The protective role of extracts from leaves and bark of Uncaria tomentosa (Willd.) DC in oxidative damage of human erythrocytes provoked by 2,4-D herbicide

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Protective activity of the *Uncaria tomentosa* extracts on human erythrocytes in oxidative stress induced by 2,4-dichlorophenol (2,4-DCP) and catechol

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ABSTRACT

The purpose of this study was to evaluate the effect of the ethanolic and aqueous extracts of *Uncaria tomentosa* on human erythrocytes and additionally the assessment of protective effect of these extracts on hemolysis induction, hemoglobin oxidation, and changes in the level of reactive oxygen species (ROS) and lipid peroxidation, which were provoked by selected xenobiotics, i.e. 2,4-dichlorophenol (2,4-DCP) and catechol.

All tested extracts, even at a very high concentration of 500 μg/ml were not toxic to the erythrocytes because they did not cause lipid peroxidation, increase methemoglobin and ROS levels nor provoked hemolysis. The results of this study also revealed protective effect of extracts of *U. tomentosa*. The extracts studied depleted the extent of hemoglobin oxidation and lipid peroxidation as well as decreased the level of ROS and hemolysis, which was provoked by 2,4-DCP. No protective activity of the extracts against catechol action, which is a precursor of semiquinones in cell was found.

A difference in the effect of the extracts studied was observed. Ethanol-based extracts revealed more pronounced ability to inhibit oxidation processes in human erythrocytes.

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1. Introduction

The progress observed in the last few decades in phytochemical and phytotherapeutic studies has been largely driven in respect to development of effective therapeutic methods, which are useful in treating of numerous diseases, especially cancer. Development of many diseases results from pollution of the environment with numerous xenobiotics including pesticides. These substances reveal noxious effect on human health. Their activity includes hepatotoxocity, changes in development of the circulatory and respiratory system, as well as development of various carcinomas. *Uncaria tomentosa* (Willd.) DC is a lignified climbing plant from South and Central America, which (under the name of "vilcacora" or "cat's claw") has become highly popular in Poland and in many other countries due to its proven immunostimulatory and anti-inflammatory activities (Akesson et al., 2003; Allen-Hall et al., 2010; Gattuso et al., 2004) and also in respect to its anticancer (Pilarski et al., 2010; Dreifuss et al., 2010) and antioxidative effects (Dreifuss et al., 2010).

A number of chemical studies on *U. tomentosa* revealed the presence of secondary metabolites, such as tetra- and pentacyclic indole and oxindole alkaloids and polyphenols (phenolic acids, flavonols, proanthocyanidins) and small concentrations of other secondary metabolites, such as quinovic acid glycosides, polyhydroxylated triterpenes and saponins (Aquino et al., 1989, 1991; Muhammad et al., 2001; Kitajima et al., 2004; Valerio and Gonzalez, 2005; Heitzman et al., 2005). Particularly noteworthy are isolated from tissues of *U. tomentosa* polyphenols, which have very interesting pharmacological properties.

According to scientific reports, aqueous and methanol extracts of different parts of *U. tomentosa* prevent the production of free radicals and lipid peroxidation *in vitro*, inhibit free radical damage to the sugar components of DNA and protect cell from death caused by various xenobiotics (Sandoval et al., 2000, 2002; Gonçalves et al., 2005; Desmarchelier et al., 1997; Pilarski et al., 2006).

Red blood cells have been extensively used to study oxidative stress. The human erythrocyte, which is destituted of nucleus and other organelles, is not a typical cell. Nevertheless, its structural and functional simplicity makes it a convenient cellular model that is particularly useful for studies of xenobiotics toxicity (Marczak and Jóźwiak, 2008; Duchowicz et al., 2005; Bukowska et al., 2007a).

Catechol and 2,4-DCP are products of 2,4-dichlorophenoxyacetic (2,4-D) transformation, which is the active ingredient of selective, hormonal herbicides used in the cultivation of cereals, rice

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and others plant cultures. These compounds are emitted into the environment mainly from anthropogenic sources and are characterized by high toxicity, persistence in the environment and ability to bioaccumulate in living organisms (Michałowicz et al., 2008, 2011; Aydin et al., 2005).

Many examples show that transformation products of organic compounds may exhibit much stronger toxicity to organisms than their parent compounds (Duchnowicz et al., 2002, 2005; Bukowska, 2003).

In the present work, the impact of extracts of *U. tomentosa* leaves and bark on human erythrocytes as well as the antioxidant properties of *U. tomentosa* extracts against oxidative stress induced by 2,4-DCP and catechol in red blood cells were evaluated. The experiments were carried out by hemolysis measurements as well as by hemoglobin oxidation and denaturation analysis. Moreover, changes in ROS and lipid peroxidation levels were evaluated.

2. Materials and methods

2.1. Plant material and chemicals

The raw material (bark and leaves) of *U. tomentosa* originated from Instituto Peruano de Investigaciones Fitoterapia Andina in Lima Peru was kindly supplied by Wilcaccora Łomianki Centre, Poland. The general characteristics of this material were described previously by Pilarski et al., 2006. The voucher material is deposited at the Laboratory of Phytochemistry, Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznań, Poland.

The 2,4-DCP was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Catechol was obtained from Sigma (Poznań, Poland). 6-Carboxy-2′,7′-dichlorodihydro-fluorescein diacetate (DCFH$_2$-DA) was purchased from Molecular Probes.

2.2. Preparation of samples

One gram of the raw material (bark and leaves) of *U. tomentosa* was extracted with 10 ml of water or 96% ethanol at 37 °C for 8 h. Then, the extracts were centrifuged at 4000 rpm for 15 min. Supernatants were evaporated on Speed-Vac to dry mass and next exsiccated with P2O5. At these conditions we recovered about 111 mg of preparation per gram of bark and about 158 mg of preparation per gram of leaves in case of aqueous extraction. During extraction with 96% ethanol we obtained 123 mg of preparation per gram of bark and 158 mg of preparation per gram of leaves. Four extracts were obtained: Bet – ethanol extract of the bark; Baq – aqueous extract of the bark; Let – ethanol extract of leaves; Laq – aqueous extract of leaves. Powdered preparations were stored in a refrigerator in tightly closed containers, from which stock solutions were prepared for each experiment.

2.3. HPLC-quantitative polyphenols analysis

The identification of the compounds was carried out on a Merck-Hitachi L-7455 liquid chromatography with a diode array detector (DAD) and quaternary pump L-7100 equipped with D-7000 HSM Multisolvent Delivery System (Merck-Hitachi, Tokyo, Japan) and autosampler L-7200. Separation was performed on a Cadenza CD C18 (75 × 4.6 mm, 5 μm) column (Imtakt Corp., Kyoto, Japan). Oven temperature was set to 20 °C. The mobile phase was composed of solvent A (4.5% formic acid, v/v) and solvent B (acetonitrile). The programme began with a linear gradient from 0% to 25% B at 36 min, followed by washing and reconditioning the column. The flow rate was 1.0 ml/min, and the runs were monitored at the following wavelengths: flavan-3-ols at 280 nm, hydroxycinnamates at 320 nm, and flavonol glycosides at 360 nm. Diode array detector (DAD) spectra were measured over the wavelength range of 200/600 nm in steps of 2 nm. Spectra were compared with those of pure standards. Calibration curves were made from (−)-epicatechin, (+)-catechin, chlorogenic acid, quercetin 3-/*O*/-glucoside and procyanidin B2, C1, B1 as standards (Oszmiański et al., 2011).

2.4. Isolation of erythrocytes

Human erythrocytes were obtained from whole blood, taken from healthy donors in the Blood Bank of Łódź, Poland. The erythrocytes were centrifuged (3000 rpm/min) at 4 °C for 10 min and washed three times with phosphate-buffer saline (PBS; 150 mmol L^{-1} NaCl, 1.9 mmol L^{-1} NaH$_2$PO$_4$, and 8.1 mmol L^{-1} Na$_2$H-PO$_4$, pH 7.4).

All of mentioned above compounds (extracts, catechol, 2,4-DCP) were dissolved in 30 μl DMSO, and then PBS was added to the final volume of 10 ml (the concentration of DMSO used in the experiments was not toxic to the cells, Felicia et al., 1997). The erythrocytes, which were only incubated with incubation buffer and DMSO were used as controls.

Erythrocyte suspension was diluted with the incubation buffer (140 mM NaCl; 10 mM KCl; 1.5 mM MgCl$_2$; 10 mM glucose; 10 mM hepes; 100 μg/ml gentamicin; 0.005 M phosphate buffer pH 7.4) to 5% hematocrit and incubated for 1 h, 5 h and 24 h at 37 °C with *U. tomentosa* (at concentrations of 5–500 μg/ml) both with and without xenobiotics: 2,4-DCP and catechol (at a concentration of 100 μg/ml – catechol and 250 μg/ml – 2,4-DCP). Then, hemolysis, hemoglobin oxidation (methemoglobin formation), parameter *T* (hemoglobin denaturation), the level of ROS formation (oxidation of fluorescent probe – 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate – DCFH$_2$-DA) and lipid peroxidation were determined.

2.5. Hemolysis

Hemolysis in the erythrocytes incubated with pure *U. tomentosa* extracts and pre-incubated with extracts and subsequently treated with 2,4-DCP, (with met-Hb content of approx. 3%, but with a very low content of released hemoglobin) was determined directly on the basis of the absorbance measurement of the released oxyhemoglobin at a wavelength of 414 nm. The measurement was performed in the Soret band because this band is 15× more sensitive than the range of 440–700 nm within which cyanohemoglobin spectrum in Drabkin's method (542 nm) is measured. The ratio of hemolysis for these samples was calculated from the equation:

$$H\ (\%) = \frac{A_{pb414nm}}{A_{water414nm}} \times 100\%$$

where: H (%) – percent of hemolysis of the erythrocytes incubated with incubation buffer, extracts or extracts and 2,4-DCP, $A_{pb414\ nm}$ – absorbance of hemoglobin released from the erythrocytes samples incubated with incubation buffer, extracts or extracts and 2,4-DCP, $A_{water414\ nm}$ – absorbance of hemoglobin released from the erythrocytes after complete hemolysis with water (100%).

In the erythrocytes incubated with catechol where the amount of the hemoglobin released was high and additionally approx. 20% met-Hb was formed, the classical method of conversion of oxy-Hb into cyanmethemoglobin and calculating the % of hemolysis from the concentration ratio was applied (Drabkin, 1946).

The ratio of hemolysis in samples with catechol was calculated from the equation:

$$H\ (\%) = \frac{C[g/100ml]_{pb542nm}}{C[g/100ml]_{542nm}} \times 100\%$$

where: H (%) – Per cent of hemolysis of the erythrocytes incubated with incubation buffer or extracts and catechol, $A_{pb542nm}$ – concentration of hemoglobin in the erythrocytes samples incubated with incubation buffer or extracts and catechol, $A_{water542nm}$ – concentration of hemoglobin in the erythrocytes samples incubated with incubation buffer or extracts and catechol after complete hemolysis with water (100%).

2.6. Percent of methemoglobin

The concentration of hemoglobin was measured by Drabkin method (1946). Absorption spectra of hemoglobin were obtained in the wavelength range from 440 to 700 nm using a spectrophotometer (Specord M40) connected to a computer. The percentage of met-Hb in the total Hb content was calculated from the absorbance at 630 and 700 nm both for hemoglobin of control erythrocytes and hemoglobin released from the erythrocytes treated with extracts and xenobiotics. Hemoglobin treated with potassium ferricyanide (100% met) was used as a positive control.

$$\% \text{ of met-Hb} = [(A_{630nm} - A_{700nm})/(A_{100\%met-Hb630nm^*} - A_{100\%met-Hb700nm^*})] \times 100\%$$

where: A_{630} – the absorbance of a control sample (with incubation buffer) and sample with extracts or/and xenobiotics at 630 nm, A_{700} – the absorbance of a control sample (with incubation buffer) and sample with extracts or/and xenobiotics at 700 nm, A_{630*} – the absorbance of a control sample (with incubation buffer) and sample with extracts or/and xenobiotics treated with potassium ferricyanide – 100% met-Hb at 630 nm, A_{700*} – the absorbance of a control sample and sample with extracts or/and xenobiotics treated with potassium ferricyanide – 100% met-Hb at 700 nm.

2.7. Flow cytometry – oxidation of DCFH$_2$-DA – the level of ROS

The rate of 6-carboxy-2′,7′-dichlorodihydrofluoresceine diacetate oxidation was measured by flow cytometer. 6-Carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCFH$_2$-DA) is a fluorescent probe widely used for the detection of intracellular oxidants production (Bartosz, 2006). The DCFH$_2$-DA diacetate was added to the erythrocytes. It diffused across the cell membrane and it was hydrolyzed by intracellular esterases to 6-carboxy-2′,7′-dichlorodihydro-fluorescein (DCFH$_2$), which, upon oxidation, yielded highly fluorescent 6-carboxy-2′,7′-dichlorofluorescein (DCF). The final concentration of DCFH$_2$-DA in samples was 20 μM. The samples after 1 h of incubation with extracts and 2,4-DCP or catechol were incubated with DCFH$_2$-DA for 0.5 h at 37 °C in the dark. After incubation, 5 μl of the erythrocytes with

xenobiotic (5% hematocrite) was added to 2 ml of incubation buffor pH 7.4. Fluorescence was measured with Becton Dickinson, LSR II flow cytometer with excitation at 488 nm (blue laser) and 530 nm emission filters.

2.8. Lipid peroxidation

Lipid peroxidation in human erythrocytes was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS) (Stock and Dormandy, 1971). The erythrocytes were mixed with 20% trichloroacetic acid (1:1). The samples were centrifuged at 4000 rpm/min at 4 °C for 10 min – Thiobarbituric acid (15%) was added to supernatant and the samples were heated at 100 °C for 15 min. The absorbance of the supernatant was measured at 532 nm. Lipid peroxidation was expressed in absorbance units.

2.9. Denaturation of hemoglobin – parameter T

Parameter T describes fairly well the degree of hemoglobin denaturation. After the total oxidation of heme iron, parameter T describes the ratio of absorbance at the wavelength of 505 nm (absorption maximum of met-Hb with intact globin) and 563 nm (hemichromogen absorption band) (Puchała et al., 1979). After the incubation, the erythrocytes with extracts of *U. tomentosa* and xenobiotics were hemolysed by the addition of water. The samples (cells' membranes) were centrifuged (12,000 rpm/min) for 15 min, and the parameter T was determined in supernatant fraction, which contained hemoglobin.

3. Statistical analyses

The statistical analysis was performed with the STATISTICA 8 data analysis software (2000 StatSoft, Inc., Tulsa, OK, USA). In this study, one-way analysis of variance (ANOVA) with *post hoc* multiple comparisons procedure (Tukey test) was used to assess statistical differences in case of normal distribution. The difference was considered to be significant for $P < 0.05$ and highly significant for $P < 0.01$.

4. Results

4.1. HPLC-quantitative polyphenols analysis

HPLC/DAD analysis revealed that bark ethanolic and aqueous extracts contained mainly Procyanidin B2 and C1 as well as (−)-epicatechin. Caffeic acid derivatives were only determined in leaves aqueous extract, whereas chlorogenic acid, flavonols and polymeric proanthocyanidins were present in all extracts studied. The highest total concentrations of free phenols were determined in bark ethanolic extracts. High content of phenolic compounds was also determined in leaves ethanolic extract, whereas lower concentrations were detected in bark aqueous extract and leaves ethanolic extracts.

4.2. The effect of U. tomentosa extracts on human erythrocytes

4.2.1. Hemolysis

At the first step, the erythrocyte suspension was incubated with *U. tomentosa* extracts (at concentrations of 5–500 µg/ml). After the incubation, hemolysis was determined. It was found that no extract of *U. tomentosa* induced hemolysis in the concentrations ranging from 5 to 500 µg/ml (Table 2). However, a slight, statistically significant decrease in hemoglobin outflow was observed in the erythrocytes incubated with ethanol extracts from the concentration of 100 µg/ml, and the cells incubated with aqueous extracts from the concentration of 250 µg/ml (Table 2).

4.2.2. Hemoglobin oxidation and denaturation of hemoglobin – parameter T

It was found that all of the analyzed *U. tomentosa* extracts, did not provoke formation of met-Hb within the concentration range of 5–500 µg/ml (Table 2).

Table 1
Polyphenols content in 100 g of the bark and leaves extracts (mg/100 g).

Polyphenols	Bark aqueous extract	Bark ethanolic extract	Leaves aqueous extract	Leaves ethanolic extract
Procyanidin B2	920.58	927.21	0.00	0.00
(−)-Epicatechin	160.40	238.08	0.00	0.00
Procyanidin C1	349.24	473.79	0.00	0.00
Caffeic acid derivatives	0.00	0.00	140.92	0.00
Chlorogenic acid	111.91	199.10	457.12	2145.78
Flavonols (quercetin and campferol derivatives)	56.61	85.31	1001.2	2434.18
Polymeric proanthocyanidins	8963.04	18298.12	4100.19	11113.47
Total concentration	10561.78	20221.61	5699.43	15693.43

No hemoglobin denaturation in human erythrocytes was found after 1, 5 and 24 h of incubation with *U. tomentosa* extracts at a concentration of 250 µg/ml (Table 3).

4.2.3. Oxidation of DCFH₂-DA – the level of ROS

U. tomentosa extract at a concentration of 250 µg/ml did not cause a statistically significant increase in the ROS level in human erythrocytes after a 1-h incubation (Fig. 1).

4.2.4. Lipid peroxidation

No increase was found in lipid peroxidation level in the erythrocytes incubated for 1 h with *U. tomentosa* extracts at a concentration of 250 µg/ml (Fig. 2).

4.3. The effect of U. tomentosa extracts on damage induced by 2,4-dichlophenol and catechol in human erythrocytes

4.3.1. Hemolysis

After the exclusion of negative effect of *U. tomentosa* extracts on hemoglobin oxidation and denaturation, the erythrocytes were pre-incubated with *U. tomentosa* extracts (at concentrations of 5–500 µg/ml) for 10 min and then they were treated with xenobiotics: 2,4-DCP and catechol for 1 h. After this time, hemolysis was determined. The applied xenobiotics concentrations (100 µg/ml for catechol and 250 µg/ml for 2,4-DCP) have been selected on the basis of the researches conducted by Duchnowicz et al., 2002 and Bukowska and Kowalska, 2004. In the above studies, it was found that catechol and 2,4-DCP in the above concentrations are capable of inducing significant changes in the structure and function of human erythrocytes.

In our study, induction of hemolysis by 2,4-DCP and catechol (Figs. 3 and 4) was confirmed. It was found that increasing concentrations of extracts reduced hemolytic effect provoked by 2,4-DCP. Aqueous extracts exhibited lower activity than ethanol extracts (Fig. 3). It was also observed that the extracts studied did not decrease hemolytic changes, which were provoked by catechol (Fig. 3).

4.3.2. Hemoglobin oxidation and denaturation of hemoglobin– parameter T

The influence of 2,4-DCP and catechol on hemoglobin was examined, and it was confirmed that they caused hemoglobin oxidation. However, it needs to be emphasized that catechol much stronger oxidized hemoglobin in comparison to 2,4-DCP (Figs. 5 and 6). It was found that *U. tomentosa* extracts decreased hemoglobin oxidation provoked by 2,4-DCP (Fig. 5). A statistically significant decrease was observed for ethanol extracts from the concentration of 100 µg/ml, and in aqueous extracts from the concentration of 250 µg/ml. No statistically significant protective

Table 2
The effect of *U. tomentosa* extracts (5–500 μg/ml) on hemolysis (n = 8) and hemoglobin oxidation (n = 15) in human erythrocytes for 1 h of incubation. The results are presented in the form of mean values ± SD.

Index	Concentration (μg/ml)	Hemolysis (%)	Met-Hb (%)
Let	Control	0.47 ± 0.04	2.68 ± 0.25
	5	0.47 ± 0.05	2.71 ± 0.28
	10	0.46 ± 0.05	2.83 ± 0.25
	50	0.44 ± 0.06	2.85 ± 0.35
	100	0.38 ± 0.02[*]	2.99 ± 0.36
	250	0.27 ± 0.04[*]	2.75 ± 0.38
	500	0.18 ± 0.03[*]	2.88 ± 0.23
	ANOVA I	$F_{6,49}$ = 56.9; $p < 0.001$	$F_{5,75}$ = 0.43; $p > 0.05$
Laq	Control	0.52 ± 0.03	2.75 ± 0.25
	5	0.50 ± 0.04	2.80 ± 0.21
	10	0.49 ± 0.05	2.81 ± 0.22
	50	0.49 ± 0.04	2.84 ± 0.23
	100	0.47 ± 0.03	3.07 ± 0.19
	250	0.43 ± 0.04[*]	3.00 ± 0.31
	500	0.33 ± 0.07[*]	2.99 ± 0.31
	ANOVA I	$F_{6,49}$ = 15.5; $p < 0.001$	$F_{5,67}$ = 0.86; $p > 0.05$
Bet	Control	0.49 ± 0.02	2.87 ± 0.28
	5	0.47 ± 0.07	2.85 ± 0.25
	10	0.47 ± 0.04	2.82 ± 0.13
	50	0.48 ± 0.06	2.94 ± 0.28
	100	0.41 ± 0.03[*]	2.82 ± 0.32
	250	0.21 ± 0.03[*]	2.91 ± 0.28
	500	0.17 ± 0.05[*]	3.05 ± 0.36
	ANOVA I	$F_{6,56}$ = 84.9; $p < 0.001$	$F_{5,69}$ = 0.30; $p > 0.05$
Baq	Control	0.50 ± 0.04	2.82 ± 0.16
	5	0.49 ± 0.04	2.80 ± 0.23
	10	0.47 ± 0.04	2.80 ± 0.32
	50	0.46 ± 0.04	2.95 ± 0.34
	100	0.46 ± 0.03	3.02 ± 0.45
	250	0.36 ± 0.03[*]	3.10 ± 0.49
	500	0.24 ± 0.04[*]	3.09 ± 0.26
	ANOVA I	$F_{6,56}$ = 53.6; $p < 0.001$	$F_{5,69}$ = 0.40; $p > 0.05$

[*] $p < 0.001$ for samples (erythrocytes treated with extracts) compared to the control (erythrocytes suspended in incubation buffer).

effect against catechol-induced hemoglobin oxidation was observed (Fig. 6).

The effect of 2,4-DCP and catechol on hemoglobin denaturation was examined and the increase in this parameter was confirmed (Table 3). Catechol caused significantly stronger changes in hemoglobin denaturation than 2,4-DCP, which increased with time. No protective effect of *U. tomentosa* extracts at the selected concentration of 250 μg/ml against hemoglobin denaturation induced by 2,4-DCP or catechol was found (Table 3).

4.3.3. Oxidation of DCFH₂-DA – the level of ROS

The observed hemolytic effects as well as the results obtained for hemoglobin oxidation (Table 2, Fig. 3 and Fig. 5) made it possible to select one extract concentration – 250 μg/ml, at which all extracts revealed a protective effect on human erythrocytes. At the above concentration, ROS formation in human erythrocytes was analyzed, which was expressed as oxidation of fluorescent probe – 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCFH₂-DA). A statistically significant decrease in 2,4-DCP-induced DCF fluorescence in the erythrocytes incubated with all extracts studied was noted. However, *U. tomentosa* extracts did not cause a statistically significant decrease in catechol-induced DCF fluorescence (Fig. 1).

4.3.4. Lipid peroxidation

The effect of 2,4-DCP and catechol on lipid peroxidation extent in human erythrocytes was examined. It was shown that 2,4-DCP increased lipid peroxidation level, whereas catechol did not provoke the above process in human erythrocytes (Fig. 2).

It was found that *U. tomentosa* extracts at a concentration of 250 μg/ml cause a statistically significant decrease in the degree of 2,4-DCP-induced lipid peroxidation (Fig. 2).
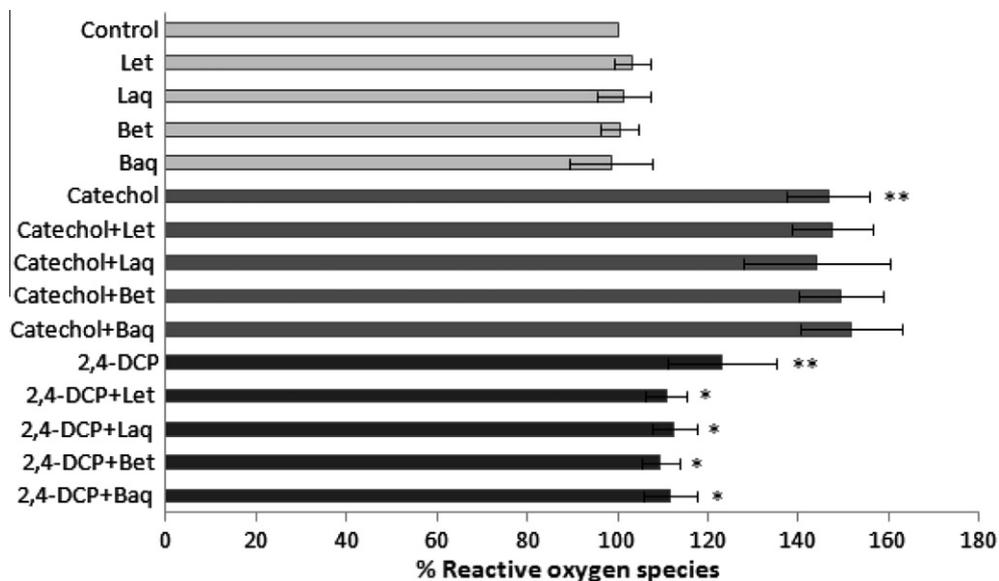
The erythrocytes incubated with *U. tomentosa* extracts and treated with catechol did not affect membrane lipid peroxidation (Fig. 2).

4.4. Discussion

Erythrocytes are used in studies of toxicity of numerous xenobiotics, e.g. anticancer drugs (Marczak and Jóźwiak, 2008), medicines used in osteoporosis treatment (Kopka et al., 2006) as well as pesticide preparations (Pieniążek et al., 2004; Szatkowska et al., 2011) and cyanotoxins (Sicińska et al., 2006).

The result obtained in this study showed that *U. tomentosa* extracts at the applied concentrations did not induce erythrocyte hemolysis (Table 2) and lipid peroxidation (Fig. 2). No enhanced formation of reactive oxygen species was found using flow cytometry (Fig. 1). No interference in hemoglobin structure and function was observed nor its oxidation or denaturation was noticed, when it was incubated with the extracts studied (Tables 2 and 3).

Therefore, the protective effect of the extracts against the exposure to the action of selected xenobiotics was examined. Two environmental transformation products of a herbicide 2,4-dichlorophenoxyacetic acid were selected, i.e. 2,4-dichlorophenol

Table 3
The effect of *U. tomentosa* (250 μg/ml) on hemoglobin denaturation (parameter *T*) in human erythrocytes incubated with by 2,4-DCP (250 μg/ml) and catechol (100 μg/ml) for 1, 5 and 24 h of incubation. The results are presented in the form of mean values ± SD (n = 15).

Index	1 h	5 h	24 h
Control	2.22 ± 0.07	2.22 ± 0.07	2.17 ± 0.05
Let	2.28 ± 0.09	2.23 ± 0.09	2.17 ± 0.05
Laq	2,24 ± 0,08	2.21 ± 0.10	2.16 ± 0.07
Ket	2.27 ± 0.10	2.25 ± 0.11	2.17 ± 0.10
Kaq	2.26 ± 0.08	2.21 ± 0.07	2.15 ± 0.05
2,4-DCP	2.11 ± 0.14[**] $F_{1,28}$ = 8.68; $P < 0.01$	2.10 ± 0.20[**] $F_{1,28}$ = 5.27; $P < 0.05$	2.05 ± 0.09[**] $F_{1,28}$ = 21.89; $P < 0.001$
2,4-DCP + Let	2.11 ± 0.21	2.11 ± 0.15	2.11 ± 0.09
2,4-DCP + Laq	2.14 ± 0.23	2.12 ± 0.18	2.10 ± 0.20
2,4-DCP + Bet	2.12 ± 0.23	2.13 ± 0.16	2.09 ± 0.04
2,4-DCP + Baq	2.13 ± 0.17	2.11 ± 0.16	2.09 ± 0.07
Catechol	2.09 ± 0.10[**] $F_{1,28}$ = 20.02; $P < 0.001$	1.98 ± 0.16[**] $F_{1,28}$ = 27.13; $P < 0.001$	1.83 ± 0.21[**] $F_{1,28}$ = 37.59; $P < 0.001$
Catechol + Let	2.09 ± 0.09	2.02 ± 0.19	1.79 ± 0.10
Catechol + Laq	2.10 ± 0.22	2.04 ± 0.13	1.77 ± 0.13
Catechol + Bet	2.10 ± 0.12	2.04 ± 0.15	1.85 ± 0.09
Catechol + Baq	2.10 ± 0.20	2.02 ± 0.20	1.84 ± 0.09

[**] $p < 0.001$ for samples (erythrocytes incubated with xenobiotic, extract or xenobiotic and extract) compared to the control (erythrocytes suspended in incubation buffer).

Fig. 1. The effect of *U. tomentosa* (250 μg/ml) on oxidation of 2′,7′-dichlorodihydrofluoresceine diacetate in human erythrocytes incubated with 2,4-DCP (250 μg/ml) and catechol (100 μg/ml) for 1 h. The results were calculated in relation to a control, which constituted 100%. The results are presented in the form of mean values ± SD. (n = 10) **$p < 0.001$ for samples (erythrocytes incubated with extract, xenobiotic or xenobiotic and extract) compared to the control (erythrocytes suspended in incubation buffer); *$p < 0.001$ for samples compared to 2,4-DCP.
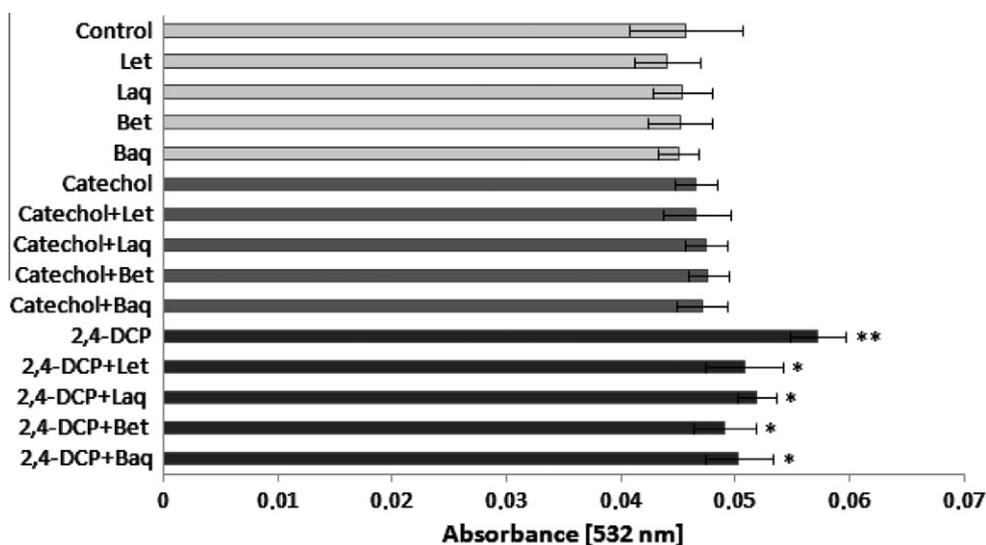
Fig. 2. The effect of *U. tomentosa* extracts (250 μg/ml) on lipid peroxidation in human erythrocytes incubated with 2,4-DCP (250 μg/ml) and catechol (100 μg/ml) for 1 h. The results are presented in the form of mean values ± SD. (n = 18) **$p < 0.001$ for samples (erythrocytes incubated with extract, xenobiotic or xenobiotic and extract) compared to the control (erythrocytes suspended in incubation buffer); *$p < 0.001$ for samples compared to 2,4-DCP.

and catechol to examine the protective properties of the extracts studied.

These xenobiotics are characterized by a different action mechanism which enables to determine what kind of damage in human erythrocytes may be decreased by *U. tomentosa* extracts.

Bukowska et al. (2007b) found an increase in ROS level in human erythrocytes treated with 2,4-DCP, and Han et al. (1998) showed that 2,4-DCP induced formation of the hydroxyl radical in ozonated water. It was also proven that 2,4-DCP caused a range of oxidative damage both to proteins and lipids. Such damage includes a change in the activity of antioxidant enzymes in human erythrocytes such as catalase and superoxide dismutase (Bukowska et al., 2000; Bukowska, 2003) as well as a decrease in reduced glutathione concentration (Bukowska, 2003) or an in-

crease in lipid peroxidation level (Duchnowicz et al., 2002). Those changes also contribute to slight hemoglobin oxidation (Duchnowicz et al., 2002), changes in oxygen affinity of hemoglobin (Bukowska et al., 1998) or changes in the parameter *T*, which determines the extent of Hb denaturation (Bukowska et al., 2007b).

Catechol oxidation leads to a formation both semiquinones and reactive oxygen species (Emdadul Haque et al., 2003; Jarabak et al., 1998), which are capable of damaging of proteins and DNA (Segura-Aguilar et al., 1997). It was also found that oxidation of –SH residues of hemoglobin leads to denaturation of this protein and to a significant depletion in the parameter *T* (Bukowska et al., 2008). On the other hand, it was noted that catechol, did not induce lipid peroxidation in human erythrocytes (Bukowska and Kowalska,
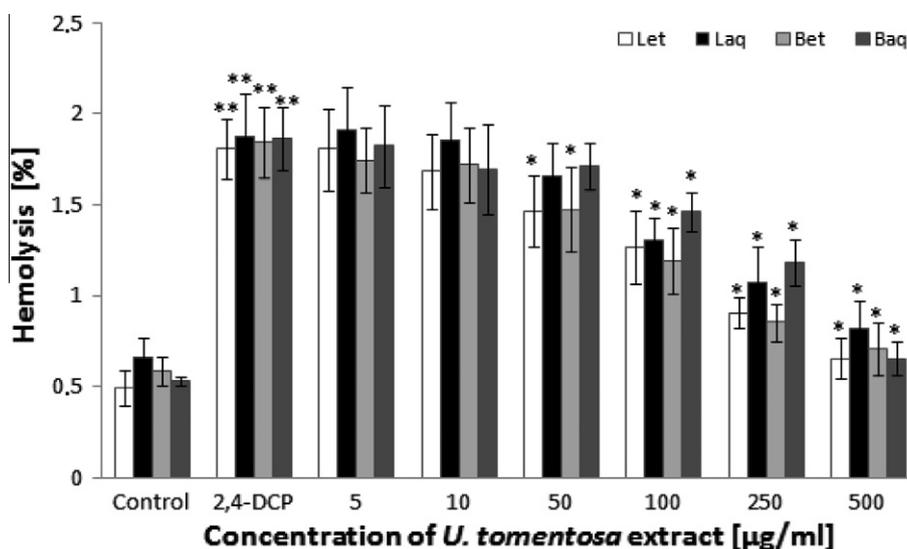
Fig. 3. The effect of *U. tomentosa* extracts (5–500 µg/ml) on hemolysis in human erythrocytes induced by 2,4-DCP (250 µg/ml) for 1 h of incubation. The results are presented in the form of mean values ± SD. ($n = 9$) **$p < 0.001$ for samples (erythrocytes incubated with 2,4-DCP or extract and 2,4-DCP) compared to the control (erythrocytes suspended in incubation buffer); *$p < 0.001$ for samples compared to 2,4-DCP.
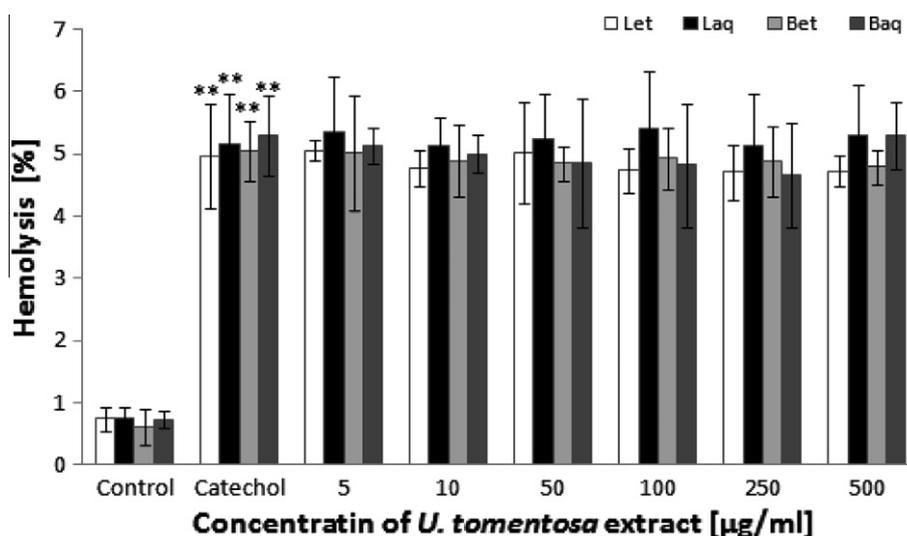
Fig. 4. The effect of *U. tomentosa* extracts (5–500 µg/ml) on hemolysis in human erythrocytes induced by catechol (100 µg/ml) for 1 h of incubation. The results are presented in the form of mean values ± SD. ($n = 12$) **$p < 0.001$ for samples samples (erythrocytes incubated with catechol or extract and catechol) compared to the control (erythrocytes suspended in incubation buffer).

2004). Stenius et al. (1989) also observed that catechol did not induce *in vivo* lipid peroxidation in rats.

In order to determine the antioxidant properties of *U. tomentosa* extracts in human erythrocytes, the fluorescent probe DCFH2-DA was used, which shows changes in reactive oxygen species (ROS) formation in cells. It was found that *U. tomentosa* extracts at the concentration of 250 µg/ml did not increase DCF fluorescence but they decrease the rise of DCF fluorescence induced by 2,4-DCP (Fig 5). The decrease in the DCF fluorescence shows that *U. tomentosa* extracts inhibited ROS formation in human erythrocytes.

On the other hand, no lowering of catechol-induced DCF fluorescence was observed in human erythrocytes incubated with *U. tomentosa* extracts (Fig. 1). This shows that the analyzed extracts were incapable of protecting red blood cells against toxic action of semiquinone radicals formed from catechol.

Literature data confirmed that aqueous and methanol extracts from various parts of *U. tomentosa* exhibited strong antioxidant properties, effectively deactivating the stable, synthetic DPPH radical (1,1-diphenyl-2-picrylohydrazyl) (Sroka et al., 2001; Sandoval et al., 2000). *U. tomentosa* extracts also prevented free radical formation (Yokoyama et al., 2004) and lipid peroxidation under *in vitro* conditions. Additionally, they inhibited free radical damage to sugar components in DNA and death of cells induced by free radicals (Desmarchelier et al., 1997; Sandoval et al., 2002).

It was also found that *U. tomentosa* extracts protected the erythrocytes against hemolysis induced by 2,4-DCP (Fig. 3), which may indicate that these extracts are effective in protection of red blood cells against ROS generated by this compound (Han et al., 1998; Bukowska et al., 2008). ROS, and in particular the hydroxyl radical, are the main factors inducing a range of oxidative processes leading to hemolysis (Bartosz, 2003). Similarly, Sekiya et al., 2002
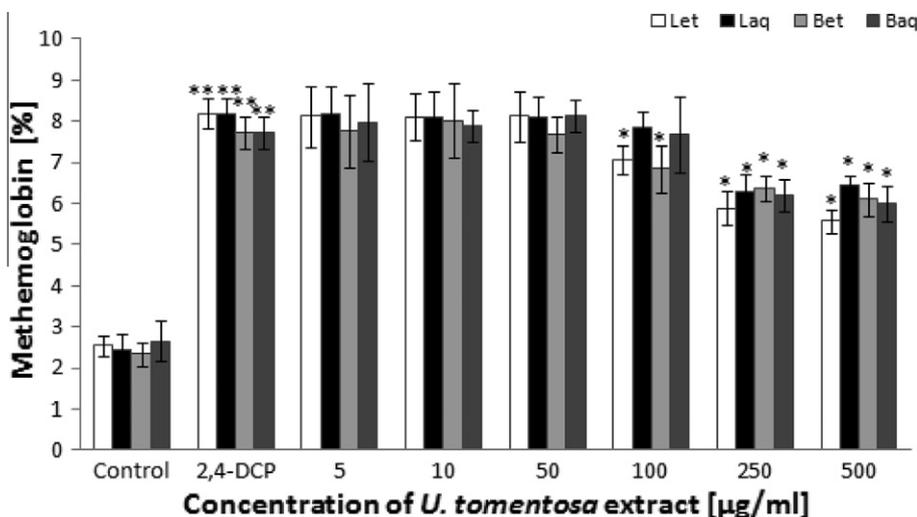
Fig. 5. The effect of *U. tomentosa* extracts (5–500 μg/ml) on hemoglobin oxidation in human erythrocytes induced by 2,4-DCP (250 μg/ml) for 1 h of incubation. The results are presented in the form of mean values ± SD. (n = 9) $^{**}p < 0.001$ for samples (erythrocytes incubated with 2,4-DCP or extract and 2,4-DCP) compared to the control (erythrocytes suspended in incubation buffer); $^{*}p < 0.001$ for samples compared to 2,4-DCP.
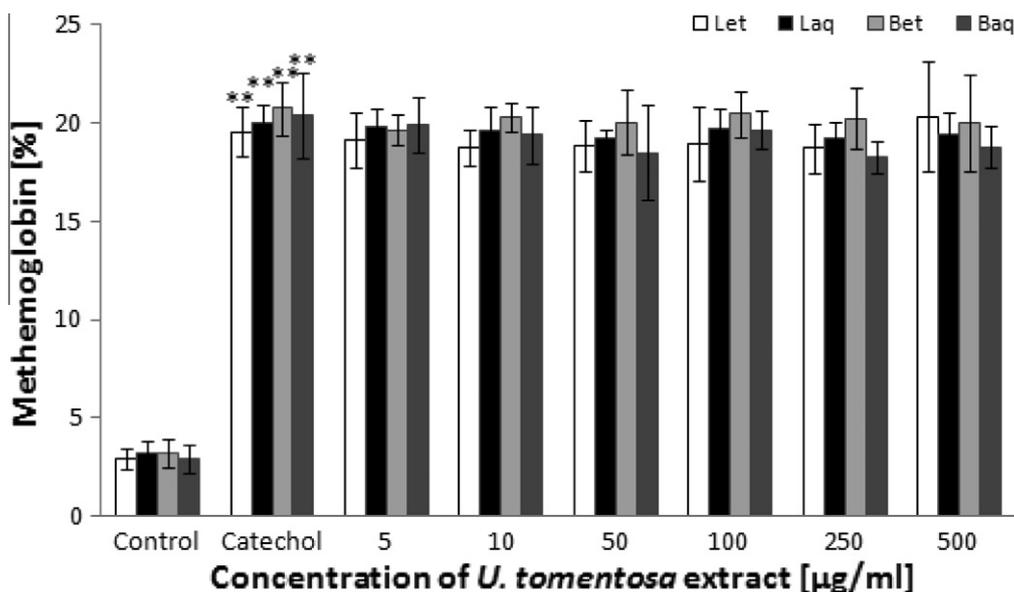
Fig. 6. The effect of *U. tomentosa* extracts (5–500 μg/ml) on hemoglobin oxidation in human erythrocytes induced by catechol (100 μg/ml) for 1 h of incubation. The results are presented in the form of mean values ± SD. (n = 9) $^{**}p < 0.001$ for samples (erythrocytes incubated with catechol or extract and catechol) compared to the control (erythrocytes suspended in incubation buffer).

showed *in vitro* and *in vivo* experiments conducted on rats that *Uncaria sinensis* extracts inhibited erythrocyte hemolysis induced by radicals generated as a result of 2,2′-azobis(2-amidinopropan) (AAPH) decomposition in a dose-dependent manner (50–1000 μg/ml). Our previous studies showed that *U. tomentosa* extracts protected against the induction of hemolysis, hemoglobin oxidation and ROS increase in human erythrocytes incubated with 2,4-dichlorophenoxyacetic acid (2,4-D) (Bors et al., 2009).

However, no protective role of the extracts was found as regards hemolysis induction by catechol (Fig. 4), which contributes to formation of semiquinones in cells (Emdadul Haqua et al., 2003; Molina Portela et al., 1996).

All *U. tomentosa* extracts, did not revealed hemolytic properties within the analyzed range of concentration after 1 h incubation with human erythrocytes, moreover, the extracts caused a statistically significant decrease in hemolysis (Table 2).

U. tomentosa extracts cause the sealing of the erythrocyte membrane and inhibit the outflow of hemoglobin. It is likely that phenolic compounds contained in *U. tomentosa* extracts coated erythrocyte membrane, thus sealing it and, as a result, inhibiting the outflow of hemoglobin. Suwalsky et al. (2006, 2008) showed that extracts from medicinal plants rich in polyphenolic compounds contributed to echinocytes formation, which was related to allocation them into the outer surface of human erythrocyte membrane. The possibility of polyphenolic compounds becoming embedded was also confirmed in research by Terao et al., 1994 and Arora et al., 2000. The inhibition of hemoglobin outflow was observed as a result of erythrocyte incubation with mannitol (Krokosz et al., 2008). It was found that mannitol decreased the above process, probably by electrostatic interaction with the membrane surface, and thus the sealing of membrane pores. It is also possible that the

compounds contained in extracts of *U. tomentosa* exhibited similar activity.

Overproduction of ROS may destroy the cell membrane directly by means of peroxidation of polyunsaturated fatty acids of membrane lipids. The prevention of the peroxidation processes by using antioxidants and free radical sweepers of plant origin becomes an important issue of clinical nature. Such attempts of protection against lipid peroxidation and other damage to erythrocyte membranes under the influence of quercetin were made, with positive results, *in vitro* studies involving erythrocytes of patients suffering from hypercholesterolemia type II (Broncel et al., 2007). Phytochemical studies are aimed at finding agents with antioxidant properties capable of reducing the degree of cell and tissue damage occurred as a result of oxidation processes. The results obtained in this study showed that lipid peroxidation caused by 2,4-DCP may be reduced from 9% to 14% by *U. tomentosa* extract at a concentration of 250 μg/ml (Fig. 2). The results quoted above are consistent with literature data confirming the fact that aqueous and ethanol *U. tomentosa* extracts prevent lipid peroxidation under *in vitro* conditions in different ranges of concentrations (IC_{50} = 10–259 μg/ml) (Desmarchelier et al., 1997; Sandoval et al., 2002; Gonçalves et al., 2005).

Iron oxidation and the formation of methemoglobin (met-Hb) is an indicator of the toxic action of xenobiotics on erythrocytes which may lead to premature removal of erythrocytes from circulation (Bartosz, 2003). Therefore, the effect of the extracts alone on met-Hb level and also their protective potential against the exposure of the erythrocytes to 2,4-DCP and catechol was examined. The results of this research showed that the extracts even at a very high concentration (500 μg/ml) did not induce hemoglobin oxidation after 1-h incubation (Table 2). Moreover, the protective role of *U. tomentosa* extracts against the formation of met-Hb in the erythrocytes treated with 2,4-DCP was observed (Fig. 5). On the other hand, the extracts did not reduce the formation of met-Hb in red blood cells exposed to catechol (Fig. 6), which may imply that *U. tomentosa* extracts do not show a protective activity against the toxic action of semiquinone radicals formed from catechol.

The parameter *T* is very significant because it determines hemoglobin denaturation. Change in the parameter *T* shows a very toxic effect of xenobiotic on hemoglobin, and significant decrease of its value may even indicate on heme release. Free heme acts as an inducer of numerous oxidation reactions in the cell (Bartosz, 2003). The parameter *T* was examined for three incubation periods (1, 5 and 24 h) to observe kinetics change. The results of our research showed that *U. tomentosa* extracts at a concentration of 250 μg/ml did not change the parameter *T* (Table 3). Thus, it may be concluded that the extracts did not cause the formation of hemichromes and globin denaturation in hemoglobin. The increase in the parameter *T* value observed after the erythrocytes treatment with 2,4-DCP and catechol was not decreased even after 24 h of incubation of the samples both with aqueous and ethanol extracts of *U. tomentosa* (Table 3). Thus, it may be concluded that the extracts studied were not capable of providing effective protection against significant changes in the protein structure of Hb, which were provoked by xenobiotics examined.

The different chemical composition of *U. tomentosa* substances (oxindole alkaloids, triterpenes, sterols, procyanidins, glycosides, quinolinic acid derivatives) may determine various pharmacological properties of the extract studied. In this study, the analysis of *U. tomentosa* extracts by means of HPLC revealed the presence of phenolic compounds responsible for the antioxidant properties of this plant. The results of our research indicate that procyanidin B2, (−)-epicatechin, procyanidin C1 and chlorogenic acid are dominant compounds in bark extracts, whereas chlorogenic acid and, secondly, caempferol derivatives and quercetin derivatives dominated in leaves extracts (Table 1).

Research on *U. tomentosa* extracts conducted by other authors also showed the presence of polyphenolic compounds of chemical structure similar to that detected in our study (Sroka et al., 2001; Sandoval et al., 2002; Gonçalves et al., 2005). The research by Gonçalves et al., (2005) corroborates the important role of proanthocyanidins detected in *U. tomentosa* extracts.

Proanthocyanidin polymers may be largely responsible for the antioxidant properties of the analyzed bark and leaf extracts, because they constitute the largest percentage of the detected phenolic compounds. Moreover, chlorogenic acid dominating in ethanol leaf extracts may also play an important antioxidant role (Table 1), because its strong antioxidant properties were corroborated by numerous studies including Sato et al., 2011. Our research has shown that ethanol extracts are characterized by a higher content of polyphenolic compounds than aqueous extracts. These results are consistent with the results obtained by Pilarski et al., 2006. They suggested that extraction with water does not allow obtaining solution, which would reveal optimal antioxidative properties. It may be supposed that the extracts are capable of counteracting further adverse effects connected with outflow of hemoglobin out of the cell due to location of phenolic compounds into erythrocyte membrane. Such adverse processes include the formation of Hb dimers or Hb cross links, phagocytosis induction or increasing the risk of bacterial infections (Everse and Hsia, 1997).

Moreover, it is probable that the compounds present in the extracts after coating of the erythrocytes can change their shape to such an extent that it will lead to premature removal of red blood cells from circulation. Nevertheless, the results of research on rats as well as on healthy volunteers who were administered *U. tomentosa* did not reveal any changes in red blood cells membrane or other changes in the erythrocytes, which rather excludes the possibility of premature removal of these cells as a result of the action of *U. tomentosa* extracts (Sekiya et al., 2002; Sheng et al., 2001).

Research by Koren et al., 2010 implies a beneficial influence of polyphenols on the total antioxidant ability of the blood, and especially of the erythrocytes, which confirmed the results obtained in the present study. They postulated that circulating erythrocytes and possibly also other blood cells might be constantly coated by polyphenols from supplemented nutrients, which act as antioxidant depots, and can thus act as protectors against the harmful consequences of oxidative stress. Further studies are needed to determine the faith of polyphenols in the circulation and their sequestration in the spleen.

It was found in the present study that *U. tomentosa* extracts change the properties of erythrocyte cell membrane making this cell more resistant to hemolysis and significantly protect eukaryotic cells against oxidation processes induced by 2,4-DCP. However, no protective role against the action of semiquinone radicals and hemoglobin oxidation was found.

No statistically significant differences in respect to protective effect between bark and leaves extracts towards 2,4-DCP action have been noted, which may be connected with the activity of some phenolic substances, e.g. flavonols, polymeric proanthocyanidins and chlorogenic acid that were present in both kind of *U. tomentosa* extracts.

Statistically significant differences have been noted between the activities of ethanolic and aqueous extracts. It was noticed that ethanolic extracts revealed stronger protective effect in comparison to aqueous extracts, which may be due to much higher content of polyphenols found in this biological matrix.

The application of *U. tomentosa* extracts as diet supplementation might lower the side-effect of xenobiotics toxicity. It must be remembered that although *in vitro* research is useful in the examination of xenobiotics toxicity, it does not take into account the effect of these compounds on whole organism because toxic

influence of the individual compound is not usually limited to one kind of cell such as erythrocyte.

In summary, it may be concluded that the extracts in a wide range of concentrations from 5 to 500 µg/ml did not cause adverse changes in human erythrocytes, however, they exhibited a protective effect against ROS formed due to 2,4-DCP action. Moreover, in this study, no protective effect of the extracts studied was observed against organic radicals, such as semiquinones, which are usually formed as a result of catechol interaction with red blood cells.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgment

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