Full Length Research Paper

The effect of *Hypericum perforatum* extract on the properties of erythrocyte membrane

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The aim of the work presented is to determine the effect of *Hypericum perforatum* extract on the properties of the erythrocyte membrane. A number of methods were applied, so that the location of polyphenols in the membrane and the changes they cause could be determined. In order to determine the location of polyphenols in the lipid bilayer of erythrocytes, their shape was investigated using optical and electron microscopes. The fluorimetric method with 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan) and diphenylhexatriene (DPH) probes were used; the effect of the plant extract on the packing order in the hydrophilic part and fluidity in the hydrophobic part of the erythrocyte membrane was investigated. Using the spectrophotometric method, the osmotic resistance of red blood cells was determined. The study has shown that the polyphenolic compounds contained in the *H. perforatum* extract are located in the hydrophilic part of the erythrocyte membrane, inducing the appearance of echinocytes. The fluorimetric investigation showed that the extract also reduces the packing density of the polar heads of the membrane lipids and also causes an increase in the fluidity of the membrane hydrophobic region. The polyphenols induce an increase in the osmotic resistance of erythrocytes, making them less sensitive to hypotonic sodium chloride solutions.

Key words: *Hypericum perforatum* extract, erythrocyte membrane, packing order, anisotropy, generalized polarization, osmotic resistance.

INTRODUCTION

Today it is known that new-generation medicines, effective in treating many diseases, often exhibit a number of side effects. These unwanted effects are usually found in the alimentary system, which exhibits limited ability to assimilate the substances and vitamins needed for proper operation of the organism. For such reasons, in many countries, herbal treatment of illnesses is on the increase and phytotherapy has made a comeback. Whole plants or plant fragments (leaves, roots, flowers, etc.) are used; or extracted biological substances, such as polyphenols, to which group the flavonoids belong. The effectiveness and scope of the therapeutic action of herbs is first of all determined by the biological activity of their natural constituents, which on their own are also used as effective medicines without any side effects. One of the oldest herbal medicines is the common *Hypericum perforatum* herb, also known as St. John’s wort after the day it flowers. It originates from Europe, though nowadays it is also found in Asia, Africa and North America (Mártonfi, 2008).

*H. perforatum* has long been used for treating alimentary duct ailments, having for example, a diastolic action on the smooth muscles of the alimentary duct and the bile tracts, and on blood vessels. It removes colic and has a pain-killing action; it also helps bile to flow to the duodenum (Dias et al., 2000). Studies have shown that it also has a beneficial effect on the nervous system, with an antidepressive effect (Erdelmeier et al., 2000; Greeson et al., 2002; Butterweck, 2003; Patocka, 2003). It has been shown that the antidepressive action of *H. perforatum* is connected with its polyphenol composition (Butterweck and Schmidt, 2007). Owing to the large

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polyphenol content in the extract (Silva et al., 2005; Biesaga et al., 2007), including flavonoids (Wilhelm et al., 2001; Cirak et al., 2007), tannins and hypericins, it has a high antioxidant activity, comparable to that of butylated hydroxytoluene (BHT), which consists of scavenging free radicals (Sagratini et al., 2008; Hernandez et al., 2010; Danila et al., 2011).

In spite of the substantial literature on the healing action of herbs, _H. perforatum_ extracts, including the effect of the compounds contained in the extracts on biological systems, is not yet fully known. The effect of _H. perforatum_ extract on the cell membrane, which is the principle site of interaction between substances and the organism, will enable the determination of whether there is any negative side-effect on the structural properties of the cell membrane and/or function impairment. As concerns the antioxidant properties of polyphenols, it is thought that they can variously bind to the biological membrane, posing restrictions on access for free radicals (Arora et al., 1998, 2000; Suwalsky et al., 2006, 2008).

The present study aimed at the determination of the molecular mechanism of the interaction between polyphenolic substances of the _H. perforatum_ extract with the biological membrane, and its lipid phase in particular. It seems likely that polyphenolic compounds, due to their chemical structure, exhibit a special affinity to the lipid phase of the biological membrane. This is due to the fact that both polyphenol and lipid molecules have a hydrophilic-hydrophobic structure. To achieve the work’s aim, the investigation was conducted on erythrocytes, treated as a model cell, and on erythrocyte extracted membranes that represented biological membranes. The effects of the interaction between polyphenols and the lipid phase of a biological membrane were determined by studying on the shape and osmotic resistance of erythrocytes, packing order of the polar heads of membrane lipids and fluidity in the hydrophobic area of the erythrocyte membrane lipid bilayer. The investigations allowed the polyphenolic compounds to be located in the erythrocyte membrane and the structural changes induced in the membrane hydrophilic and hydrophobic regions to be determined.

**MATERIALS AND METHODS**

The subject of the study was plant extracts from _H. perforatum_, which were obtained from the Department of Fruit, Vegetable and Grain Technology of Wrocław University of Environmental and Life Sciences. The method for isolating the polyphenols was described in detail in patent no. 169082 B1 (Oszmiański, 1996). Polyphenols were isolated from _H. perforatum_ (var. Shampion) by extraction with water containing 200 ppm of SO₂, the ratio of solvent to leaves being 3:1. The extract was adsorbed on Purolite AP 400 resin (UK) for further purification. The polyphenols were then eluted out with 80% ethanol, concentrated and freeze-dried. By means of the aforementioned method, a mixture of polyphenols was obtained (Gąsiorowski et al., 1997). The percentage content of polyphenols in individual preparations was determined using liquid chromatography HPLC (Skupień and Oszmiański, 2004; Oszmiański and Wodzilo, 2005; Oszmiański et al., 2008) (Figure 1 and Table 1). The study was carried out on pig erythrocytes and their membranes. Erythrocyte membranes (ghosts) were prepared by the Dodge method (Dodge et al., 1963). The pig red blood membrane is known to be the closest to the human erythrocyte membrane with respect to its lipid composition. Fresh pig blood was taken to a physiological solution with heparin added. The 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan) and 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescent probes were purchased from Molecular Probes, Eugene, Oregon USA.

**Microscopic investigation**

For investigation with the optical microscope, the red cells, when separated from plasma, were washed four times in saline solution and suspended in the same solution but containing appropriate amounts of the compounds. Hematocrit of the erythrocytes in the modifications 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 percentage share of the two basic forms (echinocytes and stomatocytes) in the population of ca. 800 cells were determined. The concentration of the extracts was 0.1 and 1 mg/ml. The individual forms of erythrocyte cells were ascribed morphological indices according to the Bessis scale (Deuticke, 2003), which for stomatocytes assumes negative values from -1 to -4 and for echinocytes from 1 to 4. The following morphological indices were ascribed to individual forms of erythrocytes: Sf(-4) spherostomocytes, SSf(-3) spherocytes, St(-2) stomatocytes, DSf(-1) discoctostomocytes, D(0) discocytes, DE(1) discoechinocytes, E(2) echinocytes, SfE(3) spherocytes, and Sf(4) spherinoechinocytes.

To investigate with the electron microscope, the red cells were fixed for 12 h in a 2.5% solution of glutaraldehyde buffered with PBS of pH 7.4 at 18°C. After fixation, the material was fixed again for 1 h in a 1% solution of osmium tetroxide in the same buffer at 4°C. Mica slides 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 CO₂ in a Balzer’s 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. Concentrations of the extracts studied were equal and amounted to 0.1 mg/ml.

**Fluorimetric studies**

Fluorescence intensity was measured by using two fluorescent probes; Laurdan and DPH. These probes were used because they incorporate into different regions of the lipid bilayer. The measurements were made with a spectrophotometer (CARY Eclipse of VARIAN) at a temperature of 37 and 23°C. 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 a fluorescence probe in a buffer solution of pH 7.4 were added appropriate amounts of the _H. perforatum_ extract at a concentration of 0.01 to 0.05 mg/ml. Laurdan probe excitation wavelength was λ = 360 nm, and the emission wavelengths were λ₂ = 440 nm and λ₁ = 490 nm. The
Figure 1. HPLC chromatogram of *H. perforatum*. Compounds are identified in the figure by numbers: 1. neochlorogenic acid; 2. p-cumaro-chinine acid; 3. chlorogenic acid; 4. (+)-catechin; 5. (−)-epicatechin; 6. rutin; 7. quercetin-3-galactoside; 8. quercetin-3-glucoside; 9. kempferol-3-glucoside; 10. biapigenin; 11. quercetin-3-rhamnoid.

Table 1. Percentage composition of *H. perforatum* extract.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neochlorogenic acid</td>
<td>0.13</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>0.46</td>
</tr>
<tr>
<td>P-Cumaro-chinine acid</td>
<td>0.10</td>
</tr>
<tr>
<td>(+) Catechin</td>
<td>2.56</td>
</tr>
<tr>
<td>(−) Epicatechin</td>
<td>5.32</td>
</tr>
<tr>
<td>Rutin</td>
<td>3.41</td>
</tr>
<tr>
<td>Quercetin-3-galactoside</td>
<td>8.60</td>
</tr>
<tr>
<td>Quercetin-3-glucoside</td>
<td>4.21</td>
</tr>
<tr>
<td>Quercetin-3-rhamnoid</td>
<td>1.45</td>
</tr>
<tr>
<td>Kemptferol-3-glucoside</td>
<td>0.50</td>
</tr>
<tr>
<td>Biapigenin</td>
<td>0.10</td>
</tr>
<tr>
<td>Procyanidine polymer</td>
<td>31.50</td>
</tr>
<tr>
<td>Total</td>
<td>58.34</td>
</tr>
</tbody>
</table>

The active part (fluorophore) of the Laurdan probe is located in the hydrophilic region of the lipid bilayer. Changes in the packing order of the hydrophilic part of the membrane were investigated using the Laurdan probe, on the basis of generalized polarization (GP), and were calculated with the formula (Parasassi et al., 1998):

$$\text{GP} = \frac{I_b - I_r}{I_b + I_r}$$  \hspace{1cm} (1)

Where $I_b$ is fluorescence intensity at $\lambda = 440$ nm and $I_r$ is...
fluorescence intensity at $\lambda = 490$ nm. Increased values of GP signify increased packing density of the membrane lipid polar heads, whereas decreased values of GP indicate decreased packing.

The excitation and emission wavelengths of DPH probe were $\lambda_{\text{ex}} = 360$ nm and $\lambda_{\text{em}} = 425$ nm. Fluorescence anisotropy (A) of DPH probe was calculated using the formula (Lakowicz, 2006):

$$A = \frac{(I_{II} - G I_{\perp})}{(I_{II} + 2G I_{\perp})} \quad (2)$$

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 polarization direction of the exciting wave. The active part (fluorophore) of DPH probe is located in the hydrophobic region of the lipid bilayer. Increased values of fluorescence anisotropy indicate increased rigidity of the membrane, whereas decreased values of fluorescence anisotropy indicate increased fluidity of the membrane.

The obtained values of GP for DPH were compared with those of unmodified ghosts.

**Osmotic resistance studies**

The experiments were performed on fresh pig blood. Full blood was centrifuged for 3 min at 2500 rev/min and 4°C to remove the plasma and leucocytes. The erythrocytes obtained were washed three times with a cooled (to ca. 4°C) 310 mosm PBS isotonic solution. Next, a 2% red cell suspension containing $H$. perforatum extract of 0.1 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 to 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 previously 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 relationship was determined between the percentage of hemolysis and NaCl concentration in the solution. Next, using the obtained plots, the NaCl percentage concentrations ($I_{50}$) that caused 50% hemolysis were found. The $I_{50}$ values were taken as the 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.

**RESULTS AND DISCUSSION**

**Microscopic investigation**

The erythrocytes were modified with the extract; the concentration of which in the suspension was 0.1 or 1 mg/l. The erythrocytes incubated in a water solution of the wort extract were viewed under an optical microscope and it was found that their shapes had changed, which proves that the substances contained in the extract penetrated the erythrocyte membrane. According to the bilayer couple hypothesis (Sheetz and Singer, 1974), the shape of red blood cells induced by incorporated substances allows one to determine the location of a substance within the lipid bilayer ($Ponder, 1948$; Isomaa et al., 1987; Danieluk et al., 1998; Wong, 1999; Deuticke, 2003; Tachev et al., 2004). The hypothesis maintains that the compounds concentrate mainly in the outer lipid monolayer of the membrane creating echinocytes, while substance accumulation in the inner lipid monolayer results in the formation of stomatocytes. $H$. perforatum extract mainly induced formation of echinocytes, indicating that the polyphenols it contains incorporate into the outer lipid layer of the erythrocyte membrane. The percentage share of specific forms of the cells was ascribed morphological indices on the Bessis and Brecher scale, and is given in Figure 2. The results also indicated that the number of spherocytes increased with increasing concentrations.

The pictures obtained from the electron microscope confirm the results of optical observations, which indicates that the compounds contained in the extract studied concentrate mainly in the hydrophilic part of lipid bilayer of the erythrocyte membrane, inducing formation of various forms of echinocytes (Figures 3 and 4).

**Fluorimetric studies**

The polar group packing arrangement of the erythrocyte membrane lipid bilayer was studied with the Laurdan probe on the basis of generalized polarization, values calculated from Formula (1). Fluidity in the hydrophobic part of the erythrocyte membrane was investigated by using the DPH probe, on the basis of fluorescence anisotropy values which was calculated using Formula (2). The values of generalized polarization (GP) and fluorescence anisotropy (A) are presented in Table 2. The fluorimetric studies with the Laurdan probe have shown that the generalized polarization decreases with increasing concentrations of the compound, a greater decrease in GP occurring at 37°C. The results indicate that the packing order of the hydrophilic part, that is, of the polar head groups of the membrane lipids, increases (Table 2).

The values of DPH fluorescence anisotropy decreased with increasing concentrations of the extract (Table 2). The changes were greater at 37°C content, which indicates that increased temperature facilitates binding between the polyphenol components and the membrane. These results testify to the fact that the polyphenols contained in the $H$. perforatum extract enter the hydrophobic region of the membrane, modifying its properties, which finds its expression in increased fluidity accompanied by increased disorder of the phospholipid alkyl chains.

Thus, the results of the study indicate that the extract polyphenols come into close interaction with the membrane hydrophilic surface and also penetrate its polyphenol hydrophobic region. The concentration in both
Figure 2. Percentage share of different shapes of erythrocytes induced by *H. perforatum* at 0.1 and 1 mg/ml concentration.

Figure 3. Unmodified erythrocytes.

Figure 4. Erythrocytes modified with *H. perforatum* extract.
The differences between controls and compounds containing samples were found to be significant on the basis of the Dunnett test. The calculations were performed using StartSoft STATISTICA 9.

**Osmotic resistance studies**

The osmotic resistance of erythrocytes subjected to the action of *H. perforatum* extract at 0.1 mg/ml concentration was investigated. To this end, the relationship between the extent of hemolysis and concentration of sodium chloride was plotted, as shown in Figure 5. Hence, the concentration of NaCl which induces 50% hemolysis of the erythrocytes was found, and this was assumed to be

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**Table 2.** Values of fluorescence generalized polarization and fluorescence anisotropy for the erythrocyte membrane at 23 and 37°C.

<table>
<thead>
<tr>
<th>Extract concentration mg/ml</th>
<th>GP±SD Temperature (°C)</th>
<th>A±SD Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>23</td>
<td>37</td>
</tr>
<tr>
<td>Control</td>
<td>0.411±0.032</td>
<td>0.338±0.028</td>
</tr>
<tr>
<td>0.01</td>
<td>0.388±0.011</td>
<td>0.354±0.004</td>
</tr>
<tr>
<td>0.02</td>
<td>0.309±0.021</td>
<td>0.289±0.027</td>
</tr>
<tr>
<td>0.03</td>
<td>0.244±0.024</td>
<td>0.227±0.035</td>
</tr>
<tr>
<td>0.04</td>
<td>0.203±0.024</td>
<td>0.164±0.020</td>
</tr>
<tr>
<td>0.05</td>
<td>0.164±0.037</td>
<td>0.135±0.015</td>
</tr>
</tbody>
</table>

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The hydrophilic and hydrophobic regions is decided by the compound’s amphiphilic properties set by the structure of the molecules; for example, epicatechin present in the wort extract exhibits a hydrophobic character (Tsuchiya, 2001). In connection with the interaction with the erythrocyte membrane, the electrostatic or the hydrophobic interaction is dominant and is thus responsible for the higher concentration of polyphenolic compounds either in the hydrophobic or hydrophilic part of the lipid bilayer of the erythrocyte membrane.

The results are presented in the form: Mean value ± standard deviation, calculated at a confidence level $\alpha = 0.05$ (p<0.05) from 5 independent measurements. Two-factor analysis of variance was carried out using ANOVA.
a measure of osmotic resistance. Such a concentration for extract modified erythrocytes is 0.65% which is markedly lower than that of the control erythrocytes, which is 0.67%. These results indicate that the wort extract causes an increase in the osmotic resistance of erythrocytes. The lower concentration of a hypotonic NaCl solution responsible for 50% hemolysis of modified erythrocytes relative to control, the higher the osmotic resistance. This means that erythrocytes treated with wort extract are less sensitive to the tonicity of the medium. It should be emphasized that cell modification was carried out in a medium where the extract concentration was 0.1 mg/ml and was thus two times higher than the highest concentration used in the fluorimetric measurements. Such a high concentration allowed us to establish that the compounds do not cause erythrocyte hemolysis, and thus are not destructive to the erythrocyte membrane.

Conclusions

On the basis of the results obtained, it can be postulated that the lipid bilayer of a biological membrane is the main site for the action of polyphenolic substances, which are, like the lipid molecules, of amphiphilic character. Therefore, the molecular mechanism of the action is connected with structural disturbances that develop in the lipid bilayer of the cell membrane due to incorporation of polyphenols. The polyphenolic compounds of the H. perforatum extract affects the shape of erythrocytes, their osmotic resistance, polar group packing arrangement and fluidity in the phospholipid alkyl chain of the erythrocyte membrane lipid bilayer. As a result, the cells transform mostly to echinocytes, indicating that the substances they contain locate mainly in the outer monolayer of the erythrocyte membrane. The fluorimetric studies showed that the compounds present in the membrane induce a decrease in the packing order of the polar heads of membrane lipids, because the GP values of the Laurdan probe decrease with increasing extract concentration. The H. perforatum extract also induced an increase in fluidity of the erythrocyte membrane in its hydrophobic region, thus decreasing the values of fluorescence anisotropy as assayed by the DPH probe. It was also found that the erythrocyte membrane becomes more resistant to osmotic pressure in hypotonic solutions of sodium chloride, as 50% of the erythrocytes undergo hemolysis at a lower concentration than the control cells.

The presence of polyphenolic compounds alters the erythrocyte membrane in its hydrophilic and hydrophobic regions; although it does not destroy the membrane as hemolysis is not induced even at high concentrations. What more, it exerts a protective action by increasing the osmotic resistance. Based on the results of the present work, one may conclude that the very good antioxidant properties of H. perforatum extract result from its ability to incorporate into the erythrocyte membrane lipid phase. Thus, the membrane embedded polyphenolic substances constitute a barrier against free radicals that attack the lipid phase of the membrane, which is the first line of attack.

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