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Antioxidant potential of *Aesculus hippocastanum* extract and escin against reactive oxygen and nitrogen species

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Abstract. – OBJECTIVE: Antioxidant, anti-inflammatory and venoconstrictor properties have been attributed to extracts from *Aesculus hippocastanum*. These unusual and diverse properties may be possibly basically linked with ability to scavenge free radicals.

MATERIALS AND METHODS: The scavenging capacity of dry horse chestnut extract of and escin have been investigated *in vitro* against superoxide anion radicals, hydroxyl radicals, nitrites and peroxynitrite.

RESULTS: In general, the activity of the whole extract against superoxide radicals did not exceed 15% at pH 7.4, but the highest inhibition (46.11%) was recorded against hydroxyl radicals at a concentration of 100 μg.ml^{-1}; however, the activity against other radicals was lower. Escin demonstrated a better ability to counteract nitric oxide oxidation products, nitrites. However, the efficiency of the whole extract completely disappeared as the concentration increased. Both extracts showed very low activity towards peroxynitrite. Escin was even able to induce peroxynitrite formation at the lower concentrations used.

CONCLUSIONS: Whole extract showed better antiradical properties compared to its main active ingredient, escin, probably due to potential synergistic interaction with a mixture of compounds present in the plant extract. These findings can be the basis of both the presentation of side-effects and the persistence of disease in spite of ongoing treatment.

Key Words:
Escin, Horse chestnut, Hydroxyl radical, Nitric oxide peroxynitrite, Superoxide.

Introduction

Plant materials rich in phenolics are of increasing interest to the food industries because they retard oxidative degradation of biomolecules such as lipids, thereby, improving the quality and nutritional value of food[1]. Phenolics are capable of scavenging free radicals. Their antioxidant activity is principally due to their redox properties, which allow them to act as reducing agents. Phenolics are especially common in leaves, flowering tissues and woody parts, such as stems and barks. A large number of aromatic, spice, medicinal and other plants were studied for antioxidant properties. However, scientific information on the antioxidant properties of plants that are less used in cuisine and medicine is still rather scarce. Therefore, the assessment of such properties remains a new area for finding sources of natural antioxidants, functional foods and nutraceuticals[2]. The chemical and biological diversity of aromatic and medicinal plants depends on factors such as the collection area, climatic conditions, local flora, genetic modification and others. Supplementation of natural antioxidants could be more effective, and also more economical than supplementation of an individual antioxidant, such as ascorbic acid or vitamin E, in protecting the body against oxidative damage under various conditions[3].

Horse chestnut and its component, escin, are used to treat chronic venous insufficiency, edemas, and also in the treatment of diabetic retinopathy. Several studies focused on their medicinal properties, such as their anti-inflammatory, antitumor, antiviral, antifungal, and antioxidant effects[4,5,6]. It was found that extract *Aesculus (A.) hippocastanum* has the potential to act as a scavenger of reactive oxygen species (ROS), and is 20 times more potent against superoxide than ascorbic acid[7]. The antioxidant

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properties depend on the hydrogen-donating and ion-chelating capability due to the presence of hydroxyl groups in the molecule. Escin is the main active compound in horse chestnut, and is responsible for most of its medicinal properties. It has been determined that escin, in particular, induces endothelial NO synthesis by making endothelial cells more permeable to calcium ions, and also induces the release of prostaglandin F2. Other possible mechanisms include serotonin antagonism, histamine antagonism and reduced catabolism of tissue mucopolysaccharides[6,8]. The effects of NO on regulatory processes are achieved at nanomole concentrations, and the substances tested are able to modulate NO production.

Therefore, we focused our work on determining the ability of dry horse chestnut extract (*A. hippocastanum)* and its main component, escin, to convert nitric oxide to nitrites or nitrates and scavenge peroxynitrite anions (ONO_2^-) *in vitro.* In addition to this, we also investigated their effect against some reactive oxygen species such as superoxide anion radicals ($O_2^{\bullet-}$) and hydroxyl radicals ($HO^\bullet$) as part of *in vitro* systems in order to link these essential qualities with the observed effects on living organisms.

Materials and Methods

Horse chestnut (*Aesculus hippocastanum* L.)-dry extract (HCE, 18 to 22% escin) and the saponin beta-escin (E) were a gift from CALENDULA, (Nová Lubovňa, a. s. Slovak Republic).

The antioxidant properties of plant extracts against $O_2^{\bullet-}$ were evaluated by the method of Beauchamp and Fridovich[9]. The reaction mixture contained 8.7 ml of 50 mmol.l^{-1} phosphate buffer solution (PBS, pH 7.4) with 0.1 mmol.l^{-1} EDTA, 13 mmol.l^{-1} L-methionine and tested extract in final concentrations of 5, 25, 50, 75 and 100 μg.ml^{-1} (PBS). Riboflavin and nitro-blue tetrazolium (NBT) were added last. The resultant mixture was, then, exposed to UV light for 10 and 20 minutes. The absorbance of the solutions in the absence of the tested substance and in the presence of the tested substance was determined at 450 nm and 560 nm before and after UV illumination. The percentage inhibition of $O_2^{\bullet-}$ generation was evaluated by comparing the absorbance values of the control and experimental tubes and was calculated according to the following equation: (%) = [(Acontrol-Asample)/Acontrol]×100.

$^\bullet OH$ was generated by a Fenton reaction system and the scavenging capacity towards the $^\bullet OH$ was measured using the deoxyribose method[10]. The reaction mixture contained 2-deoxy-D-ribose (9 mmol.l^{-1}) dissolved in 30 mmol.l^{-1} PBS (pH 7.4) containing 40 mmol.l^{-1} NaCl, 30 mmol.l^{-1} ammonium iron (II) sulphate hexahydrate, 50 mmol.l^{-1} H_2O_2 and plant extract sample at final concentrations of 5, 25, 50, 75, 100 μg.ml^{-1}. The mixture was kept in a water bath at 37°C for 10 min. After incubation, 0.5 ml thiobarbituric acid reagent (1%), TBA dissolved in 50 mmol.l^{-1} NaOH solution followed by 0.5 ml 5.6% trichloroacetic acid was added to the reaction mixture. The mixture was then heated to 100°C for 8 min and then cooled down with water. The absorbance of the solution was measured spectrophotometrically at 532 nm. The $^\bullet OH$ scavenging capacity was evaluated from the inhibition percentage of 2-deoxyribose oxidation on hydroxyl radicals. The scavenging percentage was calculated according to the following formula: (%) = $[A_0 - (A_1 - A_2)] \times 100/A_0$, where A_0 is the absorbance of the control without a sample. A_1 is the absorbance after adding the sample and deoxyribose. A_2 is the absorbance of the sample without deoxyribose.

The ability to convert NO was estimated using the Griess reaction[11]. Griess reagent was modified using naphthylethylenediamine dihydrochloride (0.1%). The reaction mixture, containing sodium nitroprusside (10 mmol.l^{-1}, 2 ml), phosphate buffer solution (1 ml) and extracts at different concentrations (5, 25, 50, 75, 100 μg.ml^{-1}), was incubated at 25 °C for 150 min in the dark. Following this, 0.3 ml sulfanilic acid reagent (1.0% in 5% H_3PO_4) was added and allowed to stand for 8 min to complete diazotization. Then 0.3 ml naphthylethylenediamine dihydrochloride was added, mixed and allowed to stand for another 8 min in darkness at 25°C until a pink colored chromophore was formed. The absorbance was measured at 546 nm.

Peroxynitrite anion (ONO_2^-) was prepared by mixing 1 volume cooled hydrogen peroxide (0.7 mol.l^{-1}) in HCl (0.6 mol.l^{-1}) and 1 volume cooled potassium nitrite (0.6 mol.l^{-1}); then, 1 volume cooled sodium hydroxide (1.2 mol.l^{-1}) was added to the mixture as prescribed by Beckman et al[12]. The reaction mixture was then left overnight below –20°C. The ONO_2^- concentration was quantified spectrophotometrically at 302 nm. The pH of the working ONO_2^- solution was adjusted to 7.4 with KH_2PO_4. The ONO_2^- yield was incubat-

ed with tested plant extracts (diluted in PBS with pH 7.4) with final concentrations as mentioned above for 15 minutes and the absorbance of the samples was recorded at 302 nm.

Statistical Analysis

All the measurements were made in triplicate. Results are represented as the mean ± SD. Statistical comparison between the extracts for individual parameters was performed using Student's *t*-test. Statistical significance within the measured activities of the individual extracts was determined by one-way ANOVA followed by Bonferroni *post hoc* test where appropriate. $p < 0.05$ was considered statistically significant.

Results

The effects of HCE and E on the inhibition of $O_2^{\bullet-}$ with the biphasic response are shown in Figure 1. The highest scavenging activity against $O_2^{\bullet-}$ was observed in the range of 25-50 $\mu g.ml^{-1}$ of extract concentration used (14.76 to 14.69%), which was followed by a decrease in activity and a second peak of radical inhibitory activity of 12.5% at $\mu g.ml^{-1}$. A similar trend was found at a concentration of 50 $\mu g.ml^{-1}$ with an inhibitory effect of 14.34%, but scavenging activity reached up to 18.24% at the highest concentration (100 $\mu g.ml^{-1}$).

There was a significant effect of concentration on $O_2^{\bullet-}$ scavenging activity for HCE at $p < 0.05$ and for E at $p < 0.001$. *Post hoc* comparison indicated that the activities at 100 $\mu g.ml^{-1}$ (likewise 50 $\mu g.ml^{-1}$ for HCE) were significantly different than those at 5 $\mu g.ml^{-1}$. Much stronger scavenging activity was detected against $HO^{\bullet}$ (Figure 2), and the activity in both exceeded 20% at 50 $\mu g.ml^{-1}$ (HCE 21% and E 23.25%). There was an upward trend in percentage inhibition of $HO^{\bullet}$ for both extracts. However, activities between the ex-

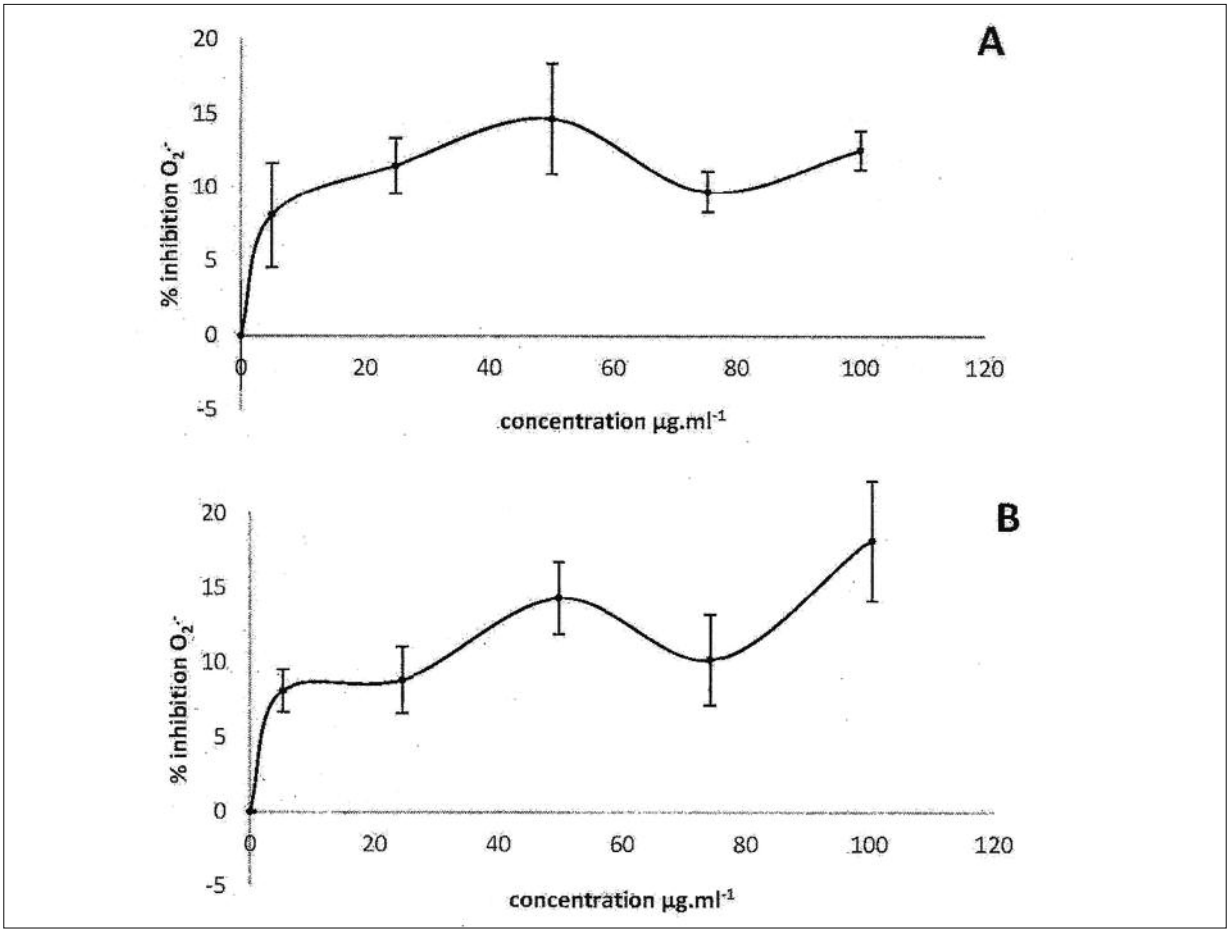

Figure 1. The percentage inhibition by horse chestnut extract ***(A)*** and escin ***(B)*** against superoxide radicals.

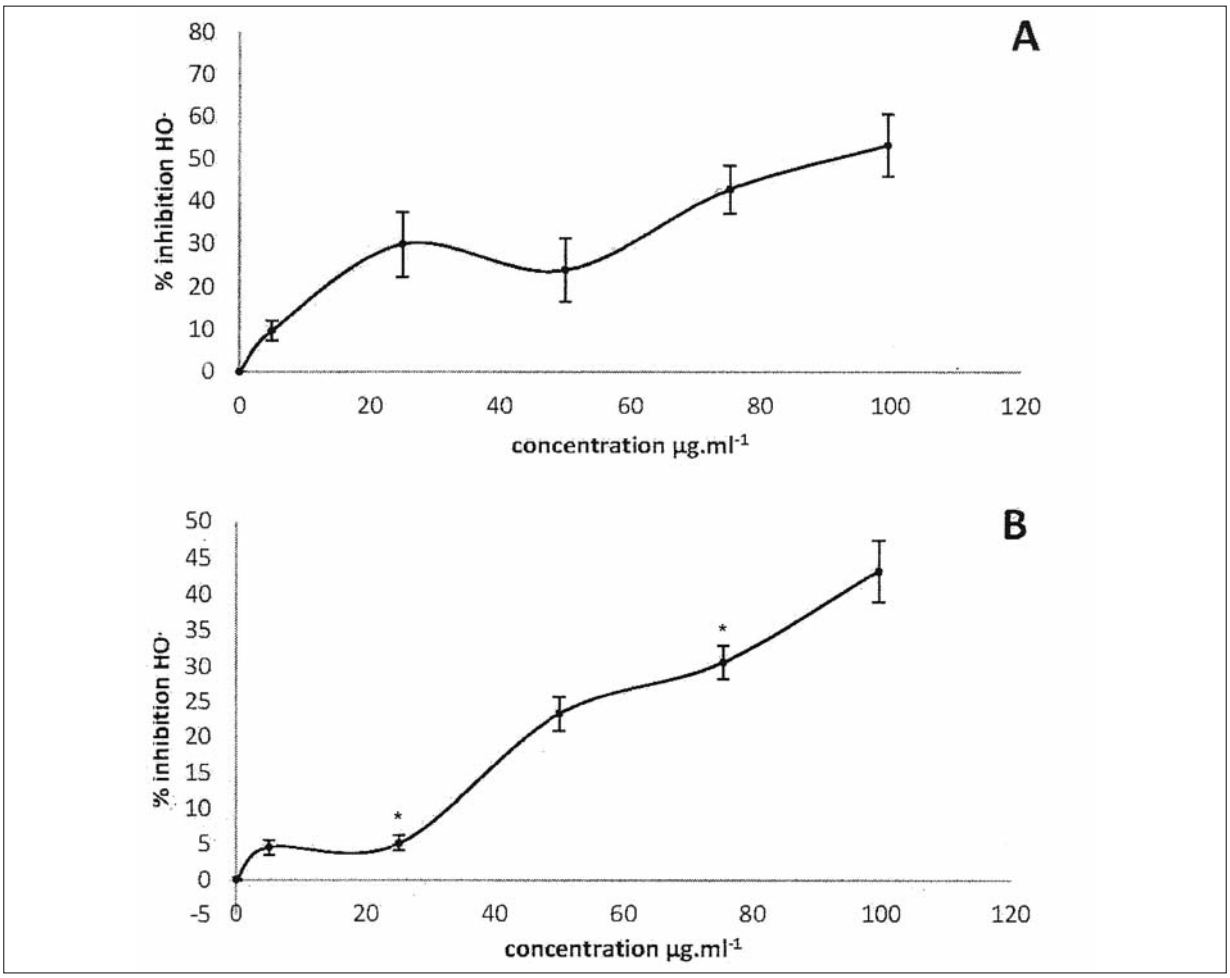

Figure 2. The percentage inhibition by horse chestnut extract ***(A)*** and escin ***(B)*** against hydroxyl radicals. *$p < 0.05$ horse chestnut extract versus escin.

tracts differed significantly at 25 µg.ml^{-1} and 75 µg.ml^{-1} in favor of HCE. The change with E ranged from 28.12% to 36.28%, while the change was much more pronounced, without significance, with whole HCE (32.1 to 46.11%). There was a significant effect of concentration on HO$^{\bullet}$ scavenging activity for both extracts at $p < 0.0005$. Bonferroni test indicated significant increase in the activities at concentrations higher than 50 µg.ml^{-1} for HCE; however, mean score for all E concentrations differed significantly.

The activity of the extracts against NO (Figure 3) was measured indirectly, because NO produced by sodium nitropruside at physiological pH interacts with oxygen to produce both nitrite and nitrate ions. We were limited to assessing the production of nitrites, because in aqueous phase, free of biological material, NO exclusively auto-oxidizes to nitrites[20]. Therefore, only nitrite determination was performed without the need to reduce nitrates prior to the assay. Percentage of NO conversion to nitrites exceeded 20% in both extracts even at a concentration of 5 µg.ml^{-1} (HCE at 21.95% with a second smaller peak at 50 µg.ml^{-1} 12.81%, and E significantly at 32%). With HCE this ability decreased with increasing concentrations (dropping to 10.37% at 25 µg.ml^{-1}), down to 0.61%. Conversely, the ability of E to convert NO to nitrite soared to 41% at a concentration of 75 µg.ml^{-1}. However, at the highest E concentrations tested, the conversion ability decreased, and was similar to HCE at 5 µg.ml^{-1} (19.51%). Differences between HCE and E activities were significant ($p < 0.01$). *Post hoc* comparison revealed significant differences within the activities of HCE between 5 and 75-100 µg.ml^{-1} ($p < 0.0005$) and 50-75 µg.ml^{-1} for E.

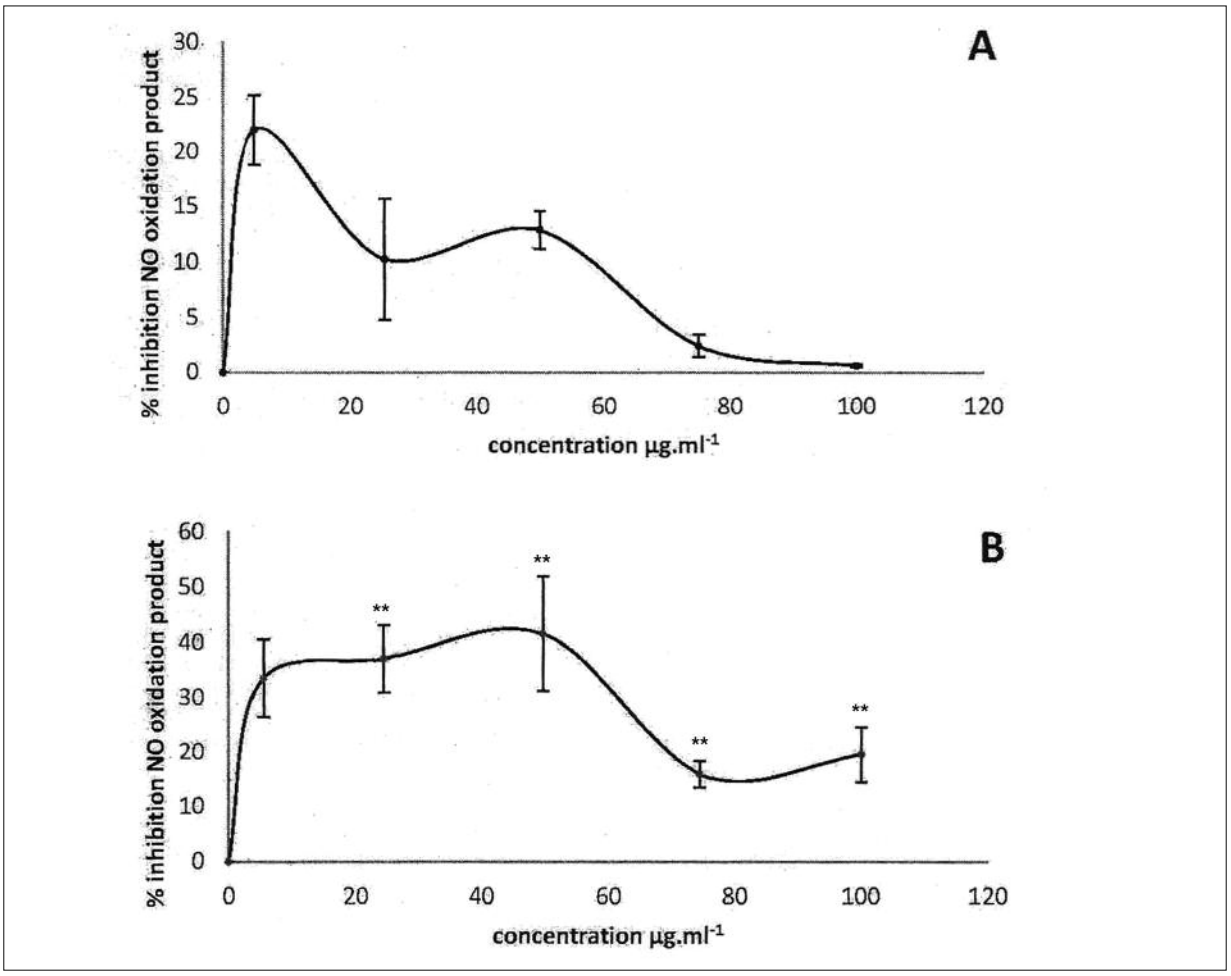

Figure 3. The percentage scavenging effect of horse chestnut extract ***(A)*** and escin ***(B)*** against the nitric oxide oxidation product, nitrite. $^{**}p < 0.01$ horse chestnut extract versus escin.

HCE has a very weak activity against ONO_2^-, which did not vary in the range of concentrations tested (from 2.21% to a maximum of 2.94%). Similarly, E exhibited a low quenching ability at a concentration of 75 µg.ml^{-1} (maximum 2.21%). Conversely, lower concentrations caused an increase in ONO_2^- which can be seen in Figure 4 as negative values. The activities of E were significantly lower, except of these measured at 50 µg.ml^{-1}, when compared to HCE, even at $p <$ 0.001 (5-25 µg.ml^{-1}). Comparison of the E values indicated significant changes between the activities under the concentration less than 50 µg.ml^{-1} as well as more than 50 µg.ml^{-1}.

Discussion

The antioxidant activity of plants, mainly attributed to the presence of active compounds, is well founded. In addition to the composition of the extracts it is, therefore, justified to deal with their basic activities and consider them in conjunction with their observed physiological and medicinal effects in living organisms. Phenolic compounds have been considered to be powerful antioxidants. *A. hippocastanum* extracts are rich in phenolics, and have been observed to possess a considerable antioxidant potential, although strong correlation has not been confirmed[13].

All aerobic organisms produce partially reduced metabolites of O_2 that have higher activities relative to O_2[14]. $O_2^{\bullet-}$ is a highly reactive molecule produced through metabolic processes that reacts with various substances. It has been reported that the antioxidant properties of some flavonoids are effective mainly via $O_2^{\bullet-}$ scavenging in the riboflavin system. Our observation allowed as to conclude that the HCE extracts had a

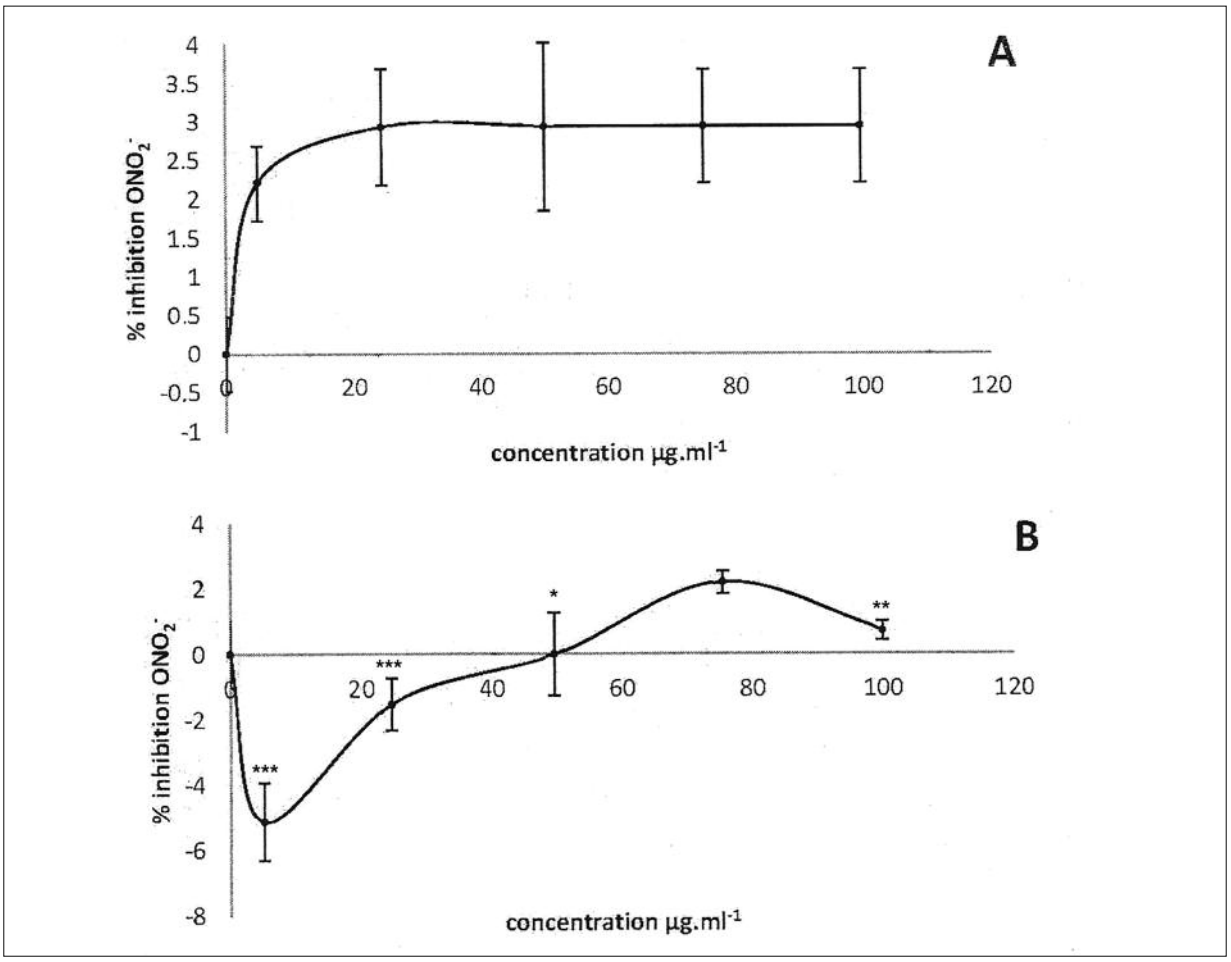

Figure 4. The percentage inhibition by horse chestnut extract ***(A)*** and escin ***(B)*** against exogenous peroxynitrite anions. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ horse chestnut extract versus escin.

greater effect against $O_2^{\bullet-}$ than its principal ingredient E (Figure 1). But at pH 7.4 (which would correspond to the pH of blood) as in our experiment, these activities were not very significant, amounting to about 14% of the scavenging activity. Kosar et al[15] observed that acidic conditions significantly increased radical-scavenging properties of selected plant extracts by increasing their reducing ability. It is possible that the compounds responsible for the antiradical activity are not stable at an alkaline pH and lose their properties after alkalization. Higher activity against $O_2^{\bullet-}$, was found at pH 6.5, even exceeding 25% in the case of HCE (data not published), which would correspond to the previously identified properties of HCE. Sefedin et al[16], however, observed an $O_2^{\bullet-}$ scavenging ability of E of up to 30% at a concentration of 100 mg.ml^{-1}, and 15% inhibitory activity at a concentration of 10 mg.ml^{-1} at pH 7.4.

$O_2^{\bullet-}$ has an approximate half-life of 2-4 µs and undergoes fast, non-enzymatic, one-electron reduction to form $HO^{\bullet}$. It has been noted that $O_2^{\bullet-}$ can undergo protonation to give up a strong oxidizing agent, the perhydroxyl radical, which directly attacks the polyunsaturated fatty acids in negatively charged membrane surfaces[17]. All the produced radicals have the potential to react with various biological substrates and have been implicated in several pathophysiological processes, if not deactivated by antioxidant systems. We used the Fenton reaction system as a source of $HO^{\bullet}$ to test the scavenging capacity of extracts. When HCE extract and E were incubated with the reaction mixture used in the deoxyribose degradation assay, they removed $HO^{\bullet}$ from the sugar and prevented its degradation. The results showed a dose-dependent response (Figure 2). Whole plant extract was more effective when compared to E and showed the highest scaveng-

ing activity 46.11% and escin 36.28% at a concentration of 100 μg.ml^{-1}. These effects can be explained by the fact that the mixture of compounds in the plant extract has better antioxidant properties than its isolated compound, both *in vitro* and *in vivo*, because of their mutually synergistic interaction[18].

The effects of compounds, and particularly that of E, are connected with the production of NO through enhancement of the cellular permeability to calcium which would increase endothelial nitric oxide synthase (NOS) activity[19]. The actual induction and increase in NOS activity in the body under certain conditions may not lead only to the increased production of NO, but also to the formation of $O_2^{\bullet-}$ [19]. This rapidly reacts with NO to form ONO_2^-, thus, creating conditions that lead to the expression of adverse effects as well as disease chronicity. It is, therefore, important to know the basic properties of the extracts against these forms of reactive species, which would lead to an explanation of mechanisms supporting the clinical indications and also the possible suppression of undesirable symptoms. Nitrites and nitrates are stable metabolites of NO, with nitrates being by far the major oxidative metabolites of NO detectable by the Griess reaction. Because nitrates themselves are biologically complete inactive, in contrast to nitrites, it was more convenient to monitor the behavior of extracts in aqueous phase, free of biological material, where NO exclusively autoxidizes to nitrites[20]. Both substances counteracted the end products of NO oxidation (Figure 3), although E showed a higher scavenging capacity. Suppression of released NO may be partially attributed to direct NO trapping, as the tested substances decreased the amount of nitrites generated from the decomposition of sodium nitroprusside *in vitro*. The percentage of NO trapped by E increased as concentrations rose from 5 to 50 μg.ml^{-1} (32%, 37%, 41% respectively) while higher concentrations decreased its ability. HCE showed about half the ability to counteract NO end products when compared to E.

Although NO is a free radical, it is remarkably unreactive towards biomolecules. The reaction of NO with O_2 or $O_2^{\bullet-}$, however, generates RNOS (especially ONO_2^-) which are highly labile and capable of modifying a wider range of biomolecules than NO itself[21]. Both extracts showed an insignificant effect on ONO_2^- scavenging ability (Figure 4). While HCE showed a very low non-concentration-dependent ability to scavenge ONO_2^- (less than 3%), E potentiated the release of ONO_2^- at concentrations lower than 25 μg.ml^{-1} (Figure 4). In the case of whole HCE, it may not be a significant finding. It was shown that even the smallest concentration of *A. hippocastanum* extract (20 μg.m l^{-1}) is still capable of counteracting $O_2^{\bullet-}$ and $^{\bullet}OH$ and, thus, prevent oxidative and nitrosative stress[22]. The formation of ONO_2^- is thus actually hindered by $O_2^{\bullet-}$ uptake. In the case of E, however, the concentration-dependent increase in ONO_2^- is a significant finding as ONO_2^- is active in oxidizing tetrahydrobiopterin, a co-factor of NOS. NOS deprived of a co-factor becomes uncoupled, producing $O_2^{\bullet-}$ in place of NO[21]. The critical concentration of NO is cell- and tissue-specific as well as process dependent. The observed conditions of ONO_2^- formation, after the induction of NOS by E in a biological system, would lead to disease chronicity rather than treatment, due to undercutting the dosage. Moreover, the production of ONO_2^- can have pathological consequences resulting from strong oxidative nitrosylation of molecular targets.

The role of free radicals is gaining increasing attention as so many pathological phenomena are related to changes in cell redox status. The oxidative stress-activated signaling cascade upregulates the gene-controlling inflammatory processes[23]. Therefore, reducing oxidative stress by controlling ROS and RNS levels with antioxidant plant extracts means interfering with the inflammatory signal transduction pathways[22]. The combination of results obtained through these *in vitro* assays may give an idea of ways to intervene in these pathways, as the tested concentration range was not random. *A. hippocastanum* extracts are not among the most commonly used herbs as part of the diet, but rather targeted medicinal herbs. The amount of active substances in the body hardly exceeds microgram quantities.

Conclusions

Our findings show that *Aesculus hippocastanum* dry extract and escin have interesting concentration-dependent activity, with more pronounced antiradical activity from the whole extract. Based on our investigation, even small concentrations of tested substances are capable of counteracting $O_2^{\bullet-}$ and $HO^{\bullet}$, although this effect is less than 15% at blood pH. We can assume that the combination of the NO induction ability *in vivo* and the following low ONO_2^- uptake or in-

creased production, coupled with a lower ability to scavenge $O_2^{\bullet-}$, may provide basis for certain types of treatment. This may also support the documented effects as functions of not only dosage but also the pH of the medium in which the effects are observed.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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