Short communication

STABILIZATION OF ERYTHROCYTES AGAINST OXIDATIVE AND HYPOTONIC STRESS BY TANNINS ISOLATED FROM SUMAC LEAVES (*Rhus typhina* L.) AND GRAPE SEEDS (*Vitis vinifera* L.)[#]

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Abstract: Erythrocytes are constantly exposed to ROS due to their function in the organism. High tension of oxygen, presence of hemoglobin iron and high concentration of polyunsaturated fatty acids in membrane make erythrocytes especially susceptible to oxidative stress. A comparison of the antioxidant activities of polyphenol-rich plant extracts containing hydrolysable tannins from sumac leaves (*Rhus typhina* L.) and condensed tannins from grape seeds (*Vitis vinifera* L.) showed that at the 5-50 µg/ml concentration range they reduced to the same extent hemolysis and glutathione, lipid and hemoglobin oxidation induced by erythrocyte treatment with 400 µM $ONOO^-$ or 1 mM HClO. However, extract (condensed tannins) from grape seeds in comparison with extract (hydrolysable tannins) from sumac leaves stabilized erythrocytes in hypotonic NaCl solutions weakly. Our data indicate that both hydrolysable and condensed tannins significantly decrease the fluidity of the surface of erythrocyte membranes but the effect of hydrolysable ones was more profound.

Abbreviations used: DPH – 1,6-diphenyl-1,3,5-hexatriene; GSH – reduced glutathione; HClO – hypochlorous acid; metHb – methemoglobin; $ONOO^-$ – peroxynitrite; TMA-DPH – 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene

In conclusion, our results indicate that extracts from sumac leaves (hydrolysable tannins) and grape seeds (condensed tannins) are very effective protectors against oxidative damage in erythrocytes.

Key words: Tannins, Erythrocytes, Oxidative stress, Peroxynitrite, Hypochlorous acid, Hemolysis, Fluorescence anisotropy

INTRODUCTION

Reactive oxygen and nitrogen species (RONS) are constantly produced in an organism as a result of metabolic processes, and they are maintained at low levels by the antioxidant system. In normal physiological conditions, transition fluctuations of RONS levels have been demonstrated. It has been shown that RONS can serve as signaling molecules, activating transcription factors which induce expression of antioxidant enzymes that help maintain the redox status in cells [1]. Under hypoxia and during exercise, the increased level of ROS induces an increase of erythrocyte mass, contributing to the organism's adaptation to such conditions. However, excessive amounts of ROS as a result of an imbalance in the pro- and antioxidant systems induce oxidative damage in cells and tissues, which is termed oxidative stress [2].

Erythrocytes, the unique carriers of oxygen, are constantly exposed to ROS due to their function in the organism. The high tension of oxygen, the presence of hemoglobin iron, and high concentration of polyunsaturated fatty acids in membrane make erythrocytes especially susceptible to oxidative stress. Oxidative damage of proteins and lipids results in disturbance of erythrocyte structure and functional properties. A correlation between the decrease of osmotic fragility induced by oxidative stress and the impaired rheological properties of blood has been demonstrated [3-5]. Hemocytes are one of the main targets for RONS produced by neutrophils as a result of respiratory burst in inflammation. When the neutrophils are activated by bacteria and their constitutive components, a series of toxic oxygen derivatives, such as $O_2^{.-}$, $NO^.$, ClO^-, H_2O_2, and $ONOO^-$, is produced. The damage of erythrocytes, for example by thermal injury-induced hemolysis, can also be accomplished by the activation of neutrophils [6]. On the other hand, in different pathological situations, erythrocytes are a potential source of RONS due to autoxidation of hemoglobin which leads to formation of superoxide anions and hydroperoxides [7-9]. Oxidative stress induces the loss of cell plasticity with impaired deformability, associated with changes in the cytoskeleton network and also suicidal death of erythrocytes, known as eryptosis [10-12], which leads to the elimination of erythrocytes and finally to anemia. Oxidative modification of erythrocytes increases their aggregability and adhesiveness to the endothelium and to other blood cells, thus contributing to vascular damage [11]. Therefore, natural antioxidant phenols with prospects for application as prophylactic and curative agents are intensely being searched for.

Tannins, polyphenols with a molecular mass ranging between 500 and 3000 Da, are esters of sugars and phenol carbonic acids or polycondensates of catechins (flavan-3-ol); based on their structure they are classified into two groups: the hydrolysable and the condensed tannins. Tannins are characterized by high chemical activity and a variety of biological effects, such as antitumor, antimutagen, antimicrobial, and antiinflammatory [13-15]. There are some publications on the protective effect of proanthocyanidins against oxidative stress in erythrocytes. Early on, it was shown that consumption of catechins and their oligomers (procyanidins) from cocoa reduced in vitro the susceptibility of human erythrocytes to AAPH-induced oxidative hemolysis [16]. It has also been found that proanthocyanidin-rich grape seed extract (*Vitis vinifera* L.) administered orally significantly improved age-associated oxidative changes in rat erythrocytes [17] and in vitro suppressed erythrocyte oxidative stress induced by UVB [18]. However, only one work regarding hydrolysable tannins has shown that non-polymeric tannins (tannic, gallic and ellagic acids) exert a protective or toxic effect depending on concentrations on stressed trout erythrocytes [19]. And recently we have shown the antiradical activity and protective effects of hydrolysable tannins from leaves of sumac (*Rhus typhina* L.) against lipid and protein oxidative damage in erythrocytes, induced by synthetic pro-oxidant *tert* butyl-hydroperoxide [20].

Here, the results of a comparative study of polyphenol-rich plant extracts containing hydrolysable tannins from sumac leaves (*Rhus typhina* L.) and condensed tannins from grape seeds (*Vitis vinifera* L.) in protection of erythrocytes against oxidative damage induced by HClO and $ONOO^-$ and by hypotonic stress are presented.

MATERIAL AND METHODS

Plant materials
Extracts from leaves of sumac (*Rhus typhina* L.) and from seeds of grape (*Vitis vinifera* L.) were prepared in the Institute of Bioorganic Chemistry of the Uzbekistan Academy of Sciences [21, 22].

Isolation of swine erythrocytes
Swine blood was collected in tubes containing 3.8% citrate as an anticoagulant (1:9). Citrated blood was centrifuged (400 g, 15 min, 4°C) and the plasma and buffy coat were removed by aspiration. The erythrocytes were washed twice with 0.9% NaCl.

Peroxynitrite-mediated oxidation of erythrocyte ghost lipids
The erythrocyte ghosts were prepared from washed cells and treated with $ONOO^-$ according to Soszynski and Bartosz [23]. The lipid peroxidation level in erythrocyte ghosts was measured as the thiobarbituric acid reactive substance (TBARS). Erythrocyte ghosts (protein content 1.5 mg/ml) were incubated with the extract from leaves of sumac (*Rhus typhina* L.) or the extract from seeds of

grape (*Vitis vinifera* L.) with or without oxidant (400 μM ONOO⁻) at 37°C for 45 min. After incubation, 200 μl of 25% trichloroacetic acid (TCA) was added and samples were centrifuged (4000g, 15 min). Then 250 μl of 0.7% thiobarbituric acid (TBA) was added to 1 ml of the supernatant. The mixture was kept in a boiling water bath for 20 min, cooled and absorbance was measured at 535 nm. TBARS concentration was estimated, using the molar extinction coefficient of 156 mM^{-1}·cm^{-1} [24].

Measurements of metHb

Erythrocyte suspension (0.25% hematocrit in PBS, pH 7.4) was incubated with the extract from leaves of sumac (*Rhus typhina* L.) or the extract from seeds of grape (*Vitis vinifera* L.) for 5 min at 37°C. Next, 400 μM of ONOO⁻ was added and absorbance was measured at 630 nm after 10 min. The amount of metHb formed in the presence of ONOO⁻ without extract was normalized and taken for 100% [25].

GSH detection in erythrocytes

Erythrocyte suspension (0.5% hematocrit in PBS, pH 7.4) was exposed to the extract of sumac (*Rhus typhina* L.) or the extract of grape (*Vitis vinifera* L.) at 37°C for 15 min. Next, the suspension was incubated for 15 min with or without oxidant (400 μM ONOO⁻ or 1 mM HClO) and then 0.2 ml of 25% trichloroacetic acid was added and samples were centrifuged. 700 μl of 0.5 M phosphate buffer (pH 7.8) and 50 μl of Ellman's reagent (5 mM) were added to 700 μl of the supernatant. After 10 min the samples were monitored spectrophotometrically at 412 nm. The concentration of GSH was calculated using the extinction coefficient $\varepsilon = 13600$ M^{-1}cm^{-1} [26].

Determination of erythrocyte oxidative hemolysis

Erythrocyte suspension (1 ml of 1% hematocrit in PBS, pH 7.4) was incubated with the extract from leaves of sumac (*Rhus typhina* L.) or the extract from seeds of grape (*Vitis vinifera* L.) for 15 min at 37°C. Next, samples were incubated with or without 1 mM HClO for 15 min. After incubation, 4 ml of buffer (150 mM NaCl; 10 mM Tris-HCl pH = 7.4) was added to the samples and they were centrifuged (400 g, 15 min). Absorbance of supernatants containing hemoglobin was measured at 540 nm. Results are presented as percent of hemolysis versus the concentration of extracts.

Measurement of erythrocyte membrane fluidity

The fluidity of the erythrocyte membranes was measured using a steady-state fluorescence polarization technique. Two different fluorescent probes were used: 1,6-diphenyl-1,3,5-hexatriene (DPH), and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH). Measurements were made with a Perkin Elmer luminescence spectrometer LS-55 equipped with a fluorescence polarization device. Finally, the concentration of erythrocyte suspension in a cuvette was 0.05% hematocrit. After the addition of 1 μM DPH (in tetrahydrofuran)

or TMA-DPH (in methanol) the sample was stirred well and incubated for 20 min in the dark at room temperature. The cuvette holder was temperature controlled by a water thermostat at 37^oC. The readings were taken at intervals of 2 s. The polarization values (r) of the samples were calculated by the fluorescence data manager program using the Jablonski equation:

$$r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$$

where I_{VV} and I_{VH} are the vertical and horizontal fluorescence intensities, respectively, of the vertical polarization of the excitation light beam. The factor $G = I_{HV}/I_{HH}$ (grating correction factor) corrects the polarizing effects of the monochromator. The excitation wavelengths were 348 nm (DPH) and 340 nm (TMA-DPH) and the fluorescence emission was measured at 426 nm for DPH and 430 nm for TMA-DPH [27].

Measurement of erythrocyte osmotic fragility

Erythrocyte suspension (1 ml of 2% hematocrit in PBS, pH 7.4) was incubated either with the extract from leaves of sumac (*Rhus typhina* L.) or the extract from seeds of grape (*Vitis vinifera* L.) for 15 min at 37^oC. Then, 4 ml of NaCl solution of different concentrations (0.3-0.8%) were added to the samples. The control sample was prepared without extracts. After incubation for 15 min the samples were centrifuged (400 g, 15 min), and absorbance of supernatants was measured at 540 nm. Results are presented as percent of hemolysis versus concentration of the hypotonic NaCl solution.

Statistical analysis

The results are presented as mean ±SE. The level of significance was analyzed using one-way ANOVA test.

RESULTS

Phytochemical screening and biological effects of extracts

Obtaining purified water-acetone extracts from leaves of sumac (*Rhus typhina* L.) and grape seeds (*Vitis vinifera* L.) and detection of compounds contained within were carried out according to the methods described by Islambekov *et al.*, 1994 [21] and Pirniyazov *et al.*, 2003 [22] respectively. Phytochemical screening revealed that extracts from sumac leaves (*Rhus typhina* L.) contain over 90% hydrolysable tannins and from grape seeds (*Vitis vinifera* L.) condensed tannins, as shown in Table 1. Furthermore, it was found that extracts from sumac leaves and grape seeds have low toxicity in administration per os (LD_{50} 5600 mg/kg and 1930 mg/kg) and exert interferon-inducing activity and an antihypoxic effect respectively [28].

Table 1. The content of polyphenols in the extract from leaves of sumac (*Rhus typhina* L.) and the extract from seeds of grape (*Vitis vinifera* L.)

Extract from leaves of sumac (*Rhus typhina* L.)		Extract from seeds of grape (*Vitis vinifera* L.)	
Components of extract	Content [%]	Components of extract	Content [%]
3,6-bis-O-di-O-galloyl-1,2,4-tri-O-galloyl-β-D-glucose	74.05	Proanthocyanidin -1	45.90
Rutin	1.15	Proanthocyanidin-2	44.10
2,3-di-O-galloyl-β-D-glucose	2.15	(±)-gallocatechin	2.30
2-O-galloyl-β-D-glucose	2.10	(-)-epicatechin	3.40
3-O-galloyl-β-D-glucose	2.20	(-)-epigallocatechin	1.80
6-O-galloyl-β-D-glucose	2.05	(+)-catechin	2.30
1,4,6-tri-O-galloyl-β-D-glucose	5.10	Chryzanthemin	0.01
1,2,3,4,6-penta-O-galloyl-β-D-glucose	10.05	Delphnidin	0.11
Quercetin	0.05	Oenin	0.02
Kaempferol	0.05	Malvin	0.03
Gallic acid	1.05	Callistephin	0.01
---	---	Pelargonin	0.02

The protective effects of extracts from leaves of sumac (*Rhus typhina* L.) and grape seeds (*Vitis vinifera* L.) against oxidative damage of erythrocytes induced by ONOO⁻ and HClO

Effects of extracts on peroxynitrite-induced lipid oxidation and metHb formation. First we studied the antioxidative effect of tannins on lipids under conditions of oxidation of ghost erythrocytes by $ONOO^-$ according to the method described by Soszynski and Bartosz, 1996 [23]. As shown in Fig. 1A, incubation of erythrocyte ghosts with extract from leaves of sumac (hydrolysable tannins) decreases the content of TBARS product to $82.33 \pm 1.23\%$, $69.15 \pm 1.19\%$ and $51.88 \pm 1.95\%$ at the concentration of 7.5, 17.5 and 30 µg/ml in comparison with samples treated with 400 µM $ONOO^-$ (100%). The values found for the extract from grape seeds (condensed tannins) in the same conditions at 7.5, 17.5 and 30 µg/ml concentration were $85.43 \pm 0.65\%$, $74.69 \pm 0.93\%$ and $62.89 \pm 1.75\%$, respectively.

The exposure of erythrocyte suspension to 400 µM $ONOO^-$ caused metHb formation and this level was taken as 100% (Fig. 1B). As shown in Fig. 1B, incubation of erythrocytes with $ONOO^-$ in the presence of the extract from sumac leaves (hydrolysable tannins) led to a decrease of metHb formation to $34.60 \pm 4.30\%$, $31.56 \pm 1.61\%$ and $27.31 \pm 1.05\%$ at the concentration of 15, 30 and 45 µg/ml respectively, in relation to the control sample containing only the oxidant (100%).The values found for the extract from grape seeds (condensed tannins) under the same conditions at 15, 30 and 45 µg/ml concentration were $41.88 \pm 2.63\%$, $37.33 \pm 0.91\%$, $31.26 \pm 1.09\%$.
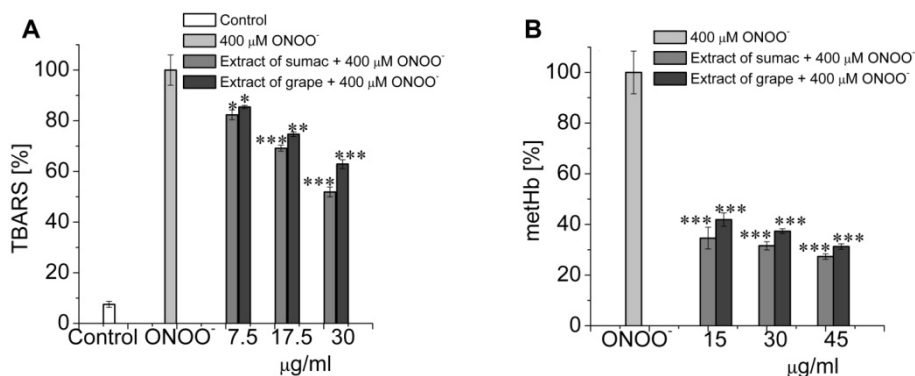
Fig. 1. Protective effect of the extracts from leaves of sumac (*Rhus typhina* L.) and from seeds of grape (*Vitis vinifera* L.) against lipid peroxidation in erythrocyte ghosts (A) and against formation of methemoglobin in erythrocyte induced by 400 μM ONOO⁻ (B). The data presented are the means ± SE. The effects of extract of sumac and extract of grape were statistically significant according to one-way ANOVA test. A − Extract of sumac vs. 400 μM ONOO⁻ treated erythrocytes (7.5 μg/ml, *p < 0.05; 17.5 μg/ml and 30 μg/ml, ***p < 0.001). Extract of grape vs. 400 μM ONOO⁻ treated erythrocytes (7.5 μg/ml, *p < 0.05, 17.5 μg/ml, **p < 0.01 and 30 μg/ml, ***p < 0.001). B − Extract of sumac vs. 400 μM ONOO⁻ treated erythrocytes (15 μg/ml; 30 μg/ml and 45 μg/ml, ***p < 0.001). Extract of grape vs. 400 μM ONOO⁻ treated erythrocytes (15 μg/ml; 30 μg/ml and 45 μg/ml, ***p < 0.001).
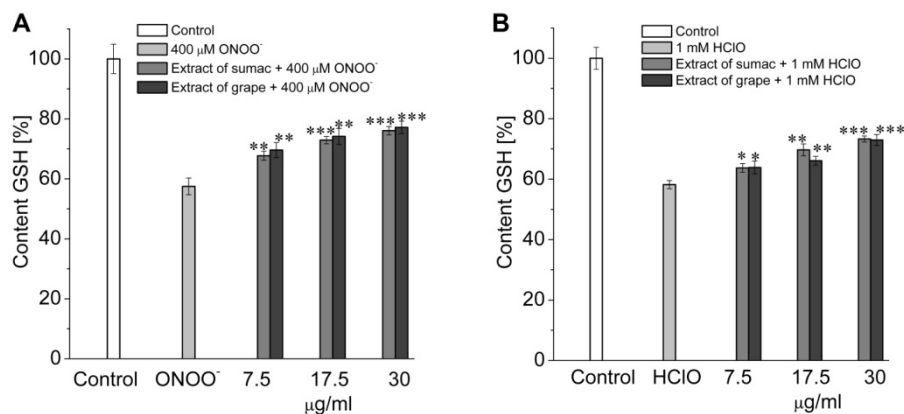

Fig. 2. Sparing effects of the extracts from leaves of sumac (*Rhus typhina* L.) and from seeds of grape (*Vitis vinifera* L.) on GSH depletion in erythrocytes induced by 400 μM ONOO⁻ (A) and 1 mM HClO (B). The data are presented as the means ± SE. The effect of extract of sumac and extract of grape were statistically significant according to one-way ANOVA test. A − Extract of sumac vs. 400 μM ONOO⁻ treated erythrocytes (7.5 μg/ml, **p < 0.01; 17.5 μg/ml and 30 μg/ml, ***p < 0.001). Extract of grape vs. 400 μM ONOO⁻ treated erythrocytes (7.5 μg/ml and 17.5 μg/ml, **p < 0.01; 30 μg/ml, ***p < 0.001). B − Extract of sumac vs. 1 mM HClO treated erythrocytes (7.5 μg/ml, *p < 0.05; 17.5 μg/ml, **p < 0.01 and 30 μg/ml, ***p < 0.001). Extract of grape vs. 1 mM HClO treated erythrocytes (7.5 μg/ml, *p < 0.05; 17.5 μg/ml, **p < 0.01; 30 μg/ml, ***p < 0.001).

Sparing effect of extracts on peroxynitrite and HClO-induced GSH depletion.
In the absence of the extracts, exposure of erythrocytes to 400 μM ONOO⁻
caused significant depletion in GSH content (to $57.48 \pm 2.79\%$). As depicted in
Fig. 2A, pretreatment of erythrocytes with sumac extract at the concentration of
7.5 μg/ml, 17.5 μg/ml and 30 μg/ml followed by exposure to ONOO⁻ led to the
increase in glutathione concentration to $67.72 \pm 1.47\%$, $72.91 \pm 1.23\%$, $76.06 \pm 1.40\%$
respectively. The same results were observed after pretreatment of erythrocytes with
grape seed extract. In this case at the same concentrations of extracts GSH content
increased to $69.61 \pm 2.52\%$, $74.17 \pm 2.67\%$ and $77.17 \pm 2.08\%$ respectively.
A significant decrease of GSH level was also observed for incubation of erythrocytes
with 1 mM HClO (Fig. 2B). The pretreatment with both extracts resulted in increase
of GSH content to the same extent as for ONOO⁻ application as an oxidant.

Protective effect of extracts on hemolysis induced by HClO. The hemolysis of
erythrocytes induced by HClO was evaluated by measuring hemoglobin release.
The incubation of erythrocytes with 1 mM HClO produced $45.76 \pm 2.01\%$ hemolysis,
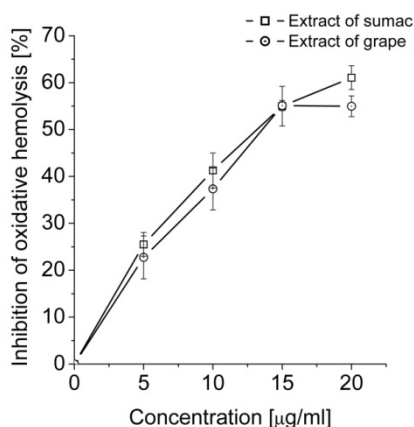which was taken as 100%.

Fig. 3. Protective effects of the extracts from leaves of sumac (*Rhus typhina* L.) and from
seeds of grape (*Vitis vinifera* L.) against oxidative hemolysis induced by 1 mM HClO. The
data presented are the means ± SE. Dose dependence is statistically significant according
to one-way ANOVA test, $p < 0.05$).

As depicted in Fig. 3, HClO-induced hemolysis was significantly reduced in
erythrocytes pretreated with both extracts in a dose-dependent manner to the same
extent. Extract from the sumac inhibited hemolysis on average by $25.50 \pm 2.54\%$,
$41.63 \pm 3.73\%$, $54.98 \pm 4.23\%$ and $61.06 \pm 2.53\%$ at the concentrations of 5, 10,
15 and 20 μg/ml, respectively. The values found for extract from grape seeds
under the same conditions were $22.74 \pm 4.58\%$, $37.34 \pm 4.47\%$, $55.10 \pm 1.06\%$
and $54.96 \pm 2.20\%$.

The effects of extracts from leaves of sumac (*Rhus typhina* L.) and from grape seeds (*Vitis vinifera* L.) on erythrocyte fragility and structure. **Osmotic stability of erythrocytes**

We also studied the effect of the extracts on erythrocyte hemolysis induced by hypotonic shock. As shown in Fig. 4A, the inhibition potency of these extracts on erythrocyte hemolysis induced by hypotonic stress differed significantly. Extract from sumac (17.5 µg/ml) increased erythrocyte stability in the whole range of hypotonic solution concentrations by $12.83 \pm 4.02\%$, $32.72 \pm 4.17\%$, $29.36 \pm 4.32\%$, $21.39 \pm 0.69\%$ at the concentrations 0.3%, 0.4%, 0.5% and 0.6% NaCl respectively.
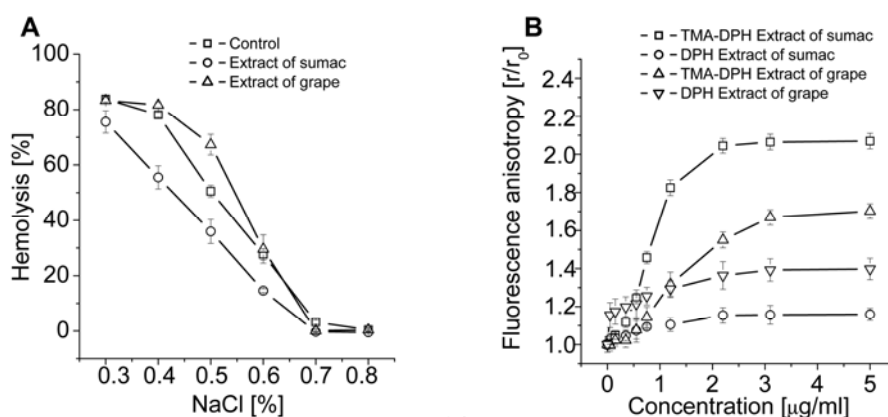

Fig. 4. Effects of the extracts from leaves of sumac (*Rhus typhina* L.) and from seeds of grape (*Vitis vinifera* L.) on hemolysis induced by hypotonic NaCl solutions (A) and fluorescence anisotropy of TMA-DPH and DPH (B) in erythrocytes. The data presented are the means ± SE. Dose dependence is statistically significant according to one-way ANOVA test ($p < 0.05$).

For the grape seed extract, its action on erythrocyte hemolysis was multidirectional. In a solution of NaCl close to isotonic (0.7-0.8%), a minimal significant protective effect against hemolysis of erythrocytes was observed. However, in strong hypotonic solutions (0.4%, 0.5%) where the integrity of erythrocytes was already disturbed, the extract displayed hemolytic action of about $5.54 \pm 0.97\%$ and $15.18 \pm 3.72\%$ in relation to the control sample without extract. It should be noted that there was also the same tendency in the effect of extracts for a lower concentration (7.5 µg/ml) but the effects were less expressed (data are not shown).

We also examined the effects of the extracts on fluidity of erythrocyte membranes at different depths of lipid bilayer, which was measured as fluorescence anisotropy values for DPH and TMA-DPH. The results are presented as the ratio (r/r_0) between the fluorescence anisotropy of probes in the presence (r) and absence (r_0) of the extracts. The TMA-DPH and DPH

fluorescence anisotropy values in the presence of increasing concentrations of extracts are shown in Fig. 4B.

A small increase was found in the anisotropy values for DPH as a result of extracts' interaction with erythrocytes, which means a decrease in fluidity in the hydrophobic region of the membrane. Thus, at 5.0 μg/ml concentration, the ratio (r/r_0) of fluorescence anisotropy for sumac extract was 1.16 ± 0.03 (compared to a control without extracts taken as 1), while for grape seed extract the corresponding value was 1.39 ± 0.06 (Fig. 4B). The alterations in TMA-DPH anisotropy values were more expressed than those for DPH for both extracts (Fig. 4B). However, in this case a significant difference between extracts in their action was revealed. The sumac extract made the surface membrane of erythrocytes more rigid. Thus, at 5.0 μg/ml, the ratio of fluorescence anisotropy TMA-DPH was increased to 2.07 ± 0.04 for sumac extract, while for grape seed extract the corresponding value was 1.70 ± 0.04.

DISCUSSION

The oxidative stress of erythrocytes accompanies many pathological conditions, such as sickle cell and iron deficiency anemias and thalassemia [8, 29, 30], as well as aging [17, 31], hypoxia [9] and obesity [32]. An intensive search has been going on for natural antioxidants to treat diseases induced by RONS. In this work, we examine the preventive activity of extracts containing polyphenol mixtures, with the main components (over 90%) being hydrolysable (sumac leaves) and condensed tannins (grape seeds), against erythrocyte oxidative damage induced by hypochlorite and peroxynitrite, typical RONS intensively produced in the organism under pathological conditions. It is believed that tannins, being highly polymerized compounds which contain many phenolic hydroxyl groups, are more effective antioxidants than flavonoids and display slight or no pro-oxidative action [33]. Furthermore, there are data showing that erythrocytes bind polyphenols and may increase the capacity of blood to scavenge oxidants. It has been shown that polymerized polyphenols are bound by proteins on the cell surface rather than intracellular structures and that erythrocyte-polyphenol complexes may serve as potent "sinks" for RONS [34-36]. We used HClO and ONOO$^-$ as oxidants that can also be transformed into other radicals, which can lead to different damage of biomolecules. HClO penetrates into the erythrocyte membrane and in a nonenzymatic reaction may be transformed into another ROS, such as $\cdot$OH and $^1O_2$ [37]. HClO induces oxidation of protein SH groups that cause S-S bridge formation and intracellular glutathione degradation. The toxic effects of HClO are connected with the formation of chloramines in reaction with the amino group of lipids, proteins, and also chlorohydrins of lipid degeneration [11, 26, 38, 39]. Erythrocyte exposure to HClO leads to cell swelling, structural rearrangement of the membrane, pore formation, and ultimately to hemolysis [11, 26, 38]. ONOO$^-$ is a reaction product of O_2^- and NO$\cdot$ that are formed in the vasculature under

normal physiological conditions. It has recently been shown that erythrocytes also express nitric oxide synthase [40] and, under normal physiological conditions, release small amounts of ONOO⁻ that can be considerably increased by oxidative stress. Peroxynitrite can oxidize lipids and proteins and also nitrosylate amino acids [41, 42].

Here we showed that extracts from leaves of sumac (hydrolysable tannins) and grape seeds (condensed tannins) in a dose-dependent manner inhibited lipid oxidation induced by ONOO⁻ and are measured as TBARS. The same authors have postulated that the oxidative effect of ONOO⁻ on lipids is indirect and depends on its decomposition to $\cdot$OH and NO_2 [42, 43]. We suggest that the tannins under investigation can protect lipids from oxidation due to their direct reaction with radicals and/or with ONOO⁻. The interaction with ONOO⁻ and $\cdot$OH was observed for some polyphenols including tannin [43-45].

As our experiments showed, preincubation of erythrocytes with ONOO⁻ also caused considerable formation of metHb. Erythrocyte preincubation with both extracts changed hemoglobin oxidation appreciably, the sumac extract exerting a somewhat stronger effect. For example, at a concentration of 30 μg/ml, the percentage of inhibition for the extract from sumac leaves was $68.44 \pm 1.61\%$, whereas it was $62.7 \pm 0.91\%$ for the extract from grape seeds.

Due to its high concentration, hemoglobin is believed to be a main target for peroxynitrite although GSH can also be oxidized directly [12]. In our experiments, the treatment of erythrocytes with ONOO⁻ also caused a decrease in the erythrocyte content of the antioxidant glutathione, which was inhibited to nearly the same extent with pretreatment by either extract. However, even at the highest concentration of 30 μg/ml, this protective effect amounted to as little as 20% for both extracts. In erythrocytes, ONOO⁻ is isomerized to NO_3 by oxyHb to form metHb and O^-_2 [46]. In the case with GSH, the products of its oxidation are nitrosoglutathione, disulfides of glutathione and thiyl radicals [23, 43]. It is plausible to hypothesize that tannins may interact with different affinity and/or rate constants with the products of GSH and Hb oxidation. This could, at least in part, account for different efficiencies of tannins in protection of Hb and GSH against ONOO⁻.

We also observed approximately the same decrease in oxidized glutathione concentration in response to treatment of erythrocytes with HClO in the presence of either extract. It is noteworthy in this connection that condensed tannins from grape seeds (*Vitis vinifera* L.) prevented hemolysis and lipid peroxidation but not intracellular GSH depletion or hemoglobin oxidation induced by UVB radiation in rat erythrocytes [18]. Hence it follows that the protective effect of antioxidants depends not only on the nature of the antioxidant itself, but also on the model of oxidative stress. This explanation agrees with data from other literature. Thus, it has been shown that from three studied lipid-soluble antioxidants only quercetin inhibited AAPH-generated oxidative hemolysis although all the compounds exhibit similar antiradical activity in a cell-free medium and almost to the same degree inhibit neutrophil oxidative bursts [47].

In contrast to ONOO⁻, HClO does not induce hemoglobin oxidation; however, it induces marked hemolysis [26, 38, 48]. It is believed that HClO-induced erythrocyte hemolysis can result from the production of phospholipid chlorohydrins in the cell membrane as well as by formation of nonselective pores [26, 38, 39]. In our experiments, erythrocyte incubation with 1 mM HClO induced $45.76 \pm 2.01\%$ cell hemolysis which was inhibited to the same extent and dose-dependently by preincubation of erythrocytes with both the extracts.

Previously it was shown that chlorohydrins exert a strong hemolytic action, proportional to the lipid:erythrocyte ratio [39]. As our extracts in low concentrations inhibited hemolysis induced by HClO, it can be assumed that they prevented lipid oxidative formation of chlorohydrins.

We also studied the effect of the extracts on the stability of erythrocyte membranes in hypotonic NaCl solutions, and in this case significant differences were found in the effects of the extracts. Our results showed that the extract from sumac leaves markedly depressed osmotic shock-induced hemolysis, whereas under low osmolarity of the medium (0.4%, and 0.5% NaCl) the extract from grape seeds had a certain hemolytic effect. These effects seem to be related to various modifications of the membranous structure by the tannins, as we showed using fluorescent anisotropy of the probes with different localizations in membranes. As was determined by the TMA-DPH fluorescent probe, both the extracts significantly decreased fluidity of the surface membrane; the effect of hydrolysable tannins was higher than that of condensed ones. Some elevated rigidity was also noted using another fluorescent probe, DPH, which is localized in the membrane's hydrophobic region. However, some difference in the effects of extracts should be noted. The ratio r/r_0 is increased a bit more in the presence of grape seed extract than sumac extract. This may be related to a more rigid configuration of the condensed tannin molecule, which enables it to penetrate more easily into the membrane.

It should be noted that the literature contains contradictory information concerning the influence of tannins on membrane structure. It has been shown that condensed tannins isolated from cocoa could interact with a polar head group of lipids, increasing membrane fluidity in the Jukart T cell line [49]. There are also findings that condensed tannins from grape seeds decrease membrane fluidity in both the hydrophobic and hydrophilic parts of erythrocyte membranes [18]. For hydrolysable tannins, increasing fluidity in the internal region of the lipid bilayer has been shown, but no changes were found on the surface of the membrane of the Chinese hamster cell line B14 [50].

It is known that the binding of tannins to the membrane surface of cells may induce cluster and raft formation [34]. It may prevent erythrocytes swelling and release of hemoglobin. Our fluorimetric studies showed that the used extracts caused an increase in the packing order in the hydrophilic area of the erythrocyte membrane. As hydrolysable tannins decreased much more the fluidity of the erythrocyte membrane surface in comparison to the condensed ones, this seems to explain their protective effect against hypotonic shock.

A comparison of the antioxidant activities of the extracts from sumac leaves (hydrolysable tannins) and grape seeds (condensed tannins) showed that they reduced to the same extent hemolysis and glutathione, lipid and hemoglobin oxidation induced by erythrocytes' treatment with 400 µM ONOO⁻ or 1 mM HClO. These results support the suggestion [33] that manifestation of the radical scavenging activity of tannins depends more on the large number of aromatic rings and hydroxyl groups and their proximity than on the specificity of functional groups.

Summary. We have shown that extracts from sumac leaves (hydrolysable tannins) and grape seeds (condensed tannins) are very effective protectors against the oxidative damage of erythrocytes and may have potential therapeutic value as water soluble, low toxicity, highly efficient antioxidants.

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