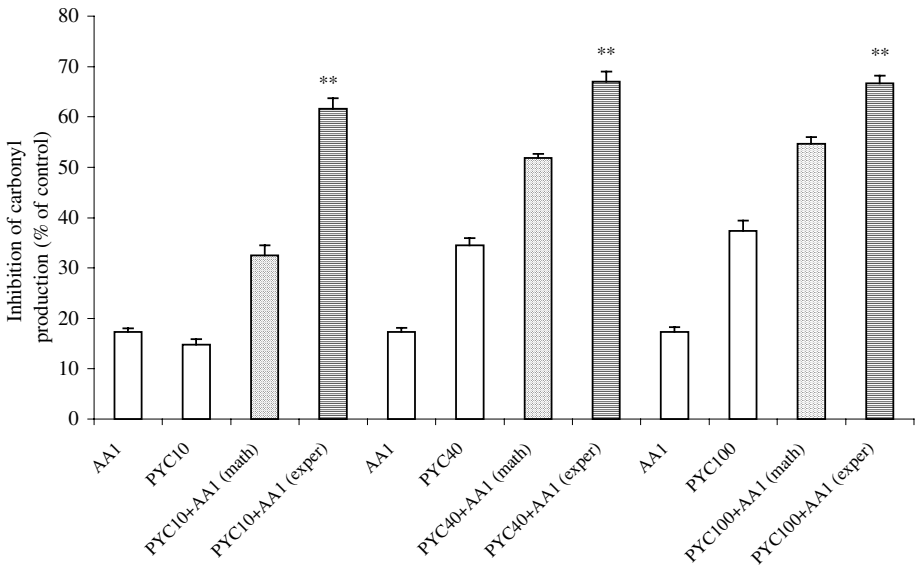

Figure 3. PYC, ascorbic acid and their combination-mediated inhibition of carbonyl production (initiated by oxidative system $H_2O_2/FeSO_4$) in plasma proteins. Plasma samples were incubated with mentioned antioxidants or phosphate buffer (control) at 37°C for 30 min, then incubated with H_2O_2 (10^{-2} mol/l) and $FeSO_4$ (80 μmol/l) at 37°C for 60 min. Ascorbic acid was used at the constant concentration of 1 μmol/l (AA1) and PYC at different concentrations – 10, 40, 100 μg/ml (PYC10, PYC40, PYC100, respectively). Results are expressed as percentage inhibition of carbonyl production. Inhibition in the absence of antioxidants (control) is 0%. PYC10/PYC40/PYC100 + AA1 (exper) express the combination effect of PYC10, PYC40 and PYC100 with ascorbic acid on carbonyl production obtained experimentally and PYC10/PYC40/PYC100 + AA1 (math) calculated mathematically as a sum of percentage inhibition of individuals agents. Values are mean ± SEM of eight independent experiments. ** means significant differences between values obtained experimentally and mathematically ($p < 0.01$, for concentration of PYC10, PYC40, and PYC100).

Similarly trolox at concentrations 50, 100 and 500 μmol/l, which decreased the protein carbonyls in HOCl-oxidized BSA by 4.8 ± 1.7%, 6.9 ± 1.0% and 13.4 ± 1.8% in combination with concentrations of PYC mentioned above, had only additive effects (not shown).

Scavenging of hypochlorous acid

TNB was used as a detector molecule for scavenging HOCl. The absorbance of TNB at 412 nm without the presence of HOCl was set as 100%. Addition of HOCl, in the final concentration of 55 μmol/l, to TNB (84 μmol/l) decreased the TNB content to approximately 50–55%. This oxidation was inhibited in a concentration-dependent manner by ascorbic acid (Fig. 5). PYC and trolox did not exhibit any scavenging

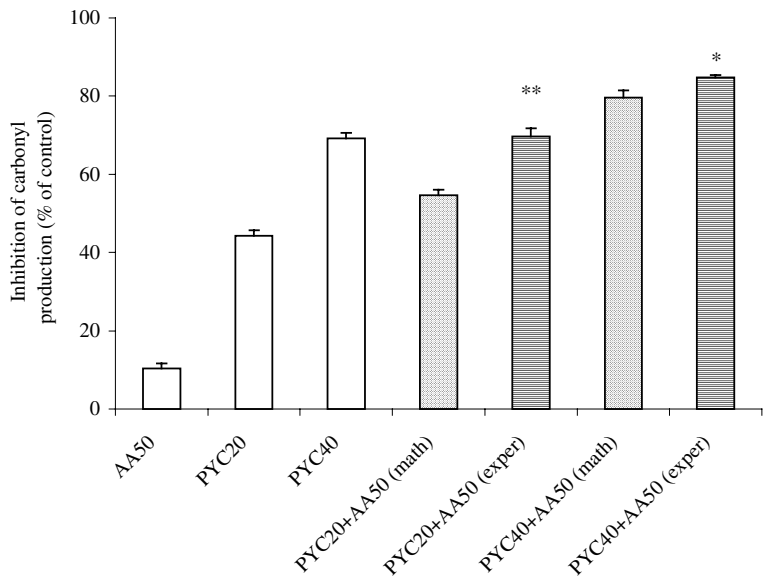

Figure 4. Inhibition of carbonyl production (initiated by oxidative agent HOCl) in BSA by PYC and ascorbic acid and their combination. BSA was incubated with mentioned antioxidants or phosphate buffer (control), then incubated with HOCl (200 μmol/l) at 37 °C for 30 min. Ascorbic acid was used at the constant concentration of 50 μmol/l (AA50) and PYC at different concentrations – 20 μg/ml (PYC20), 40 μg/ml (PYC40). Results are expressed as percentage inhibition of carbonyl production. Inhibition in the absence of antioxidants (control) is 0 %. PYC20/PYC40 + AA50 (exper) express the combination effect of PYC20 and PYC40 with AA50 on carbonyl production obtained experimentally and PYC20/PYC40 + AA50 (math) calculated mathematically as a sum of percentage inhibition of individuals agents. Values are mean ± SEM of at least three independent experiments. * means significant differences between values obtained experimentally and mathematically ($p < 0.05$ for PYC40); ** means significant differences between values obtained experimentally and mathematically ($p < 0.01$ for PYC20).

effect against HOCl. Lipoic acid was used as comparable standard compound with known scavenging properties against HOCl (Ching et al. 1994).

Interaction of PYC with BSA

The interaction of PYC with BSA was determined by measuring their individual absorption spectra and a spectrum of their mixture (BSA+PYC). The spectrum of the given mixture was compared with that mathematically added from individual spectra. A significant decrease in absorbance (52.5 %) measured experimentally in comparison with their mathematical sum was observed. Percentages were calculated from two independent measurements. The spectra of a representative experiment of the interaction of PYC with BSA are depicted in Fig. 6.

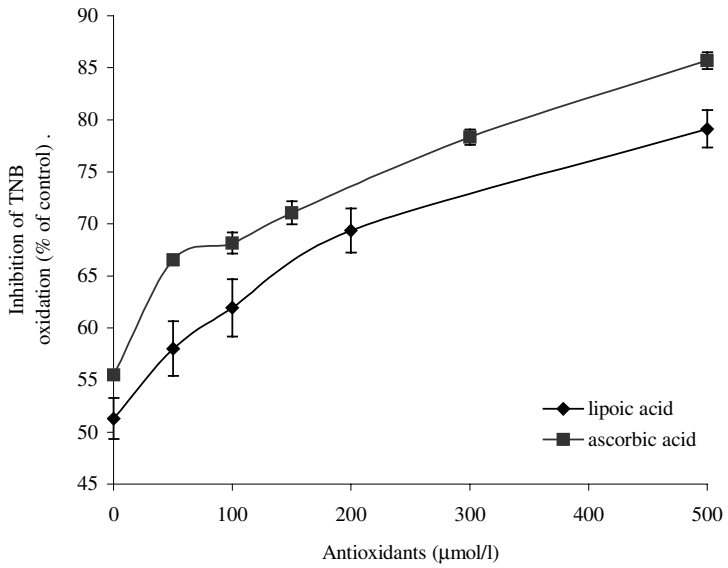

Figure 5. Influence of ascorbic acid (■) and lipoic acid (●) on oxidation of 5-thio-2-nitrobenzoic acid (TNB) by HOCl. Addition of HOCl decreased the content of TNB to approximately 50–55 %. Antioxidants were added before HOCl and increased the content of TNB. Values are the mean ± SEM of three independent experiments measured in at least two parallel measurements.

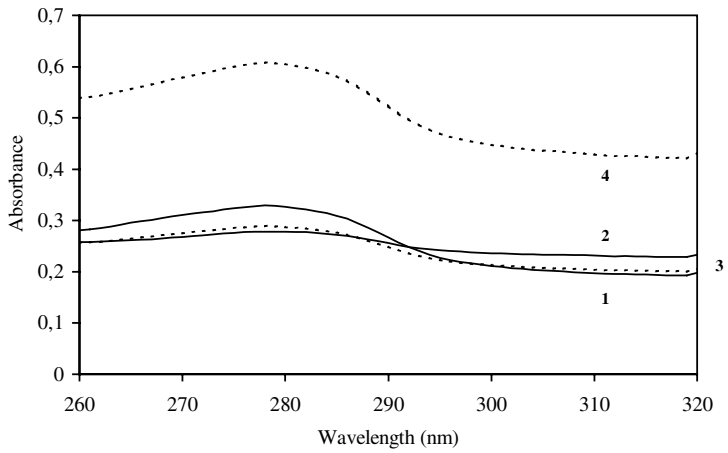

Figure 6. Spectra of individual agents (continuous lines) and their mixtures (interrupted lines). The numbers of individual lines mean spectra of: 0.2 mg/ml BSA (1); 6.8 μg/ml PYC (2); 0.2 mg/ml BSA + 6.8 μg/ml PYC measured experimentally (3); 0.2 mg/ml BSA + 6.8 μg/ml PYC calculated mathematically as a sum of the spectra of individual agents (4). Individual agents or mixtures were incubated for 90 min in phosphate buffer pH 7.4.

Discussion

In our previous study we have shown that PYC has the protective effect against lipid peroxidation, TBARS production and significantly increased the membrane fluidity predominantly at the erythrocyte membrane surface (Sivoňová et al. 2004).

In this project we have studied the effect of PYC, trolox and ascorbic acid on lipid peroxidation induced by hydroxyl radicals produced *via* Fenton's reaction. Based on our results, PYC proved to have a pronounced antioxidant effect on lipid peroxidation of phosphatidylcholine unilamellar liposomes and production of conjugated dienes. We suppose that PYC containing procyanidins and phenolic acids was able to scavenge a very reactive hydroxyl radical. PYC may act as an antioxidant agent also by chelating transition metals, thus preventing formation of hypervalent forms involved in the initiation of peroxidative process. Arora and co-workers (1998) have shown that flavonoids tested in *in vitro* liposomal system exhibited higher antioxidant efficacies against metal-ion-induced peroxidation than against peroxyl-radical-induced peroxidation.

On the other hand, it has mainly been shown that flavonoids may act as pro-oxidants in the presence of the transition metal Cu^{2+}. Both the antioxidant and the copper-initiated pro-oxidant activities of a flavonoid depend upon the number of hydroxyl substitutions in its backbone structure, which has neither antioxidant nor pro-oxidant action (Lebeau et al. 2000).

It is not clear, whether PYC reduces lipid peroxidation either by quenching free radicals or by chelating metal ions, or both. In addition, we assume that protective effect of PYC might be ascribed to the ability of PYC to interact and/or penetrate lipid membrane and change its properties. The presence of different substituents in the backbone structure of flavonoids modulates their incorporation, orientation or interaction with the lipid phase of biomembranes (Lebeau et al. 2000; Sivoňová et al. 2004).

We have confirmed the protective effect of trolox on the lipid peroxidation of unilamellar liposomes. However, we have found that ascorbic acid alone had no antioxidant effect on the lipoperoxidation of unilamellar liposomes in our *in vitro* conditions and a possible co-operation of ascorbate with PYC was not able to study using of this system (Cu^{2+}/*tert*-butylhydroperoxide). Ascorbic acid with metal ions Fe^{3+}/Cu^{2+} is often used to induce the peroxidation of liposome phospholipids (Ratty and Das 1988; Greenspan et al. 1996; Panasenko 1997). On the other hand, when ascorbate was trapped within the unilamellar liposomes and α-tocopherol was incorporated into the liposomal membrane, intravesicular ascorbate prevented oxidation of α-tocopherol and protected membranes from oxidation by oxidants (Waters et al. 1997).

PYC is composed of hydrophilic and lipophilic compounds. We examined its cooperation with ascorbic acid and trolox. In our model system of unilamellar liposomes, PYC with trolox together had the additive effect. We suppose that the cooperation of PYC with trolox depends on the location of these antioxidants in the membrane.

Increased production of reactive oxygen species can lead to the oxidative damage not only to lipids but also to proteins and DNA. Oxidative modification of proteins may occur in a variety of physiological and pathological processes (Ciolino and Levine 1997). The formation of carbonyl groups has been widely used as a convenient index of oxidative modification of proteins leading to the loss of their functions. We decided to examine the protective effect of PYC together with other synthetic antioxidants – ascorbic acid and trolox also on the oxidative damage to plasma proteins represented by carbonyl compounds when Fe^{3+}/Cu^{2+} was used as an oxidant system. In all cases we found that PYC acts in a synergistic or an additive way with mentioned antioxidants in a concentration-dependent manner. Previously, other investigators also used PYC or other natural antioxidants in *in vivo* experiments to study their protective effects on oxidative damage to proteins. Horáková et al. (2003) investigated *in vitro* effect of PYC on the formation of protein carbonyls in experiments studying the effect of different concentrations of H_2O_2 on rat pheochromocytoma (PC12) cell viability. The concentration of PYC 100 μg/ml increased the viability of PC12 cells oxidized by H_2O_2 but did not prevent protein carbonyl oxidation. On the other hand, O'Byrne et al. (2002) investigated the protective effect of Concord grape juice (CGJ) – a rich source of flavonoids, after its supplementation to healthy adults. In their experimental study, protein carbonyl concentration in native plasma decreased by 20 % after CGJ supplementation.

Metal-catalyzed modification of proteins under physiological conditions is relatively insensitive to inhibition by free radical scavengers (Stadtman 1993). Trolox and PYC mildly inhibited protein carbonyl formation in BSA induced by Fe^{2+}/H_2O_2 in a concentration-dependent manner. This inhibition did not reach 50 % inhibition. PYC with trolox together did not prevent BSA from oxidation by Fe^{2+}/H_2O_2, neither synergistically nor additively.

Hypochloric acid, a product of myeloperoxidase is also able to modify proteins oxidatively. Electrophilic substitutions are the dominant mechanisms of hypochlorous acid-induced injury, but free radical reactions may also be involved (Panasenko 1997; Hawkins and Davies 1998). Under pathological conditions, HOCl can be found in body fluids at concentrations of 5–50 μmol/l (Davies et al. 1993) or even at 200 μmol/l in some tissues (Favero et al. 1998). In our experiments the carbonyl content of BSA increased linearly with increasing HOCl concentration up to 200 μmol/l. This is the concentration, which does not induce fragmentation of BSA (Yan et al. 1995) and would lead to formation of chloramines and these spontaneously decompose to corresponding aldehydes. Ascorbic acid in our experiments inhibited protein carbonyl formation by IC_{50} of 305 ± 29 μmol/l, IC_{50} for PYC was 29.8 ± 2.7 μg/ml. Trolox only non significantly inhibited protein carbonyl formation at concentrations tested (50–1000 μmol/l). According to TNB test based on the ability of HOCl to oxidize TNB, ascorbic acid exerted concentration-dependent HOCl scavenging activity. No scavenging effect of HOCl by trolox and PYC was observed. Thus, the preventive effect against protein carbonyl generation was probably caused by interaction of PYC with BSA, as shown by decrease in absorbance (BSA+PYC) by

52.5 % in comparison with the mathematical sum of their absorbances. The additive or synergistic effects of ascorbic acid and PYC or additive effects of trolox and PYC might be caused by ability of flavonoids with higher redox potential (540–700 mV) to be reduced by compounds with lower redox potential, ascorbic acid (300 mV) and vitamin E (480 mV). In addition, also different preventive mechanisms against HOCl-induced injury may be involved in synergistic effects of ascorbic acid and PYC, i.e. the ability of ascorbic acid to scavenge HOCl and ability of PYC to bind to BSA.

We studied oxidative injury of different substrates (liposomes, pure protein (BSA) and plasma lipoproteins) induced by several oxidants generating hydroxyl radicals. Cooperation of PYC with trolox under conditions of BSA oxidation induced neither synergistic nor additive preventive effects. In liposomes and plasma lipoproteins, simultaneous addition of these antioxidants induced an additive preventive effect. Synergistic preventive effects were found only in the system of plasma lipoproteins when combining PYC and ascorbic acid. An additive or synergistic effect of PYC with trolox or ascorbic acid may be caused not only by the radical scavenging activity of PYC but also by its heavy metals chelatation ability as well as by its ability to change properties of lipid membranes. By means of these abilities, PYC can cooperate with radical scavenging activity of ascorbic acid or trolox.

PYC, in cooperation with trolox or ascorbic acid, was studied also in oxidation of BSA by HOCl. Under these conditions, PYC with trolox exerted an additive effect and PYC with ascorbic acid a synergistic effect. In addition to radical scavenging effects of these antioxidants also HOCl scavenging effect of ascorbic acid and binding of PYC to BSA might be involved in synergistic effects of these agents.

Based on our results from *in vitro* study we can conclude that PYC used together with ascorbic acid or trolox at appropriate concentrations may provide the organism an efficient antioxidant protection. However, this protection is dependent on the type of oxidant as well as on the type of oxidized substrate. More experiments using PYC, ascorbic acid and trolox as food supplements are needed to study their effects in *in vivo* conditions.

Acknowledgements. This study was supported partly by VEGA grant of Ministry of Education of Slovak Republic, No. 1/1157/04, and by Drug Research Institute, Modra, Slovak Republic.

References

Arora A., Nair M. G., Strasburg G. M. (1998): Structure-activity relationships for activities of a series of flavonoids in a liposomal system. Free Radic. Biol. Med. **24**, 1355—1363

Babincová M., Machová E., Kogan G. (1999): Carboxymethylated glucan inhibits lipid peroxidation in liposomes. Z. Naturforsch., C: Biosci. **54**, 1084—1088

Ciolino H. P., Levine R. L. (1997): Modification of proteins in endothelial cell death during oxidative stress. Free Radic. Biol. Med. **22**, 1277—1282

Cossins E., Lee R., Packer L. (1998): ESR studies of vitamin C regeneration, order of reactivity of natural source phytochemical preparations. Biochem. Mol. Biol. Int. **45**, 583—597

Chen S. S., Chang L. S., Wei Y. H. (2001): Oxidative damage to proteins and decrease of antioxidant capacity in patients with varicocele. Free Radic. Biol. Med. **30**, 1328—1334

Ching T. L., de Jong J., Bast A. (1994): A method for screening hypochlorous acid scavengers by inhibition of the oxidation of 5-thio-2-nitrobenzoic acid: application to anti-asthmatic drugs. Anal. Biochem. **218**, 377—381

Davies J. M., Horwitz D. A., Davies K. J. (1993): Potential roles of hypochlorous acid and N-chloroamines in collagen breakdown by phagocytic cells in synovitis. Free Radic. Biol. Med. **15**, 637—643

Deiana L., Carru C., Pes G., Tadolini B. (1999): Spectrophotometric measurement of hydroperoxides at increased sensitivity by oxidation of Fe^{2+} in the presence of xylenol orange. Free Radic. Res. **31**, 237—244

de Zwart L. L., Meerman J. H., Commandeur J. N., Vermeulen N. P. (1999): Biomarkers of free radical damage applications in experimental animals and in humans. Free Radic. Biol. Med. **26**, 202—226

Drozdz R., Guevara I., Naskalski J. W. (1995): Immunoglobulin cleavage by hypochlorous acid treatment. Clin. Chim. Acta **236**, 155—160

Favero T. G., Colter D., Hooper P. F., Abramson J. J. (1998): Hypochlorous acid inhibits Ca^{2+}-ATPase from skeletal muscle sarcoplasmic reticulum. J. Appl. Physiol. **84**, 425—430

Filípek J., Uhríková D., Slosarčík P., Balgavý P. (2001): Effect of cholesterol on egg yolk phosphatidylcholine peroxidation in multilamellar liposomes. Pharmazie **56**, 953—957

Fitzpatrick D. F., Bing B., Rohdewald P. (1998): Endothelium-dependent vascular effects of Pycnogenol. J. Cardiovasc. Pharmacol. **32**, 509—515

Greenspan P., Yu H., Gutman R. L., Mao F., Ryu B. H., Lou P. (1996): Iron-ascorbate-phospholipid mediated modification of low density lipoprotein. Biochim. Biophys. Acta **1301**, 242—248

Grimm T., Schäfer A., Högger P. (2004): Antioxidant activity and inhibition of matrix metalloproteinases by metabolites of maritime pine bark extract (Pycnogenol). Free Radic. Biol. Med. **36**, 811—822

Hawkins C. L., Davies M. J. (1998): Degradation of hyaluronic acid, poly- and monosaccharides, and model compounds by hypochlorite: evidence for radical intermediates and fragmentation. Free Radic. Biol. Med. **24**, 1396—1410

Horáková Ľ., Licht A., Sandig G., Jakstadt M., Ďuračková Z., Grune T. (2003): Standardized extracts of flavonoids increase the viability of PC12 cells treated with hydrogen peroxide: effects on oxidative injury. Arch. Toxicol. **77**, 22—29

Lebeau J., Furman C., Bernier J. L., Duriez P., Teissier E., Colette N. (2000): Antioxidant properties of di-*tert*-butylhydroxylated flavonoids. Free Radic. Biol. Med. **29**, 900—912

Liao K., Yin M. (2000): Individual and combined antioxidant effects of seven phenolic agents in human erythrocyte membrane ghosts and phosphatidylcholine liposome systems: importance of the partition coefficient. J. Agric. Food Chem. **48**, 2266—2270

Liptáková A. (1999): Aterosclerosis and free radicals. In: Free Radicals and Antioxidants in Medicine (II). (Eds. Z. Ďuračková, Ľ. Bergendi and J. Čársky), pp. 135—176, Slovak Academic Press, Bratislava (in Slovak)

Lyras L., Evans P. J., Shaw P. J., Ince P. G., Halliwell B. (1996): Oxidative damage and motor neurone disease difficulties in the measurement of protein carbonyls in human brain tissue. Free Radic. Res. **24**, 397—406

Naskalski J. W. (1994): Oxidative modification of protein structures under the action of myeloperoxidase and the hydrogen peroxide and chloride system. Ann. Biol. Clin. (Paris) **52**, 451—456 (in French)

Nelson A. B., Lau B. H., Ide N., Rong Y. Q. (1998): Pycnogenol inhibits macrophage oxidative burst, lipoprotein oxidation and hydroxyl radical-induced DNA damage. Drug Dev. Ind. Pharm. **24**, 139—144

O'Byrne D. J., Devaraj S., Grundy S. M., Jialal I. (2002): Comparison of the antioxidant effects of Concord grape juice flavonoids α-tocopherol on markers of oxidative stress in healthy adults. Am. J. Clin. Nutr. **76**, 1367—1374

Ozgová Š., Heřmánek J., Gut I. (2003): Different antioxidant effects of polyphenols on lipid peroxidation and hydroxyl radicals in the NADPH-, Fe-ascorbate- and Fe-microsomal systems. Biochem. Pharmacol. **66**, 1127—1137

Packer L., Rimbach G., Virgili F. (1999): Antioxidant activity and biologic properties of a procyanidin-rich extract from pine (*Pinus maritima*) bark, Pycnogenol. Free Radic. Biol. Med. **27**, 704—724

Panasenko O. M. (1997): The mechanism of the hypochlorite-induced lipid peroxidation. Biofactors **6**, 181—190

Pietta P. G. (2000): Flavonoids as antioxidants. J. Nat. Prod. **63**, 1035—1042

Ratty A. K., Das N. P. (1988): Effects of flavonoids on nonenzymatic lipid peroxidation: structure-activity relationship. Biochem. Med. Metab. Biol. **39**, 69—79

Reznick A. Z., Packer L. (1994): Oxidative damage to proteins: spectrophotometric method for carbonyl assay. Methods Enzymol. **233**, 357—363

Rice-Evans C. A., Miller N. J., Paganga G. (1997): Antioxidant properties of phenolic compounds. Trends Plant Sci. **2**, 152—159

Rohdewald P. (2005): Pycnogenol®, french maritime pine bark extract. In: Encyclopedia of Dietary Supplements (Eds. P. M. Coates, M. R. Blackman, G. Cragg, M. Levine, J. Moss and J. White), pp. 545—553, Marcel Dekker, Ltd., New York

Rong Y., Li L., Shah V., Lau B. H. (1995): Pycnogenol protects vascular endothelial cells from *t*-butyl hydroperoxide induced oxidant injury. Biotechnol. Ther. **5**, 117—126

Santos M. R., Mira L. (2004): Protection by flavonoids against the peroxynitrite-mediated oxidation of dihydrorhodamine. Free Radic. Res. **38**, 1011—1018

Silliman K., Parry J., Kirk L. L., Prior R. L. (2003): Pycnogenol does not impact the antioxidant or vitamin C status of healthy young adults. J. Am. Diet. Assoc. **103**, 67—72

Singleton W. S., Gray M. S., Brown M. L., White J. L. (1965): Chromatographically homogenous lecithin from egg phospholipids. J. Am. Oil. Chem. Soc. **42**, 53—56

Sivoňová M., Waczulíková I., Kilanczyk E., Hrnčiarová M., Bryszewska M., Klajnert B., Ďuračková Z. (2004): The effect of Pycnogenol® on the erythrocyte membrane fluidity. Gen. Physiol. Biophys. **23**, 39—51

Stadtman E. R. (1993): Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. Annu. Rev. Biochem. **62**, 797—821

Stadtman E. R. (2002): Importance of individuality in oxidative stress and aging. Free Radic. Biol. Med. **33**, 597—604

Tang L., Zhang Y., Qian Z., Shen X. (2000): The mechanism of Fe^{2+}-initiated lipid peroxidation in liposomes: the dual function of ferrous ions, the roles of the pre-existing lipid peroxides and the lipid peroxyl radical. Biochem. J. **352**, 27—36

Tesoriere L., Butera D., Pintaudi A M., Allegra M., Livrea M. A. (2004): Supplementation with cactus pear (*Opuntia ficus-indica*) fruit decreases oxidative stress in healthy humans: a comparative study with vitamin C. Am. J. Clin. Nutr. **80**, 391—395

van Acker S. A., Tromp M. N., Haenen G. R., van der Vijgh W. J., Bast A. (1995): Flavonoids as scavengers of nitric oxide radical. Biochem. Biophys. Res. Commun. **214**, 755—759

Vasiljeva O. V., Lyubitsky O. B., Klebanov G. I., Vladimirov Y. A. (2000): Effect of the combined action of flavonoids, ascorbate and α-tocopherol on peroxidation of phospholipid liposomes induced by Fe^{2+} ions. Membr. Cell Biol. **14**, 47—56

Vinson J. A., Jang J. (2001): *In vitro* and *in vivo* lipoprotein antioxidant effect of a citrus extract and ascorbic acid on normal and hypercholesterolemic human subjects. J. Med. Food **4**, 187—192

Virgili F., Kobuchi H., Packer L. (1998): Procyanidins extracted from *Pinus maritima* (Pycnogenol®): scavengers of free radical species and modulators of nitrogen monoxide metabolism in activated murine RAW 264.7 macrophages. Free Radic. Biol. Med. **24**, 1120—1129

Waters R. E. 2^{nd}, White L. L., May J. M. (1997): Liposomes containing α-tocopherol and ascorbate are protected from an external oxidant stress. Free Radic. Res. **26**, 373—379

Yan L. J., Traber M. G., Packer L. (1995): Spectrophotometric method for determination of carbonyls in oxidatively modified apolipoprotein B of human low-density lipoproteins. Anal. Biochem. **228**, 349—351

Final version accepted: August 24, 2006