

THE INFLUENCE OF BLACK CURRANT (*RIBES NIGRUM*) SEED EXTRACT ON EFFECTIVENESS OF HUMAN CERULOPLASMIN IN Fe(II) IONS ELIMINATION

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Abstract

Ions of irons, especially ferrous ions may be harmful for living organisms, because they generate reactive oxygen species like $O_2^{\bullet-}$ or $\bullet OH$. Probability of the risk rises especially in pathological conditions, in which high level of iron is observed. For this reason scientists try to establish new methods that can support organism in eliminating reactive ferrous ions.

Nowadays, attention focuses on substances present in plants, especially polyphenols, whose administration prevents oxidative damages in iron overloading.

This new approach requires some research on behavior of plant-derived compounds in human organism, within a system containing other biomolecules involved in iron metabolism. The aim of this study has been to investigate the influence of black currant (*Ribes nigrum*) seed extract, a source of polyphenols, on the activity of ceruloplasmin, an enzyme participating in Fe(II) elimination from blood plasma in human organism. Depletion of Fe(II) caused by ceruloplasmin isolated from healthy blood donors was compared to its decrease in a system containing both ceruloplasmin and the extract. The results have shown that addition of a particular amount of the extract elevates the effectiveness of ceruloplasmin in eliminating Fe(II) from the sample but only under physiological condition (pH 7.4; T 37°C). In a weak acidic solution, addition of the extract does not lead to any change in Fe(II) concentration.

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WPLYW EKSTRAKTU Z PESTEK PORZECZKI CZARNEJ (*RIBUS NIGRUM*) NA SKUTECZNOŚĆ LUDZKIEJ CERULOPLAZMINY W ELIMINOWANIU Fe(II)

Abstrakt

Jony żelaza, szczególnie jony żelazawe Fe(II), ze względu na wysoki potencjał oksydo-redukcyjny mają zdolność generowania wolnych rodników, takich jak $O_2^{\bullet-}$ i $\bullet OH$. W pewnych stanach patologicznych, którym towarzyszy przeładowanie organizmu żelazem, ryzyko pojawienia się wolnych jonów Fe(II) inicjujących wiele procesów wolnorodnikowych jest bardzo prawdopodobne. W tym celu dąży się do opracowania terapii, które w chorobach żelazozależnych wspomogą naturalne mechanizmy usuwania reaktywnych jonów żelaza z organizmu. Ostatnio dużą uwagę poświęca się aktywnym substancjom obecnym w roślinach, szczególnie związkom polifenolowym, ze względu na ich silne właściwości chelatujące. Ten nowy kierunek badań wymaga jednak wnikliwej analizy, która wyjaśni mechanizm współdziałania substancji roślinnych z innymi biomolekułami zaangażowanymi w prawidłowy metabolizm żelaza w organizmie oraz rozstrzygnie o bezpieczeństwie ich stosowania. Celem badań było ustalenie wpływu ekstraktu z pestek porzeczki czarnej (*Ribes nigrum*), bogatego w związki polifenolowe, na aktywność ludzkiej ceruloplazminy, enzymu uczestniczącego w usuwaniu Fe(II) w organizmie ludzkim. Ubytek Fe(II) obserwowany w obecności ceruloplazminy porównywano z jego ubytkiem w mieszaninie ceruloplazmina-ekstrakt. Stwierdzono, że dodatek ekstraktu podnosi skuteczność ceruloplazminy w eliminowaniu Fe(II) ze środowiska. Istnieje więc przypuszczenie, że związki pochodzenia roślinnego takie jak polifenole mogą wspomagać naturalne mechanizmy eliminowania Fe(II) w organizmie.

Słowa kluczowe: ekstrakt z pestek porzeczki czarnej (*Ribes nigrum*), polifenole, ceruloplazmina, aktywność ferroksozydazowa, jony żelazawe.

INTRODUCTION

Iron is an essential element in human organism, necessary for such basic processes as oxygen transport or cell respiration. This element is an important component of many enzymes and metalloproteins, which are involved in DNA synthesis, cholesterol metabolism and processes of detoxification. Despite beneficial effects of iron, there are some dangers connected with the presence of unbound iron ions in biological fluids or tissues. Ions of irons, especially ferrous ions, may generate reactive oxygen species (ROS) in Fenton, Haber-Weiss reactions or during the process of their non-enzymatic oxidation. The level of reactive iron in human organism is precisely regulated by ceruloplasmin (ROESER et al. 1970, STOCKS et al. 1974) blue plasma copper protein, composed of three 42-45 kDa domains that are homologous to each other (ORTEL et al. 1984) and similar to domains in factors V and VIII of coagulation cascade (CHURCH et al. 1984). Ceruloplasmin catalyzes the oxidation of ferrous ions into less reactive ferric ions, subsequently bind-

ed by transport and storage proteins: apotransferrin and apoferritin (RYDEN 1984). Ferroxidase activity of the protein, prevents organism from ROS generation catalyzed by free ferrous ions.

In some pathological condition related to disorders of iron absorption and metabolism, regulation mechanism may fail and lead to uncontrolled increase of iron concentration in human organism. It is observed during the course of a such diseases as aceruloplasminemia, atransferrinemia, beta-talasemia, obesity, hypertension or diabetes type II (POWELL 2002). In the case of patients suffering from iron overload diseases, the production of ROS, especially hydroxyl radical is highly probable (SOCHASKI et al. 2002, MCCULLOUGH, BARTFAYB 2007). Free radicals can cause oxidative modification of biomolecules, mainly lipids peroxidation, protein injury, DNA damage and lead to dysfunction of many organs or cancerogenesis. Under these circumstances it is necessary to take up some precautions which can support organism in eliminating reactive ferrous ions and prevent their dangerous effects. Nowadays, patients with iron overload diseases are treated with bloodletting or chelating therapy using synthetic, hard-assimilate compounds (DAGG 1974). Invasiveness of these methods and number of side effects force us to search for safer therapies.

Recently, many researches have focused on active substances derived from plants and supplied to organism in daily diet. Fruit and vegetables are rich in vitamin C, tokoferols, karetonids and polyphenols, which act as antioxidants. This important antioxidant action of the compounds is based on their ability to sequester metal ions, therefore some researchers pay attention to their use in metal poisoning.

It was indicated that the administration of polyphenols like quercetin, rutin and silibin reduces the oxidation processes in iron overload diseases (ZHAO et al. 2005, ZHANG et al. 2006) resulting from strong chelating activity of these compounds towards ferrous ions as well as from the ability of polyphenols to eliminate free radicals generated by metal (AFANAS·EV et al. 1989, MORAN et al. 1997, YOSHINO et al. 1998, ZHAO et al. 1998, MIRA et al. 2002). *In vitro*, radical scavenging and iron-chelating activities are also observed for plant extracts containing polyphenols, like black currant, (*Ribes nigrum*) rich in anthocyanins and flavonols (LUGASI, HOVARI 2003, BENVENUTI et al. 2004)

Pharmaceuticals enriched with some plant extracts might support traditional therapies in iron overload diseases, but their safe use should be preceded some laboratory tests checking their properties under physiological conditions and their cooperation with other biomolecules involved in iron metabolism. The present study was focused on the influence of black currant seed extract, rich in polyphenols, on the activity of ceruloplasmin, the enzyme participating in one of the most important processes of Fe(II) elimination in human organism.

MATERIALS AND METHODS

MATERIALS

Ceruloplasmin (Cp) was isolated from serum of healthy blood donors and subjected to the process of purification (HILEWICZ-GRABSKA et al. 1988). In the first stage the protein was filtered through the column DEAE – Sephadex A – 25 using 0.2 M acetate buffer (pH 5.5) followed by precipitation with ammonium sulphate. The protein solution was then denaturated in chloroform-ethanol mixture (1:9 v/v). The protein pellet was reextracted and dialyzed to the 0.05 M phosphate buffer with addition of sodium chloride. Particular volume of 6 M protein solution: 10; 20; 30; 40 or 50 μ l, was diluted in phosphate buffer (pH 7.4 or 6.0) to the final volume of the sample (525 μ l). Different concentrations of Cp were tested (0.12; 0.24; 0.4; 0.5 and 0.61 μ M) separately or in system including the extract.

Black currant seed extract (BcE) was obtained from seeds obtained as a waste product in the fruit industry. Seeds of black currant were dried, crumbled, degreased and threefold extracted using 80% aqueous ethanol. Dry mass obtained after evaporation was dissolved in 96% ethanol. The total content of phenolic compounds in the extract of seed was 340 +/- 20 mg/100 g d.m. and was determined by Folin-Ciocaltau method, using caffeic acid as a calibration standard (ROURA et al. 2006). The BcE was diluted in phosphate buffer (pH 7.4 or 6.0) to the final concentration 166.67 mg d.m. l⁻¹. Particular volume of the extract: 10, 20, 40, 50, 100 or 150 μ l was added to the main sample and diluted to 525 μ l. Different concentrations of extract (3.18; 6.36; 9.54; 12.72; 16; 31.75; 47.62 mg d.m. l⁻¹) were tested separately or in system including ceruloplasmin.

METHODS

Reagents

Phosphate buffer pH 7.4 and pH 6.0; 3-2-Pyridyl-5,6-diphenyl-1,2,4-triazine-4,4-disulfonic acid sodium salt (ferrozine); histidine; ferrous-ammonium sulfate (Mohr's salt). The chromogen solution was made as follows: 0.0249 g of 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4,4-disulfonic acid sodium salt was dissolved in 25 ml of phosphate buffer (pH 7.4). The ferrous ions solution was made as follows: 0.0216 g (NH₄)₂ Fe (SO₄)₂·6H₂O was dissolved in 250 ml of deionized water. Histidine solution was made as follows: 0.0170g of histidine was dissolved in 100 ml of deionized water. All the reagents were stored for a maximum of one week in a shaded place. Before every experiment, ferrous ions were added to the reaction mixture as a complex with histidine, in ratio 1:1 (v/v). Histidine protects ferrous ions against the change of oxidation state.

Ferrozine assay

The ability of Cp and BcE to eliminate ferrous ions was determined using ferrozine, a chromogen that forms a highly colored complex with ferrous ions (EREL 1998). Concentration of Fe(II) ions in the reaction mixture was always constant but concentration of ceruloplasmin and extract were changed. The incubation of Fe(II) ions with ceruloplasmin and/or extract was conducted at constant temperature 37°C and pH 7.4 for 1 minute to create conditions close to physiological ones. In the second stage of the method only pH was lowered to the value 6.0. After 1 minute of incubation ferrozine was added and the resulting colour was measured spectrophotometrically at 564 nm. The ability of the tested compounds to eliminate Fe(II) was estimated by comparing the value of absorbance of a sample without any biomolecules participating in iron transformation (blank sample) with absorbance of a sample containing different concentrations of ceruloplasmin and/or the extract. Results were expressed as a depletion of Fe(II) ions in μM according to the formula:

$$\Delta\text{Fe(II)}=(A_0-A_n)/27,9 \quad (1)$$

where:

- A_0 – blank sample,
- A_n – sample,
- 27,9 – molar coefficient of absorbancy ($27\,900\text{ mM}^{-1}\text{cm}^{-1}$).

In every single experiment one concentration of ceruloplasmin and/or extract was prepared in constant volume of sample 0.525 ml. Ferrous ions mixture was added to the solution of ceruloplasmin and/or extract 1 ml of histidine. All the reagents were mixed together and incubated for 1 minute. After 1 minute incubation, 900 μl of ferrozine was added to the 100 μl of reaction mixture.

Statistical analysis

Statistical analysis was carried out using the Instat Sigma software. The results were compared by both ANOVA test and Student's T-test. Statistical significance was set at the level of $p=0.05$.

RESULTS AND DISCUSSION

Ribes nigrum belongs to the berries which are a rich source of bioactive compounds possessing important biological properties. It has been demonstrated that extracts and juices from *Ribes nigrum* have antiviral, anti-inflammatory, antitumor abilities (DECLUME 1989, YOKO et al. 2003, KONNO, OKUBO 2005). Studies of biochemical profiles of *Ribes nigrum* berries and black cur-

rant juices by High Performance Liquid Chromatography (HPLC) revealed abundance of polyphenols, mainly anthocyanins (NIELSEN et al. 2003) and flavonols such as quercetin, kaempferol and myricetin (MIKKONEN et al. 2001). *Ribes nigrum* seeds, which are often a waste product from food industry, can be a source of many substances such as fatty acids: oleic, linoleic, stearidonic acids, α -tocopherol and carotenes (PICURIC-JOVANOVIC et al. 2002). Like in berries, different classes of phenolic compounds are also detected in seed content (LU, YEAP FOO 2003). Some research suggested that 70% of the antioxidant capacity of *Ribes nigrum* extract could be attributed to its phenolic compounds content.

Polyphenols have been known as compounds advantageous for human organism, preventing many illnesses such as heart diseases or cancer (STONER, MUKHTAR 1995, DUTHIE et al. 2000). Recent studies show they may also be used as therapeutics in treatment of some iron overload diseases (AFANAS'EV 1995, PIETRANGELO et al. 2002, ZHAO et al. 2005, ZHANG et al. 2006). The beneficial effect of polyphenols is based on their ability to inhibit oxidation processes catalyzed by ferrous ions, either through their chelation and/or the elimination of metal-induced ROS. There are suggestions that orally administered polyphenols individually or in varied combinations might enhance the efficacy of prevention of the oxidative damage caused by the excess of iron. Plant extracts which are condensed mixture of phenolic compounds can be used as therapeutics, however, it is vital to first determine the influence of their components on other mechanisms occurring in human organism and participating in the maintenance of the proper iron level. The present study tried to define the effect of *Ribes nigrum* seed extract on human ceruloplasmin, the enzyme involved in one of the most important mechanism of Fe(II) elimination.

The results suggest that increased concentration of Cp cause a proportionally higher depletion of Fe(II) in the reaction mixture (Figure 1). A similar effect was noticed for the BcE, which effectively eliminates Fe(II) from the environment in a dose-depend manner (Figure 2). The behavior of the extract is probably connected with the presence of polyphenols as its major component, although the influence of other substances like ascorbic acid and tocopherols cannot be excluded.

In the first part of the experiment, the solutions of Cp and BcE were studied separately, whereas in the second part they were combined as a mixture to check their joint effect on Fe(II) elimination. Three concentrations of Cp were prepared (0.12; 0.40 and 0.61 μ M) and each was submitted to the reaction with different concentration of the BcE.

The loss of Fe(II) was greater in the mixture containing both Cp and the BcE than in the solution of Cp only (Figures 3a,b,c), so it was stated that components of BcE cooperate with Cp in the process of eliminating of Fe(II). Supporting effect of the BcE on the activity of Cp was observed in all the tested concentrations of the enzyme, although the most noticeable re-

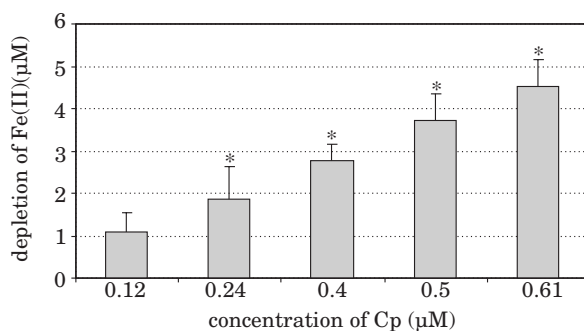


Fig. 1. Depletion of Fe(II) in the presence of different concentrations of Cp after 1 minute of incubation. Results were expressed as means \pm SD. * $p \leq 0.05$ (concentration of Cp vs. the nearest neighbor concentration of Cp)

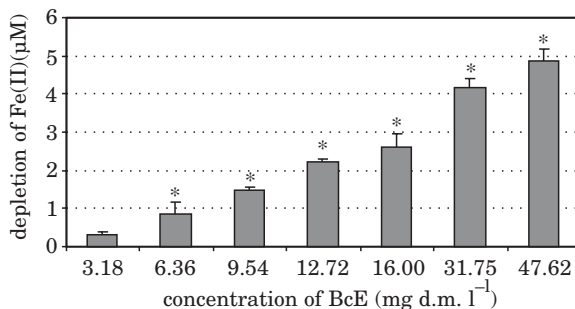


Fig. 2. Depletion of Fe(II) in the presence of different concentrations of BcE after 1 minute of incubation. Results were expressed as means \pm SD. * $p \leq 0.05$ (concentration of BcE vs. the nearest, neighbor concentration of BcE)

sult appeared at the lowest Cp concentration (Figure 3a). Presumably, it is a consequence of the competition between Cp and the extract for the substrate in the reaction mixture. In a range of higher concentrations, Cp gains an advantage over the extract and reduces its participation in the process.

In the present study, all the experiments were conducted at physiological pH, but in some stage of the research the pH value of a reaction mixture was lowered to 6.0. Solutions of one concentration of Cp (0.61 μM) and BcE (12,72 mg d.m. l⁻¹) were chosen and tested separately and in mixture. A change of pH did not affect the effectiveness of Cp but dramatically lowered the extract involvement in Fe(II) elimination (Figure 4). For this reason, addition of the extract to Cp solution at pH 6.0 did not cause significant difference in the amount of eliminated Fe(II), observed previously at pH 7.4. This observation might be attributable to the influence of pH on the ability of polyphenols to form complexes with iron ions. This process has been described in earlier publications (MORAN et al. 1997, MIRA et al. 2002),

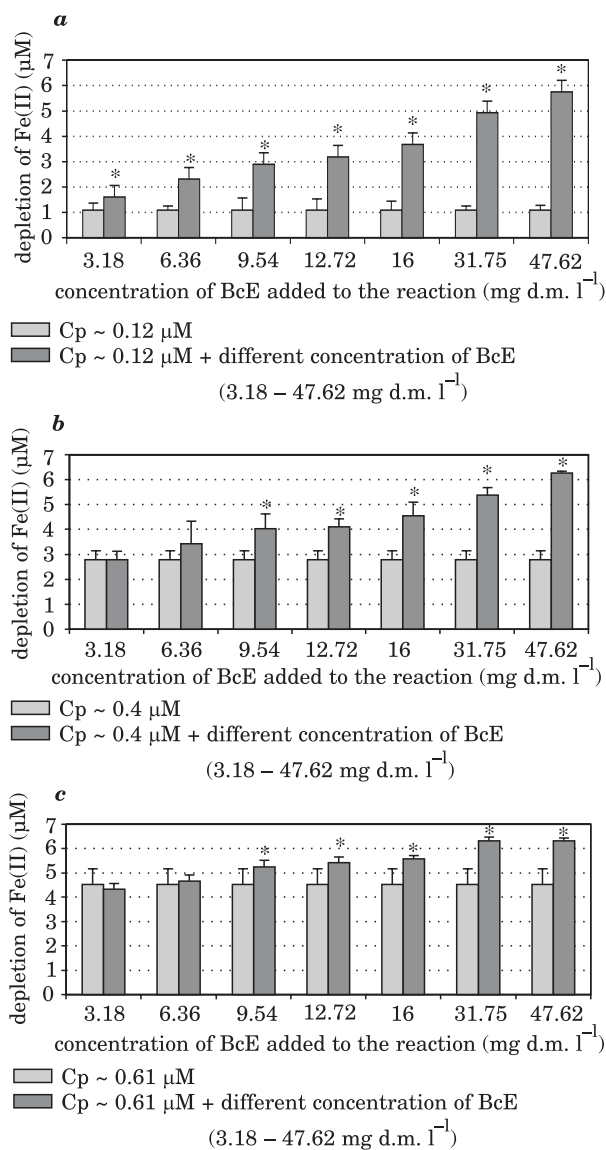


Fig. 3. Depletion of Fe(II) in the mixture containing constant concentration of C_p (*a* – 0.12 μM , *b* – 0.40 μM , *c* – 0.61 μM) and different concentrations of BcE (3.18-47,62 mg d.m. l^{-1}). Results were expressed as means \pm SD.

* $p \leq 0.05$ (C_p vs. $C_p + \text{specified concentration of BcE}$)

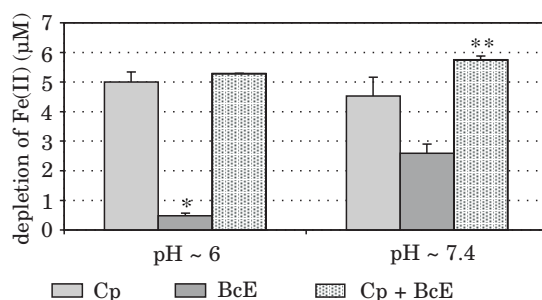


Fig. 4. Influence of pH on Cp, BcE and mixture containing both (Cp + BcE) in Fe(II) elimination. Results were expressed as means \pm SD.

* $p \leq 0.05$ (* BcE at pH 6 vs. BcE at pH 7.4; **Cp at pH 7.4 vs. Cp + BcE at pH 7.4

in which the authors indicate that chelating properties of polyphenols in acidic pH are decreased .

Although our studies were carried out under *in vitro* conditions, they show that black currant extract rich in polyphenols raise the effectiveness of ceruloplasmin in Fe(II) elimination. The results could be more reliable if the natural processes occurring in human organism such as absorption and metabolism affecting natural abilities of nutritional compounds were taken into account. Apart from ceruloplasmin, it is necessary to focus on other endogenous antioxidants present in human organism, that could be either positively or negatively affected by active substances provided with food.

Plant extract are a mixture of different compounds, therefore it is difficult to analyse and determine the effect caused by individual substances. Iron elimination performed by BcE can result from the chelating activity of polyphenols or other compounds or their combination. Thus, this aspect must be carefully verified in the future studies.

Development of new approaches using plant compounds for treatment of iron overload diseases is a very important issue for future investigations concerning their safety, toxicity and their combined use with traditional medicines.

CONCLUSIONS

1. *Ribes nigrum* seed extract effectively eliminates Fe(II) from the environment in a dose-dependent manner. This effect may be connected with the presence of polyphenols as its major component. However, influences of other substances cannot be excluded.

2. The addition of *Ribes nigrum* seed extract elevates the effectiveness of ceruloplasmin in removal of Fe(II) from a sample provided it occurs under physiological conditions (pH 7.4; T 37°C).

3. *Ribes nigrum* seed extract rich in active polyphenols can support natural mechanisms regulating proper iron level, although this aspect should be confirmed by *in vivo* studies.

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