

Antioxidant Activity of Anthocyanin Glycoside Derivatives Evaluated by the Inhibition of Liposome Oxidation

Janina Gabrielska^{a,*} and Jan Oszmiański^b

^a Department of Physics and Biophysics, Agricultural University, Norwida 25, 50-375 Wrocław, Poland. Fax: (+48) 71-3205172. E-mail: jaga@ozi.ar.wroc.pl

^b Department of Fruit and Vegetable Technology, Agricultural University, Norwida 25, 50-375 Wrocław, Poland

* Author for correspondence and reprint requests

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Cyanidin-3-glycosides (arabinoside, rutinoside, galactoside and glucoside) and delphinidin-3-rutinoside were examined for their ability to inhibit lipid peroxidation induced either by Fe(II) ions, UV irradiation or 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) peroxyl radicals in a liposomal membrane system. The antioxidant abilities of anthocyanins were compared with a water-soluble tocopherol derivative, trolox. The antioxidant efficacies of these compounds were evaluated by their ability to inhibit the fluorescence intensity decay of the extrinsic probe 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl] phenylpropionic acid, caused by the free radicals generated during peroxidation. All the anthocyanins tested (at concentrations of 15–20 μ M) exhibited higher antioxidant activities against Fe(II)-induced peroxidation than UV- and AAPH-induced peroxidation, suggesting that metal chelation may play an important role in determining the antioxidant potency of these compounds. It was also found that delphinidin-3-rutinoside had a higher antioxidant activity against Fe(II)-induced liposome oxidation than cyanidin-3-rutinoside, which indicates an important role of the OH group in the B ring of delphinidin-3-rutinoside in its antioxidant action. The antioxidant activity of all the anthocyanins studied was higher than that of trolox in the case of Fe(II)-induced liposome oxidation and was comparable with the action of trolox in the case of UV- and AAPH-induced liposome membrane oxidation.

Key words: Anthocyanin, Liposome, Antilipoperoxidative Agents

Introduction

Colours in plants essentially consist of only three major pigments, chlorophylls, carotenoids and flavonoids. Anthocyanins as coloured flavonoids are prominent in flower petals or fruit peels. Their intense colours, such as red, violet or blue, have been widely used as colour agents for food additives or dyeing. Recently, many data suggest that anthocyanins are not only static colouring agents but also exhibit an active biochemical function in plant cells. There are few reports of the antioxidant activity of anthocyanins as another possible function in cells (Lapidot *et al.*, 1999). The reported beneficial effects of anthocyanins are associated with the inhibition of carcinogenesis (Formica and Regelson, 1995; Bomsey *et al.*, 1996), atherosclerosis (Ghiselli *et al.*, 1998), and inflammation (Moroney *et al.*, 1988), which are connected with their antioxidant function. Synthetic antioxidants such as butylated hydroxytoluene (BHT) have been used as such since the beginning of this century. Restrictions on the use of these

compounds, however, are being imposed because of their toxicity (Faure *et al.*, 1990; Wu *et al.*, 1994). Thus, the interest in natural antioxidants has increased considerably. Several researchers have investigated the antioxidant activity of flavonoids, including anthocyanins, and have attempted to define the structural characteristics of the compounds that contribute to this activity. It has been shown, among others, that the antioxidant activity of those natural compounds is connected with the number and conformation of *o*-dihydroxy groups in the B ring, with the presence of the C2,C3 double bond in conjunction with 4-oxo in ring C and 3- and 5-hydroxy groups, and the 4-oxo function in the A and C rings. There are also scarce reports on the role of glycoside groups in the activity of flavonoids (Tsuda *et al.*, 1994, 1996; Rice-Evans *et al.*, 1997; Terao and Piskula, 1998).

The earlier studies we conducted indicated a high antioxidant activity of anthocyanin extracts from chokeberry, honeysuckle and sloe (Gabrielska *et al.*, 1999). These extracts also exhibit a great

antiradical activity with respect to 1,1-diphenyl-2-picrylhydrazyl. The studies confirm the beneficial effects of fruit consumption, as well as preparations from fruits that may effectively supplement the nonenzymatic system of protection against oxidative stress.

The aim of the present investigation was to determine the antioxidant activity of five anthocyanin glycosides (Fig. 1) in the process of nonenzymatic oxidation of phosphatidylcholine liposome membranes and to compare this activity with that of a water-soluble tocopherol derivative, trolox. The objective was also to investigate the role of the additional OH group in ring B of delphinidin-3-rutinoside and to compare its antioxidant effect on a liposome membrane with cyanidin-3-rutinoside.

Materials and Methods

Materials

Egg yolk phosphatidylcholine (PC) was obtained from Avanti Polar Lipids (Alabaster, AL). The lipid stock solution in chloroform was maintained at -20°C in glass vials that were layered with nitrogen. The fluorescence probe 3- $[p$ -(6-phenyl)-1,3,5-hexatrienyl] phenylpropionic acid (DPH-PA) was obtained from Molecular Probes (Eugene, OR). 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) was from Wako Chemical Company (Richmond, VA). $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ of 99% purity and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) of 97% purity were obtained from Aldrich (Milwaukee, WI). 1,1-Diphenyl-2-picrylhydrazyl (DPPH $^{\bullet}$) was obtained from Sigma (St. Louis, MO). All other reagents were of the highest grade available. The DPH-PA stock solution was prepared in *N,N*-dimethylformamide (DMF). The FeCl_2 , AAPH and anthocyanin pigments were stock solutions prepared immediately before use in double-distilled water and maintained on ice. All other chemicals were of highest grades from Sigma (Deisenhofen). Light-induced lipid oxidation was carried out by the exposure of liposome suspension to UV radiation (3.0 mW/cm^2 at the sample). Light quanta (90%) were emitted at 253.7 nm.

Preparation of anthocyanin pigments

The anthocyanin pigments cyanidin-3-galactoside (C3-gal) and cyanidin-3-arabinoside (C3-ara) were purified from chokeberry (*Aronia melano-*

carpa Elliot) fruit (Oszmiański and Sapis, 1988), cyanidin-3-rutinoside (C3-rut) and cyanidin-3-glucoside (C3-glu) from bird cherry (*Prunus padus*) fruit (Kucharska and Oszmiański, 2002) and delphinidin-3-rutinoside (D3-rut) from black current fruit (*Ribes nigrum*) by column chromatography using Purolite AP400 resins (Purolite, UK), MN Polyamid SC6.G (Machery Nagel), Lichroprep (TM) RP-18 (Merck) and Toyopearl HW-40(S) (TOSOH, Japan) (Oszmiański *et al.*, 2004). The structures and purities were confirmed by ^1H NMR, MS, UV-VIS, GC and HPLC analysis before and after hydrolysis (Kucharska and Oszmiański, 2002). The aglycons and sugars were identified by comparisons with standards after being separated by GC and HPLC analysis. The purities of the anthocyanins were more than 98% each.

Liposome preparation

The lecithin was dried under vacuum onto the wall of a round-bottom flask. The resulting lipid film was hydrated in 50 mM of Tris [(hydroxymethyl)-aminomethane]-HCl buffer at pH 7.4 and shaken for 15 min. The suspension was then sonicated for time spans up to 10 min, using a 20 kHz sonicator with a titanium probe. During the sonication the samples were thermostated at $0-2^{\circ}\text{C}$. Sonication was carried out intermittently for 30 s, followed by a 30 s resting period. 4 min before the end of sonication a DPH-PA probe was added to the liposome suspension. The sonicated sample was then centrifuged for 5 min at $690 \times g$ to remove any particles (Gabrielska *et al.*, 1999).

Antioxidant evaluation of anthocyanins

The fluorescence intensity assay (described by Arora and Strasburg, 1997; Arora *et al.*, 1998) was used to evaluate the antioxidant efficacy of the anthocyanins. In the assay, the peroxidative degradation of the probe DPH-PA, indicated by a decrease in its fluorescence, is used to monitor the sensitivity of the membrane towards oxidative stress. A 40- μl aliquot of the liposome suspension (1 mg of lipid per 1 ml of the buffers and $0.6\text{ }\mu\text{M}$ of fluorescence probe) was diluted to 2 ml in a buffer containing 50 mM Tris-HCl (pH 7.4). Each sample of the liposome suspension was prepared shortly before measurements were made, and kept on ice during experiments. Before each fluorescence measurement the diluted phosphatidylcholine li-

posome suspension was thermally equilibrated in a cuvette holder to obtain stable fluorescence (about 3 min). The anthocyanins glycosides were then added in water to the stirred sample of the vesicle suspension. The concentration of the anthocyanin pigment studied varied in the range 2.5–20 μM . The final lipid concentration of each sample was 0.02 mg PC/ml. Following a 3 min incubation of the sample after the addition of an antioxidant to allow partitioning of the anthocyanin within the membrane, peroxidation was initiated either by the addition of 0.02 μM FeCl_2 or by UV radiation at room temperature or by 20 μM of AAPH at 37 °C. The control samples did not contain any peroxidation initiator or anthocyanin under study. The decay in fluorescence intensity was monitored over 25 min, with readings taken at 0, 1, and every 3 min thereafter. The fluorescence experiment was conducted using a spectrofluorometer with a built-in polarization attachment (SFM-25, Kontron Instruments, Zürich, Switzerland). The excitation (λ_{EX}) and emission (λ_{EM}) wavelengths were as follows: $\lambda_{\text{EX}} = 354 \text{ nm}$ and $\lambda_{\text{EM}} = 430 \text{ nm}$. Fluorescence intensities were corrected for the inner filter and dilution effects (Lakowicz, 1999). The decrease in relative fluorescence intensity (F/F_0 ; where F_0 is the fluorescence at time equal zero, and F the fluorescence after a time) with time was indicating the rate of peroxidation. The percentage of inhibition of the lipid oxidation was calculated using the equation:

$$\% \text{ inhibition} = \{[(F_{\text{REL}})_{\text{OA}} - (F_{\text{REL}})_0] / [(F_{\text{REL}})_C - (F_{\text{REL}})_0]\} \times 100\%, \quad (1)$$

where $(F_{\text{REL}})_{\text{OA}}$ is the relative fluorescence for the oxidized sample [initiated by Fe(II) or AAPH or UV] in the presence of anthocyanin at 25 min, $(F_{\text{REL}})_0$ is the relative fluorescence for the oxidized sample [initiated by Fe(II) or AAPH or UV] in the absence of anthocyanin at 25 min, and $(F_{\text{REL}})_C$ is the relative fluorescence for the control sample at 25 min.

Antiradical measurement

The free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH \cdot) is highly stable, and therefore commonly used in measurements as a model radical (Morazioni and Malandrino, 1988). The effect of anthocyanin glycosides on the reduction of free DPPH \cdot radicals was measured spectrophotometrically as described previously by Brand-Williams *et al.* (1995) with small modifications. In short, 2 ml of

280 μM DPPH \cdot solution in methanol was mixed with an appropriate amount of anthocyanin pigment or trolox (final concentration 5–25 μM) and immediately placed in a specol (A_{0A}). The loss in absorbance at 517 nm by DPPH \cdot was also measured after a 60 min incubation of the mixture in dark (A_A). As a control, the absorption of DPPH \cdot (without the addition of an antioxidant) was measured at time $t = 0$ ($A_{0,\text{DPPH}}$), and again after 60 min (A_{DPPH}). The degree of DPPH \cdot reduction in the sample after a 60 min incubation with an antioxidant (of fixed concentration) was determined using the formula:

$$\text{DPPH}\cdot \text{ reduction } [\%] = \{1 - [(A_{0A} - A_A) / (A_{0,\text{DPPH}} - A_{\text{DPPH}})]\} \times 100\%. \quad (2)$$

Results

The inhibitory effects of cyanidin-3-glycosides (C3-glycosides): cyanidin-3-arabinoside (C3-ara, **1a**), cyanidin-3-rutinoside (C3-rut, **1b**), cyanidin-3-galactoside (C3-gal, **1c**), cyanidin-3-glucoside (C3-glu, **1d**); delphinidin-3-rutinoside (D3-rut, **2**) and trolox (**3**) (see the chemical structures in Fig. 1), at a chosen 20 μM concentration, on the rate of Fe(II)-induced peroxidation are illustrated in Fig. 2A. The efficacies of these compounds as antioxidants were evaluated as the degree of inhibition of the fluorescence intensity of the probe DPH-PA. All the C3-glycosides examined in the

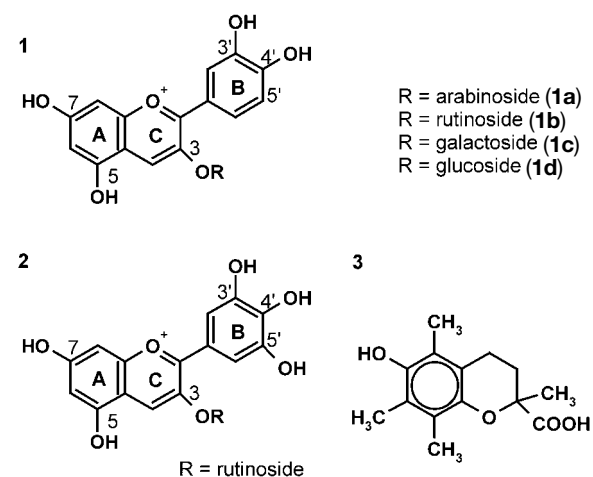


Fig. 1. The chemical structure of the compounds studied: cyanidin-3-glycosides: cyanidin-3-arabinoside (**1a**), cyanidin-3-rutinoside (**1b**), cyanidin-3-galactoside (**1c**), cyanidin-3-glucoside (**1d**); delphinidin-3-rutinoside (**2**); trolox (**3**).

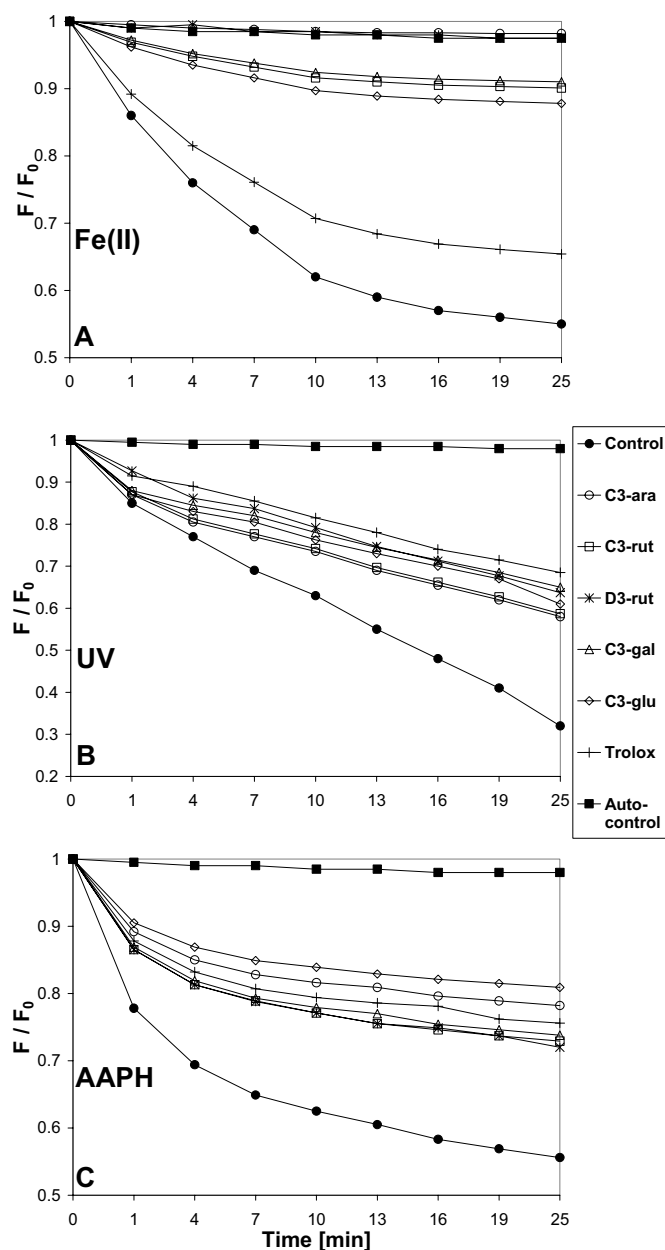


Fig. 2. Dependence of the relative fluorescence intensity (F/F_0) of the DPH-PA probe in the PC liposome membranes (denotes the rate of peroxidation of PC) from the time of incubation in the presence of oxidant agents: Fe(II) ions (A), UV radiation (B) and AAPH (C) and antioxidants (cyanidin-3-glycosides, delphinidin-3-rutinoside and trolox). Peroxidation was initiated by the addition of 20 nM of FeCl_2 or 10 mM of AAPH (at 37 °C) or by UV irradiation at intensity 3.5 mW/cm² of liposome suspension containing 20 μM lipid, 600 nM of DPH-PA probe and 20 μM test compounds suspended in 2 ml of 50 mM Tris-HCl buffer. Values represent the means of triple measurements.

study exhibited a drop in intensity to some degree. However, the rate of inhibition did not vary widely. The highest inhibition of the relative intensity of DPH-PA fluorescence was caused by compounds **1a** and **2**. The other anthocyanins, **1b**, **1c** and **1d**, caused the inhibition to a similar extent in liposome membranes oxidized by Fe(II) ions, with

only the inhibition of the DPH-PA probe caused by trolox being vividly lowest. Figs. 2B and 2C illustrate the inhibitory effects of the C3-glycosides, D3-rut and trolox against peroxidation in the liposome membrane, induced by UV radiation (Fig. 2B) and generated at a constant rate in the aqueous phase by thermal decomposition of the

azo-compound AAPH (Fig. 2C). All the compounds studied in the two cases and at the concentration $20\text{ }\mu\text{M}$, exhibited weaker antioxidant potencies against UV- and AAPH-induced peroxidation, compared with the inhibitory effect when oxidation was induced by Fe(II). The antioxidant trends observed earlier against Fe-ions-induced peroxidation were maintained here. However, there were only small differences between the antioxidant activities of the different C3-glycosides. It should be noted that the action of anthocyanin **2** does not markedly differ from the other C3-glycosides, both in the case of liposome oxidation by UV radiation and by the AAPH compound. A compilation of the inhibitory effects of the anthocyanins studied and of trolox, for comparison, for PC liposome membranes oxidized with Fe(II), UV light and AAPH is given in Figs. 3A, B and C. The percentage of oxidation inhibition shown in Fig. 3A, B and C was determined using formula (1) (see Materials and Methods) – based on data from Fig. 2 for the chosen $20\text{ }\mu\text{M}$ concentration of the anthocyanins and similar plots made for the following anthocyanins concentrations: 2.5, 5, 10 and $15\text{ }\mu\text{M}$. As stated earlier, the highest inhibitions amounting to 98, 78, 79, 73, 98 and 22% were found for compounds **1a**, **1b**, **1c**, **1d**, **2** and **3** (for the highest used concentration $20\text{ }\mu\text{M}$ and after 25 min of oxidation), respectively, in a membrane oxidized by Fe(II) (Fig. 3A). Significantly lower inhibitions, in the range 37–55% and about 35–45%, were exhibited by the anthocyanins studied in the case of membranes oxidized with UV light (Fig. 3B) and AAPH (Fig. 3C), respectively. From the plots, shown in Figs. 3A, B and C, concentrations were read that caused 20% inhibition of membrane peroxidation ($\text{IC}_{20}^{\text{PC}}$) for the membrane oxidized by Fe(II) ($\text{IC}_{20}^{\text{Fe}}$), UV light ($\text{IC}_{20}^{\text{UV}}$) and AAPH ($\text{IC}_{20}^{\text{AAPH}}$). It was possible to determine the $\text{IC}_{50}^{\text{PC}}$ parameter for the antioxidants studied only in the case when membrane oxidation was induced by Fe(II) ions ($\text{IC}_{50}^{\text{Fe}}$). Its values are 13.6, 16.0, 15.3, 15.6, 11.8 and $>> 20\text{ }\mu\text{M}$ for compounds **1a**, **1b**, **1c**, **1d**, **2** and **3**, respectively. The values of the parameters $\text{IC}_{20}^{\text{PC}}$ ($\text{IC}_{20}^{\text{Fe}}$, $\text{IC}_{20}^{\text{UV}}$ and $\text{IC}_{20}^{\text{AAPH}}$) and TEAC (trolox equivalent antioxidant capacity) are given in Table I. The parameter TEAC gives the number of micromoles of trolox needed to balance the antioxidant activity of an anthocyanin of $1\text{ }\mu\text{M}$ concentration.

It should be noted, when comparing $\text{IC}_{20}^{\text{PC}}$ for various factors inducing oxidation, that the lowest

values of these parameters refer to UV radiation (from 1.7 to 3.6), while in the remaining two cases they are several times higher (5.5–12.8). This indicates a large predominance of the protective properties of anthocyanins used at low concentrations (up to $5\text{ }\mu\text{M}$) with respect to membranes oxidized with UV radiation, when compared with their much weaker action towards membranes oxidized with Fe(II) ions and an AAPH compound. At the largest concentration ($20\text{ }\mu\text{M}$) a high antioxidant efficacy (reaching 100%) was shown by the anthocyanins only towards membranes oxidized with Fe(II) ions, since in the other cases it did not exceed 55%.

Comparison of the antioxidative action of anthocyanins with respect to a hydrophilic standard, trolox, is shown in Fig. 4. The graphic collection of the parameters TEAC exhibits a predominance of anthocyanins (especially D3-rut) over trolox with respect to antioxidative properties towards PC liposome membranes exposed to oxidative stress induced by Fe(II) ions. However, trolox is a more suitable (or comparable) antioxidant than the anthocyanins studied when protection of PC liposomes against peroxidation induced by UV light and also AAPH compound is concerned.

The percentage of degree of DPPH• quenching by the anthocyanins after an hour incubation with DPPH•, calculated according to formula (2) (see Materials and Methods) as a function of antioxidant concentration is presented in Fig. 3D. As follows from the plot, reduction of the DPPH free radical in the sample increased with the increase in concentration of all the antioxidants studied. The antiradical activity of anthocyanins (or antioxidative potency) expressed (from the curves in Fig. 3D) as concentrations required to eliminate half of the free DPPH• radicals present in the sample ($\text{RC}_{50}^{\text{DPPH}}$) are given in Table I. There is also the TEAA (trolox equivalent antiradical activity) parameter which gives the number of micromoles of trolox needed to balance the affinity for quenching the DPPH• radical by an anthocyanin of $1\text{ }\mu\text{M}$ concentration. The data in Table I indicate the following sequence of anthocyanins antiradical activity with respect to DPPH•: **2** > **1a** > **1d** \cong **1c** > **1b** > **3**. It is in fairly good accordance with the obtained antioxidative activity of the anthocyanins with respect to liposomes whose oxidation was induced by Fe(II) ions (**2** > **1a** > **1c** \cong **1b** \cong **1d** > **3**).

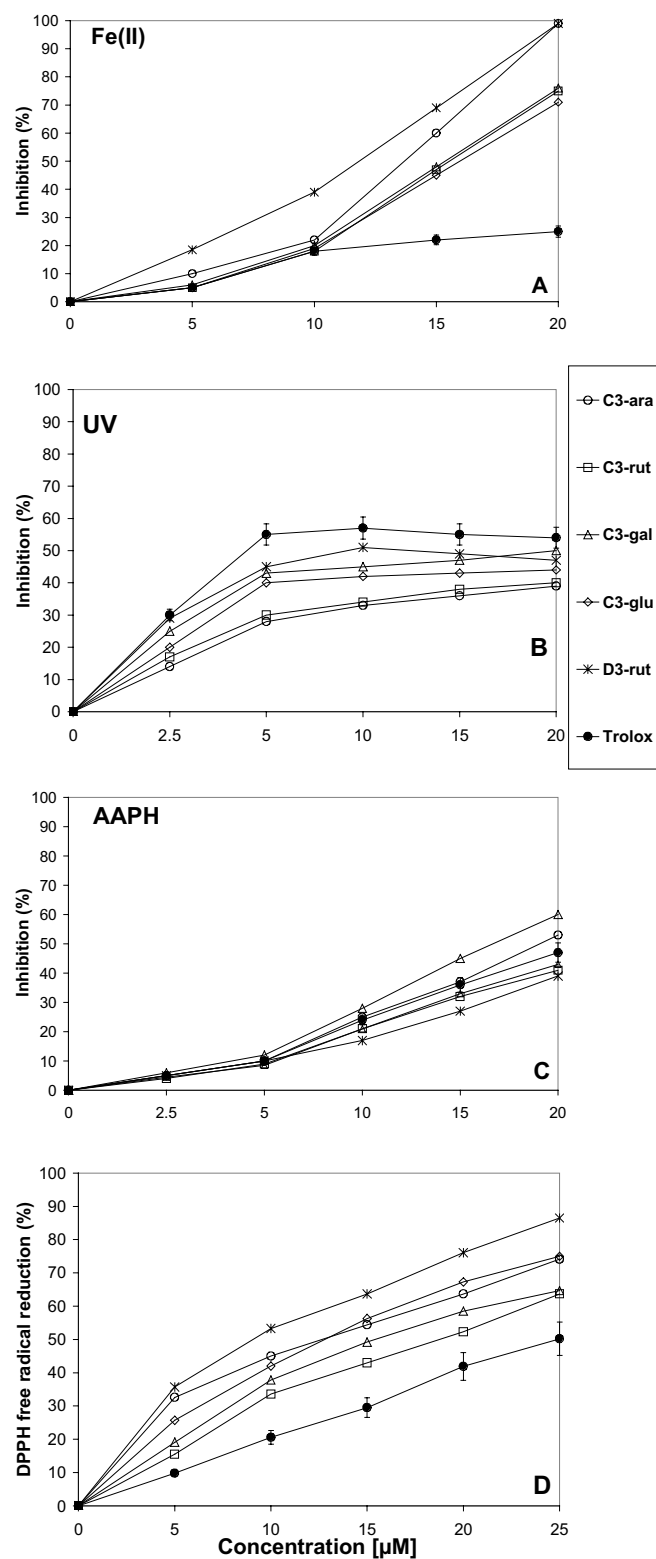


Fig. 3. Percent inhibition of PC oxidation induced by Fe(II) ions (A), UV light (B), AAPH (C) and disappearance of the DPPH[•] free radical (D) for studied cyanidin-3-glycosides, delphinidin-3-rutinoside and trolox. The results (A, B and C) were obtained by using equation 1 (see Materials and Methods) (on the basis of the example data for 20 μM concentration of antioxidants presented in Fig. 2). Values represent the means of triple measurements.

Table I. Compared antioxidant activity of studied compounds. The inhibition of PC liposome oxidation (fluorimetric method) and antiradical activity (DPPH• measurements) are presented. The inhibition potency was determined for the concentration (in micromol) at which phospholipid oxidation was reduced by 20% (IC_{20}^{Fe} , IC_{20}^{UV} , IC_{20}^{AAPH}), or at which the DPPH• radicals were eliminated from the liquid phase by 50% (RC_{50}^{DPPH}). The TEAC (trolox equivalent antioxidant capacity) and TEAA (trolox equivalent antiradical activity) parameters presented mean the number of micromoles of trolox needed to balance the antioxidant activity of an anthocyanin of 1 μM concentration, and to balance the antiradical activity of an anthocyanin of 1 μM concentration against DPPH• radical, respectively.

	IC_{20}^{Fe}	$TEAC^{Fe}$	IC_{20}^{UV}	$TEAC^{UV}$	IC_{20}^{AAPH}	$TEAC^{AAPH}$	RC_{50}^{DPPH}	$TEAA^{DPPH}$
C3-ara (1a)	9.0	1.42	3.6	0.47	8.3	1.02	12.8	1.84
C3-rut (1b)	10.5	1.22	3.0	0.57	9.5	0.90	18.0	1.31
C3-gal (1c)	10.0	1.28	2.0	0.85	9.5	0.90	15.3	1.54
C3-glu (1d)	10.3	1.24	2.5	0.68	7.5	1.13	12.6	1.91
D3-rut (2)	5.5	2.33	1.7	1.0	11.5	0.74	9.0	2.61
Trolox (3)	12.8		1.7		8.5		23.5	

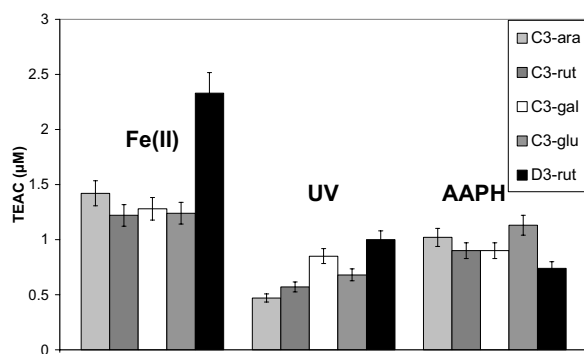


Fig. 4. Comparison of TEAC parameters for the compounds studied (cyanidin-3-glycosides, delphinidin-3-rutinoside) obtained for PC liposomes whose oxidation was induced by Fe(II) ions, UV light and AAPH. Standard deviation not exceeds 8%.

Discussion

In this study, the protective effects of a few anthocyanin glycosides (compounds **1** – see Fig. 1) against oxidation by Fe(II) ions, UV light and aqueous phase azo generators AAPH of lipid membrane peroxidation were assessed. The aim of our investigation was to determine the antioxidant activity of compounds **1** (C3-glycosides – arabinoside, rutinoside, galactoside, gluconoside) in the process of peroxidation and to determine the role of the type of glycoside group in that activity. The objective was also to investigate the role of an additional OH group in ring B of D3-rut compared with C3-rut in the antioxidant effect on the liposome membrane. The activity of the anthocyanins studied was compared with that of trolox. The TBA method is popular in determining lipid per-

oxidation levels, but this assay is not specific for MDA and is interfered with in the presence of anthocyanin pigments because the measured wavelength of the TBA reactive substance is similar to the absorption of the pigment. Therefore, we used the fluorimetric method to determine the oxidation level (Arora *et al.*, 1997, 1998). In general, all the anthocyanins examined at lower concentrations ($\leq 5 \mu M$) were more effective at inhibiting UV-induced peroxidation than AAPH- and Fe(II)-induced lipid oxidation. The high antioxidative activity of the anthocyanins at low concentrations, even decreasing, for example, in the case of D3-rut at higher concentration may be partly a consequence of the natural function of flavonoids consisting in UV absorption (Bonina *et al.*, 1996). However, the applied *in vitro* UV-C radiation at wavelength $\lambda = 253.7$ nm is to a certain degree destructive towards the molecules of anthocyanins. This was confirmed by the lowered absorption (maximum being anthocyanin absorption) after 30 min irradiation with UV light of the compounds at 20 μM concentration. The percent of degree of photodestruction was 19, 17.5, 12.7, 14.5, and 12% for compounds **1a**, **1b**, **1c**, **1d**, and **2**. A fairly good reversed relationship can be observed between the increase in destruction degree (**1a** > **1b** > **1d** > **1c** > **2**) and decrease in antioxidant activity of the compounds studied (**1a** < **1b** < **1d** < **1c** < **2**). It cannot be excluded that the products of anthocyanin photodegradation assume various forms, including, for example, free radicals. The degenerated molecules may bind to each other and also react with undamaged anthocyanin molecules, thus reducing their numbers. This results in a decrease in their antioxidative activity.

It can also be seen that all the anthocyanins examined at higher concentration (15–20 μM) were more effective at inhibiting Fe(II)-induced peroxidation than AAPH- and UV-induced lipid oxidation. These results induced the suggestion that metal chelation may play a larger role in determining the antioxidant potency of the compounds (van Acker *et al.*, 1998). As a result of the complex formation of anthocyanin molecules with transition metals such as Fe(II) ions, the ions' ability to catalyse the oxidation reaction is restricted. This is very probable due to the favourable position of the dihydroxyl group (C3, C4) in the B ring of C3-glycoside molecules. Some investigators maintain (Rice-Evans *et al.*, 1995; Moridani *et al.*, 2003; Souza *et al.*, 2003) that the *ortho* position of the groups is responsible, among others, for the formation of complexes of polyphenol compounds and metals. The results of our experiments indicate that in that complex formation the most active was C3-ara and the weakest was C3-rut. Such differentiation of antioxidant activity probably follows from the different roles of the sugar groups in the compounds that more or less restrict the activity and mobility of protons in the hydroxyl groups of molecules responsible for the antioxidant effect. A similar reduction in antioxidant activity was observed in the case of quercetin derivatives, of which rutin, being a quercetin rutinoside, shows activity lower than quercetin and its glucoside with one sugar molecule (Pratt and Hudson, 1990).

Studies also indicated another important role of the molecular hydroxyl groups in the process of Fe(II) ions chelating (Moridani *et al.*, 2003). It follows from comparing the antioxidative activity of compounds **1b** and **2** with respect to liposome oxidized with Fe(II) ions. The presence in an anthocyanin molecule **2** of additional OH groups in the

B ring, compared with two groups possessed in that ring by compound **1b**, enables anthocyanin **2** to bind better with Fe(II) ions (compared with the chelating properties of **1b**). The weak relative antioxidative activity of trolox (possessing one hydroxyl group) seems to additionally confirm the thesis about the importance of the number of the ligands in the process of Fe(II) ions binding.

In the case of UV- and AAPH-induced peroxidation, all the anthocyanins studied at a higher concentration were less effective at inhibiting a process than the metal-ion-induced peroxidation. Also, in two cases of oxidation induction, only weak differences in antioxidant activity of the compounds were noted. The mechanism of antioxidant action of the compounds in that case (also in Fe-induced oxidation) is, most probably, connected with free radical scavenging, because DPPH \cdot (a stable model of free radical) reduction (Fig. 3D) was increased with increasing concentrations of pigment and trolox present in the medium with DPPH \cdot . The increase of DPPH \cdot reduction is connected with the reaction of a hydrogen atom transfer from the hydroxyl group of the antioxidant to DPPH \cdot which thus assumes a non-radical state. The concentration of antioxidants that caused 50% reduction in DPPH \cdot ($\text{RC}_{50}^{\text{DPPH}}$ – Table I) is a measure of the antiradical activity of the compounds, which is also an antioxidant potential connected with the effective scavenging of radicals (Bors *et al.*, 1990). This probably indicates that the compounds studied may act as preventive or chain-breaching antioxidants towards lipid peroxidation by decreasing the effective concentration of radicals such as hydroxyl (induced by UV light), alkoxy and peroxy (induced by AAPH and Fe ions), which can initiate, reinitiate and promote peroxidation.

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