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EVALUATION OF ANTIOXIDANT PROPERTIES OF METHANOLIC EXTRACTS FROM LEAVES AND ROOTS OF *REHMANNIA GLUTINOSA* LIBOSCH. IN HUMAN BLOOD

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Abstract: Compounds with antioxidant activity may protect different elements of blood from damaging effects of oxidative stress. The present *in vitro* study was designed to examine the antioxidant activity of methanolic extracts from leaves and roots of *R. glutinosa* plants against damages induced by oxidative stress. Oxidative stress was stimulated in human plasma and human blood platelet samples by the strong biological oxidant – hydrogen peroxide (H_2O_2) or H_2O_2/Fe (the donor of hydroxyl radicals). In experimental trials, the level of biomarker of lipid peroxidation – TBARS was significantly decreased by the action of methanolic extracts from *R. glutinosa* leaves. Therefore, it seems that the methanolic extract from leaves of the plants may be a new source of bioactive antioxidant natural compounds. It may be also an active pharmacological agent or a food supplement for healthy subjects and for people with different diseases (cardiovascular diseases and cancer) induced by oxidative stress.

Keywords: antioxidant activity, iridoid glycosides, lipid peroxidation, oxidative stress, phenylethanoid glycosides, *Rehmannia glutinosa*

Medicinal plants are a great source of secondary metabolites with antioxidant activities. Plant extracts containing natural antioxidants are often more effective than the synthetic antioxidants or even isolated pure compounds due to synergistic interactions between plant compounds that improve the bioavailability of the individual antioxidant agents (1). The second reason for the upsurge of interest in plants as a source of natural antioxidants is the fact that the commercial synthetic antioxidants such as butylated hydroxytoluene (BHT) and gallic acid esters have been associated with negative effects on health (2).

Rehmannia Libosch. ex Fisch. et Mey is a small genus consisting of 6 species belonging to the Orobanchaceae family. Five of them are endemic to China. The best known species is *Rehmannia glutinosa* Libosch. which occurs in China as well as in Korea and Japan. It was recorded in Chinese medical classics "Shennong's Herba" and is thought as a "top grade" herb in China (3). Roots of *R. glutinosa* are listed in the Chinese Pharmacopoeia (4) and are widely used in traditional chinese medicine as very active agents affecting the blood system, cardiovascular system, nervous system, immune system and bone metabolism (5). The properties have been attributed to secondary metabolites occuring in the species. Iridoid glycosides, such as catalpol, aucubin, loganin and phenylethanoid glycosides such as verbascoside, isoverbascoside and echinacoside as well as polysaccharides have been isolated from the plant roots (5, 6). Little is known about the compounds occurring in aerial parts. Albach et al. (7) have described the presence of iridoid (catalpol, ajugol, 8-epiloganic acid) and phenylethanoid glycosides - verbascoside and trace of rehmannoside A in ethanolic extracts from dry leaves of *R. glutinosa*. Zhang et al. (8) have isolated three ursane-type triterpenes (glutinosalactone A-C) from leaves of the plant species collected from natural locations in China. Moreover, small amounts of phenolic acids (cinnamic, salicylic, ferulic, syringic, caffeic, p-

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coumaric, chlorogenic, p-hydroxybenzoic acids) and flavonoids (hesperidin, naringin, myricetin and traces of quercetin, naringenin and kaempferol) were found in *R. glutinosa* young plants by Chung et al. (9). Most of studies have reported the antioxidant activity of the roots from field-growing plants (10-13). Piątczak et al. (14) have recently reported the total flavonoid and phenolic compound contents in extracts from leaves and roots of *R. glutinosa* and the antioxidant activity of using several *in vitro* assays: scavenging of free radicals (DPPH and ABTS), metal transition reduction (FRAP) and total antioxidant power (P-Mo) in the tested extracts were also determined. In the present study, other tests were used to estimate antioxidant activity of leaf and root extracts of the plant species. We have tested the antioxidant activity of the methanolic extracts against the effect of strong biological oxidant – hydrogen peroxide (H_2O_2) or H_2O_2/Fe (the donor of hydroxyl radicals) on human plasma proteins or lipids, and the superoxide anion ($O_2^{\cdot-}$) generation in resting blood platelets *in vitro*. The experimental models used in the study were similar to the reactions which take place in human plasma and platelet under oxidative stress conditions. Moreover, further more detailed information on the type and quantity of phytochemicals from iridoid and phenylethanoid groups present in extracts tested were included in the paper.

MATERIAL AND METHODS

Chemicals

Cytochrome c, dimethyl sulfoxide (DMSO), 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), thiobarbituric acid (TBA), H_2O_2 were purchased from Sigma (St. Louis, MO., USA). All other reagents were of analytical grade and were provided by commercial suppliers. Stock solution of plant extracts tested was made in 50% DMSO. The final concentration of DMSO in samples was lower than 0.05% and in all experiments its effects were determined.

Extract preparation

Leaves and roots of 12-month old *R. glutinosa* plants derived from seeds, growing in a field were used in the experiments. Plant materials (leaves and roots, separately) were powdered, lyophilized and extracted with methanol (40 mL) as it was described earlier (26). Dry plant tissue (500 mg) was used for antioxidant experiments and 100 mg was weighted for UHPLC analyses. The yields of extracts were as follows: 42.8% (leaf extract) and 50.8% (root extract).

Blood platelet and plasma isolation

Fresh human plasma was obtained from medication free, regular donors at the blood bank (Łódź, Poland) or peripheral blood was obtained from non-smoking men (collected into ACD solution (citric acid/citrate/dextrose; 5 : 1; v/v; blood/ACD); the protocol was passed by the Committee for Research on Human Subjects of the University of Lodz number KBBN-UŁ/I/4/2011. Platelet-rich plasma (PRP) was prepared by centrifugation of fresh human blood at 250 × g for 10 min at room temperature. Platelets were then sedimented by centrifugation at 500 × g for 10 min at room temperature. Platelet pellet was washed twice with Tyrode's buffer containing 10 mM HEPES, 140 mM NaCl, 3 mM KCl, 0.5 mM $MgCl_2$, 5 mM $NaHCO_3$, 10 mM glucose, pH 7.4 and then platelets were suspended in Tyrode's buffer. Concentration of platelets in platelet suspensions estimated spectrophotometrically (16) was about 5×10^8/mL.

Suspensions of blood platelets or plasma were incubated with:

- plant extract at the final concentrations of 0.5-50 μg/mL (15 min, at 37°C);
- plant extract at the final concentrations of 0.5-50 μg/mL plus 2 mM H_2O_2 (15 min, at 37°C);
- plant extract at the final concentrations of 0.5-50 μg/mL plus 4.7 mM H_2O_2/3.8 mM $FeSO_4$/2.5 mM EDTA (15 min, at 37°C).

Lipid peroxidation measurement

Lipid peroxidation was quantified by measuring the concentration of TBARS. Incubation of plasma (control, plant extract and H_2O_2- or H_2O_2/Fe-treated plasma) was stopped by cooling the samples in an ice-bath. Samples of plasma were transferred to an equal volume of 20% (v/v) cold trichloroacetic acid in 0.6 M HCl and centrifuged at 1200 × g for 15 min. One volume of clear supernatant was mixed with 0.2 volume of 0.12 M thiobarbituric acid in 0.26 M Tris at pH 7.0 and immersed in a boiling water bath for 15 min, then absorbance at 532 nm (Spectrophotometer UV/Vis Helios alpha Unicam) was measured (17). The TBARS concentration was calculated using the molar extinction coefficient (ε = 156,000 $M^{-1} cm^{-1}$).

Thiol group measurements

Thiol group content was measured spectrophotometrically (Spectrophotometer UV/VIS Helios alpha Unicam) with Ellman's reagent. The thiol group concentration was calculated by using the molar extinction coefficient (ε = 13,600 $M^{-1}cm^{-1}$) (18, 19).

Superoxide anion measurement

Cytochrome c reduction method as described earlier (20) was used to test the $O_2^{\cdot-}$ generation in control and in blood platelets incubated with tested compounds. Briefly, an equal volume of modified Tyrode's buffer, containing cytochrome c (160 µM), was added to suspension of platelets. After incubation, the platelets were sedimented by centrifugation at 2000 × g for 5 min and the supernatants were transferred to cuvettes. Reduction in cytochrome c was measured spectrophotometrically at 595 nm. To calculate the molar concentration of $O_2^{\cdot-}$, the molar extinction coefficient for cytochrome c of 18,700 M^{-1} cm^{-1} was used.

Chemical analysis

The UHPLC method was used to determine iridoid (catalpol, aucubin, loganin, harpagide, harpagoside, catalposide) and phenylethanoid glycoside (verbascoside, isoverbascoside) contents in leaf and root extracts. The analyses were carried out on Agilent Technologies 1290 Infinity apparatus equipped with a diode array detector (DAD), binary solvent delivery pump, vacuum degasser, autosampler and thermostated column compartment. The separation was performed on a Zorbax Eclipse Plus C18 column (100 × 3.1 mm i.d.; 1.8 µm Agilent Technologies) at 27°C. The mobile phase consisted of 0.1% formic acid in acetonitrile (v/v) (solvent A) and 0.1% formic acid (v/v) in water (solvent B). A gradient program was applied as follows: 0–1 min 1% A; 1–4 min 1–15% A; 4–6 min 15–21% A; 6–9 min 21% A; 9–11.5 min 21–90% A; 11.5–15 min 90% A. The column was equilibrated with 99% A for 2 min between injections. The flow rate was 0.4 mL/min. The injection volume was 0.3 µL. The detection wavelength was set at 204 nm (aucubin, catalpol and harpagide), 237 nm (loganin), 265 nm (catalposide), 280 nm (harpagoside) and 320 nm (verbascoside, isoverbascoside). The identification of determined compounds was made by comparing their UV spectra and the retention times of the peaks with those of the reference compounds purchased from PHYTOPLAN (Diehm & Neuberger GmbH, Heidelberg, Germany) and Roth (Karlsruhe, Germany) and by spiking the sample with standard solutions. The compound concentrations were estimated by the interpolation of the peak areas with calibration curves constructed for standard analytes. The content of all analytes in the samples was expressed as mg of the compound per gram of dry weight (mg/g DW).

Data analysis

The statistical analysis was done by several tests. In order to eliminate uncertain data, the Q-

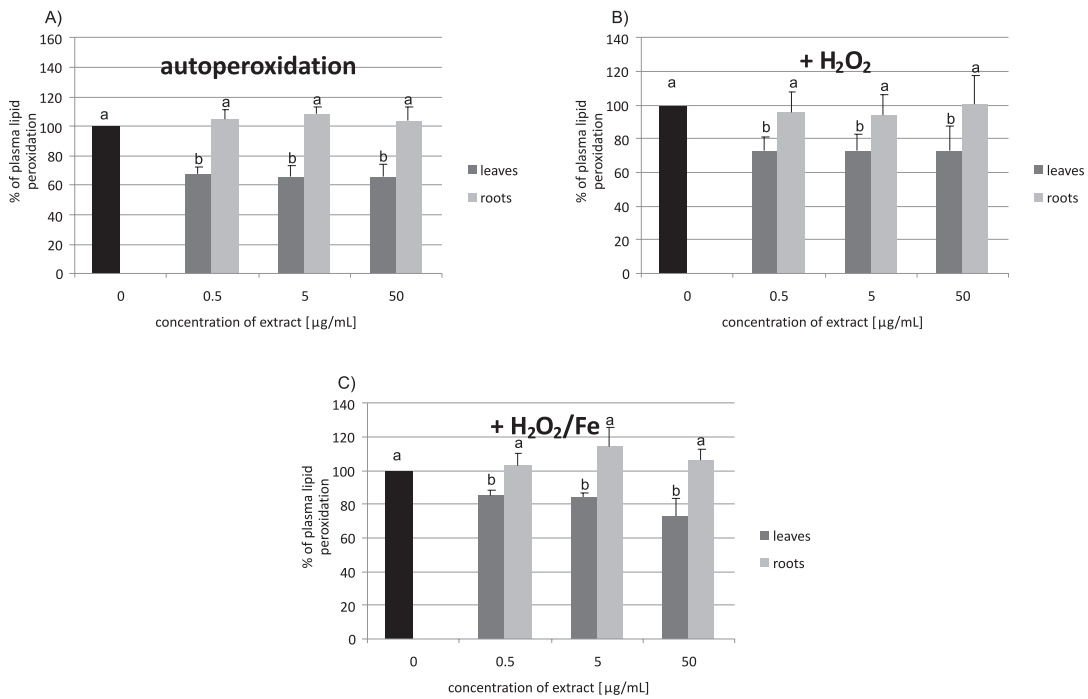

Figure 1. The effects of methanolic extracts from leaves and roots of *R. glutinosa* (0.5–50 µg/mL; 15 min) on plasma lipid autoperoxidation (**A**), plasma lipid peroxidation induced by H_2O_2 (**B**) and plasma lipid peroxidation induced by H_2O_2/Fe (**C**). Data represent the means ± standard error (SE) of 8-12. Bars with the same letter are not statistically different in Kruskal-Wallis test ($p \leq 0.05$)

Dixon test was performed. All the values in this study were expressed as the mean ± standard error (SE) of 2–12 independent experiments (for antioxidant properties) and 3 samples for each extract (for UHPLC analyses). The statistically significant differences were also assessed by applying the Kruskal-Wallis test at significance level $p \leq 0.05$ using STATISTICA 10 (STATSoft) software.

RESULTS

The effect of leaf and root methanolic extracts on the oxidative stress

The antioxidative properties of methanolic extracts from leaves and roots of *R. glutinosa* plants were studied *in vitro*. The plant extracts were tested at a dose range of 0.5–50 µg/mL. As shown in Fig. 1, the extract from leaves of *R. glutinosa* reduced autoperoxidation of plasma lipids and plasma lipid peroxidation induced by H_2O_2 or H_2O_2/Fe. The reduction in TBARS level in plasma treated with the extract at the presence of H_2O_2 or H_2O_2/Fe reached about 30% when the highest concentration (50 µg/mL) of extract was used (Figs. 1b and 1c). Compared to the extract from leaves, the extract from roots of *R. glutinosa* did not have effect on plasma lipid peroxidation *in vitro* (Fig. 1).

In experiments (determined of thiols) with plasma and the extracts only (without adding H_2O_2 or H_2O_2/Fe), plant extracts tested changed slightly the level of thiol groups in plasma proteins ($p \leq 0.05$) (Fig. 2a) and reduced the level of thiol groups in plasma proteins caused by H_2O_2 (Fig. 2b). However, the results included in Fig. 2c show that the extract from roots of *R. glutinosa* tested at the lowest concentration of 0.5 µg/mL effectively diminished the oxidation of thiol groups in plasma proteins treated with H_2O_2/Fe by approximately 30% ($p \leq 0.05$) (Fig. 2c).

Comparative analysis of the effects of extracts from leaves and roots of *R. glutinosa* (at the concentrations of 0.5–50 µg/mL) on inhibition of $O_2^{\cdot-}$ production in resting blood platelets is presented in Figure 3. Generation of $O_2^{\cdot-}$ in blood platelets was reduced only by 50 µg/mL concentration of root extract ($p \leq 0.05$) (Fig. 3).

Analysis of extracts by UHPLC

UHPLC analysis revealed the presence of four or five iridoid glycosides: catalpol, aucubin, loganin, catalposide, harpagide, harpagoside and two phenylethanoid glycosides: verbascoside and isoverbascoside in 1-year-old *R. glutinosa* plants. The compounds were identified by comparison of

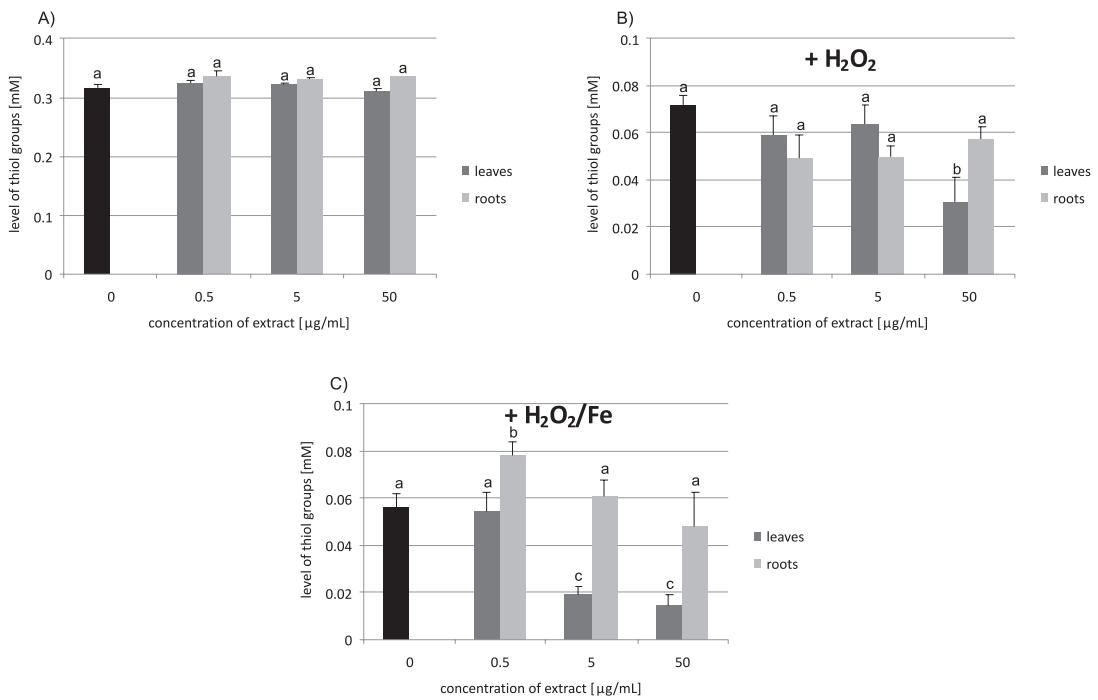

Figure 2. The effects of methanolic extracts from leaves and roots of *R. glutinosa* (0.5–50 µg/mL; 15 min) on plasma protein thiol (**A**), plasma protein thiol oxidation induced by H_2O_2 (**B**) and plasma protein thiol oxidation induced by H_2O_2/Fe (**C**). Data represent the means ± standard error (SE) of 6-10. Bars with the same letter are not statistically different in Kruskal-Wallis test ($p \leq 0.05$)

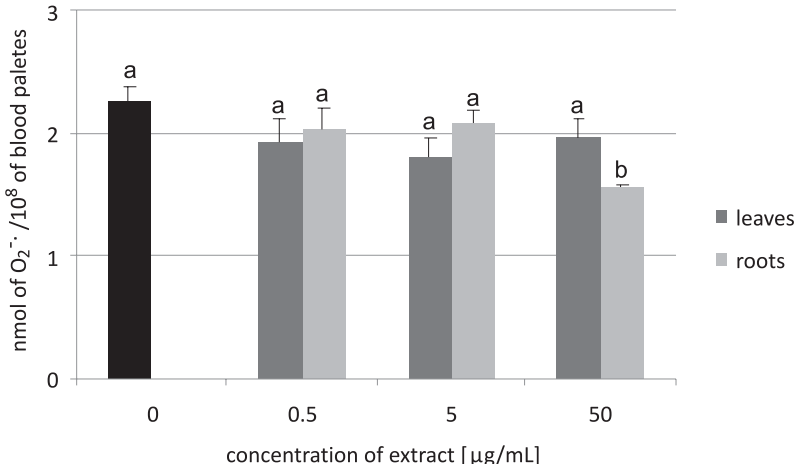

Figure 3. The effects of methanolic extracts from leaves and roots of *R. glutinosa* (0.5–50 µg/mL; 15 min) on $O_2^{\cdot-}$ generation in resting blood platelets. Data represent the means of 2 donors ± standard error (SE). Bars with the same letter are not statistically different in Kruskal-Wallis test ($p \leq 0.05$)

Table 1. Iridoid (catalpol, aucubin, loganin, catalposide, harpagide, harpagoside) and phenylethanoid glycoside (verbascoside, isoverbascoside) contents in leaf and root methanolic extracts of field-grown *R. glutinosa* plants.

Metabolite	Metabolite content (mg/g DW) ± SE in	
	leaves	roots
Catalpol	10.7292 ± 0.9	36.1074 ± 0.14
Aucubin	0.0401 ± 0.002	0.0406 ± 0.001
Loganin	0.4998 ± 0.03	0.1489 ± 0.01
Catalposide	-	0.6565 ± 0.002
Harpagoside	0.0261 ± 0.0006	0.0607 ± 0.001
Harpagide	2.4485 ± 0.15	2.9145 ± 0.14
Verbascoside	18.1252 ± 1.0	2.9469 ± 0.01
Isoverbascoside	7.5093 ± 0.006	6.6829 ± 0.0004

- not detected; presented data are the means ± standard error (SE) of three samples.

their retention times, UV spectra and ion mass spectra with authentic standards, as it was described previously (15). As shown in Table 1, production of individual compounds was different in leaf and root extracts. It was found that verbascoside was a predominant phenylethanoid glycoside in leaf extracts. Its amount (18 mg/g DW) was 6 times higher than in root extracts, while the content of isoverbascoside in both extracts was similar (6.9 and 7.5 mg/g DW) (Table 1).

The highest iridoid contents were recorded in root extracts. The roots accumulated 36 mg/g DW of catalpol i.e., 6-times more than leaves. 6-O-ester of catalpol (catalposide) was detected only in root extracts. Aucubin was present in roots as well as in leaves but at much lower concentrations (0.04 mg/g DW) than catalpol (Table 1). Another iridoid, loganin was present both in roots and leaves, but a higher amount of the iridoid was determined in leaf extracts than in root extracts (0.5 mg/g DW *vs*. 0.15 mg/g DW). Two other iridoids: harpagide and harpagoside were also detected in leaf and root extracts of *R. glutinosa*. The contents of harpagide in both extracts were similar (2.45 and 2.91 mg/g DW), whereas the quantitative differences in harpagoside content were found. The harpagoside level in roots was two times higher than in leaves (Table 1).

DISCUSSION

The present study provides more information on biological activity of methanolic extracts from leaves and roots of *R. glutinosa* plants. The results

demonstrate for the first time the antioxidative properties of the extracts when the oxidative stress model of blood components (plasma and blood platelets) was used. The oxidative stress was studied by using the measurement of the level of well known oxidative markers – the concentration of TBARS (the marker of lipid peroxidation), the concentration of protein thiol groups (the marker of oxidative damages in proteins), and the $O_2^{\cdot}$ generation. We measured not only autoperoxidation of plasma lipids, but we also provoked the lipid peroxidation and the oxidation of protein thiols by using H_2O_2 (the physiological bacteriostatic agent) and H_2O_2/Fe (the donor of $OH^{\cdot}$). H_2O_2 is also involved in various pathological processes such as cancer or atherosclerosis (21). Moreover, we studied the effect of tested methanolic extracts on $O_2^{\cdot}$ production in resting blood platelets, which play an important function in the pathogenesis of thrombosis, atherosclerosis and metastasis. In the present experiments, the methanolic extract from leaves exhibited a stronger antioxidant activity than root extracts, which was mostly attributed to its direct hydroxyl radical scavenging potency. Leaf extract was also able to protect plasma lipid against peroxidation caused by hydrogen peroxide. Earlier, Piątczak et al. (14) have found that methanolic extracts from *R. glutinosa* leaves also possessed stronger antioxidant activity in DPPH, ABTS, FRAP and P-Mo *in vitro* assays than root extracts of the plant. It was also indicated that the higher antioxidant activity of leaf extracts could be connected with higher total levels of flavonoids and total phenolic compounds. It is known that the phenolic compounds possess a large capacity to neutralize free radicals to trap singlet oxygen and to reduce and chelate transition metal ions (22). The antioxidative properties of phenolic compounds are believed to be partly responsible for the beneficial effects of these compounds on human health. Phenolic compounds may protect various elements of circulatory system and prevent cardiovascular diseases (23, 24). The comparative analysis performed in the present study showed that the leaf extracts of *R. glutinosa* were also characterized by a higher level of phenylethanoid glycosides (calculated as the sum of verbascoside and isoverbascoside) than root extracts. It is also known that verbascoside and isoverbascoside revealed antioxidant activity and a wide range of free radical scavenging against DPPH, hydroxyl and superoxide anion activity (25-30). Georgiev et al. (31) reported that verbascoside isolated from *Harpagophytum procumbens* cell suspension had strong antioxidative potential, similar to that of the positive control - superoxide dismutase. Moreover, the authors reported that concentrations of verbascoside higher than 20 µg/mL displayed higher superoxide anion scavenging activity than rosmarinic acid used as a positive control (31). Verbascoside isolated from *Sideritis trojana* was also tested for their antioxidant activity by *in vitro* TEAC assay by Kirmizibekmez et al. (32). The authors reported strong activity of the compound (1.03 mM) when compared with the reference compound (quercetin – 1.86 mM). Shikanga et al., (33) indicated that the EC_{50} values of verbascoside (89 µg/mL) and isoverbascoside (101 µg/mL) isolated from aerial parts of *Lippia* sp. were close to those of ascorbic acid (75 µg/mL), confirming reports that caffeoyl-containing compounds such as verbascoside and isoverbascoside are particularly powerful antioxidant agents (34). Moreover, neuroprotective effect of verbascoside due to antioxidant properties was also described by other authors (35, 36). Isoverbascoside was also found to reduce intracellular reactive oxygen species (ROS) production induced by t-BOOH (a known prooxidant agent) (32).

On the other hand, root extracts of *R. glutinosa* produced more iridoid glycosides than leaf extracts. The differences were observed mainly in respect to concentration of catalpol (10.8 mg/g DW in leaves *vs*. 36.1 mg/g DW in roots). Other iridoids were present in lower amounts. Literature data indicated that several iridoids were weakly active in DPPH-radical scavenging assay (37). However, among iridoids detected in *R. glutinosa* only information about antioxidant activity of harpagoside are available in the literature (38). These authors reported that the administration of harpagoside isolated from *Scrophularia buergeriana* roots to mice caused a significant fall in TBARS (marker of lipid peroxidation) concentration and scavenged ROS. This iridoid exerted a protective effect against oxidative damage induced by scopolamine by diminishing the reduction in the activities of glutathione reductase and superoxide dismutase (SOD) in both cortex and hippocampus of mice. Moreover, the authors reported that treatment with harpagoside elevated SOD activity significantly higher than that found in normal, control mice (38). The presence of harpagoside in the tested *R. glutinosa* extracts suggest that it might be partly responsible for decreasing plasma lipid peroxidation, measured by the reduction in TBARS concentration, showed in the study. However, further investigations are needed for confirming the antioxidant activity of the compound.

The values of concentration of the *R. glutinosa* extracts used in our experiments (5-50 µg/mL) cor-

respond to the range of physiological compounds after supplementation or dietary intake, therefore may be potentially therapeutically useful in the prevention of "Western diseases" (cardiovascular diseases and cancers), which are often induced by the oxidative stress.

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Declaration of interest

None to declare.

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