# RASPBERRY SEED EXTRACT IMPROVES THE FERROXIDASE ACTIVITY OF CERULOPLASMIN IN PATIENTS WITH LOWER ARTERY CHRONIC TOTAL OCCLUSION*

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## Abstract

Ceruloplasmin (Cp) is the major antioxidant in plasma, a protein that carries about 95% of the total copper. Multiple biochemical activities of ceruloplasmin have been described, including copper transport or oxidation of Fe(II) to Fe(III) for subsequent uptake by transferrin and ferritin. Reduction of the pool of free Fe(II) ions by Cp prevents the generation of free radicals and reactive oxygen species by oxidation of Fe(II), thus inhibiting the Haber-Weiss reaction. The removal of both free Fe(II) and Fe(III) ions from blood plasma by polyphenols enhances the antioxidant system of the living organism. However, the mechanism of interactions between exo- and endogenous antioxidants is still under consideration.

The effect of raspberry seed extract (RSE) on the ferroxidase activity of ceruloplasmin isolated from plasma of patients with chronic arterial occlusion of the lower limbs due to ath-

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erosclerosis ($Cp_{AO}$) was investigated. Moreover, the effect of RSE on the ferroxidase activity of Cp isolated from healthy volunteers ($Cp_C$) was also estimated. The ferroxidase activity of Cp, expressed as $\Delta Fe(II)$, was determined by spectrophotometry with the use of the Fe(II) – histidine complex and ferrozine as a chromogenic reagent. The addition of RSE to samples with the same amount of both $Cp_{AO}$ or $Cp_C$ in each caused an increase in $\Delta Fe(II)$. The polyphenol-rich RSE may assist Cp in the fight against free radicals and reactive oxygen species when the disease occurs due to an excessive use or reduced production of endogenous antioxidants.

**Key words:** raspberry seed extract, atherosclerosis obliterans, ceruloplasmin, ferroxidase activity, natural antioxidants.

# INTRODUCTION

Particularly elevated production of free radicals and reactive oxygen species (ROS) occurs as a result of upsetting the balance between antioxidant and pro-oxidant processes. This imbalance, accompanied by a lower activity of antioxidant enzymes and a reduced concentration of low-molecular-weight antioxidants, plays a significant role in the pathogenesis of many diseases. Oxidative stress, together with other risk factors, is the background of many pathological conditions, including atherosclerosis (MAJEWSKI et al. 2007). The occlusion of the lower limbs due to atherosclerosis may lead to moderate or critical ischaemia of the lower limbs. Reperfusion injuries and ischaemic changes in tissues entail the formation of various reactive oxygen species. The latter are resistant to elimination, due to insufficient adaptive mechanisms of the organism, and are produced when the body's antioxidant status is significantly reduced (HARRIS 1992).

Inflammatory reactions accompanying ischaemia increase the concentration of Cp and its activity in serum (MAJEWSKI et al. 2007). The results of their study revealed that patients with critical limb ischaemia, especially those with necrotic changes, had a significantly higher concentration and oxidase activity of Cp compared to controls (median: 164.8 U $dm^{-3}$ and 216.6 U $dm^{-3}$, respectively). ISKRA and MAJEWSKI (2001) demonstrated that ischaemia affects the concentration of Cu ions, the oxidase activity of Cp in serum and the activity of superoxide dismutase (SOD) in the erythrocytes of men with critical and moderate ischaemia of the lower limbs due to atherosclerosis obliterans. Furthermore, the concentration of Cu and the activity of Cp increased with the stage of ischaemia and appeared to be higher in critical ischaemia than in a moderate stage of the disease. A reverse pattern was observed with regard to SOD activity in erythrocytes, namely it was higher in moderate and lower in critical ischaemia. Probably, the activity of SOD in erythrocytes reaches the upper normal level. It suggests that inflammatory reactions accompanying ischaemia depress the synthesis of SOD.

The biological effects of ROS are controlled *in vivo* by the antioxidant defense system including enzymes such as ceruloplasmin (SHUKLA et al. 2006) and superoxide dismutase (YU et al. 2007). Ceruloplasmin (Cp) is the

main plasma protein with multiple biochemical activities including copper transport, oxidation of various amines, antioxidant activity against lipid peroxidation, and finally oxidation of Fe(II) to Fe(III), for subsequent uptake by transferrin and ferritin. Fe(II) oxidation by Cp prevents the generation of oxygen species otherwise produced in the Haber-Weiss reaction. Cp is an acute-phase plasma protein, whose relative concentration and ferroxidase activity increase during inflammation (LOURO et al. 2000), in critical lower limb ischaemia (ISKRA, MAJEWSKI 1999), colorectal cancer (ZOWCZAK et al. 2001), pregnancy (FOSET et al. 2004), after trauma (**JOUNG** et al**.** 1998) and in atherosclerosis (PIORUŃSKA-STOLZMANN et al. 2001).

The enzymatic antioxidant system of the human body may be supported by exogenous antioxidants delivered with food. Polyphenols, components of various dietary products, may inhibit the metal-dependent processes generating reactive oxygen species and exhibit the antioxidant activity. Polyphenols from catechol or gallol groups are effective metal chelators and the most potent antioxidants (PERRON, BRUMAGHIM 2009). Due to a high redox potential Fe(II) ions have the ability to generate free radicals ($O_2 \cdot^-$ or $\cdot OH$) and the risk increases in pathological conditions often accompanied by a high concentration of unbound Fe(II) ions.

Fruits and their extracts are efficient sources of polyphenols. In the previous studies, the effect of raspberry seed extract (RSE) (GRYSZCZYŃSKA et al. 2009) and blackcurrant seed extract (BcsE) (BUDZYŃ et al. 2009) on the removal of Fe(II) ions from the reaction moiety was studied *in vitro*. Both RSE and BcSe affected the antioxidant properties of Cp by increasing its ferroxidase activity of the model mixture.

The interaction between polyphenolic compounds and Cp, or any other endogenous antioxidants of the human body, is underestimated. The study was carried to find to what extent polyphenolic compounds might support endogenous antioxidants to prevent generation of free radicals and reactive oxygen species. The prolonged state of arterial occlusion of lower limbs represents a pathological process, causing an excessive use or insufficient production of endogenous antioxidants in living organisms. The aim of the study was to investigate the effect of RSE on the ferroxidase activity of Cp isolated from plasma of healthy volunteers and patients with chronic arterial occlusion of lower limbs due to atherosclerosis (atherosclerosis obliterans, AO).

## MATERIAL AND METHODS

### Chemicals

$(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ (Mohr's salt), $(NH_4)_2SO_4$, NaCl, $KH_2PO_4$, $K_2HPO_4$, $CH_3COOH$, $CH_3COONa$, chloroform and ethanol were purchased from POCh (Gliwice, Poland). 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulphonic acid sodium salt (ferrozine), histidine, hexane, Folin & Ciocalteu's phenol

reagent, DEAE-Sephadex A-25 chloride form, caffeic acid, ellagic acid, quercetine were purchased from Sigma-Aldrich (St. Louis, MO, USA). All reagents and solvents were of analytical grade of purity.

## Preparation of raspberry seed extract

Raspberry seeds, obtained as waste material from a food processing company in Kotlin, Poland, were first dried, comminuted, defatted with hexane and finally extracted by using 80% aqueous ethanol (GRYSZCZYŃSKA et al. 2009). For further studies, the raspberry seed extract was prepared as a solution diluted in PBS (0.05 mol $dm^{-3}$, pH 7.38) in a 1:100 ratio (v/v). The ethanolic extracts was characterized in terms of their total phenolic content measured by Folin Ciocalteau (SINGLETON, ROSSI 1965). The content of flavonoids and flavanols was determined by the Deleu's method (DELEU et al. 2000). Determination of anthocyanins was made by the Wrolstad's method (WROLSTAD et al. 2005) according to the Wiesenborn and co-workers' procedure (WIESENBORN et al. 1993). The high-performance liquid chromatography (HPLC) analysis of flavonoids and ellagic acid present in the ethanolic extract of analysed seeds was performed at room temperature on a Waters 600 high-performance liquid chromatographer (Waters, Milford, MA, USA) equipped with a Symmetry $C_{18}$ column (150 x 3.9 mm, 5μm) and fitted with a μBondapak $C_{18}$ guard column (Waters, Milford, MA, USA). For the simultaneous determination of flavonoids and ellagic acid in ethanolic extract, a gradient of mobile phase: acetonitrile (solvent A) and water adjusted to pH 2.5 with trifluoroacetic acid (solvent B) was developed and used according to the following program: isocratically 15% A for 15 min, linear increment starting with 15- 35% in 18 min and the return to the initial conditions within the next 10 min at a flow rate of 1 ml $min^{-1}$. Flavonoids and ellagic acid were detected using a Waters 996 photodiode-array detector set at 370 nm. The total procyanidin content was measured by the method given by Ph. Eur.7 (European Pharmacopoeia 7.0), Hawthorn berries 01/2008:1220 corrected 6.0. The ethanolic extract was first purified and then phenolic acids were isolated on a quaternary amine Bakerbond SPE column using the modified methodology proposed by Głowniak (GŁOWNIAK et al. 1996). The modification consisted in omitting preliminary purification on an octadecyl ($C_{18}$) column (octadecyl, 500 mg, Baker, Phillipsburg, NJ, USA) and increasing the polarity of $H_3PO_4$-MeOH mixture in order to elute the absorbed fraction of phenolic acids. A BAKER SPE 12G (J.T. Baker, Philipsburg, NJ, USA) system with SPE Bakerbond columns filled with quaternary amine (500 mg) was used for the isolation of phenolic acid fraction. Analysis of the phenolic acid content in the ethanolic extract was performed by HPLC according to the Klimczak's procedure (KLIMCZAK et al. 2002).

The analysed compounds were identified on the basis of their absorption spectra and retention times compared with corresponding standards. Quantitative analyses were made using the external standard method. All analyses

were performed in triplicate and the results are reported as means±standard deviation.

## Composition of raspberry seed extract

The total content of phenolic compounds in the raspberry seeds expressed in mg of caffeic acid equivalents per 100 g of dry matter of seeds was $2370.5 \pm 110$ mg 100 $g^{-1}$ d.m, and is given in Table 1.

Table 1

The content of phenolic compounds in raspberry seed extract

| Phenolic compounds | Raspberry seeds (mg 100 $g^{-1}$ d.m.) |
|---|---|
| Total phenolic content [1] | $2370.5 \pm 110$* |
| flavonoids | $464.2 \pm 12.5$ |
| flavanols[2] | $383.7 \pm 12.0$ |
| anthocyanins | $8.7 \pm 0.3$ |
| remaining flavonoids[3] | $71.8 \pm 3.5$ |
| procyanidins[4] | $772.5 \pm 0.3$ |
| phenolic acids[5] | $110.5 \pm 3.7$ |
| * mean ± standard deviation [1] calculated as caffeic acid [2] calculated as (-)epicatechin [3] calculated as rutin [4] calculated as cyanidin chloride [5] analyzed by HPLC | |

## Isolation and purification of ceruloplasmin from human plasma samples

For the purpose of the study, 100 ml of blood plasma from healthy volunteers (control, C) and 100 ml of blood plasma from patients with atherosclerosis obliterans (AO) were used for the isolation of Cp. $Cp_C$ and $Cp_{AO}$ were isolated and purified according to the procedure detailed in the previous paper (GRYSZCZYŃSKA et al. 2009). The purification procedure yielded an essentially pure preparation of $Cp_C$ and $Cp_{AO}$, and the absorbance $A_{610}/A_{280}$ ratio reached the value of 0.036 and 0.031, respectively.

## The effect of raspberry seed extract on the ferroxidase activity of $Cp_{AO}$ and $Cp_C$

The ability of RSE alone to eliminate Fe(II) was analyzed within its concentration range of 1.2-36.0 μg d.m. $ml^{-1}$. The effect of RSE on the ferroxidase activity of $Cp_{AO}$ and $Cp_C$ was measured in a solution containing RSE within the range of 1.20-36.0 μg $ml^{-1}$ d.m. and Cp at chosen concentrations

(22, 33, 44, and 66 mg dm$^{-3}$). In the presence of Cp or RSE, Fe(II) ions are oxidized to Fe(III) and the remaining amount of Fe(II) forms a more stable complex with ferrozine in the oxidation state specific reaction, yielding a product measured spectrophotometrically (Juan, Aust 1998). A change in Fe(II) concentration was measured according to the procedure described by Gryszczyńska et al. (2009). The solutions studied contained appropriate amounts of Cp and RSE, Fe(II) ions (71.4 µmol dm$^{-3}$), histidine (1.1 mmol dm$^{-3}$), ferrozine (4.0 mmol dm$^{-3}$) and were incubated at the temperature 37ºC for 1 minute. The ferroxidase activity of Cp and the effect of RSE were expressed as a change in the Fe(II) ions concentration [$\Delta$Fe(II)] in a solution.

### Statistical analysis

All measurements were carried out 8 times and the results were expressed as means± standard deviation. Statistical differences between the compared groups of results were estimated by using Student's $t$-test. The significance level was accepted at p<0.01.

## RESULTS AND DISCUSSION

The study showed the effect of both RSE and chronic disease (AO) on the ferroxidase activity of Cp. Within the range of Cp concentration of 22 to 66 mg dm$^{-3}$, the ferroxidase activity of $Cp_{AO}$ was found higher than that of $Cp_{C}$ (Figure 1). The data presented in Table 2 confirm the effect of Cp concentra-
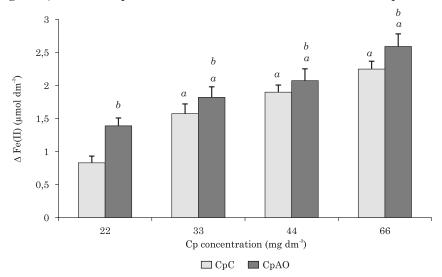


Fig 1. Effect of $Cp_{AO}$ and $Cp_{C}$ concentration on the ferroxidase activity (expressed as $\Delta$Fe(II) (µmol dm$^{-3}$): $a$ – significant difference vs. concentration of Cp 22 mg dm$^{-3}$, $b$ – significant difference between $Cp_{AO}$ and $Cp_{C}$, $p < 0.001$)

Table 2

The effect of atherosclerosis obliterans (AO) and Cp concentration on Fe(II) ion elimination (ΔFe(II))

| Cp (mg dm$^{-3}$) | $Cp_C$ | | $Cp_{AO}$ | |
|---|---|---|---|---|
| | Δ Fe(II) (μmol dm$^{-3}$) | Fe(II) (%) | Δ Fe(II) (μmol dm$^{-3}$) | Fe(II) (%) |
| 22 | 0.83 | 100* | 1.39 | 100* |
| 33 | 1.08 | 130 | 1.79 | 129 |
| 44 | 1.84 | 222 | 2.05 | 147 |
| 66 | 2.27 | 273 | 2.55 | 183 |

* ΔFe(II) obtained for $Cp_C$ or $Cp_{AO}$ at concentration of 22 mg l$^{-1}$ was estimated as 100%

tion and chronic disease on its activity. However, for higher Cp concentration, i.e. 44 and 66 mg dm$^{-3}$, the ability of $Cp_{AO}$ to oxidize Fe(II) to Fe(III), expressed as Fe(II)%, was found lower in comparison to Cpc.

The ferroxidase activity of Cp depends on some factors, and is related mainly to the oxidative status of copper ions present in the copper centers, the conformation of the polypeptide chain of Cp. It was expected that the ferroxidase activity of $Cp_{AO}$ may differ from that of $Cp_C$ due to effect of ischaemia and excessive usage of Cu(II)). The maximum concentration of Fe(II) eliminated by $Cp_{AO}$ or $Cp_C$ alone was estimated. The parameters of the regression between the reciprocal of the concentration of $Cp_{AO}$ or $Cp_C$ and the reciprocal of ΔFe(II) were calculated. The highest decrease in Fe(II) found for $Cp_{AO}$ and $Cp_C$ was 4.43 and 45.05 μmol dm$^{-3}$, respectively (Table 3).

Table 3

The maximum concentration of Fe(II) ions eliminated by $Cp_{AO}$ and $Cp_C$

| $Cp_C$ | | $Cp_{AO}$ | |
|---|---|---|---|
| $y = ax + b$ | $1/b = \Delta Fe(II)_{max}$ | $y = ax + b^*$ | $1/b = \Delta Fe(II)_{max}$ |
| $y = 26.619x + 0.0222$ | 45.05 | $y = 10.86x + 0.2258$ | 4.43 |

* Double reciprocal curve: $1/\Delta Fe(II) = a (1/Cp_C) + b$ or $1/\Delta Fe(II) = a (1/Cp_{AO}) + b$. When $x = 0$ then $y = b$, and $b = 1/\Delta Fe(II)$.
$1/b = \Delta Fe(II)_{max}$ is the maximum possible decrease in Fe(II) concentration

The assumption that some exogenous antioxidants may improve the ferroxidase activity of Cp was a good reason for evaluating the cooperation between them in the Fe(II) removal from a solution. The ability of RSE to eliminate Fe(II) was analyzed first. RSE was proved to be capable of eliminating Fe(II) ions within the studied range 1.2 to 36.0 μg ml$^{-1}$ d.m. (Figure 2 and Figure 3). The addition of varied amounts of RSE to the mixtures with a constant amount of $Cp_{AO}$ or $Cp_C$ caused a significant decrease in Fe(II), shown as ΔFe(II) in Figure 2 and 3, respectively. The effect of RSE on the ferroxidase activity of $Cp_{AO}$ or $Cp_C$ was dosedependent for each Cp concentration. However, the addition of higher concentrations of RSE (12.0 μg ml$^{-1}$ d.m.

Fig. 2. Effect of raspberry seed extract (RSE) concentration on the ferroxidase activity (expressed as $\Delta$Fe(II)) of mixture of ceruloplasmin ($Cp_{AO}$) and RSE: ($a - p < 0.001$, $b - p < 0.01$ – significant differences $vs$ samples without RSE or significant differences $vs$ samples with the lowest concentration of RSE without $Cp_{AO}$)



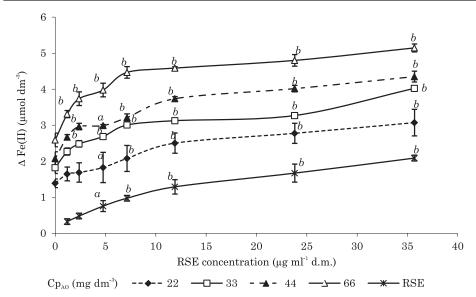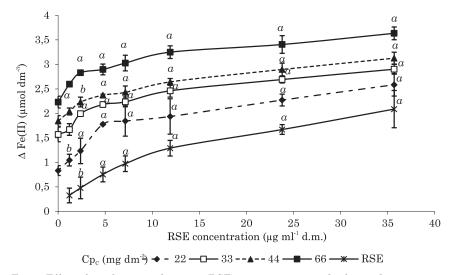Fig. 3. Effect of raspberry seed extract (RSE) concentration on the ferroxidase activity (expressed as $\Delta$Fe(II)) of mixture of ceruloplasmin ($Cp_C$) and RSE: $a - p < 0.001$, $b -$ p<0.01 – significant differences $vs$ samples without RSE or significant differences $vs$ samples with the lowest concentration of RSE without $Cp_C$)

and more) to the samples with a constant amount of $Cp_{AO}$ or $Cp_C$, especially within a higher concentration range, i.e. 44 and 66 mg dm$^{-3}$, caused less efficient Fe(II) removal in both groups.

The study revealed that the ferroxidase activity of $Cp_{AO}$ increases in patients with AO in comparison to control subject. This implies the participation of Cp in the acute phase response and the antioxidant barrier, which is in agreement with the previous study (MAJEWSKI et al. 2007). However, since $Cp_{AO}$ is required to act under oxidative stress conditions, the efficiency of Fe(II) oxidation may be changed. It has been shown that $\Delta$Fe(II) of $Cp_{AO}$ is significantly higher than $\Delta$Fe(II) of $Cp_C$, but the relative increase in the ferroxidase activity of $Cp_{AO}$ is lower in comparison with $Cp_C$, particularly at Cp concentration of 44 and 66 mg dm$^{-3}$ (lower Fe(II)%$_{AO}$ than Fe(II)%$_C$, Table 2). Although further studies are required to find the reason for differences between Fe(II)%$_{AO}$ and Fe(II)%$_C$, it may be suggested that the development of AO causes more intensive usage of antioxidants and a higher demand for the activity of antioxidant enzymes. The efficacy of endogenous antioxidants system may be increased by supplements with exogenous antioxidant acting similarly.

In the present study, the substrate concentration, i.e. Fe(II) ions, was the same (71.4 µmol dm$^{-3}$) in all model solutions. For the evaluation of the maximum amount of Fe(II) ions that could be eliminated by RSE with or without Cp, the regression parameters of the reciprocal of $\Delta$Fe(II) and RSE concentrations were calculated. The maximum decrease in Fe(II) caused by Cp studied alone showed that $\Delta$Fe(II)$_{maxAO}$ was ten times lower than $\Delta$Fe(II)$_{maxC}$ (Table 3). Similar calculations were carried out for RSE alone (1.87 µmol dm$^{-3}$) and revealed that the value of $\Delta$Fe(II)$_{max}$ were higher (9-44%) for samples with RSE and $Cp_{AO}$ than for $Cp_C$ (Table 4). It may be suggested that the oxidative stress during chronic disease can diminish the ability of $Cp_{AO}$ to oxidize Fe(II) ions and/or modify both the structure of Cp and the oxidative status of copper ions as well.

Table 4

Assessment of the maximum amounts of Fe(II) ions ($\Delta$Fe(II)$_{max}$) eliminated by RSE with and without Cp

| Cp (mg dm$^{-3}$) | $Cp_C$ | | $Cp_{AO}$ | |
|---|---|---|---|---|
| | $y = ax + b^*$ | $1/b = \Delta$Fe(II)$_{max}$ | $y = ax + b^*$ | $1/b = \Delta$Fe(II)$_{max}$ |
| RSE without Cp | $y = 3.1909x + 0.5340$ | 1.87 | $y = 3.1909x + 0.5340$ | 1.87 |
| 22 | $y = 0.6859x + 0.4294$ | 2.33 | $y = 0.3237x + 0.3919$ | 2.55 |
| 33 | $y = 0.2836x + 0.3758$ | 2.66 | $y = 0.1979x + 0.2963$ | 3.38 |
| 44 | $y = 0.1818x + 0.3564$ | 2.80 | $y = 0.1567x + 0.2613$ | 3.83 |
| 66 | $y = 0.1173x + 0.2977$ | 3.36 | $y = 0.1245x + 0.2067$ | 4.84 |

* Double reciprocal curve: $1/\Delta$Fe(II) $= a (1/RSE) + b$. When $x=0$ then $y=b$, and $1/\Delta$Fe(II) $= b$.
$1/b = \Delta$Fe(II)$_{max}$ is the maximum possible decrease in Fe(II) concentration

The possibility of oxidative modification of human enzymes has been implicated in a variety of studies. Kang (2006) demonstrated that Cp incubated with methylglyoxal, an endogenous physiological metabolite, loses its ferroxidase activity and releases copper ions. Methylgloxal probably reacts with Cp, in particular with lysine and arginine residues, leading to the formation of cross-linked products. Kang's results suggest that the decrease in Cp ferroxidase activity by methylgloxal is associated with enzymatic protein modification. Furthermore, copper ions released from a modified Cp molecule can enhance metal-catalyzed reactions that generate free radicals and reactive oxygen species and then induce the oxidative modifications of macromolecules. Musci et al. (1993) noticed an age-related difference in the redox state of Cp. They found that the type 1 Cu(II) ions had a tendency to be reduced in Cp isolated from young donors and to stay oxidized in Cp of elderly people. Spectroscopic analyses suggest that Cp can be oxidatively modified around copper sites during aging. However, in the study of hydrogen peroxide effect on Cu,Zn-superoxide dismutase, Choi et al. (1999) observed that the incubation of Cu,Zn-SOD with $H_2O_2$ at a physiological concentration led to the fragmentation and lower activity of the enzyme. Furthermore, the authors demonstrated an increase in the $^.$OH concentration during the incubation of Cu,Zn-superoxide dismutase with $H_2O_2$, which may suggest that the fragmentation of the enzyme results from $^.$OH formation in Cu,Zn-SOD/$H_2O_2$ mixture. Two possible mechanisms were provided to explain the involvement of copper ions in this process. One assumes that $^.$OH reacts with Cu,Zn-SOD directly, leading to a decrease in the enzyme's activity and a release of copper ions. The other one suggests that released copper ions can enhance the Fenton reaction to produce $^.$OH radicals and increase the damage of the enzyme.

Several reports have indicated the antioxidant activity of polyphenol-rich berries. Bowen-Forbes et al. (2010) demonstrated that the content of antioxidants in fresh or processed berries (*Rubus acuminatus, Rubus idaeus, Rubus racemosus, Rubus rosifolius*) was in the range of 146-2199 mg 100 g$^{-1}$ fresh weight, and their extracts showed good antioxidant activity and the ability to inhibit lipid peroxidation. Van Acker et al. (2000) suggested that flavonoids can even replace α-tocopherol as an antioxidant, and proved the ability of flavonoids to cooperate with gluthatione and to replace α-tocopherol as a chain-breaking antioxidant in hepatic microsomal membranes. It is not uncommon for fruit or any other plant material to contain different levels of polyphenolic compounds and hence varied antioxidant capacity, depending on many factors, such as cultivation conditions, insolation, collecting time, storage time and conditions (Bobinaite et al. 2012). In the present study, the RSE demonstrated the ability to eliminate Fe(II) ions in a dose-dependent way. However, addition of higher concentrations of RSE (12.0 μg ml$^{-1}$ d.m.) to samples with a constant amount of Cp, caused less efficient oxidation/chelation of Fe(II) in both groups. It may be suggested that the highest efficiency of oxidation/chelation reaction is reached and addition of more Cp and RSE

does not cause any further change in the Fe(II) concentration because of the competition for the substrate, i.e. Fe(II) ions, whose level *in vitro* is limited.

Although the ferroxidase activity of $Cp_{AO}$ is higher than that of $Cp_C$, it seems that the impact of RSE on eliminating Fe(II) ions is stronger in the presence of $Cp_{AO}$. The study revealed that the values of $\Delta Fe(II)_{max}$ for samples with RSE alone were usually lower than in the presence of $Cp_{AO}$ or $Cp_C$. It may be suggested that polyphenolic compounds and other components of the extract, such as vitamin C (BOWEN-FORBES et al. 2010), which create a dynamic redox system, can probably affect the value of $\Delta Fe(II)_{max}$. Moreover, the extract components are capable of lowering the concentration of Fe(II) ions by oxidation or chelation, but it would be difficult to find out which one of the two processes is more efficient in an experimental model.

Polyphenols may act as antioxidants by metal chelation and/or the scavenging of free radicals (JIA et al. 2012, TÖRRÖNEN et al. 2012) and reactive oxygen species (ANDJELKOVIĆ et al. 2006). It seems that the ability of phenolic compounds to oxidize unbound Fe(II) ions (CHVÁTALOVÁ et al. 2008), followed by Fe(III) ions binding to apotransferrin and ferritin, may represent a beneficial mechanism that could assist in the elimination of unbound Fe(II) ions by ceruloplasmin in the human body. The enhancement of the ferroxidase activity of Cp in the presence of RSE shows the cooperation of both types of antioxidants in alleviating effects of the oxidative stress. Therefore, the beneficial influence of RSE, rich in polyphenols, results from the ability to support mechanisms regulating the protein bound-iron level in the human body.

## CONCLUSION

The present study shows that the development of chronic arterial occlusion of the lower limbs due to atherosclerosis increases the ferroxidase activity of $Cp_{AO}$ in comparison with $Cp_C$. The addition of RSE improved the antioxidant capacity of $Cp_{AO}$ and $Cp_C$, but the increase for $Cp_{AO}$ was observed to be higher. Polyphenol-rich RSE may assist Cp, or even dominate when the disease causes an excessive use or inferior production of endogenous antioxidants.

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