

Short communication

INTERACTION BETWEEN PLANT POLYPHENOLS AND THE ERYTHROCYTE MEMBRANE

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Abstract: The purpose of these studies was to determine the effect of polyphenols contained in extracts from apple, strawberry and blackcurrant on the properties of the erythrocyte membrane, treated as a model of the biological membrane. To this end, the effect of the substances used on hemolysis, osmotic resistance and shape of erythrocytes, and on packing order in the hydrophilic region of the erythrocyte membrane was studied. The investigation was performed with spectrophotometric and fluorimetric methods, and using the optical microscope. The hemolytic studies have shown that the extracts do not induce hemolysis at the concentrations used. The results obtained from the spectrophotometric measurements of osmotic resistance of erythrocytes showed that the polyphenols contained in the extracts cause an increase in the resistance, rendering them less prone to hemolysis in hypotonic solutions of sodium chloride. The fluorimetric studies indicate that the used substances cause a decrease of packing order in the hydrophilic area of membrane lipids. The observations of erythrocyte shapes in a biological optical microscope have

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Abbreviations used: cAK – catalytic subunit of cyclic activated protein kinase (AMP); GP – generalized polarization; HPLC – high performance liquid chromatography; HPLC/DAD – high performance liquid chromatography/diode array detector; Laurdan – fluorescent probes 6-dodecanoyl-2-dimethylaminonaphthalene; PBS – phosphate buffer solution; UPLC/ESI/MS – ultra performance liquid chromatography/electrospray ionization/mass spectrometry

shown that, as a result of the substances' action, the erythrocytes become mostly echinocytes, which means that the polyphenols of the extracts localize in the outer lipid monolayer of the erythrocyte membrane. The results obtained indicate that, in the concentration range used, the plant extracts are incorporated into the hydrophilic area of the membrane, modifying its properties.

Key words: Erythrocyte membrane, Plant polyphenols, Hemolysis, Osmotic resistance, Echinocytes, Generalized polarization, Lipid packing order

INTRODUCTION

Plant extracts contain a number of biologically active compounds, which either protect the organism against the toxic effect of various physicochemical agents or enhance treatment of many diseases. Such compounds include alkaloids, glycosides, flavonoids and anthocyanins. They protect living organisms, exhibiting anticancer, antiatherosclerotic, antioxidant and anti-inflammatory actions, which make them very helpful in proper functioning of living organisms [1]. A rich source of the protective and healing substances is the leaves of fruit trees and shrubs. The subject of the present study is extracts from leaves of blackcurrant, strawberry and apple, which exhibit a series of positive actions with respect to biological systems.

Extracts from currant leaves, aside from phenolic compounds that mainly belong to quercetin and kaempferol derivatives [2, 3], also contain large amounts of prodelphinidins, and can thus be used for treating osteoarthritis [4]. Declume [5] showed that a water-alcohol extract from currant leaves has an anti-inflammatory effect, and the tannins it contains are a competitive inhibitor of cAK with respect to both ATP and synthetic peptide substrate [6]. Moreover, it has been shown that the biological activity of a mixture of flavonoids extracted from currant leaves is higher than the activity of its two main constituents, i.e. rutin and isoquercetin [7].

The phenolic content of strawberry leaves to a large extent depends on variety, development stage and cultivation site [8-11]. It was also found that young strawberry leaves contain much more polyphenolic compounds than the fruits and mature leaves [12], and are a rich source of flavonoids and procyanidins. Results of studies also indicate that the total phenolic content (TPC) and antioxidant activity of extract from strawberry leaves is very high, occupying 11th place among 70 plant species and varieties [13]. Moreover, it has been shown that water extract from strawberry leaves lowers the risk of circulatory system disease [14]. Strawberry leaves were also studied with respect to preservation and storage of food. It was found that 5% strawberry leaf extract in fish oil markedly improves its quality and durability by protecting its lipids against oxidation [15]. The phenolic compounds occurring in apple leaves, aside from their antioxidative properties, also exhibit antibacterial action [16].

Extract from leaves of trees and shrubs protect biological systems against the harmful effect of free radicals [17, 18]. In particular, the protection of biological

membranes, which are the main site of attack by free radicals, is directly due to the presence of polyphenols in the membrane and indirectly due to scavenging of free radicals in the medium. It has been shown that extracts from leaves of strawberry, blackcurrant and apple have very good antioxidant properties with respect to biological membranes, the strawberry extract exhibiting the highest activity, comparable to that of Trolox[®] [3]. Plant extracts as substances of high biological activity have long been used in prevention and treatment, though the mechanism of their action at the cell and molecular level is not yet fully explained. The aim of the present study was to determine the changes induced by extracts from leaves of blackcurrant, strawberry and apple in the biological membrane. We also wanted to elucidate the mechanism of their interaction with the membrane, assessing whether the substances induce any negative side effects, aside from the protective and healing activity.

MATERIALS AND METHODS

Plant extracts were obtained from the Department of Fruit, Vegetable and Grain Technology, Wrocław University of Environmental and Life Sciences. The percent content of polyphenols in the extracts was determined with the liquid chromatography (HPLC) method described by Oszmiański *et al.* [19]. Phenolic compounds were identified with the HPLC/DAD method described by Oszmiański *et al.* [20] and the method of UPLC/ESI/MS analysis described by Cyboran *et al.* [3].

Polyphenols were isolated from leaves by extraction with water containing 200 ppm of SO₂, the ratio of solvent to leaves being 3:1. The extract was absorbed on Purolite AP 400 (UK) for further purification. The polyphenols were then eluted out with 80% ethanol, concentrated and freeze-dried. By means of the above method a mixture of polyphenols was obtained by Gąsiorowski *et al.* [21]. The percent content of polyphenols in individual preparations was determined by means of liquid chromatography (HPLC [22-24] and HPLC/DAD [20]). Detailed quantitative and qualitative contents of phenolic compounds in the extracts from leaves of apple, blackcurrant and strawberry are given in Tab. 1.

The investigation was conducted with erythrocytes and their membranes (ghosts) obtained from fresh heparinized pig blood according to the Dodge *et al.* method [25]. Membrane protein concentration was assayed using Bradford's method [26] and it was 1 mg/ml. The fluorescence probe 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan) was purchased from Molecular Probes, Eugene, OR (USA).

Hemolysis and osmotic resistance of erythrocytes

The experiments were conducted on fresh, heparinized pig blood. For washing the erythrocytes, and in the experiments performed, an isotonic phosphate solution of pH 7.4 (131 mM NaCl, 1.79 mM KCl, 0.86 mM MgCl₂, 11.79 mM Na₂HPO₄·2H₂O, 1.80 mM Na₂H₂PO₄·H₂O) was used. Upon removing from plasma, the erythrocytes were washed four times in phosphate solution and then incubated in the same

Tab. 1. Content of polyphenolic compounds in extracts from leaves of apple tree, blackcurrant and strawberry.

Phenolic compound [%]	Apple leaves ¹	Blackcurrant leaves ²	Strawberry leaves ²
chlorogenic acid	0.92	1.15	0
neochlorogenic acid	0	0.1	0
cryptochlorogenic acid	0	0.11	0
ellagic acid	0	0	0.89
derivate of caffeic acid	0.12	0	0
derivate of p-coumaric acid	0.29	0	0
p-coumaroyl-glucoside	0.16	0	0.45
quercetin-3-O-galactoside	3.40	2.52	0
quercetin-3-O-glucoside	1.40	0	0
quercetin-3-(6"-malonyl) glucoside	0	1.91	0
quercetin-3-O-arabinoside	1.39	0	0
quercetin-3-O-xyloside	2.44	0	0
quercetin-3-O-rhamnoside	8.54	0	0
quercetin-3-O-glucosyl-6"-acetate	0	5.33	0
quercetin-3-O-rutinoside	0	0.33	5.23
quercetin-3-O-glucoside glucuronide	0	0	0.27
quercetin-3-O- glucuronide	0	0	2.81
phloretin-2'-O-xyloglucoside	5.38	0	0
phloridzin	2.98	0	0
kaempferol-3-O-rutinoside	0	0.23	0.99
kaempferol-3-O-galactoside	0	0.41	0
kaempferol-3-O-glucosyl-6"-acetate	0	0.96	0
kaempferol-3-O-glucuronide	0	0	0.12
TOTAL	27.02	13.25	10.76

The percent content of polyphenols was determined by means of liquid chromatography:¹ – HPLC, ²– HPLC/DAD

solution but containing appropriate amounts of the compounds studied. The modification was conducted at 37°C for 0.5 h, each sample containing 10 ml of erythrocyte suspension of 2% hematocrit, stirred continuously. After modification 1 ml samples were taken, centrifuged and the supernatant assayed for hemoglobin content using a spectrophotometer (Specord 40, AnalytikJena) at 540 nm wavelength. Hemoglobin concentration in the supernatant, expressed as percentage of hemoglobin concentration in the supernatant of totally hemolyzed cells, was assumed as the measure of the extent of hemolysis.

Osmotic resistance assay was performed on fresh pig blood. Full blood was centrifuged for 3 min, 2500 r.p.m. at 4°C, to remove the plasma and leucocytes. The erythrocytes obtained were washed thrice with a cool (ca. 4°C),

310 mOsm PBS isotonic solution. Next, a 2% red cell suspension containing extracts of 0.01 mg/ml concentration was prepared and left for 1 h at 37°C with continuous stirring. After this modification, the suspension of erythrocytes was centrifuged for 15 min at room temperature in order to remove the cells from the extract solution. From the cell sediment were taken 100 µl samples of the extract-modified cells and suspended in test tubes containing NaCl solutions of 0.5-0.86% concentration and to an isotonic (0.9%) NaCl solution. In solutions of the same concentrations were also suspended unmodified red blood cells that constituted the control for osmotic resistance determinations. Then, the suspension was stirred and centrifuged under the above stated conditions. After that the percentage of hemolysis was measured with a spectrophotometer at $\lambda = 540$ nm wavelength. On the basis of the results obtained, the relation was determined between the percentage of hemolysis and NaCl concentration in the solution. Next, using the obtained plots, the NaCl percent concentrations (C_{50}) that caused 50% hemolysis were found. The C_{50} values were taken as a measure of osmotic resistance. If a determined sodium chloride concentration is higher than that of control cells, the osmotic resistance of the erythrocytes is regarded to be lower, and vice versa.

Packing order in the hydrophilic region on the membrane

Packing density in the hydrophilic part of lipids of erythrocyte membrane was studied with the fluorimetric method, using the Laurdan fluorescent probe. The measurements were done with a spectrophotometer (Cary Eclipse, Varian). The study was carried out on isolated unsealed erythrocyte membranes. The amount of erythrocyte ghosts in the samples was determined on the basis of protein concentration, which was about 100 µg/ml, while the concentration of the fluorescence probe was ca. 1 µM. To samples containing erythrocyte ghosts and the probe in a buffer solution of pH 7.4 were added appropriate amounts of apple, strawberry and blackcurrant extracts to obtain concentrations from 0.01 to 0.1 mg/ml. The Laurdan probe excitation wavelength (λ_{exc}) was 360 nm, and the emission wavelengths were $\lambda_b = 440$ nm and $\lambda_r = 490$ nm. The erythrocyte membrane fluidity was determined on the basis of the generalized polarization (GP) of Laurdan [27], calculated with the formula:

$$GP = \frac{I_b - I_r}{I_b + I_r} \quad (1)$$

where I_b is the fluorescence intensity at $\lambda = 440$ nm and I_r is the fluorescence intensity at $\lambda = 490$ nm.

The obtained values of GP were compared with those of unmodified ghosts. Increased values of GP signify increased packing density of the membrane lipid polar heads, whereas decreased values of GP indicate decreased polar group packing arrangements of the erythrocyte membrane lipid bilayer.

Shapes of erythrocytes

For investigation with the optical microscope, the red cells on separation from plasma were washed four times in saline solution and suspended in the same solution but containing an appropriate amount of the compounds 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 the two basic forms (echinocytes and stomatocytes) in the population of ca. 500 cells was determined. The concentration of extracts was 0.01 and 0.1 mg/ml. Individual forms of erythrocyte cells were ascribed morphological indices according to Bessis' scale [28], which for stomatocytes assume negative values from -1 to -4 and for echinocytes from 1 to 4.

Statistical analysis

The results obtained were statistically interpreted using STATISTICA 9.0, on the basis of the analysis of variance (ANOVA). The Dunnett test was used for difference estimation at confidence level $\alpha = 0.05$.

RESULTS AND DISCUSSION

Hemolysis and osmotic resistance of erythrocytes

The spectrophotometric method was used to investigate the hemolytic activity of the extracts and their influence on osmotic resistance of erythrocytes. The hemolytic studies have shown that polyphenolic extracts from leaves of apple, strawberry and blackcurrant do not induce hemolysis when used at 0.1 to 0.5 mg/ml concentration.

Based on the results obtained from osmotic resistance studies, curves were drawn of the relation between percentage of hemolysis of the extract-modified erythrocytes and percent concentration of sodium chloride. From the plots, NaCl concentrations were found at which 50% hemolysis occurred. Those concentrations were assumed as a measure of osmotic resistance of the erythrocytes and were denoted as C_{50} . Fig. 1 presents representative curves of the osmotic resistance of erythrocytes modified with strawberry and apple extracts of 0.01 mg/ml concentration. In the presence of these extracts a marked decrease in red blood cell hemolysis occurs relative to control. A similar curve was obtained for blackcurrant leaf extract.

The extracts studied cause a shift in osmotic resistance curve towards lower C_{50} values (Fig. 1). This means that the extracts increase the osmotic resistance of

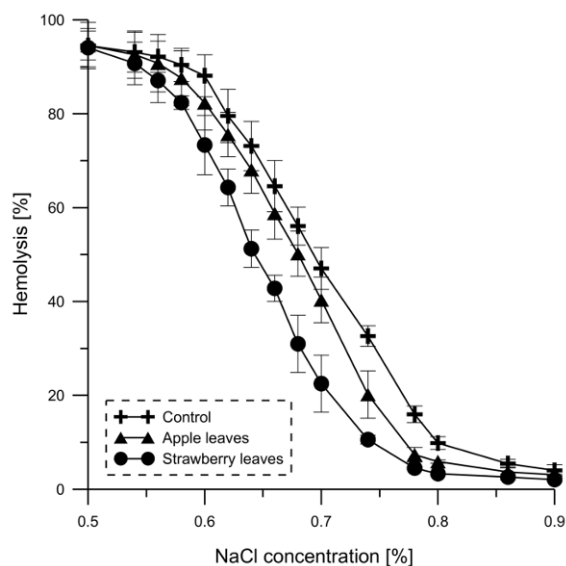


Fig. 1. Hypotonic hemolysis of erythrocytes in the presence of 0.01 mg/ml strawberry or apple leaf extract.

the cells, making them less susceptible to hemolysis in hypotonic solution of sodium chloride. Studies performed with other extracts [29, 30] confirm the results obtained here. They indicate that the erythrocyte membrane becomes more resilient under the effect of the extracts, yielding to disruption at a lower (compared to the control) concentration of a hypotonic sodium chloride solution. The observed changes in the properties of the erythrocyte membrane may be due to changes in membrane permeability or an increased surface/volume ratio caused by incorporation of polyphenols in the membrane [31]. The results of spectrophotometric measurements indicate that the extracts do not induce hemolysis in direct interaction with erythrocytes, at the concentrations used here, and have a positive effect on the erythrocytes, which become less sensitive to changes in osmotic pressure in the medium.

Packing order in the hydrophilic region of the membrane

Changes in the packing order of the hydrophilic phase of the membrane have been studied with the fluorimetric method, using the Laurdan probe, which is incorporated into the membrane at the level of the glycerol cytoskeleton of phospholipids [32, 33]. The extracts used at 0.01-0.06 mg/ml concentrations caused a marked decrease in GP values (Fig. 2), which is connected with a decrease in the packing order in the hydrophilic layer of the membrane. Additionally, Fig. 2 shows that with increasing extract concentration the value of generalized polarization decreases.

The greatest changes in the packing order of the polar heads of lipids were induced by strawberry leaf extract, with apple and blackcurrant extracts causing

lesser changes. These results agree with those of other authors, and they testify that natural polyphenolic compounds, due to their amphiphilic character, localize mainly in the hydrophilic membrane layer, where they cause changes in the polar group packing arrangement. However, no changes were found in fluidity of the hydrophobic membrane region, which was confirmed by experiments with probes emitting fluorescence from that region [30, 34, 35].

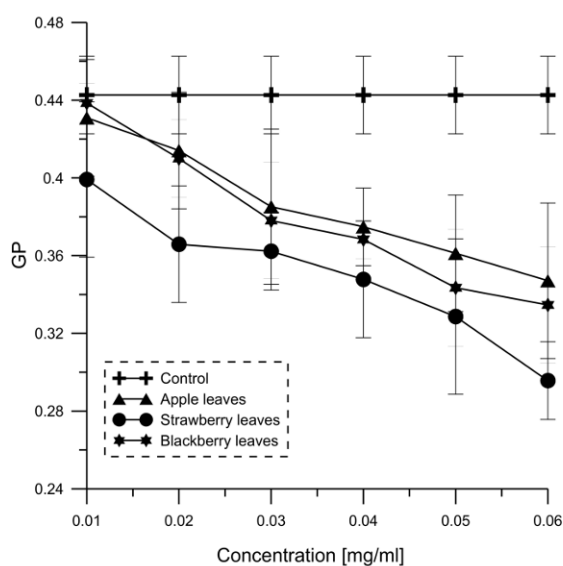


Fig. 2. Relationship between GP and concentration of extracts from strawberry, apple and blackcurrant leaves.

Shapes of erythrocytes

In order to confirm the results from the fluorimetric method, which indicate an interaction between phenolic compounds and the outer layer of a lipid membrane, we observed erythrocytes modified with extracts from the leaves of apple, strawberry and blackcurrant. Their effect on the shapes of erythrocytes was observed using an optical microscope, connected to a digital camera. The photographs obtained enabled us to calculate the share of the respective forms of erythrocytes in a population of 500 cells. The shapes were classified according to Bessis and Brecher's scale [28], where various shapes are given morphological indices as follows: spherostomatocytes (-4), stomatocytes II (-3), stomatocytes I (-2), discostomatocytes (-1), discocytes (0), discoechinocytes (1), echinocytes (2), spheroechinocytes (3), spherocytes (4).

Fig. 3 shows a representative relation between the percent share of individual forms of erythrocytes and concentration of apple leaf extract. As seen in the figure, in the presence of the extract, echinocytes are the dominant form of erythrocytes, the share of spheroechinocytes and spherocytes increasing with the extract concentration. However, the share of the respective shapes depends on the kind of extract, which is probably connected with the content of polyphenols.

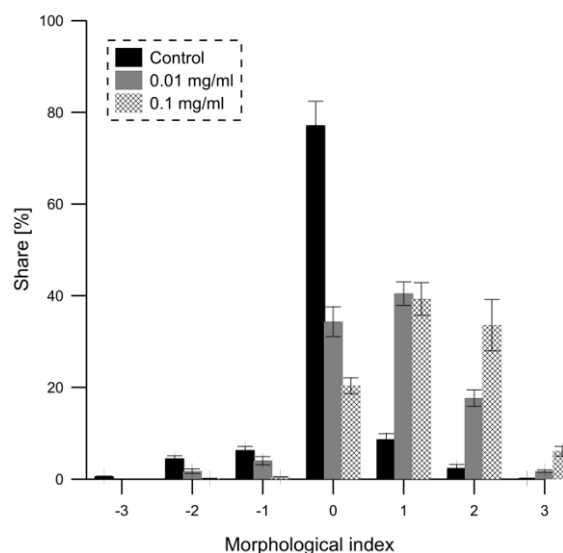


Fig. 3. Shapes of erythrocytes induced in the presence of apple leaf extract (-3, -2, -1 – stomatocytes; 0 – discocytes; 1, 2, 3 – echinocytes).

The induction of mostly echinocytes indicates that the substances, according to the bilayer couple hypothesis [36], are present in the outer lipid layer of the membrane. Similar changes in the erythrocyte shapes were observed for natural and synthetic phenolic compounds [30, 34, 35].

The results obtained in the microscopic investigation are in accord with the results of fluorimetric studies and prove that the phenolic compounds contained in the extracts from strawberry, apple and blackcurrant permeate the hydrophilic lipid layer of the erythrocyte membrane.

CONCLUSION

The extracts from leaves of strawberry, apple and blackcurrant, aside from the very good antioxidative properties relative to membrane lipids [3], also cause alterations in the properties of the cell membrane. The changes, which mainly occur in the lipid phase of the erythrocyte membrane and are caused by the presence of polyphenols in that hydrophilic part, are beneficial and connected with, e.g., increased osmotic resistance of red blood cells.

In particular, the extracts tested have no destructive effect on the membrane of erythrocytes, and do not cause hemolysis at concentrations higher than those that are effective in protecting erythrocyte membranes against oxidation. Their beneficial effect on osmotic resistance, making the membrane more resilient to changes in osmotic pressure, has been demonstrated. Moreover, the results of the fluorimetric measurements indicate that the extracts studied cause a decrease in the polar group packing arrangement of the erythrocyte membrane. Modulation of the packing in the hydrophilic part of a lipid membrane may be due to

membrane dehydration [37] and erythrocyte shape alteration connected with incorporation of polyphenols into the membrane. Interaction of the extract with the hydrophilic layer of the membrane has been proved by the investigation with the optical microscope. These results show that the polyphenols contained in the extracts induced an alteration in the erythrocyte morphology from the normal discoid shape to an echinocytic form, which indicates that polyphenol molecules localize mainly in the outer lipid monolayer of the red cell membrane.

The results obtained testify that the presence of apple, strawberry and blackcurrant leaf extracts makes the erythrocytes less susceptible to changes in the medium tonicity. Their presence in the membrane may also prevent the membrane from stiffening in some pathological states, e.g. in diabetes.

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