Research article

INTERACTION OF SELECTED ANTHOCYANINS WITH ERYTHROCYTES AND LIPOSOME MEMBRANES [#]

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Abstract: Anthocyanins are one of the main flavonoid groups. They are responsible for, e.g., the color of plants and have antioxidant features and a wide spectrum of medical activity. The subject of the study was the following compounds that belong to the anthocyanins and which can be found, e.g., in strawberries and chokeberries: callistephin chloride (pelargonidin-3-O-glucoside chloride) and ideain chloride (cyanidin-3-O-galactoside chloride). The aim of the study was to determine the compounds' antioxidant activity towards the erythrocyte membrane and changes incurred by the tested anthocyanins in the lipid phase of the erythrocyte membrane, in liposomes composed of erythrocyte lipids and in DPPC, DPPC/cholesterol and egg lecithin liposomes. In particular, we studied the effect of the two selected anthocyanins on red blood cell morphology, on packing order in the lipid hydrophilic phase, on fluidity of the hydrophobic phase, as well as on the temperature of phase transition in DPPC and DPPC/cholesterol liposomes. Fluorimetry with the Laurdan and Prodan probes indicated increased packing density in the hydrophilic phase of the

Abbreviations used: AAPH – 2,2′-azobis(2-methylpropionamidine) dihydrochloride; DPH – 1,6-diphenyl-1,3,5-hexatriene; DPH-PA – (1,6-diphenyl-1,3,5-hexatrienyl) propionic acid; DPPC – 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine; DSC – differential scanning calorimetry; egg-PC – L-α-phosphatidylcholine from egg yolk; GP – generalized polarization; Laurdan – 6-dodecanoyl-2-dimethylaminonaphthalene; Prodan – 6-propionyl-2-dimethylaminonaphthalene; TMA-DPH – 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate

membrane in the presence of anthocyanins. Using the fluorescence probes DPH and TMA-DPH, no effect was noted inside the hydrophobic phase of the membrane, as the lipid bilayer fluidity was not modified. The compounds slightly lowered the phase transition temperature of phosphatidylcholine liposomes. The study has shown that both anthocyanins are incorporated into the outer region of the erythrocyte membrane, affecting its shape and lipid packing order, which is reflected in the increasing number of echinocytes. The investigation proved that the compounds penetrate only the outer part of the external lipid layer of liposomes composed of erythrocyte lipids, DPPC, DPPC/cholesterol and egg lecithin lipids, changing its packing order. Fluorimetry studies with DPH-PA proved that the tested anthocyanins are very effective antioxidants. The antioxidant activity of the compounds was comparable with the activity of Trolox$^{®}$.

Key words: Anthocyanins, Fluorescent probes, Liposomes, Erythrocyte membrane, Echinocytes, DSC, Anisotropy, Generalized polarization, Phase transition

INTRODUCTION

Anthocyanins are polyphenolic compounds that endow plants with their original and unique color. Their ability to absorb UV-VIS radiation protects plants against photo-oxidation and photodegradation. The compounds also protect plants against fungi and pests, against freezing and overheating, properly controlling their internal osmotic pressure, and they are also able to chelate metals [1].

The interest in these natural antioxidants is nowadays very great, as they prevent many serious diseases, connected with oxidative stress caused, among others, by lipid peroxidation [2-7]. Anthocyanins exhibit many properties that are favorable to human health, such as immunostimulation, anticancer, antibacterial, and anti-inflammatory activity. They are used in preventive treatment of heart disease, digestive and circulatory system disease, diabetes, and also ophthalmology. It is thought that such beneficial and protective properties of anthocyanins with respect to human physiology are mainly connected with reduction of free radicals, including reactive forms of oxygen. That is why they are regarded as antioxidants, which are called free radical scavengers [8-13].

The antioxidant activity of some flavonoids, including anthocyanin, is markedly higher than the activity of vitamin E, and its synthetic equivalent Trolox$^{®}$. They also effectively inhibit erythrocyte hemolysis induced by free radicals, protecting the erythrocyte membrane against oxidative destruction [5, 10, 13-18].

An exposed and very good site of attack by free radicals in an organism is the cell membrane. Oxidation of its constituents, the membrane lipids in particular, by free radicals results in structural disturbances and impairs the function of biological membranes, resulting in pathological changes in the organism. In spite of many reports on the protective and healing properties of polyphenolic compounds, including anthocyanins, with respect to biological systems, their mechanism of interaction with biological membranes has not yet been explained.

Owing to the anthocyanins' chemical structure, the compounds should mainly be bound to the lipid phase of membrane. The degree of their adsorption seems to depend both on the strength of electrostatic interactions with the polar groups of phospholipids and hydrophobic interactions with their alkyl chains. Anthocyanins with numerous hydroxyl groups bind electrostatically with the membrane surface, whereas others that have fewer such groups can partly penetrate into the hydrophobic region, changing the membrane fluidity [19-21]. Anthocyanins when bound to a membrane can effectively protect it against oxidation, reducing free radicals, thanks to the hydroxyl group, both in the medium and the membrane interior.

In order to determine the effects of polyphenolic extracts on biological membranes, two compounds of the anthocyanin group were chosen, callistephin chloride (pelargonidin-3-O-glucoside chloride) and ideain chloride (cyanidin-3-O-galactoside chloride), which are present in many plants, and in particular are found in large quantities in fruits of strawberry and chokeberry. The effects of these fruits on the structure of biological and lipid membranes were described by the authors in a previous paper [22]. Fruit extracts constitute, however, a mixture of various polyphenols, and thus it is difficult to determine the effect and mechanism of their constituents on the biological membrane.

Therefore, studies (not performed before) were undertaken on the effect of selected anthocyanins on the structure of erythrocyte membranes, treated as a molecular model of biological membrane and on model lipid membranes; their antioxidant properties were also studied. Lipid membranes were composed of lipids extracted from erythrocyte membranes, synthetic DPPC and DPPC with cholesterol added, and also of egg lecithin.

In the investigations we employed microscopic, spectroscopic and calorimetric methods. The results of the interaction of the compounds with biological membrane were assessed on the basis of erythrocyte shape alteration, packing order of the polar heads of membrane lipids, membrane fluidity in the hydrophobic region and change in phase transition temperature of lipid membranes. The antioxidant activity of anthocyanins on membrane lipids was determined using the fluorimetric method.

The research techniques and molecular models of biological membranes selected and applied in the experiments allow one to determine the changes anthocyanins induce in the biological membrane, especially in its lipid phase, and assess their antioxidant activity with respect to membrane lipids.

MATERIALS AND METHODS

The anthocyanins callistephin chloride (pelargonidin-3-O-glucoside chloride) and ideain chloride (cyanidin-3-O-galactoside chloride) were purchased from Extrasynthese® of France.
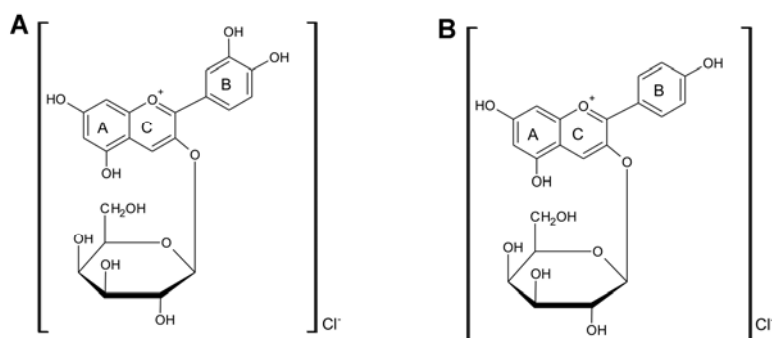
Fig. 1. Chemical structures of anthocyanins. A – Callistephin chloride (pelargonidin-3-O-glucoside chloride). B – Ideain chloride (cyanidin-3-O-galactoside chloride).

The fluorescent probes 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan), 6-propionyl-2-dimethylaminonaphthalene (Prodan), 1,6-diphenyl-1,3,5-hexatriene (DPH), 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluene-sulfonate (TMA-DPH), and (3-(p-(6-phenyl)-1.3.5-hexatrienyl) propionic acid (DPH-PA) were purchased from Molecular Probes, Eugene, Oregon, USA.
The lipids 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) and L-α-phosphatidylcholine from egg yolk (egg-PC) and cholesterol were purchased from Sigma Aldrich, Steinheim, Germany.
The studies were conducted on pig erythrocytes and isolated erythrocyte membranes, which were obtained from fresh blood using the Dodge *et al.* (1963) method [24]. The choice of pig erythrocytes was dictated by the fact that this cell's percentage share of lipids is closest to that of the human erythrocyte, and the blood was easily available. Fresh blood was taken each time to a physiological solution of sodium chloride with heparin added. The lipids were extracted from erythrocyte membranes according to the method described by Maddy *et al.* [23]. Erythrocyte ghosts were obtained according to Dodge *et al*. [24]. Membrane protein concentration was assayed using Bradford's method [25] and it was 1 mg/l.

Microscopic investigation
For investigation with the optical microscope, the red cells separated from plasma were washed four times in saline solution and suspended in the same solution but containing 0.01 and 0.1 mg/ml of the anthocyanins studied. Hematocrit of the erythrocytes in the modification solution was 2%, the modification lasting 1 h at 37°C. After modification the erythrocytes were fixed with a 0.2% solution of glutaraldehyde. After that the red cells were observed under a biological optical microscope (Nikon Eclipse E200) equipped with a digital camera. The photographs obtained made it possible to count erythrocytes of various shapes, and then the percent share of two basic forms (echinocytes and stomatocytes) in a population of ca. 800 cells was determined.

The individual forms of erythrocyte cells were ascribed morphological indices according to the Bessis scale [26], which for stomatocytes assume negative values from –1 to –4, and for echinocytes from 1 to 4.

For investigation with the electron microscope, the red cells separated from plasma were washed four times in saline solution and suspended in the same solution but containing 0.01 mg/ml of the anthocyanins studied. Hematocrit of the erythrocytes in the modification solution was 2%, the modification lasting 1 h at 37°C. After modification the erythrocytes were fixed for 12 h in a 2.5% solution of glutaraldehyde, buffered with PBS of pH 7.4 at 18°C. Then the material was fixed again for 1 h in a 1% solution of osmium tetroxide in the same buffer at 4°C. Mica slates covered with the red cells were dehydrated with solutions of alcohol and acetone of increasing concentrations. The preparations were then dried with a method based on the critical point of CO_2 in a Balzers instrument CP-010. Afterwards, mica fragments covered with cells were placed on metal plates and sprayed with carbon and silver in a sputter coater (VEB Hochvakuum Dresden B30.1). The preparations were viewed and photographed in a scanning electron microscope (Tesla BS 300) at 20 kV.

Fluorimetric method
The effect of polyphenols on the packing arrangements of lipids in the erythrocyte membrane (ghosts) and a model lipid membrane was investigated using the fluorimetric method. Fluorescence intensity was measured by using fluorescent probes: Laurdan, Prodan, DPH and TMA-DPH. These fluorescent probes were used because each of them is incorporated into different regions of the lipid bilayer. The TMA-DPH probe is incorporated at the fourth carbon atom in the transient region between hydrophobic and hydrophilic parts of the bilayer. The active part (fluorophore) of DPH and TMA-DPH probes is located in the hydrophobic and that of Laurdan and Prodan in hydrophilic regions of the bilayer, respectively. Such differentiated incorporation of the probes gives an insight into the structural changes caused by incorporation of callistephin and ideain chloride [27-29, 33-34].

The ghosts were suspended in an isotonic phosphate solution of pH 7.4, in a quantity such that the protein concentration in the samples was approximately 100 mg/ml. The control samples contained an erythrocyte ghost suspension and a fluorescent probe, while the investigated samples contained in addition appropriate concentrations of the compounds studied. Fluorescence intensity was measured by using four fluorescent probes, Laurdan, Prodan, DPH and TMA-DPH, whose concentration in the samples was 10 μM, while concentrations of the compounds were within the range 0.005-0.05 mg/ml at a temperature of 37°C. The measurements were conducted with a fluorimeter (CARRY Eclipse of VARIAN) equipped with a Peltier temperature controller DBS (temp. accuracy ± 0.1°C). The excitation and emission wavelengths were as follows: for probe DPH, λ_{ex} = 360 nm, λ_{em} = 425 nm; and for probe TMA-DPH, λ_{ex} = 358 nm,

λ_{em} = 428 nm. The excitation wavelength for Laurdan and Prodan was 360 nm, and the emitted fluorescence was recorded at two wavelengths, 440 and 490 nm. Unilamellar liposomes were composed of dipalmitoyl phosphatidylcholine (DPPC), DPPC/cholesterol and egg-PC. The lipids and cholesterol were dissolved in chloroform and a suitable probe was added and very carefully evaporated to dryness under nitrogen. Then phosphate buffer of pH 7.4 was added. The lipid film was dispersed by agitating the flask on a vortex mixer to give a milky suspension of liposomes, at a temperature above the main phase transition (for liposomes composed of DPPC and of DPPC/cholesterol). Small unilamellar liposomes (SUVs) with probes were formed by sonication of lecithin dispersion in a buffer for 15 min at 20 kHz. Control samples contained lipid suspension and a suitable fluorescence probe at 100:1 molar ratio, and the appropriate compound at a concentration 0.005-0.05 mg/ml was added to the remaining samples. Fluorescence intensity was measured with the Laurdan, Prodan, TMA-DPH and DPH probes. The measurements were made at different temperatures. For liposomes composed of one kind of lipid, the measurements were made above and below the main phase transition.

Small unilamellar liposomes were composed of lipids extracted from erythrocytes. The natural lipids were dissolved in a chloroform:methanol solvent and evaporated to dryness under nitrogen. Subsequently, a phosphate buffer of pH 7.4 was added to the obtained film and liposomes were formed by mechanical shaking. Then SUVs were formed using a sonicator in the presence of fluorescent probes. Control samples contained only lipid suspension with fluorescence probes at 1000:1 (lipids:fluorescent molar ratio), the appropriate compound at a concentration of 0.005-0.05 mg/ml being added to the remaining samples.

Fluorescence anisotropy (A) for probes DPH and TMA-DPH was calculated using the formula [27]:

$$A = \frac{(I_{II} - GI_{\perp})}{(I_{II} + 2GI_{\perp})} \qquad (1)$$

where I_{II} and $I_{\perp}$ are fluorescence intensities observed in directions parallel and perpendicular, respectively, to the polarization direction of the exciting wave. G is an apparatus constant dependent on the emission wavelength.

Changes in the polar group packing arrangement of the hydrophilic part of the membrane were investigated using the Laurdan or Prodan probe, on the basis of generalized polarization (GP), and were calculated with the formula [27, 29]:

$$GP = \frac{(I_b - I_r)}{(I_b + I_r)} \qquad (2)$$

where I_b is fluorescence intensity at λ = 440 nm, and I_r is fluorescence intensity at λ = 490 nm.

Fluorimetric method in antioxidative tests
The DPH-PA probe was used in the fluorimetric experiments. Erythrocyte ghosts, with and without (control) additions of the extracts, were suspended in a phosphate buffer of pH 7.4 and UVC irradiated or treated with the chemical oxidation inducer 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) for 30 min. Free radicals, released in the process of membrane lipid irradiation, cause quenching of the DPH-PA fluorescence, decreasing the fluorescence intensity. As a measure of the extent of lipid oxidation we used relative fluorescence, i.e. the ratio of UVC-oxidized probe fluorescence to the initial fluorescence of the probe. Here, as a control we used the relative fluorescence of an erythrocyte ghost suspension that contained the DPH-PA probe, oxidized with UVC or AAPH radical, while the blank was the relative fluorescence of a suspension of the same concentration but not oxidized.

A Cary Eclipse (Varian) spectrofluorimeter was used to measure free radical concentrations in the samples. Excitation and emission wavelengths were $\lambda_{ex} = 364$ nm and $\lambda_{em} = 430$ nm. The measure of lipid oxidation was the relative change of fluorescence intensity, F/F_0, where F_0 is the initial fluorescence and F the one measured during an oxidation procedure [35]. The percentage of lipid oxidation inhibition was calculated from the following formula:

$$inhibition \ \% \ = \frac{(F_x - F_u)}{(F_k - F_u)} \cdot 100 \ \%, \qquad\qquad (3)$$

where: F_X = relative fluorescence of a UVC irradiated sample, or oxidized by AAPH, for 30 min in the presence of the compounds, F_U = relative fluorescence of the control sample, oxidized by AAPH or UVC irradiated, without the compounds, measured after 30 min. F_K = relative fluorescence of the blank sample, not subjected to oxidation procedure, measured after 30 min. The results of the assay were expressed relative to Trolox®, in terms of TEAC (Trolox® Equivalent Antioxidant Capacity).

Calorimetric studies
In the calorimetric studies the effect of the anthocyanins on the pre-transition (T_p) and main transition (T_m) temperature of DPPC and the main transition temperature (T_m) of DPPC/cholesterol was analyzed. For that purpose differential scanning calorimetry (DSC) was used. The measurements were made with a calorimeter of Mettler Toledo Thermal Analysis System D.S.C. 821^e (scanning rate 2ºC/min). The samples contained multilamellar liposomes (MLV) composed of DPPC and DPPC/cholesterol in the presence of the anthocyanins. The lipids were dissolved in chloroform and then evaporated for approx. 2 h under nitrogen to dryness. The obtained film was admixed with anthocyanins dissolved in phosphate buffer of pH 7.4 and liposomes were formed by mechanical shaking at a temperature above the main phase transition. The lipid concentration in the samples was 25 mg/ml and the cholesterol concentration was 10 mol%. The prepared dispersion of pure lecithin (control

sample) and lecithin with the compounds added were encapsulated in 40 μl volumes and left for 24 h at 4ºC before measurement [35].

Statistical analysis was carried out using Statistica 9.0 (StatSoft Inc.). All the experiments were performed at least in triplicate unless otherwise specified. Analysis of variance was carried out and significance between means was determined using Dunnett's post-hoc test. Results are presented as mean ± SD. Significant levels were defined at $p < 0.05$.

RESULTS

Microscopic studies
Fig. 2 shows the erythrocyte shapes as observed in the scanning electron microscope. Both the studied anthocyanins mostly induced formation of various forms of echinocytes. Callistephin chloride was responsible for creation of the most varied forms of echinocytes (Fig. 2C).
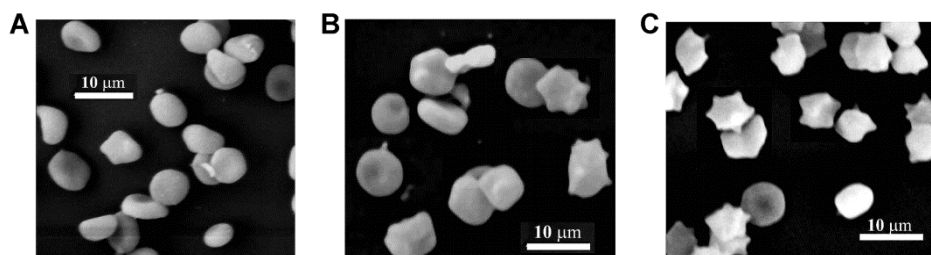

Fig. 2. Changes of erythrocyte shapes caused by the anthocyanins tested: A – control, B – ideain chloride, C – callistephin chloride. Scanning electron microscope images, for details see Materials and Methods.

Fig. 3 shows the percent share of the various forms of cells in a population of erythrocytes modified with callistephin and ideain chloride at 0.1 and 0.01 mg/ml concentration. As seen in the figures, both anthocyanins mostly induce various forms of echinocytes, whose percent share in a population depends on the type of compound. Proportions of the different populations of red cells, identified by an optical microscope using the Bessis morphological index, are presented in Fig. 3 for both anthocyanins at both concentrations.

Studies of Iglic *et al*. [29] and Isomaa *et al*. [30] have shown that formation of echinocytes occurred when amphiphilic molecules were incorporated into the outer monolayer of the erythrocyte membrane. Compounds penetrating to the inner monolayer of the membrane induced formation of stomatocytes. We can therefore assume that the tested compounds concentrated mainly in the outer monolayer of the erythrocyte membrane.

Fluorimetric measurements
The effect of callistephin and ideain chloride on fluidity of the lipid phase of erythrocyte and liposome membranes was studied on the basis of fluorescence anisotropy measured with the two fluorescence probes DPH and TMA-DPH. An

investigation was also conducted on lipid fluidity in the hydrophobic region of erythrocyte ghosts and liposome membrane composed of lipids extracted from erythrocyte membranes. The results of the DPH and TMA-DPH probe fluorescence anisotropy for such a membrane are presented in Table 1. These results indicate minor changes caused by the compounds in the hydrocarbon chains of membrane lipids, testifying that the anthocyanins do not penetrate deep into the lipid hydrophobic region.
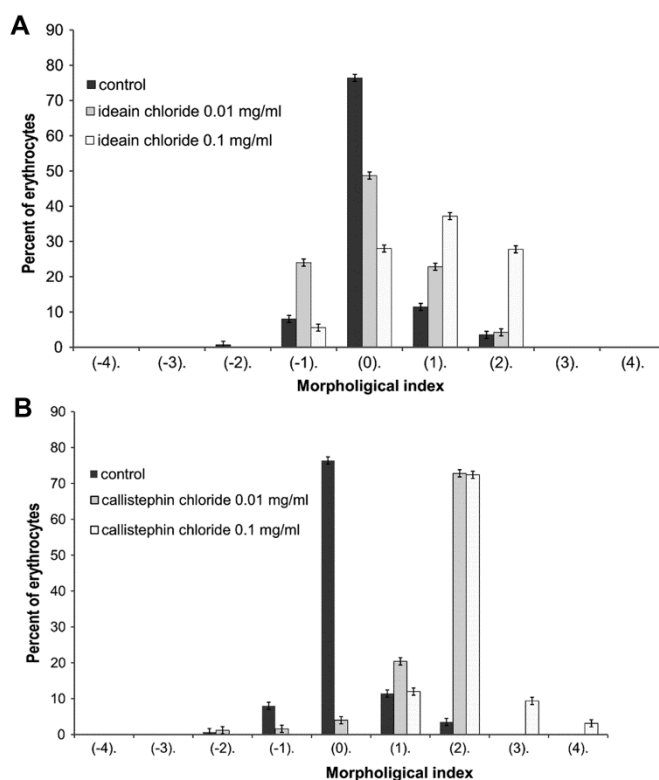

Fig. 3. Percent share of different shapes of erythrocytes induced by ideain chloride (A) and callistephin chloride (B) at 0.01 and 0.1 mM concentration. On the abscissa there are morphological indices for the respective shapes of cells: spherostomatocytes (-4), stomatocytes II (-3), stomatocytes I (-2), discostomatocytes (-1), discocytes (0), discoechinocytes (1), echinocytes (2), spheroechinocytes (3), and spherocytes (4).

As indicated by the values of fluorescence anisotropy, at the interphase between the hydrophilic and hydrophobic parts of the membrane – the area where the fluorophores of the TMA-DPH probes are incorporated – the ideain chloride induced a small increase in fluidity of the lipid layer. Practically, relative to the control, negligible effects are observed in the hydrophobic region where the signal is from DPH and TMA-DPH. It can thus be postulated that the compounds practically do not concentrate in the hydrophobic lipid phase of the erythrocyte membrane [4-5].

Table 1. Values of fluorescence anisotropy of the DPH and TMA-DPH probes for the erythrocyte membrane and liposomes composed of erythrocyte lipids modified by the tested compounds at 37°C.

DPH				
Membrane	Erythrocyte ghosts		Liposomes from erythrocyte lipids	
Compound	Callistephin chloride	Ideain chloride	Callistephin chloride	Ideain chloride
Concentrations [mg/ml]	Anisotropy (A) ± SD			
Control	0.239 ± 0.002		0.226 ± 0.002	
0.005	0.232 ± 0.007	0.237 ± 0.006	0.216 ± 0.002	0.215 ± 0.003
0.0075	0.234 ± 0.006	0.236 ± 0.014	0.215 ± 0.003	0.212 ± 0.001
0.01	0.233 ± 0.006	0.240 ± 0.006	0.211 ± 0.002	0.211 ± 0.002
0.025	0.234 ± 0.006	0.240 ± 0.012	0.216 ± 0.005	0.211 ± 0.001
0.05	0.236 ± 0.014	0.241 ± 0.005	0.215 ± 0.003	0.210 ± 0.002
TMA-DPH				
Membrane	Erythrocyte ghosts		Liposomes from erythrocyte lipids	
Compound	Callistephin chloride	Ideain chloride	Callistephin chloride	Ideain chloride
Concentrations [mg/ml]	Anisotropy (A) ± SD			
Control	0.268 ± 0.001		0.245 ± 0.001	
0.005	0.269 ± 0.007	0.274 ± 0.011	0.247 ± 0.003	0.247 ± 0.001
0.0075	0.268 ± 0.004	0.267 ± 0.003	0.248 ± 0.001	0.248 ± 0.002
0.01	0.269 ± 0.002	0.271 ± 0.003	0.247 ± 0.002	0.247 ± 0.002
0.025	0.272 ± 0.005	0.268 ± 0.010	0.254 ± 0.004	0.256 ± 0.003
0.05	0.271 ± 0.002	0.273 ± 0.007	0.257 ± 0.004	0.263 ± 0.003

Using the TMA-DPH probe, it was checked how the fluidity of a lipid membrane (from egg-PC) was modified with both the anthocyanins by 0.05 mg/ml concentration changes. In the area of the fourth carbon of the hydrocarbon chains, no effect of the anthocyanins was observed in egg-lecithin liposomes. The presence of the compounds causes only a slight increase in the fluorescence anisotropy at a physiological temperature of 37°C. The anisotropy of the fluorescence for pure egg-PC was 0.193 ± 0.003, with callistephin chloride it was 0.201 ± 0.039, and with ideain chloride it was 0.209 ± 0.022. We have also investigated, using the Laurdan probe, the degree of order in the hydrophilic part of liposomes composed of lipids extracted from erythrocyte membranes. The calculated values of general polarization (GP) slightly decreased with increasing concentration of callistephin chloride (Fig. 4.), which is indicative of increasing disorder in the hydrophilic part of the lipid layer and presence of the compounds in that area. Though the changes induced by the anthocyanins in liposome membranes are small, the conviction remains that the

anthocyanin compounds are incorporated into erythrocyte and liposome membranes, concentrating mainly in their hydrophilic part.

The investigation of the lipid phase packing order in the hydrophilic region of the erythrocyte membrane, using the Laurdan probe, showed distinct changes induced by the tested anthocyanins. The measured values of GP decreased slightly with increasing concentration of the compounds, which indicates increasing disorder of the polar heads of membrane lipids induced by incorporation of the anthocyanins. The most effective in inducing disorder in the hydrophilic region of the erythrocyte membrane is callistephin chloride, its GP value decreasing with the highest concentration of 0.05 mg/ml. Thus, it can be inferred that the callistephin and ideain chlorides are incorporated into and concentrate mainly in the hydrophilic region of the membrane.
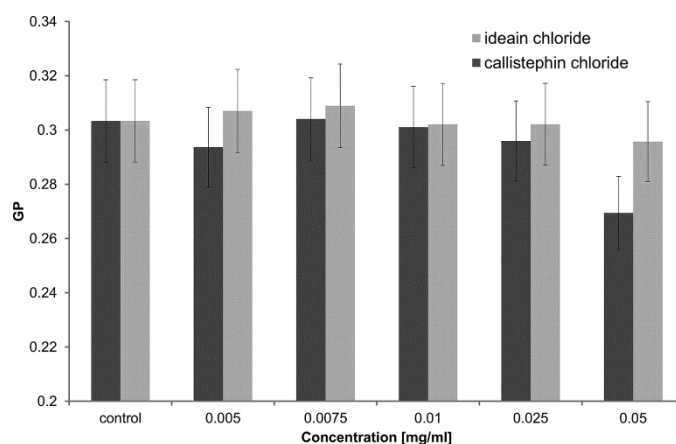

Fig. 4. Values of generalized polarization (GP) of the Laurdan probe for liposomes from erythrocyte lipids modified with the anthocyanins at 37ºC.

Table 2. Values of GP for Laurdan and Prodan at different temperatures for liposomes from egg-PC with addition of the compounds tested at 0.05 mg/ml concentration.

	GP ± SD		
	15 [°C]	25 [°C]	37 [°C]
	Laurdan		
Egg-PC	-0.0452 ± 0.0010	-0.1943 ± 0.0045	-0.3310 ± 0.0036
Ideain chloride	0.0482 ± 0.0119	-0.0942 ± 0.0156	-0.2424 ± 0.0040
Callistephin chloride	0.0521 ± 0.0037	-0.0690 ± 0.0044	-0.2078 ± 0.0145
	Prodan		
Egg-PC	-0.1623 ± 0.0023	-0.3070 ± 0.0028	-0.4230 ± 0.0087
Ideain chloride	-0.1135 ± 0.0100	-0.2628 ± 0.0096	-0.3889 ± 0.0134
Callistephin chloride	-0.0486 ± 0.0102	-0.1925 ± 0.0089	-0.3142 ± 0.0083

Values of general polarization for Laurdan and Prodan (Table 2) indicate that the liposomes composed of egg-PC are in the liquid-crystallized phase. The presence of the studied compounds causes an increase in GP for the three temperatures used, which indicates that the packing order in the hydrophilic phase of the membrane increases, the increase being however larger for callistephin chloride.
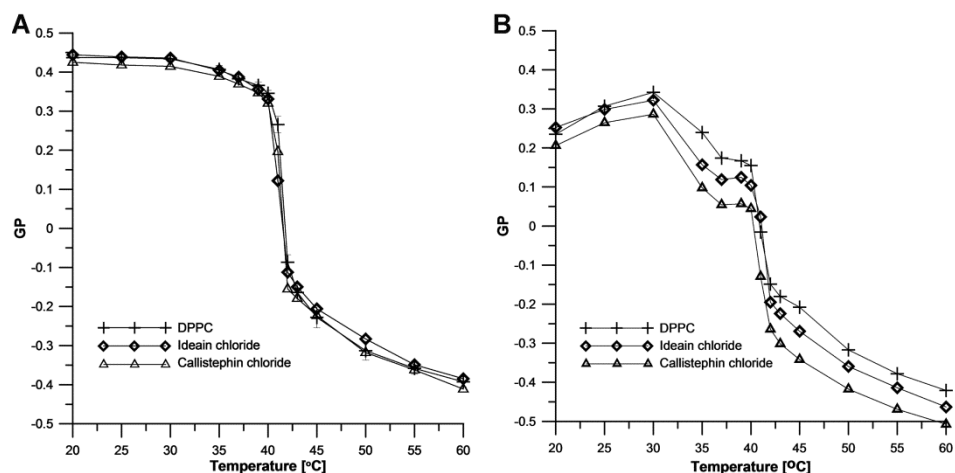

Fig. 5. Values of GP for Laurdan (A) and Prodan (B) at different temperatures for DPPC liposomes with the tested compounds added at 0.05 mg/ml.
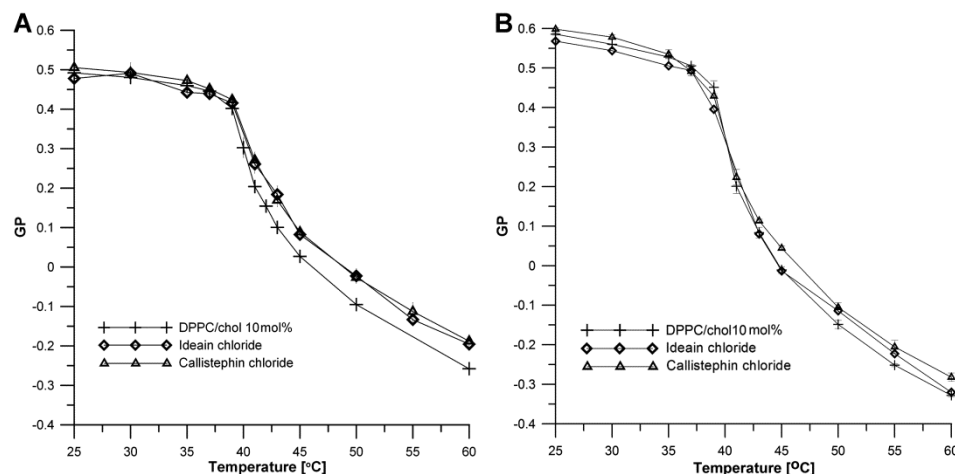

Fig. 6. Values of GP for Laurdan (A) and Prodan (B) at different temperatures for PPC/10 mol% cholesterol with the tested compounds added at 0.05 mg/ml.

By using the Prodan probe, we investigated the packing order of the hydrophilic phase of lipid membranes composed of DPPC (Fig. 5), DPPC/cholesterol (Fig. 6) and lipids from erythrocytes (Fig. 7). The measurements were carried

out at different temperatures and the results are shown for selected concentrations (0.01 mg/ml for liposomes from erythrocyte lipids and 0.05 mg/ml for DPPC liposomes). The effect of the anthocyanins on the temperature of the main phase transition of DPPC found by the fluorimetric method using the Laurdan and Prodan probes was also studied. From the fluorimetric spectra, GP values were calculated and are presented in Figs. 5, 6, 7 for different temperatures.
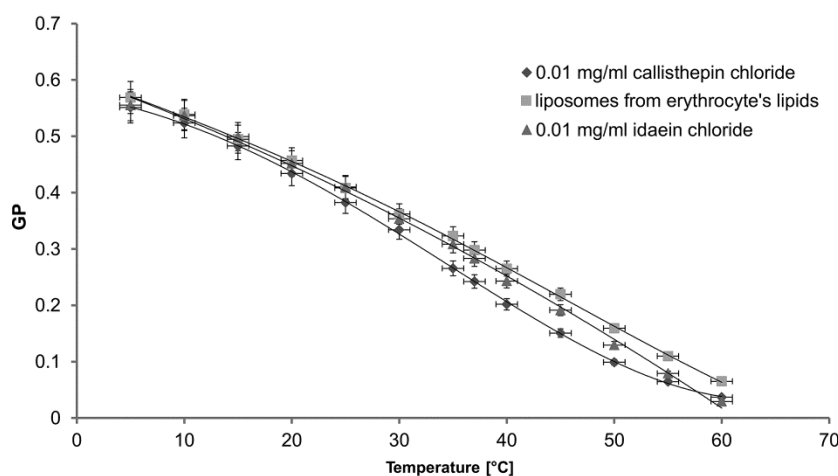

Fig. 7. Values of GP for Prodan at different temperatures for liposomes from erythrocyte lipids with addition of the compounds tested.

In liposome membranes composed of DPPC only the presence of callistephin chloride caused negligible changes at the level of the glycerol residue in the hydrophilic part of the membrane, which is confirmed by the GP values from the Laurdan probe. For Prodan the changes in GP in the presence of the compounds are much larger, both in the gel and liquid-crystalline phases. No significant changes were observed at the main phase transition temperature. Larger effects appear at the pre-transition for callistephin chloride than for ideain chloride. When the DPPC membrane contains cholesterol, anthocyanins make it more ridged, especially in the liquid-crystalline phase, this being indicated by the results obtained with both Laurdan and Prodan.
In liposomes composed of erythrocyte lipids the anthocyanins induced small changes in the packing order of the hydrophilic part of the membrane. Greater changes in GP values of Prodan were induced by the pelargonidin derivative at higher temperatures.

Calorimetric studies
The calorimetric studies investigated the effect of the anthocyanins on the temperature of the phase pretransition (T_p) of DPPC and temperature of the main phase transition (T_m) of DPPC and DPPC/cholesterol. The measurements were made for a number of selected concentrations of the extracts within the range

0.1-5.0 mg/ml (Fig. 8). With increasing concentration the polyphenols caused a slight decrease in the phase transition temperature (T_m) and an increase in the peak half-width. The changes were greatest for callistephin chloride, which affected the lipid phase mostly, compared with the others. This compound removed the pre-transition at the lowest concentration, lowered the main transition temperature more than the others, and diminished cooperativity of that transition.
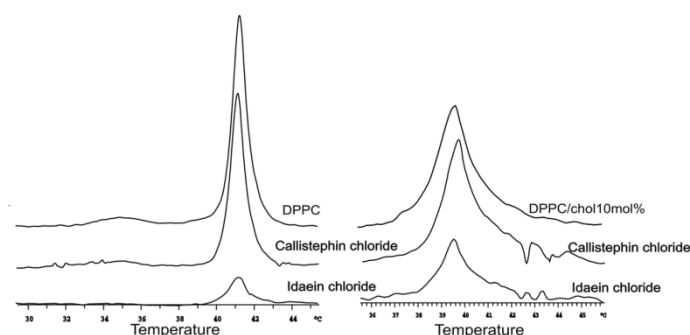

Fig. 8. Influence of anthocyanins on the temperature of the main phase transition of the lipids, at concentration of the compounds 1 mg/ml, scan 2 degree/min, accuracy of temperature 0.2 degree.

Fluorimetric method in antioxidative tests

The antioxidative activity of the anthocyanins was investigated with the fluorimetric method. Fig. 9 shows a representative relation between relative fluorescence and time of oxidation of erythrocyte ghosts with UVC radiation, for samples containing callistephin chloride at 0.005, 0.01, 0.05 mg/ml.

As seen in Fig. 9, the relative fluorescence for the studied concentrations of pelargonidin-3-glycoside decreases with oxidation time, which shows that the degree of lipid oxidation inhibition increases, and it reaches the level of ca. 70% at 0.05 mg/ml. Quenching of the DPH-PA fluorescence in the presence of the anthocyanins at different concentrations was also observed when lipid oxidation was induced in erythrocyte membranes with the chemical radical AAPH at 60 μM. Based on the kinetics of the oxidation curves obtained for various concentrations of both the compounds and the antioxidant Trolox®, the concentration responsible for 50% inhibition of the membrane lipids (IC_{50}) was found. The results from the fluorimetric method are given in Table 3.

Both the compounds, as indicated by the IC_{50} values, protect membrane lipids against oxidation. The results obtained have shown that pelargonidin-3-glucoside and cyanidin-3-galactoside protect the erythrocyte membrane against UVC-induced oxidation as well as Trolox®. For protecting membrane against AAPH-induced oxidation, the more potent compound proved to be ideain chloride, whose antioxidant activity was over two times higher than that of callistephin chloride. Such results suggest that the reaction with free radicals depends on the oxidation inducer. In the case of photo-oxidation, the percentage
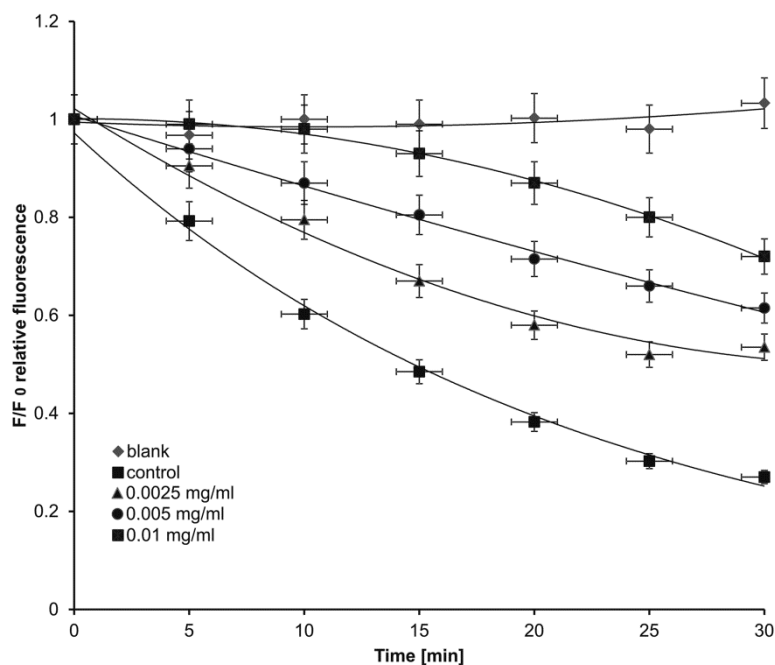
Fig. 9. Relation between relative fluorescence intensity and time of UVC irradiation of erythrocyte ghosts for control and test sample that contained various concentrations of callistephin chloride (0.005-0.05 mg/ml).

Table 3. Values of IC_{50} for callistephin and ideain chlorides, and Trolox[®], that inhibit erythrocyte membrane lipid oxidation by 50%, determined with the fluorimetric method. The oxidation was induced with UVC and AAPH radical.

Compound/Inducer	IC_{50} [mg/ml] $\pm$ SD	
	UVC	AAPH
Callistephin chloride	0.0177 ± 0.0016	0.0058 ± 0.0005
Ideain chloride	0.0146 ± 0.0013	0.0017 ± 0.0002
Trolox[®]	0.0146 ± 0.0013	0.0039 ± 0.0003

of inhibition and kinetics of the process are similar for both the compounds, whereas in the case of the AAPH inducer, much faster and more efficient in inhibiting oxidation is the cyanidin derivative. Greater activity is shown by ideain chloride, which possesses two hydroxyl groups in the B ring. This increases its ability to scavenge free radicals compared with callistephin chloride, which has one hydroxyl group in the B ring.

DISCUSSION

The results of the present research show that both the tested compounds, pelargonidin-3-O-glucoside and cyanidin-3-O-galactoside, induce changes in biological and lipid membranes. Their amphiphilic character and chemical structure enable their interaction with the lipids of biological membranes. Various membrane models were used in the experiment, from the simple one-lipid DPPC and DPPC/cholesterol membrane through more complicated membranes of liposomes composed of egg-PC and lipids extracted from erythrocytes, up to the most complicated model membrane, which is the erythrocyte ghost. Both the anthocyanins tested when interacting with specific model membranes show membrane changes which are of the same character.

Changes occurring in the hydrophobic part of the membrane were determined on the basis of fluorescence anisotropy of the probes DPH and TMA-DPH. They refer to various depths of the hydrophobic region, determined by localization of the probe's chromophore. The lack of essential changes with respect to the control in this interphase region could mean that the compounds interact with the membrane quite shallowly, and this interaction has an interfacial character. A similar character of the interaction between polyphenolic compounds and membranes was documented in other studies [16, 17, 21].

This conclusion is confirmed by the fluorimetric studies with the Laurdan and Prodan probes. These probes are particularly sensitive to polarity of the environment and monitor relevant differences in polarity of the different phase states of lipid bilayers. The Laurdan probe completely partitions into the membrane, while Prodan partitions between water and polar head groups of the membrane, which strongly depends on the state of the membrane phase [29].

Laurdan is a fluorescent probe whose fluorophore becomes located in the phospholipid glycerol backbone and is sensitive to polarity changes and dynamic properties in the hydrophilic region of the membrane. Decreased GP values in the tested membranes seem to indicate increased lipid-water interface hydration of the membrane [27-29, 33, 34].

A significant decrease in the generalized polarization of the Laurdan probe was observed only with callistephin chloride at different concentrations in all the membranes tested, while ideain chloride changed the GP value insignificantly. Such changes in GP of the Laurdan probe may result from different strength of binding between the studied molecules and water, owing to the presence of sugar residues. The presence of glucose in the pelargonidin molecule increases its affinity for water, which may result in greater water content in the membrane surface than in the case of cyanidin, which has water insoluble galactose.

The intensity of the Prodan probe, like that of Laurdan, depends on the polarity of the medium. A slight decrease in GP was recorded in the presence of pelargonidin-3-galactoside for liposomes composed of erythrocyte lipids. For DPPC membranes, differences occurred in GP values both for the gel and liquid-crystalline phases on addition of the compounds studied. Greater changes were

observed for callistephin chloride. They, however, disappeared on addition of cholesterol to the DPPC membrane; cholesterol imposes order on the hydrophilic phase of the membrane, and in the presence of anthocyanins the order increases slightly [28, 29, 36]. For the Prodan probe a high intensity of polarity is recorded in an aqueous medium at 512 nm. Due to the dense packing of the erythrocyte membrane components at physiological temperature, the Prodan probe did not give fluorescence spectra that would indicate its presence in the membrane – the external aqueous medium spectrum was obtained instead. The anthocyanins had little impact on the phase transition temperature, as seen by differential scanning calorimetry or in fluorimetric GP studied at different temperatures with DPPC liposomes [7, 35, 37].

The anthocyanins induced a modification of the shape of erythrocytes towards echinocytes. Callistephin chloride is responsible for creation of the most varied forms of echinocytes. It can thus be assumed that the compounds concentrate mainly in the outer monolayer of the erythrocyte membrane when inducing echinocytes, and practically do not permeate into the inner monolayer of the membrane [26, 30, 31].

Microscopic, fluorimetric and calorimetric experimental results show that the changes are greatest in the hydrophilic part of erythrocyte membranes and lipid membranes composed of lipids extracted from erythrocytes and different lipids. The tested compounds incorporated mainly into the outer, hydrophilic part of the membranes. They had practically no influence on fluidity in the hydrophobic region of the membranes, i.e. in the area of their hydrocarbon chains. On the basis of the results obtained, one can infer that the protective action of anthocyanins with respect to biological membranes depends on the extent of their incorporation into the hydrophilic part of the membrane.

The location of the compounds in the hydrophilic part of the membrane seems to constitute a protective shield of the cell against other substances, free radicals in particular, which is reflected in their antioxidant properties as tested by the fluorimetric method. Both the anthocyanins exhibited a high antioxidant activity towards free radicals, which developed as a result of membrane photo-oxidation induced by UVC radiation. A greater antioxidant activity in the oxidation process induced by the AAPH radical was shown by ideain chloride, apparently because of its two hydroxyl groups in the aromatic ring B (Fig. 1).

The results of the present study on the interaction of selected anthocyanins with the erythrocyte membrane and lipid membranes have shown that the substances exhibit an affinity for membrane lipids. They successfully protect membrane lipids against oxidation induced by both chemical (AAPH) and physical (UVC radiation) agents, modifying the lipid phase negligibly both within erythrocytes and in pure lipid membranes. Thus they can be considered to cause insignificant changes in the membrane structure. Their action is limited to the membrane surface, which indicates that the substances are effective and safe antioxidants.

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