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Lipophilicity of flavonoid complexes with iron(II) and their interaction with liposomes

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ABSTRACT

We studied complex formation of flavonoids quercetin and taxifolin with iron(II) and the complex influence on phase transitions of phospholipid bilayer. UV–Vis spectroscopy revealed that the stoichiometry of flavonoid/iron complexes was equal to 3:2 and 2:1. Molecular modeling and experimental measurements demonstrated the increase of flavonoids lipophilicity after the complex formation. A considerable influence of quercetin–iron complex on Palmitoyl–Oleoyl–Phosphatidylethanolamine transitions from bilayer to hexagonal H_{II} phase was detected by differential scanning calorimetry. The obtained data are related to flavonoid/iron complexes bioavailability, their influence on cell membrane functioning, and should be considered in designing liposomal vehicles for drug and gene delivery.

1. Introduction

Flavonoids attract great attention because they are present in regularly consumed fruits and vegetables. Moreover, these compounds are regarded not only as powerful antioxidants, but also as important biological regulators capable of preventing various diseases including cardiovascular, autoimmune, inflammatory, neurodegenerative and carcinogenic ones [1].

It is known that flavonoids can interact with transient metal cations and prevent their involvement in peroxidation processes [2]. The protection is explained by the ability of flavonoids to bind iron and copper cations and prevent their participation in production of reactive hydroxyl radicals through Fenton reaction [3,4]. Besides, complexes of flavonoids with iron or copper can decompose superoxide radical to oxygen and hydrogen similarly to enzyme superoxide dismutase [5]. It is known that the unsaturated double bonds of carbohydrate chains present in hydrophobic area of biological membranes may undergo peroxidation [6]. Thus the lipophilicity of flavonoids responsible for their penetration into the hydrophobic region of the lipid bilayer may contribute to membrane protection against peroxidation [7]. Moreover, the lipophilicity of the compounds is regarded as an important factor responsible for their transport into the cell and expression of their biological activity in the cytoplasm [8]. Recently presented analyses of computer models suggest the dependence of lipophilicity from stoichiometry of the flavonoid complexes with iron [9]. It was suggested that metal chelation may promote flavonoid penetration into the hydrophobic region of biological membranes.

Here we studied the ability of flavonoid–iron complexes to interact with lipids. We used flavonoids representing different groups (Fig. 1A and B). Thus, quercetin belongs to flavonols, while taxifolin – to flavanonols.

2. Materials and methods

Lipids 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) were purchased from Avanti Polar Lipids, Inc. (USA), quercetin and taxifolin were from Sigma–Aldrich (USA), TRIS–HCl was from Serva (Germany). All the solutions were prepared on deionized water (Milli-Q, Arium 611VF, Germany).

Abbreviations: DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DSC, differential scanning calorimetry; PCS, photon correlation spectroscopy; POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine.

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2.1. Liposome preparation

Phospholipids were dissolved in chloroform, dried under argon stream and kept under vacuum overnight to remove the solvent. Then lipids were hydrated in 10 mM Tris–HCl buffer (pH 7.4) by mechanical shaking at 35 °C. Taxifolin dissolved in 70% ethanol or quercetin dissolved in 50% DMSO was added to suspension of liposomes at the quantity such that the final amount of solvents in the samples was less than 0.1%. After addition of polyphenolic compounds the samples were vortexed and heated to 35 °C. In some experiments we used large unilamellar vesicles (LUV) of 100 nm prepared with mini-extruder (Avanti Polar Lipids, USA) according to standard procedure [18].

2.2. Differential scanning calorimetry (DSC)

The excess heat capacity of multilamellar liposomes prepared from DMPC or POPE was recorded with differential adiabatic scanning microcalorimeter DASM-4 (IBP RAS, Pushchino, Russia). The heating rate was 1 K/min. A suspension of multilamellar liposomes was prepared on 10 mM Tris–HCl buffer (pH 7.4) and contained 0.2 mg/ml DMPC, or 2 mg/ml POPE. The thermograms were analyzed with Microcal Origin software (Northampton, MA).

2.3. Photon correlation spectroscopy (PCS)

The size of flavonoid–Fe^{2+} complexes was measured on N4 PLUS Submicron Particle Size Analyzer (Beckman Coulter, USA) equipped with Size Distribution Processor (SDP) that was used for a detailed analysis of the particle size distribution. 20 μl of flavonoid (10^{-3} M) and appropriate amount of $FeSO_4$ were added in 2 ml cuvette with 10 mM Tris–HCl (pH 7.4) and measured at the angle of 90°. To avoid possible dust contamination the buffer solution was preliminarily extruded through 10 μm carbon filter. After filtration the control samples of buffer did contain particles.

2.4. Partition coefficient of flavonoids and their complexes with iron

To measure the partition coefficient of flavonoids or their complexes with iron in the octanol–water system we used the earlier described method [10] with some modifications. 10 mM Tris–HCl buffer (pH 7.4) was used as an aqueous phase. To achieve saturation of water with octanol and octanol with water, both the compounds were mixed as 1:1, shacked for a few hours and kept at room temperature overnight. A flavonoid was added to the system, vortexed during half an hour, then iron was added and vortexed again. Then system was left for 4–5 h for phase segregation and centrifuged for 15 min at 1500 rev/min on 5414 Eppendorf (Germany). After that the aqueous phase was retrieved by syringe and the flavonoid concentration was measured on spectrophotometer Shimadzu UV–VIS 1800. The $\log P$ in the octanol/buffer system was calculated as $\log[C_o/C_b]$, where $[C_o]$ is the concentration of the flavonoid in the octanol phase, $[C_b]$ is the concentration of the flavonoid in buffer. The value of $[C_o]$ was calculated using the equation $[C_o] = [C_i] - [C_b]$ where $[C_i]$ is the initial concentration of a flavonoid added in buffer.

2.5. UV–Vis spectroscopy determination of flavonoid/iron stoichiometry

For spectral analyses of samples we used spectrophotometer Shimadzu UV–VIS 1800. Two approaches were used:

a. Job's continuous variation method (mole ratio method) [11]. The solutions of a flavonoid (20 mM) and $FeSO_4$ with identical concentrations (20 mM) were mixed in varying amounts, but their sum (total volume) remained constant. We prepared nine samples with flavonoid/iron ratios equal to 9:1; 8:2; 6:4; 5:5; 4:6; 2:8; 1:9 in 10 mM Tris–HCl, pH 7.4 buffer, at 20 °C. Then the maximal absorbance value of the flavonoid–iron complex (D) was plotted against the mole fraction of the reactants. The resultant curve has a maximum at the flavonoid/iron ratio that corresponds to the compounds stoichiometry.
b. Titration of flavonoids with increasing concentrations of $FeSO_4$ [11]. The maximal absorbance of the flavonoid–iron complex (D) was measured for a series of solutions in 10 mM Tris–HCl, pH 7.4 buffer containing 20 mM flavonoid and $FeSO_4$ varying from 1 mM to 80 mM. The value of (D) was plotted against the concentration of the added iron. The experimental points obtained were approximated by a straight line with a break located at the value of the flavonoid/iron stoichiometry.

2.6. Computer calculations of lipophilicity

Lipophilicity was calculated with ChemBio3D Ultra 12.0 software package (PerkinElmer, GB) on the basis of the chemical formula of the compounds. The calculated lipophilicity ($C\log P$) was determined as a logarithm of the partition coefficient in n-octanol/water. The calculation accuracy of this software was tested on various flavonoids and good agreement with experimental data was demonstrated [12].

3. Results

3.1. Determination of the octanol:water partition coefficient for flavonoid–iron complexes

Computer analyses of molecular models of flavonoid–iron complexes suggested by Ren and colleagues revealed 3-hydroxy-4-carbonyl group as the most probable chelation site for Fe cations. Slightly less preferable were 4-carbonyl-5-hydroxyl and 3′-4′-hydroxyl groups. The quercetin/iron ratio equal to 2:1 was found to be the most thermodynamically stable [4]. A detailed analysis of quercetin–iron complexes presented by Guo and colleagues [13] revealed that the preferable quercetin/iron ratios were 1:1, 2:1 and 3:2 where 3-hydroxy-4-carbonyl and 3′-4′-hydroxyl groups were predominantly involved (Fig. 1C and D). According to our calculations based on computer models, the lipophilicity of flavonoid–iron complexes ($C\log P$) with flavonoid/iron ratio 1:1 is lower than that of a free flavonoid, while 2:1 and 3:2 complexes are more lipophilic (Table 1).

Our experimental values of free flavonoids lipophilicity ($\log P$) are similar to the values presented in Table 1 and those available in literature, though the literature data vary in different studies [14–17]. We found that the lipophilicity of both quercetin and taxifolin increases after addition of iron (Fig. 2). The calculated values of 3:2 complexes presented in Table 1 are considerably larger than the values obtained in the experiment.

3.2. Determination of flavonoid–iron complexes stoichiometry with UV–Vis spectroscopy

Quercetin adsorption with maximum 373 nm is designated as band I while adsorption maximum at 255 nm is designated as band II [18]. When a flavonoid and iron are mixed, the complex is formed for less than one minute. During titration of quercetin with increasing quantities of iron the absorption maximum at 373 nm decreases while a new maximum at 425 nm appears and grows which demonstrates formation of a quercetin–iron complex

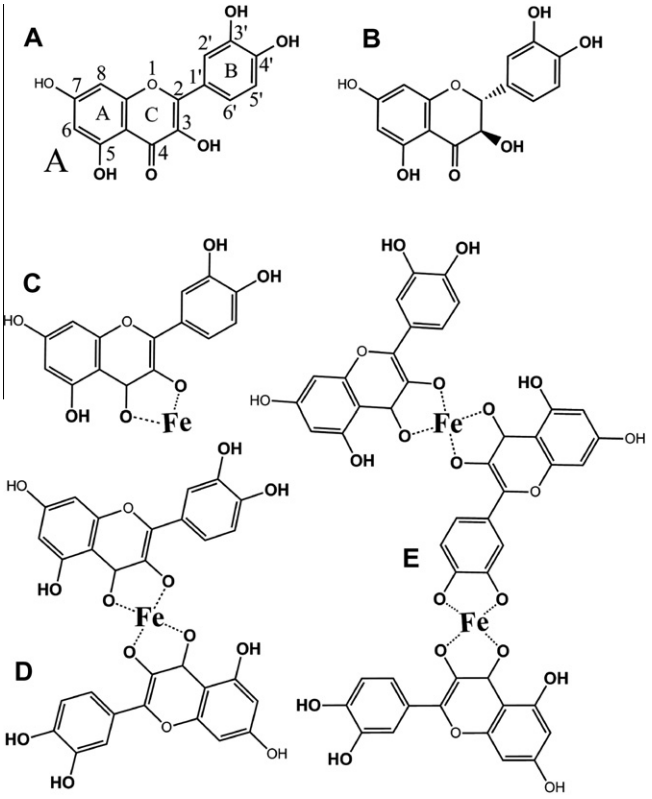

Fig. 1. Flavonoids and their complexes with iron used in the present study. (A) Quercetin, (B) taxifolin. Hydroxyl moieties able to interact with iron cations are highlighted by larger letters. The conventional numeration of carbon atoms and literal notation of phenolic rings is also presented. (C–E) Structure of quercetin–iron complexes suggested by Guo and colleagues according to UV–Vis, NMR and EPR spectrometry [21]. The quercetin/iron stoichiometry of complexes is 1:1 (C); 2:1 (D); 3:2 (E).

Table 1
Calculated lipophilicity (*C*log*P*) of free flavonoids and their complexes with iron designated as distribution coefficients in octanol–water system. For calculation we used complexes of quercetin presented in Fig. 1 and similar complexes of taxifolin. The lettering of complexes presented in the Fig. 1 and in the table is identical.

Flavonoid	Complex	Flav:Fe	*C*log*P*
Quercetin	A (Free)	1:0	1.5037
	C	1:1	0.8663
	D	2:1	3.4775
	E	3:2	5.5498
Taxifolin	B (Free)	1:0	0.7710
	C	1:1	0.1103
	D	2:1	1.9655
	E	3:2	3.2818

(Fig. 3A,a). The plot of adsorption at 425 nm against the flavonoid/iron ratio reveals a break at Fe(II) concentration equal to 10 μM (Fig. 3A,b). Since the concentration of quercetin in this experiment was 20 μM we have the ratio of quercetin/iron equal to 2:1. We obtained similar results in experiments with taxifolin, though the UV–Vis spectrum of taxifolin was different (not presented). Spectra of quercetin–iron complexes obtained at different molar ratios of the compounds gives a curve with maximum at 3:2 ratios (Fig. 3B). A similar result was obtained in an experiment with taxifolin (not presented). The results obtained demonstrate that the interaction of quercetin or taxifolin with iron(II) cations may result in the formation of complexes with stoichiometry flavonoid/iron equal to 2:1 or 3:2.

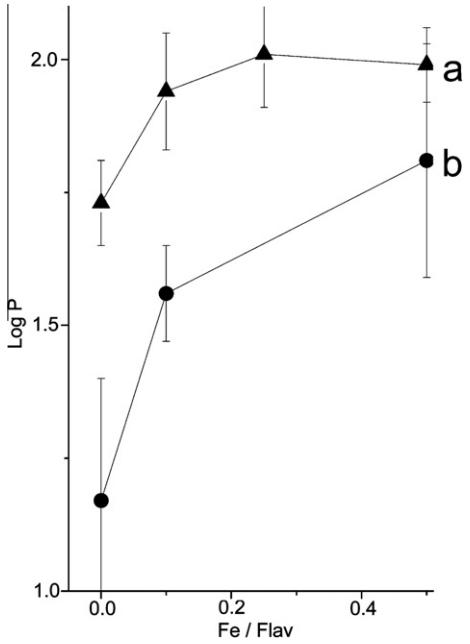

Fig. 2. Distribution coefficient (log*P*) dependence from flavonoid/iron ratio experimentally obtained in octanol:water system for quercetin (a) and taxifolin (b).

3.3. Influence of quercetin/iron(II) complexes on phase transitions of lipids

It is well known that flavonoids can interact with the phospholipid bilayer of liposomes and influence lipid phase transitions observed by DSC [7,19–24]. We suppose that changes in the lipophilicity of flavonoids after iron chelation may influence their interaction with the phospholipid bilayer and modify phase behavior of lipids. In calorimetric studies we used DMPC and POPE phospholipids because the temperature of their transitions is suitable for DSC studies. It is known that DMPC has a phase transition at about 24 °C related to melting of lipid hydrocarbon chains and transition from gel to a liquid crystal. This transition can be registered by DSC as a narrow maximum on the thermogram (Fig. 4A). Addition of iron(II) sulfate (10^{-6} M) which is approximately close to the iron concentration in the human blood [25] had no significant influence on the lipid melting while free quercetin decreased the temperature, decreased the height, and increased the transition width of DMPC melting. After consecutive addition of quercetin followed by iron, the quercetin–iron complex initiated downshift of the transition maximum and initiated changes in the transition shape similar to those of free quercetin. Formation of quercetin/iron(II) complex in these conditions is confirmed by UV–Vis spectroscopy (not presented). If a preliminarily prepared complex of quercetin and iron was added to DMPC no changes in lipid melting were detected. This phenomenon can be explained by lipophilicity and low solubility of the quercetin–iron complex in water. PCS analyses revealed that in the conditions of our experiments the quercetin–iron complex produced insoluble particles of 10–20 nm and 1–5 μm (not presented) that were unable to interact with liposomes.

DSC analyses of POPE liposomes reveals two phase transitions (Fig. 4B). A low-temperature transition observed at about 25 °C is similar to that of DMPC liposomes observed at about 24 °C. As it was mentioned above, the transition is explained by melting of hydrocarbon chains of lipids. A high-temperature transition of POPE appears at about 69 °C and is explained by transformation of the bilayer structure to the hexagonal H_{II} phase where lipid produces bundles of inverted tubules arranged into a hexagonal lattice [26,27]. We found that separately added quercetin, iron, or consec-

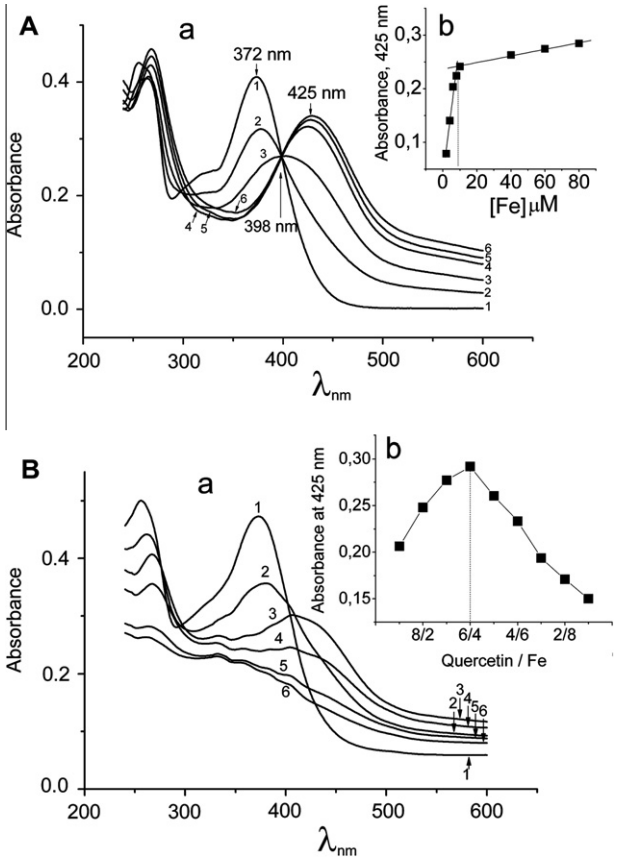

Fig. 3. Determination of quercetin/iron(II) complexes stoichiometry with UV–Vis spectroscopy. (A) Titration approach: (a) UV–Vis spectra of 20 μM quercetin (1), and complexes of quercetin/iron obtained by addition of 2 μM, 4 μM, 6 μM, 8 μM and 10 μM of iron to 20 μM of quercetin (numbers 2–6 correspondingly); (b) dependence of adsorption value at 425 nm from concentration of added iron determined from the spectra presented on the panel (a). The curve break appears at about 10 μM of iron and corresponds to stoichiometry quercetin/iron = 2:1. (B) Method of molar ratio: (a) UV–Vis spectra of quercetin (1) and complexes of quercetin/iron obtained by mixing of quercetin and iron in molar ratio 8:2, 6:4, 4:6, 2:8; 1:9 (numbers 2–6 correspondingly); (b) dependence of adsorption at 425 nm from quercetin/iron ratio obtained from the panel (a). Maximum appears at ratio quercetin/iron = 6:4 (or 3:2).

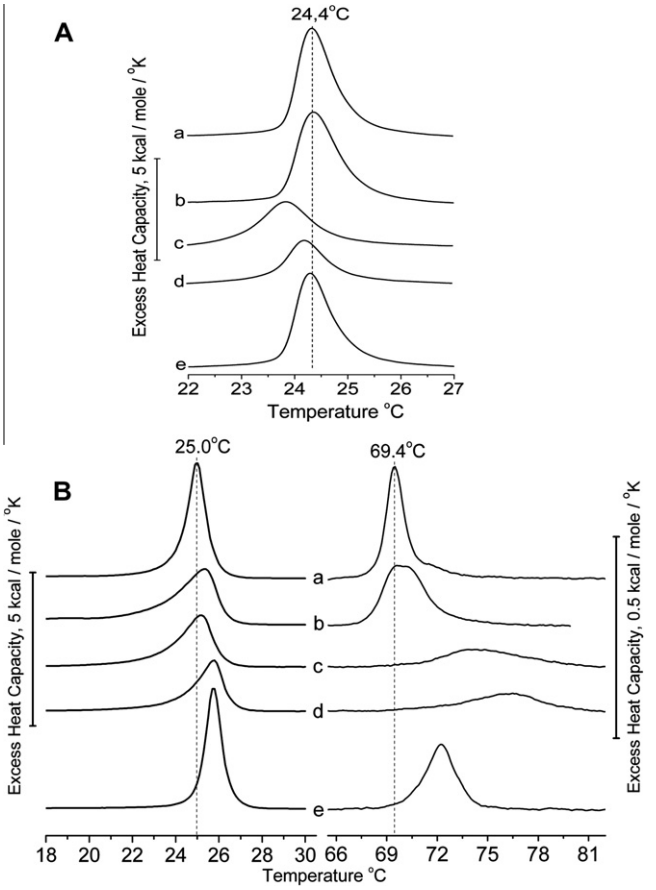

Fig. 4. Influence of quercetin and quercetin/iron complexes on DSC thermogram of: (A) DMPC liposomes. Concentration of lipid was 295×10^{-4} M (0.2 mg/ml), quercetin: 4×10^{-5} M, $FeSO_4$: 4×10^{-6} M; (B) POPE liposomes. Concentration of lipid was 195×10^{-3} M (2 mg/ml), quercetin: 4×10^{-4} M, $FeSO_4$: 4×10^{-5} M. The thermograms presented here are of untreated liposomes (a); liposomes treated by quercetin (b); treated by iron (c); treated by quercetin followed by iron added 30 min later (d); treated by preliminary produced quercetin/iron complex (e).

utively added quercetin followed by iron produced similar changes in the low-temperature transition. The height of the transition decreased while the width increased. As in experiments with DMPC liposomes, the effect on the transition shape of a preliminarily prepared quercetin–iron complex was small, though a one-degree shift of the transition maxima towards higher temperatures was observed.

Changes in the high-temperature transition of POPE liposomes were much more pronounced than the changes in the low-temperature transition. In samples treated by iron the maximum of the transition shifted 5 °C towards higher temperatures while the height of the transition decreased and the width of the transition increased. The largest changes were observed in the samples treated by quercetin followed by iron. The effect of a preliminarily formed quercetin–iron complex was considerably smaller. As it was mentioned above, the decreased effect of a preliminarily formed complex could be explained by its low solubility in water and formation of insoluble particles.

4. Discussion

Flavonoids, like some other plant compounds, have versatile influence on cell. It is possible that the broad spectrum of flavonoids action could be explained by their ability to modulate phase transitions of lipid bilayer were numerous membrane receptors and signal transducers are integrated. In the presented study we want to emphasize, that not free flavonoids but mostly flavonoid–iron complexes participate in regulation of cell metabolism. Earlier, using molecular models, we suggested that the flavonoids lipophilicity may considerably increase after iron chelation [9]. Here we presented experimental evidence of this increase after addition of iron(II) cations to quercetin and taxifolin. Our results demonstrate formation of flavonoid:iron complexes with stoichiometry 2:1 and 3:2 (Fig. 3). According to calculations with the use of computer models the lipophilicity of complexes is considerably larger than the lipophilicity of free flavonoids (Table 1). The experimental analyses also demonstrate the lipophilicity ($\log P$) increase when iron(II) was added to quercetin or taxifolin (Fig. 2). The observed changes of lipophilicity could be explained by charge redistribution in molecules after the complex formation as we discussed earlier [9]. We also found that the lipophilic complexes of flavonoids with iron cannot interact with the lipid bilayer because they produce insoluble particles. To provide conditions necessary for complexes to interact with the phospholipid bilayer, flavonoids should first be added to liposomes, and later the iron should be added to produce complexes inside the hydrophobic region of the bilayer.

Phosphatidylcholine and phosphatidylethanolamine are often used for preparation of liposomes applicable for drugs and genes

delivery. The delivery efficiency depends on the phase transitions of lipids [28]. The ability of flavonoid–metal complexes to influence the phase behavior of phosphatidylethanolamine could be especially important in liposomes containing this lipid, for example those used in gene therapy [29]. Liposomes loaded with flavonoids may considerably increase the flavonoids concentration in blood and facilitate their bioavailability [30]. Lipophilic complexes of flavonoids with iron could be useful for development of liposomal vehicles for drug and gene delivery.

The DSC study of lipid phase transitions reveals that the quercetin–iron complex mainly influences the phase transition of POPE from the bilayer to the hexagonal H_{II} phase (Fig. 4). The reviled ability of flavonoid–iron complexes to influence phase transitions of phosphatidylethanolamine is very interesting because this lipid is responsible for maintenance of functional conformation of membrane proteins. In membranes of living cells this transition of phosphatidylethanolamine could influence functioning of proteins responsible for methabolism regulation. For example, it is known that functioning of protein kinase C is modulated by lipid phases [31], though the influences of flavonoid-iron complexes on this process have not been studied yet.

The interaction of G-proteins with phospholipid bilayer is also controlled by phase transitions of phosphatidylethanolamine [32]. The involvement of plant polyphenols in regulation of G-proteins through lipid surrounding is responsible for prevention of numerous age-relative diseases [33], control of blood pressure [34], protection against cancer [35] and Alzheimer disease [36].

It is also necessary to mention the phosphatidylethanolamine-binding protein (PEBP) involved in functioning of cardiovascular system [37], carcinogenesis [38,39], cell apoptosis [40] tumor necrosis [41], protection against bacterial infection [42], and Alzheimer disease [43].

Acknowledgments

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