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Oxidation of liposomal membrane suppressed by flavonoids: Quantitative structure–activity relationship

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Abstract—Antioxidant activity of the set of 12 flavonoids in heterogeneous membrane system of dioleoyl phosphatidylcholine liposomes stressed by peroxidative damage induced by 2,2′-azobis-(2-amidinopropane)hydrochloride azoiniciator and evaluated by the thiocyanate method was assessed. Antioxidant activity (pIC_{50}) was correlated with 19 molecule parameters calculated for the minimum energy conformers of the compounds tested. The linear regression analysis revealed that the parameter of hydration energy E_{HYDR} ($R = -0.747$) was the best predictor of antioxidant activity in a liposomal system.

1. Introduction

The largest group within the plant polyphenols including over 8000 well-known compounds is represented by flavonoids. The flavonoids are formed biosynthetically from a phenylpropanoid precursor, linked to three malonyl coenzyme A units. These polyphenols are structurally derived from flavone, the parent compound, bearing a tricyclic (C_6–C_3–C_6) skeleton.[1] According to oxidation level of the central pyrane ring, flavonoids have been distributed into seven groups: flavanols, flavanonols, flavanones, flavones, flavonols, isoflavones, and anthocyanins.[2] They are produced as plant secondary metabolites and play an important role in biological processes, such as pigmentation, germination, pollination attraction, and protection against UV light. They act in the plant defense system and as signal molecules[3,4] as well.

As many flavonoids are found in fruit, vegetables, cereals, tea, and wine, they are an integral part of human diet. An estimated daily intake of flavonoids is 0.023–1 g, which is positively associated with prevention against several disorders.[2] Some flavonoids have been found to possess antitumor,[5–7] anti-diabetic,[8] anti-atherosclerotic and cardioprotective,[9–11] anti-inflammatory,[12,13] anti-peroxidant,[14,15] anti-osteoporotic,[16] anti-microbial,[17] and anti-viral[18,19] characteristics. The most beneficial and the most studied health effect of flavonoids is their antioxidant impact.[20–23] In addition, the aforementioned activities may be basically ascribed to the antioxidant abilities of flavonoids. This points to a very promising role of these compounds in the search for effective preventive and therapeutic agents application, offering a host of health benefits apart from their natural inherent character.

The definition and quantification of structure–activity relationship (SAR) may be a very useful tool for the pre-

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diction of biological activities within this group of compounds. There are quite a few studies reporting on SAR of flavonoids focusing on the radical scavenging activity of flavonoid antioxidants.[24–27] However, much less attention has been paid to the relationship between structure and inhibition of lipid peroxidation, and quantitative evaluation of the relationship between antioxidant efficiency and relevant molecule parameters. Considering this fact, the objective of the present study was to investigate the influence of flavonoid structure-related parameters on lipid peroxidation inhibition of a series of flavonoids (Table 1) in a model membrane system of DOPC liposomes in terms of quantitative structure–activity relationship (QSAR) analysis.

2. Results

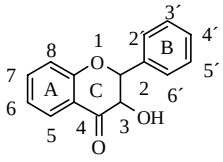
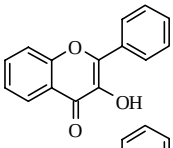
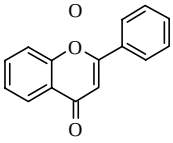
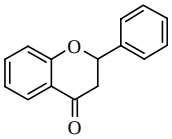
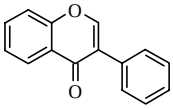
2.1. Inhibition of lipid peroxidation

A set of flavonoids **1**–**12** (Table 1) was assessed for their ability to suppress the oxidation of liposomal membrane. Peroxidation of the unilamellar dioleoyl phosphatidylcholine (DOPC) liposomes was triggered by thermal decomposition of the hydrophilic compound 2,2′-azobis(2-amidinopropane)hydrochloride (AAPH) in the presence of flavonoid antioxidant. Our previous results showed that in a complete reaction system of DOPC liposomes/AAPH/buffer, lipid peroxidation proceeded at a constant rate, and an approximately linear time-dependent increase of lipid hydroperoxides was observed without any induction period.[28] No accumulation of hydroperoxides was observed in the absence of AAPH or liposomes. The inhibitory effect of each flavonoid antioxidant was expressed as the value IC_{50} or pIC_{50} (−log value of the concentration inhibiting DOPC-hydroperoxide production at 80-min incubation time by 50%), respectively. The hierarchy of antioxidant activities of flavonoids and their structural features are shown in Table 1. The most potent antioxidants were flavonol quercetin (**2**) and flavanonol taxifolin (**1**) with IC_{50} values of 11.7 μmol/l and 37.3 μmol/l, respectively.

2.2. Computations

Nineteen different molecular parameters were calculated for the minimum energy conformers of the compounds tested by the program Hyperchem[29] to evaluate the quantitative relationship between the structural parameters and the antioxidant activity of the set of flavonoids. These molecular parameters can be divided into physicochemical (ΔE, ΔH, ε(HOMO), ε(LUMO), ε(LUMO)r, $\Delta\varepsilon$, $\log P$, Σq(O), Σq(H), q(O), q(H), s(O), and α) and structural (I, V, S, E_{HYDR}, nOH, and MR) representations. The calculated parameters were correlated with dependent variables, antioxidant activity expressed in pIC_{50} value. At first, a simple linear regression analysis was used to find out the best descriptor from among all the parameters evaluated. The calculated values of the parameters E_{HYDR} and ε(LUMO)r that yielded the best values of correlation coefficients are given in Table 1 along with values of antioxidant activity (IC_{50}). The values showing the difference between the heat of formation of a parent molecule (H_F) and its phenoxyl radical

Table 1. The structures of flavonoid subclasses and their representatives tested **1**–**12**, values of their antioxidant activity (expressed as IC_{50}) in peroxidatively damaged DOPC liposomes and calculated values of parameters hydration energy (E_{HYDR}) and energy of the lowest unoccupied molecule orbital pertinent to this radical (ε(LUMO)r) yielding the best values of correlation coefficients

Compound	Ring structure	–OH position	IC_{50} (μmol/l)[a]	E_{HYDR} (kcal/mol)	ε(LUMO)r (eV)
Flavanol					
Taxifolin (**1**)		3, 5, 7, 3′, 4′	37.30 ± 0.11	−32.73	−0.89
Flavonol					
Quercetin (**2**)		3, 5, 7, 3′, 4′	11.66 ± 0.09	−32.83	−0.99
Morin (**3**)		3, 5, 7, 2′, 4′	57.23 ± 0.26	−32.10	−1.01
Galangin (**4**)		3, 5, 7	119.59 ± 1.94	−20.57	−0.97
Flavone					
6-Hydroxyflavone (**5**)		6	190.63 ± 2.01	−11.46	−1.09
7,8-Dihydroxyflavone (**6**)		7, 8	60.49 ± 0.56	−16.18	−0.92
Chrysin (**7**)		5, 7	67.92 ± 0.49	−17.13	−1.13
Baicalein (**8**)		5, 6, 7	158.49 ± 0.98	−21.88	−1.12
Flavanone					
Naringenin (**9**)		5, 7, 4′	68.64 ± 1.23	−23.35	−0.80
Hesperetin (**10**)		5, 7, 3′,	52.33 ± 0.16	−23.95	−0.79
6-Hydroxyflavanone (**11**)		4′-OCH_3, 6	136.77 ± 1.47	−11.49	−0.61
Isoflavone					
Daidzein (**12**)		7, 4′	79.05 ± 0.33	−19.40	−0.93

[a] The data (IC_{50} values) are presented as the means ± SEM for at least three independent experiments.

Table 2. Calculated values of the parameter ΔH for all the possible radical formations in the molecule of the flavonoids **1**–**12**

Compound	ΔH (kcal/mol)							
	3-OH	5-OH	6-OH	7-OH	8-OH	2′-OH	3′-OH	4′-OH
Taxifolin (**1**)	66.72	46.97	—	45.01	—	—	32.70[a]	36.27
Quercetin (**2**)	31.70[a]	47.56	—	46.36	—	—	33.36	37.28
Morin (**3**)	32.14	46.49	—	47.80	—	41.09	—	42.14
Galangin (**4**)	31.64	47.47	—	46.12	—	—	—	—
6-Hydroxyflavone (**5**)	—	—	37.17[a]	—	—	—	—	—
7,8-Dihydroxyflavone (**6**)	—	—	—	37.52	32.43[a]	—	—	—
Chrysin (**7**)	—	47.77	—	44.62[a]	—	—	—	
Baicalein (**8**)	—	39.54	33.20[a]	39.00	—	—	—	—
Naringenin (**9**)	—	47.42	—	44.46	—	—	—	37.9[a]
Hesperetin (**10**)	—	47.42	—	44.48	—	—	—	35.75[a]
6-Hydroxyflavanone (**11**)	—	—	35.38[a]	—	—	—	—	—
Daidzein (**12**)	—	—	—	41.67	—	—	—	37.59[a]

[a] The lowest value of ΔH in the molecule indicating the preference for radical formation.

(H_A) derived from one of the alternative-OH groups (ΔH) are shown in Table 2.

A list of structural parameters, as well as the values of correlation coefficients for the linear regression analysis between pIC_{50} values and these parameters, are shown in Table 3. As shown in Table 3 and Figure 3, the best obtained correlation (1) was that obtained between the antioxidant activity and the parameter of hydration energy (E_{HYDR}).

$$pIC_{50} = -0.0319 * E_{HYDR} + 3.46 \quad (n = 12, R = -0.747, p < 0.005, s = 0.227) \tag{1}$$

Including the parameters such as ΔH, ε(LUMO)r, and hydration energy (E_{HYDR}) in the two-parameter Eqs. 2,3 resulted in slightly increased R values (Fig. 4).

$$pIC_{50} = -0.035 * E_{HYDR} + 0.012 * \Delta H + 2.99 \quad (n = 12, R = 0.756, p < 0.022, s = 0.235) \tag{2}$$

$$pIC_{50} = 0.033 * E_{HYDR} + 0.29 * \varepsilon(\text{LUMOr}) + 3.72 \quad (n = 12, R = 0.759, p < 0.021, s = 0.234) \tag{3}$$

Table 3. Values of correlation coefficients (R) of linear regression analysis between values pIC_{50} and various molecule parameters

Molecule parameter	R
ΔE	−0.25
ΔH	−0.23
C log P	−0.58
ε(HOMO)	0.29
ε(LUMO)	−0.26
E_{HYDR}	−0.75
V	0.63
S	0.59
Σq(O)	−0.71
Σq(H)	0.70
MR	0.63
α	0.63
nOH	0.72
I	0.58
q(O)	0.05
q(H)	0.27
s(O)	−0.36
ε(LUMO)r	0.05
$\Delta\varepsilon$	0.35

Notes: ΔE, difference between total energy of a parent molecule and its radical formed; ΔH, difference between heat of formation of a parent molecule and its radical formed; ε(HOMO), energy of the highest occupied molecule orbital; ε(LUMO), energy of the lowest occupied molecule orbital; $\Delta\varepsilon$, HOMO–LUMO gap; C log P, partition coefficient; E_{HYDR}, hydration energy; V, volume; S, surface; Σq(O), Σq(H), sums of the partial charges of oxygen and hydrogen atoms pertinent to OH groups; α polarizability; nOH, number of OH groups; I, indicator variable (which is the sum of the following indicators: presence of the C2,C3-double bond ($I = 1$) or two of C3,C5,C7-OH groups ($I = 1$) or two of C3′,C4′,C5-OH groups ($I = 1$), or absence of above situations ($I = 0$)); q(O), q(H), partial charges of the oxygen and hydrogen atoms pertinent to OH group showing the lowest value of ΔH and ΔE; s(O), spin density pertinent to the oxygen radical derived from this group; ε(LUMO)r, energy of the lowest unoccupied molecule orbital pertinent to this radical.

3. Discussion

The flavonoids have been shown to exert effective inhibitory properties in many lipid peroxidation models.[25,30–35] Several studies have been carried out on the structure–activity approach.[25,33–35] Vaya et al.[34] reported on the quantitative relationship between the enthalpy of phenoxy-radical formation (ΔH) and the inhibitory activity of flavonoids tested in a low-density lipoprotein (LDL) oxidation model. However, there is dearth of knowledge with regard to the examining the contribution of other structural parameters to the inhibitory effect of lipid peroxidation multiple regression analysis approach. A more specific method than thiobarbituric acid reactive substances (TBARS) measurement is needed to assess the lipid peroxidation of the model membrane system.[36]

A series of flavonoids **1**–**12** (Table 1), comprising of the representatives from the five different subclasses, was selected based on their structural differences to allow the attribution of specific activity to specific structural variations and functional groups. From among the 12 flavonoids tested, four compounds (**5**–**8**) possessed a flavone structure; three flavonols (**2**–**4**), three flavanones (**9**–**11**), one isoflavone (**12**) and one flavanonol structures (**1**).

The best inhibitors of lipid peroxidation in a liposomal membrane system were flavonol quercetin (**2**) and flavanonol taxifolin (**1**). In general, the highest inhibitory effect on DOPC membrane oxidation was attributed to those compounds possessing two hydroxyl groups on the ring B in the mutual *ortho* position. This finding is in accordance with the results of LDL oxidation inhibition, published by Vaya et al.[34] and Dugas et al.[37] in which C3′, C4′-catechol structure in the B ring was considered as particularly significant. The presence of this catecholic moiety probably weighed down the lack of C2,C3-double bond of taxifolin (**1**), which has been shown to be prerequisite for good antioxidant effect of flavonoids.[38,39] Thus, quercetin (**2**) and taxifolin (**1**) showed the best antioxidant activities. The substitution of the –OH group at the position C4′ by a methyl moiety reduced the antioxidant potential of catecholic moiety as documented by IC_{50} of hesperetine (**10**). A slightly reduced activity was also shown for morin (**3**) possessing two hydroxyl groups on the ring B in the mutual *meta* position. The double-hydroxylated analogues (**6**) and (**7**) containing both hydroxy groups on the ring A in the mutual *ortho* and *meta* positions (without any additional hydroxy-moieties on ring B) exhibited lower antioxidant activities.

Though the flavonoids quercetin (**2**) and morin (**3**) differed only in the position of a phenolic hydroxyl substitution on the ring B, quercetin was five times a more potent inhibitor of lipid peroxidation than morin. In accordance with the other authors,[33,35,40,41] we came to the conclusion that hydroxyls on the B ring might be considered as primary active sites in a radical scavenging process in the liposomal membrane. Zhang[41,42] has proved that three factors contributed to this phenomenon: namely, free radicals derived from -OH groups on the ring B can create intramolecular hydrogen bonds, thus increasing the stability of the radical, and they are able to transform themselves to an *ortho*-benzoquinonoid free radical through resonance, paving the way for proper distribution of unpaired electrons on the atoms, thereby reducing the internal energy.

However, electron-attracting property of the chromone ring of flavonoids on the ring A makes it less active,[41,42] as documented by the reduced values of antioxidant efficiency of the compounds **5**, **6**, **7**, **8**, and **11**, in which B ring could not contribute to the antioxidant action of a flavonoid molecule. In those cases, ring A turned out to be a determining factor of the antioxidant effect. From among the structures bearing the OH groups on the ring A, hydroxylation on C8 was shown to be crucial for good antioxidant activity, as long as 7,8-dihydroxyflavone (**6**) exerted better activity than chrysin (**7**, 5,7-dihydroxyflavone), 6-hydroxyflavone (**5**), and 6-hydroxyflavanone (**11**). By contrast, baicalein (**8**), containing pyrogallol moiety on the ring A, exhibited a remarkably low antioxidant activity.

Unlike in other reports,[37,38] lack or presence of a C3-OH moiety with or without the combination with an unsaturated C2,C3 bond did not significantly affect the antioxidant efficiency. It was probably due to additional processes that may control the protective action of flavonoid antioxidant in an oxidatively damaged DOPC membrane.

On the other hand, inhibitory effect was apparently dependent on the number of OH moieties. In agreement with this assumption and the results obtained by Lien et al.[43] for trolox equivalent antioxidant activity (TEAC) of a set of flavonoids, we achieved quite a good correlation between pIC_{50} values and values of parameters, i.e., the number of OH groups (*n*OH) ($R = 0.72$) and indicator variable ($R = 0.58$), which is the sum of the following indicators: presence of the C2,C3-double bond ($I = 1$) or two of the C3,C5,C7-OH groups ($I = 1$), eventually two of the C3′,C4′,C5′-OH groups ($I = 1$), or absence of the abovementioned situations ($I = 0$). In agreement with this result, 6-hydroxyflavone (**5**) and 6-hydroxyflavanone (**11**) possessing the only one OH moiety on the ring A showed the least activity.

van Acker et al.[44] considered ΔH to probably be the best parameter to predict antioxidant activity. This value may represent the relative stability of a radical with respect to its parent compound, enabling a comparison to be made between the stabilization, effected by hydrogen abstraction from alternative positions within an individual molecule, as well as between different molecules. In agreement with the results published by Vaya et al.[34] from among the ΔH values calculated for alternative OH groups (Table 2) of the flavonoids tested, the lowest values of ΔH were obtained for radical formation at position C3 on the ring C of the compounds quercetin (**2**), morin (**3**), and galangin (**4**), indicating its preference for oxidation. This does not hold, if the double bond between C2 and C3 is not simultaneously present on the same ring of flavonoid, as documented by ΔH for 3-OH of taxifolin (**1**). The next most stable radicals could be attributed to *ortho*-hydroxyl groups of the ring B, as supported by the isosurface 3D maps of the highest occupied molecular orbital (HOMO), developed for the most potent antioxidant quercetin (**2**) (Fig. 1). This is in agreement with the above-postulated hypothesis on the preferential oxidation of catecholic moiety of the ring B. In addition, the maps of spin densities showed potential stabilization of the phenoxyl C-3 radical, formed by delocalization through rings B and C (Fig. 2). *meta*-Hydroxyl groups of morin (**3**) possessed relatively high values of ΔH however, they were lower than those pertaining to positions C5 and C7

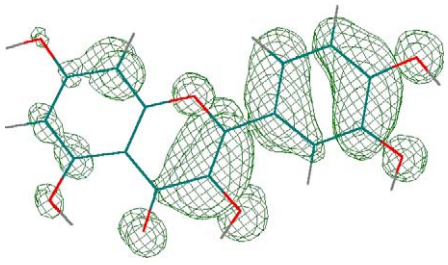


Figure 1. Isosurface 3D maps for the highest occupied molecular orbital (HOMO) density squared with a contour value of 0.01 of the optimal conformers of quercetin (**2**).

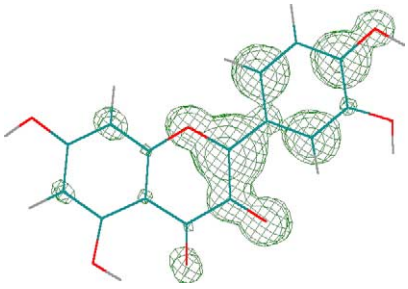


Figure 2. Spin density isosurface maps with a contour value of 0.002 of the optimal conformers of quercetin (**2**) in the form of radicals derived from position 3.

on the ring A. On the other hand, positions C6 and C8 in flavones (**8**) and (**6**) gave ΔH values comparable to those on the ring B and position 3 on the ring C of the compounds **1**–**4**, corroborating the good antioxidant activity of 7,8-dihydroxyflavone (**6**).

For the simple regression analysis, we achieved the best value of $R = -0.747$, using the parameter hydration energy E_{HYDR} (Table 3, Fig. 3). The highest values of hydration energy were obtained for the most potent antioxidants possessing the highest number of OH moieties, as shown for taxifolin (**1**, −32.73 kcal/mol), quercetin (**2**, −32.83 kcal/mol), and morin (**3**, −32.73 kcal/mol), while the lowest values E_{HYDR} were attributed to the mono-hydroxylated flavonoids, 6-hydroxyflavone (**5**, −11.46 kcal/mol) and 6-hydroxyflavanone (**11**, −11.49 kcal/mol), which exerted low antioxidant activities. With regard to the partitioning properties, we obtained a poor value of $R = -0.58$ for a simple correlation involving the parameter C log P, which reflects the lipophilic properties of the compounds tested. This deficiency did not particularly improved even with using the available experimental values of log P,[36] which provided a R value = 0.43. Results of other studies on QSAR analysis of antioxidants in membrane, systems[45] indicate that increased lipophilicity of compounds could be the basic reason for their good antioxidant activity in the liposomal membrane, however, our results on the simple correlation between biological activities and partitioning properties of flavonoids did not provide unambiguous and convincing evidence of a simple relationship. As it was recently mentioned by Erlejman et al.[36] different flavonoid families with a protective effect against lipid-soluble oxidant-induced damage may be active at various levels and locations, which could be the reason for multivalent meaning of partitioning for flavonoids antioxidant activities.

Since the parameter E_{HYDR} is closely associated with a number of OH groups, as documented by its colinearity with the parameter nOH ($R = -0.99$), it most probably reflects the radical scavenging properties of the compounds. As discussed above, these improve with an increasing number of OH groups. On the other hand, our results are in agreement with the work of Terao et al.,[46] which demonstrated that the flavonoids tested showed a higher efficiency in oxidatively damaged liposomes than in the more lipophilic structural analogue, α-tocopherol. These authors have assumed that the radical scavenging activity in AAPH induced peroxidation of unilamellar liposomes may be significantly influenced by location of the antioxidant in the membrane and the site of the radicals to be generated. As previously reported by Ratty et al.,[47] flavonoids are located in the polar surface region of the phospholipid bilayer. Therefore, they can easily trap aqueous peroxyl radicals and may be better accessible to chain-initiating peroxyl radicals than the more lipophilic antioxidants located within the membrane. Therefore, we assume that the parameter E_{HYDR}, which reflects the hydrophilicity properties of the compounds, most probably determines the availability of the compound in

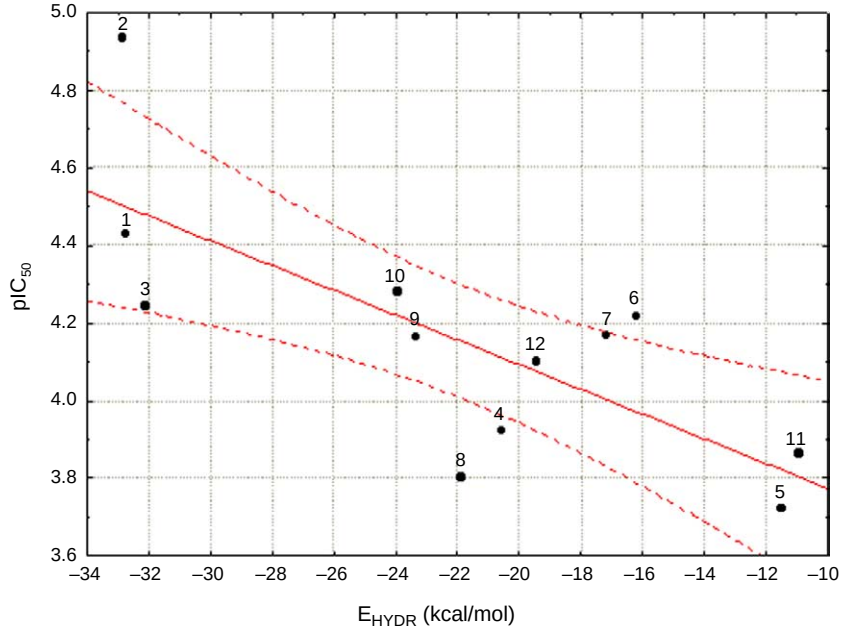


Figure 3. The regression line, 95% confidence interval, and data points for radical scavenging efficacy of flavonoids **1**–**12** as a function of hydration energy (E_{HYDR}; $n = 12$, $R = 0.747$).

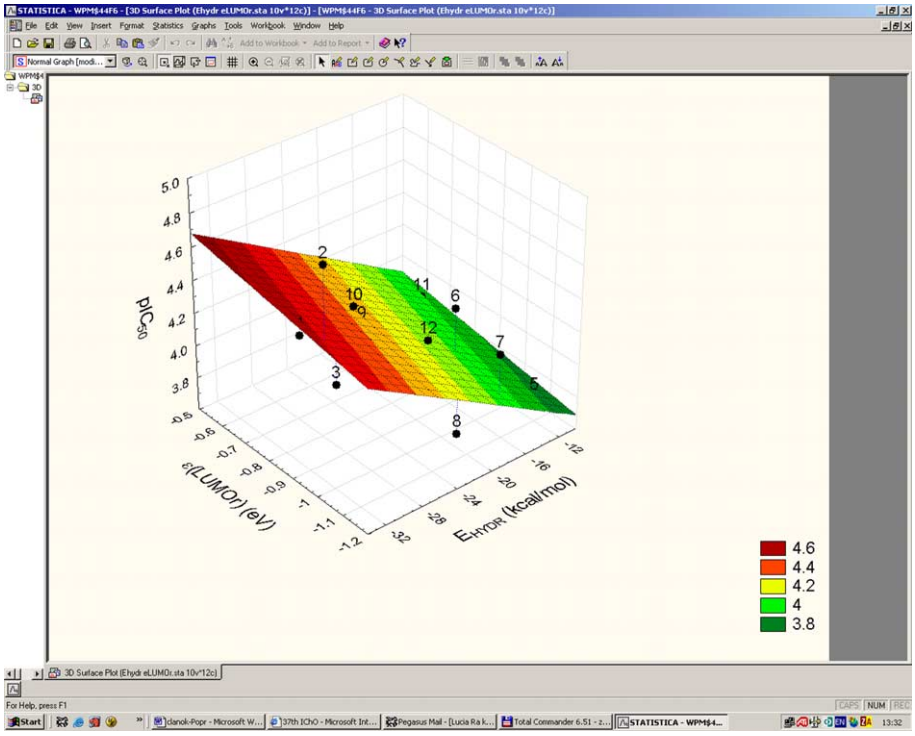


Figure 4. The regression plane and data points of antioxidant efficiency of flavonoids **1**–**12** in lipid peroxidation of DOPC liposomes as a function of parameters, hydration energy (E_{HYDR}) and energy of the lowest occupied molecule orbital (LUMO), pertinent to the radical derived from the OH group showing the lowest value of ΔH (ε(LUMO)r, $n = 12$, $R = 0.759$).

the polar region of the liposomal bilayer, defining the antioxidant properties of the flavonoid. This may explain the best antioxidant properties of the most hydrophilic compounds **2**, **3**, and **4**.

Moderately lower values of R were obtained for parameters Σq(O) (−0.71) and Σq(H) (0.70), reflecting the charge distribution at OH groups, which may determine their readiness of hydrogen bonding required for the good hydrophilicity of the compound. As expected, the squared correlation matrix among the parameters E_{HYDR}, Σq(O), Σq(H), and nOH showed their interdependency (data not shown). Relatively good correlations were also shown for parameters S, V, α, and MR ($R = 0.59-0.63$), which could also be explained by their colinearity with E_{HYDR} ($R = -0.59$, -0.77, -0.81, and -0.83).

For multiple regression analysis, independent parameters ε(LUMO)r and ΔH in conjunction with the hydration energy E_{HYDR} yielding a slight increase in R (0.759 and 0.756, respectively, Fig. 4) were used further on. This result indicates that electronic properties of the molecule are conditional for radical scavenging activity of the compounds and are ensured by availability of the molecules in the polar surface region of the model membrane reflected by the parameter, hydration energy.

4. Conclusions

Our results indicate that good antioxidant efficiency of flavonoids in an oxidatively damaged DOPC liposomal membrane is ensured by the increased number of OH groups on the skeleton and attenuation of the formation of phenoxyl radicals derived from these groups. The most stable radicals originating in the course of antioxidative action may be attributed to OH groups at the position C3 on the ring C, hydroxy-groups at mutual *ortho* positions at C3′, C4′ or C4′, C5′ on the ring B, and at positions C6 and C8 on the ring A. Therefore, we hypothesize that the new flavonoid derivative possessing improved antioxidant efficiency in the system of unilamellar liposomes should bear –OH groups at all these sites.

5. Experimental

5.1. Chemicals

Initiator 2,2′-azobis(2-aminidopropane)hydrochloride (AAPH) and cumene hydroperoxide (80% in cumene) were obtained from Fluka Chemie GmbH (Buchs, Switzerland). The set of tested flavonoids, as well as 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (C18:1, *cis*-**9**, DOPC, 99% grade) and 2,6-di-*t*-butyl-*p*-cresol (BHT), were purchased from Sigma Chemical (St. Louis, MO, USA). Flavonoids were of the highest grade available. All other chemicals were purchased from local commercial sources and were of analytical grade quality. All solvents used for lipid peroxidation studies were de-aerated under nitrogen.

5.2. Inhibition of lipid peroxidation

Unilamellar DOPC liposomes were used so as to evaluate the antioxidant activity of the tested flavonoids.

Peroxidation of the liposomal membrane was triggered by thermal decomposition of the hydrophilic compound, AAPH. Methods of liposome preparation have been described previously.[48,49] The liposomes (final concentration 0.8 mM DOPC) were incubated in the presence of different concentrations of the antioxidants tested and of the water-soluble initiator AAPH (final concentration 10 mM) at 50 °C for a time period of 80 min.

Aliquots (1 ml) of the incubation mixtures were extracted with 2 ml portions of an ice-cold mixture $CHCl_3$/MeOH (2:1, v/v), containing BHT (0.05%). Lipid hydroperoxide content was determined by the thiocyanate method according to Mihaljević et al.[50] The reagent was prepared by mixing equivalent volumes of methanolic solution of KSCN (3%) and ferrous-ammonium sulfate solution (45 mM in 0.2 mM HCl). After the mixture had been left at ambient temperature for at least 5 min, the absorbance at 500 nm was recorded on a Hewlett-Packard Diode Array spectrophotometer 8452 A. The lipid peroxide value was determined using a calibration curve prepared with standard cumene hydroperoxide.

The antioxidant activity was evaluated as $pIC_{50} = -\log(IC_{50})$, where IC_{50} (μM) is the concentration of a flavonoid required to inhibit peroxidation of DOPC liposomes by 50% at 80-min incubation time.

5.3. Statistical analysis

Statistical analysis of the correlation equations was evaluated by means of the program Statistica.[51]

5.4. Computational methods

The lowest energy molecular conformations of the flavonoids tested were calculated using Conformational Search module in HyperChem,[29] molecular modeling software using Austin model 1, and Polak-Ribiere conjugate gradient algorithm with 0.01 convergence limit in vacuum. For optimal conformers of antioxidants, the heat of formation H_A and total energies E_A were calculated. Geometric optimization of the radicals formed, with the abstraction of hydrogen, from a phenolic oxygen belonging to all alternative OH groups was performed, the corresponding H_F and E_F were calculated, thus creating the theoretical measures of antioxidant activity Eqs. 4 and 5.

$$\Delta H = H_F - H_A \quad (4)$$

$$\Delta E = E_F - E_A \quad (5)$$

Further calculated parameters of the tested compounds included energy values of the highest occupied molecular orbital ε(HOMO), energy of the lowest occupied molecular orbital ε(LUMO), HOMO–LUMO gap (Δε), partition coefficients (C log *P*), hydration energies (E_H), volume (*V*), surface (*S*), and sum of the partial charges of oxygen (Σ*q*(O)) and hydrogen (Σ*q*(H)), belonging to the OH groups, polarizability (α), number of OH groups (*n*OH), indicator variable (the sum of the following indicators: presence of the 2,3-double bond (*I* = 1) or two of 3,5,7-OH groups (*I* = 1) or two of 3′,4′,5′-OH groups (*I* = 1), or absence of above situations (*I* = 0)), partial charges of the oxygen and hydrogen belonging to the OH group showing the lowest value of Δ*H* and Δ*E*, and spin density *s*(O) belonging to the oxygen radical derived from this group, energy of the lowest unoccupied molecule orbital relevant to this radical ε(LUMO)r.

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