Statistical Analysis of the Relationship Between Antioxidant Activity and the Structure of Flavonoid Compounds

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Using different methods of statistics, this paper aims to highlight the potential link between the antioxidant activity of flavonoids and the corresponding molecular descriptors. By calculating the descriptors (van der Waals surface (A), molar volume (V), partition coefficient (LogP), refractivity (R), polarizability (a), forming heat ($H_{formation}$), hydration energy (E_{hidr}), the dipole moment (m_y)), together with antioxidant activities (RSA) calculated or taken from the literature, number of phenolic -OH groups and the presence (2) or absence (1) of $C_2=C_3$ double bond) for 29 flavonoid compounds and by intercorrelation between the studied parameters, the link between the number of phenolic groups grafted to the basic structure of flavonoids and their antioxidant activity was confirmed. Simultaneously, by using the chi-squared test and the intercorrelations matrix, a satisfactorily correlation coefficient ($r^2=0.5678$; $r=0.7536$) between the structure of the flavonoids and their activity was obtained, fact that confirms the correlation of the antioxidant activity with the number of -OH phenolic groups.

Keywords: flavonoids, free radicals, molecular descriptors, antioxidant activity, statistics

Flavonoids are a group of phenolic compounds, found in fruits, vegetables, as well as in beverages (tea, wine and beer) and other types of food, as well as in food supplements [1-6]. The basic structural backbone of flavonoids consists of 15 carbon atoms, arranged in the form C6-C3-C6 (aryl-propyl-aryl), which corresponds to two aromatic rings linked by a unit of three carbon atoms [7]. Normally, plants synthesize them as a protection mechanism to environmental attack (microbial infection, UV exposure). Over the years, the flavonoids were found to play an important role on the human health due to their antimicrobial, antioxidant, anti-inflammatory and anticancer properties [8-12].

The inhibition of the oxidative processes generated by the release of free radicals implies the action of a control agent represented by antioxidants. They function properly in close correlation with the structure of the free radical, its properties and level of action and, of course, the concentration of harmful agent.

Antioxidants are substances capable of retarding or inhibiting the oxidation processes of various atmospheric oxygen systems or reactive chemical species derived therefrom. Due to oxidative stress, the cell is damaged and the human body begins to age and face a number of serious diseases such as: diabetes, cancer, cardiovascular diseases [13-18]. The increased interest in the replacement of synthetic antioxidants from food with the natural ones has supported the search of plant sources and the choice of raw materials for new antioxidants identification. Because, until now, about 10000 flavonoids have been recorded [9], the researchers have focused their work on finding relationships between flavonoids structure and their antioxidant activity, in order to identify the most valuable ones [19-22].

Studies have shown that phenolic compounds reduce *in vitro* the oxidation of low-density lipoproteins, especially those polyphenols with many -OH groups, which are more effective for preventing the oxidation of lipids and lipoproteins with low density. Verzelloni et al. show the relationship between the antioxidant properties and the phenolic and flavonoid content in traditional balsamic vinegar [23]. The ability of phenolic compounds to act as antioxidants depends on their molecular structure. The anti-lipo-peroxide effect depends on the number and position of the -OH and -OCH_3 groups grafted on the benzene ring and on the possibility of electrons delocalization on the double bonds [24].

The aim of this study is to establish the relationship between the molecular structure of a series of structurally related flavonoids and their antioxidant activity also using different methods of statistical analysis.

Experimental part

The antioxidant properties data used in this study were antioxidant activity of flavonoids, a set of twenty-nine derivatives of flavonoids. Experimental values of antioxidant activity are taken from the bibliography [25] in order to determine a quantitative structure-activity relationship (QSAR) between antioxidant activity and the structure of these molecules that are described by their substituents 3, 5, 7, 8, 2', 3', 4' and 5'. The chemical structure and numbering of substituents in flavonoid derivatives studied had the following common structure shown in figure 1 [26].

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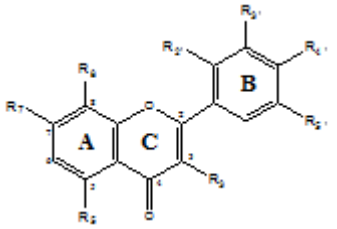

Fig. 1. The general molecular structure and substitution mode for flavonoids

The structure of all flavonoids is based on the C_{15} skeleton of the chromatic structure for which the secondary ring (B) is attached (fig. 1) [14,27]. Flavonoids are divided according to the substitution profile of the heterocyclic ring. In the classification of flavonoids are taken into account the oxidation state of the heterocyclic ring as well as the position of the secondary aromatic ring are taken into account. A total of about 12 subgroups of flavonoids are distinguished. The secondary (B) ring may be in position 2 (flavones, flavonols, dihydroflavonols, catechins, flavans, and anthocyanidins), position 3 (isoflavonoids), or position 4 (4-phenyl-coumarins, neoflavonoids). In a few cases, the six-membered heterocyclic ring occurs in an open isomeric form (chalcones and dihydrochalcones) or is replaced by a five-membered ring. The chemical structures of 29 compounds of flavonoids used in this study are included in Table 1.

Calculation of molecular descriptors

The QSAR study was conducted through molecular modeling of structures, which was performed using the HyperChem version 7.1 program. The basic structure of the flavonoids was the starting point for the calculations. Based on this, for 29 flavonoids were built the 2D structures and introduced into the molecular modeling program. The geometric arrangement characterized by minimal energy (most stable) was determined by semi-empirical PM3, RHF molecular orbital calculations, in vacuo, using the Polak-Ribere minimization algorithm with the RMS energy gradient of 0.01 Kcal/Å×mol [28]. A correlation between the antioxidant activity of flavonoids and the number of phenolic -OH groups was performed using a chi-squared test. Also, the statistical relationship between the molecular descriptors and the antioxidant activity was performed using the Pearson correlation.

Results and discussions

To ascertain the relationship between chemical structures of the flavonoids and their radical scavenging activities (RSA), various molecular features can be analyzed. Table 2 contains data corresponding to the calculated properties (structures of the flavonoids used in the analysis): van der Waals surface (A), molar volume (V), partition coefficient (LogP), refractivity (R), polarizability (α), forming heat ($H_{formation}$) energy of hydration (E_{hydr}), the dipole moment (μ_t), along with the antioxidant activities calculated or taken from the literature (the experimental values of related free scavenging activities), the number of phenolic –OH groups and the presence (2) or absence (1) of $C_2=C_3$ double bond.

Generally, the number of –OH groups has a great influence on the antioxidant activity of flavonoids [29]. In our case, the presence of -OH groups directly linked to the carbon atoms of the benzene ring (3, 5, 7 and 3', 4'-dihydroxy substitution pattern) determines the antioxidant role of flavonoids. The majority of compounds (from 1 to 16) had a very high antioxidant activity ranging between a minimum value of 66 % for 3-hydroxyflavone and a maximum of 96.5 % for morin. The position of -OH group could also affect the antioxidant activity of flavonoids. It is known that the 3-OH group at ring C played an important

Table 1

POSITION OF SUBSTITUENTS AT THE BASE STRUCTURE RINGS

No.	Compound	R_3	R_5	R_7	R_8	$R_{2'}$	$R_{3'}$	$R_{4'}$	$R_{5'}$
1	Morin	OH	OH	OH	H	OH	H	OH	H
2	Taxifolin	OH	OH	OH	H	H	OH	OH	H
3	Kaempferol	OH	OH	OH	H	H	H	OH	H
4	Fustin	OH	H	OH	H	H	OH	OH	H
5	Galangin	OH	OH	OH	H	H	H	H	H
6	Rutin	O-$C_{12}H_{21}O_9$	OH	OH	H	H	OH	OH	H
7	Quercetin	OH	OH	OH	H	H	OH	OH	H
8	Luteolin 7-gl	H	OH	O-$C_{12}H_{21}O_9$	H	H	OH	OH	H
9	Quercetin 3,7-digl	O-$C_{12}H_{21}O_9$	OH	O-$C_{12}H_{21}O_9$	H	H	OH	OH	H
10	Laricytin	OH	OH	OH	H	H	OH	OH	O-CH_3
11	Laricytin 3`-gl	OH	OH	OH	H	H	O-$C_{12}H_{21}O_9$	OH	O-CH_3
12	Robinetin	OH	H	OH	H	H	OH	OH	OH
13	Fisetin	OH	H	OH	H	H	OH	OH	H
14	Myricetin	OH	OH	OH	H	H	OH	OH	OH
15	Kaempherol 3,7-digl	O-$C_{12}H_{21}O_9$	OH	O-$C_{12}H_{21}O_9$	H	H	H	OH	H
16	3-Hydroxyflavone	OH	H	H	H	H	H	H	H
17	Apigenin 7-gl	H	OH	O-$C_{12}H_{21}O_9$	H	H	H	OH	H
18	Hesperetin	H	OH	OH	H	H	OH	O-CH_3	H
19	Vitexin	H	OH	OH	O-$C_{12}H_{21}O_9$	H	H	OH	H
20	3,5,7,3`,4`,5`-Hexametoxyflavone	O-CH_3	O-CH_3	O-CH_3	H	H	O-CH_3	O-CH_3	O-CH_3
21	Naringenin	H	OH	OH	H	H	H	OH	H
22	Naringin	H	OH	O-$C_{12}H_{21}O_9$	H	H	H	OH	H
23	7-Hydroxyflavone	H	H	OH	H	H	H	H	H
24	Flavanone	H	H	H	H	H	H	H	H
25	Flavone	H	H	H	H	H	H	H	H
26	Chrysin	H	OH	OH	H	H	H	H	H
27	Apigenin	H	OH	OH	H	H	H	OH	H
28	8-Metoxyflavone	H	H	H	O-CH_3	H	H	H	H
29	5-Hydroxyflavone	H	OH	H	H	H	H	H	H

Table 2
CALCULATED PROPERTIES OF STUDIED FLAVONOIDS

No. of compound	A [Å²]	V [Å³]	Log P	R [Å³]	α [Å³]	$H_{formation}$ [kcal/mol]	E_{stab} [kcal/mol]	μ_t [D]	Antiox. activity [%]*	No. of -OH phenolic groups	C_2=C_3
1	451.74	738.33	-3.11	81.49	27.9	-184.2058	26.73	4.365	96.5	5	2
2	473.58	777.01	-3.02	81.2	28.73	-232.1405	-30.12	1.115	94.8	5	1
3	450.04	737.21	-2.99	81.56	27.9	-181.6742	-27.01	4.123	93.5	4	2
4	473.23	764.81	-1.99	79.6	28.1	-190.429	-26.06	2.559	91.9	4	1
5	442.37	723.53	-1.96	79.96	27.27	-137.1021	-21.83	3.384	91.8	3	2
6	783.58	1320.04	11.21	97.01	44.18	-598.683	-22.54	2.162	90.9	4	2
7	443.05	704.27	2.52	77.12	23.99	150.6105	-10	2.352	89.9	5	2
8	643.8	1108.09	-4.58	113.92	41.33	-393.7498	-39.13	3.791	87.6	3	2
9	799.79	1453.95	-5.7	146.05	55.58	-480.8171	-46.73	5.458	86.8	3	2
10	498.91	830.07	-5.01	89.54	31.01	-261.27	-32.52	3.603	84.6	5	2
11	673.1	1163.31	-5.57	120.29	43.8	-425.3688	-35.7	1.287	83.8	4	2
12	464.04	762.88	-4.01	83.17	28.54	-217.6325	-31.5	3.844	82.3	5	2
13	447.78	727.99	-2.09	79.88	27.27	-136.0538	-23.65	1.848	79	4	2
14	479.13	784.03	-5.04	84.77	29.18	561.943	-39.49	2.343	72.8	6	2
15	811.5	1451.42	-5.91	146.43	54.75	-508.4698	-48.08	5.366	70.6	2	2
16	428.29	690.42	0.09	76.75	25.99	-42.027	-9.55	3.901	66	1	2
17	584.83	1023.38	-3.23	110.64	40.05	-301.1446	-28.77	1.741	34.8	2	2
18	496.14	815.59	-2.56	84.63	29.93	-197.2303	-23.73	2.808	30	3	1
19	576.04	1022.56	-4.26	112.24	40.69	-344.9095	-31.26	6.222	21	3	2
20	736.49	1228.71	-4.85	113.39	40.19	-58.8665	-11.15	6.744	12.6	0	2
21	463.44	742.22	-1.56	78.26	27.46	-154.3086	-21.71	3.311	6.3	3	1
22	603.08	1034.07	-2.71	109.01	40.24	-326.5001	-29.42	3.101	4.7	2	1
23	439.29	704.81	-0.76	77.36	26.54	-57.50176	-15.47	0.444	2.8	1	2
24	425.92	682.02	1.51	73.44	25.55	-20.6583	-4.13	2.146	2.6	0	1
25	417.47	664.73	0.99	75.07	25.36	-1.88294	-4.64	3.43	1.5	0	2
26	434.3	705.36	-1.06	78.28	26.63	-89.5137	-15.52	4.119	1.1	2	2
27	441.01	721.73	-2.09	79.88	27.27	-142.7774	-23.86	2.593	0.7	3	2
28	449.53	737.62	-0.01	81.44	27.83	-36.6453	-4.82	3.948	0.7	0	2
29	423.07	683.33	-0.04	76.67	25.99	-43.3665	-8.98	3.444	0.6	0	2

role in the antioxidant activity of a flavonoid. In flavonol, the most preferred position was 3-OH position, which indicated the importance of 3–OH group in reacting with free radicals [21].

The expressed activity is shown by compounds with two -OH groups, arranged as for catechol, and three -OH groups arranged as in pyrogallol. Also, the most effective radical scavengers are flavonoids with the 3',4'-dihydroxy substitution pattern on the B-ring and/or hydroxyl group at the C-3 position. The catechol moiety in combination with a C2-C3 double bond and a 4-keto function are the essential structural elements for a potent antioxidant activity. This is not necessarily true, as flavanols - without the C2-C3 double bond, are among the most potent compounds. The C2-C3 double bond is not necessary for a high activity, but the presence of a 3 -OH group significantly enhances the antioxidant activity [30].

In flavonoids that have only one -OH group in ring B or none at all, the rest of the flavonoid appears to become more important for the scavenging activity than in the case of catechol flavonoids. The flavanol galanin, which lacks hydroxyl substitution at the B-ring, demonstrated high activity. This is probably caused by the combination of the C2-C3 double bond with the 3-OH groups. Flavonoids which lack catechol -OH groups on ring B but possess a 3-OH next to the 4-keto group, show a high scavenging activity [31].

As polyphenolic compounds, flavonoids have the ability to act as antioxidants by a free radical scavenging mechanism with the formation of less reactive flavonoid phenoxy radicals [31]. Amic et al. suggest in their studies the possible mechanism of free radical scavenging of flavonoids lacking -OH groups on ring B [19].

Combining the results of our study and general theory and researches from the literature, we conclude that there is much discussion about the mechanisms of the antioxidative action of flavonoids.

Statistical analysis

Chi-squared test. The question is whether the value of the antioxidant activity of flavonoids is influenced by the number of phenolic -OH groups or it is due to other factors. Using the chi-squared test (χ^2), it is checked whether the calculated probability of the antioxidant activity of flavonoids differs significantly from the theoretical probability of their values. So, the chi square test (χ^2) can be used to evaluate a relationship between two of these categorical variables [32].

The 29 studied flavonoids were divided according to the number of phenolic groups: 5 have no phenolic groups, 2 have only one -OH group, and 4 have two flavonoid groups grafted to the basic molecular structure. Most of the studied flavonoid compounds, namely 7, have three -OH groups, 5 compounds have four -OH groups and 5 compounds have five -OH groups per structure. One compound has six-OH phenolic groups. For each category, the average of the antioxidant activity was calculated. The results are shown in Table 3.

If, according to the hypothesis, the basic molecular structure of flavonoids is the same, then from all seven substitution possibilities, the probability that one of them exists is p=0.143. The mathematical expectation for the value of the antioxidant activity of the flavonoid compounds was found to be M(n)=51.76. The criterion used to measure and test the significance of the antioxidant activity deviation from the mathematical expectation of antioxidant activity was:

$$\chi^2 = \frac{(F - n \cdot p)^2}{n \cdot p} \quad (1)$$

where F - the frequency of antioxidant activity values, n - the sum of the mean values of antioxidant activities.

For ν=7-1=6 degrees of freedom we find for the significance level α =0.05 the value $\chi^2\alpha$=12.6, so in this case the critical region is the interval [12.6, +∞) [33,34]. Therefore, we had reason to reject the hypothesis of zero, H_0 with a risk of error of the first case ≤α, χ^2>>12.6 so that the zero hypothesis H_0 was rejected with a lower risk of 5% and there could be admitted that the difference between the averages of antioxidant activity values was not accidental. In conclusion, antioxidant activity could be associated with the increase of the number of -OH groups, in a proportional manner.

The Pearson correlation. In order to correlate the antioxidant activity with the molecular descriptors, the intercorrelation matrix for the correlation coefficients was generated. The multiple linear regression method is used to study the relation between one dependent variable and several independent variables. The analysis were generated to predict antioxidant activities of flavonoids. Equations were justified by the coefficient of determination (r^2). The Statistica 10.0 program was used. The results are presented in table 4.

In order to correlate the values of antioxidant activity (j) with molecular descriptors, several forms of mathematical relations were analyzed. Following the processing and value analysis of the parameters, it is observed that the best linear correlation is found between RSA and the number of phenolic -OH groups. The statistical mathematical model found to describe the relation between the antioxidant activity of the studied compounds and the number of -OH phenolic groups is presented in equation (1). In order to estimate the adequacy of the model, the following indicators were calculated: the coefficient of determination (r^2), the correlation coefficient (r), the Fischer test (F) and the significance level (p) [35-39].

Table 3

RESULTS OF THE χ^2 TEST CALCULATION

Number of phenolic -OH groups	0	1	2	3	4	5	6	Total
Average antioxidant activity [%]	3.60	34.40	27.80	46.31	87.82	89.62	72.80	362.35
The mathematical probability of appearance	51.76	51.76	51.76	51.76	51.76	51.76	51.76	362.35
F-n·p	-48.16	-17.36	-23.96	-5.45	36.06	37.86	21.04	0.00
$\frac{(F - n \cdot p)^2}{n \cdot p}$	44.82	5.83	11.09	0.57	25.11	27.68	8.55	123.65

Table 4

THE INTERCORRELATION MATRIX (PEARSON (n)) FOR CORRELATION COEFFICIENTS, r

	x	y	z	q	r	s	v	w	j	k	i
x											
y	1.00										
z	-0.14	-0.19									
q	0.91	0.94	-0.44								
r	0.96	0.98	-0.28	0.98							
s	-0.65	-0.67	0.02	-0.62	-0.69						
v	-0.52	-0.55	0.43	-0.61	-0.60	0.36					
w	0.36	0.37	-0.33	0.43	0.37	-0.17	-0.01				
j	0.22	0.22	-0.12	0.18	0.20	-0.23	-0.29	-0.07			
k	0.01	0.01	-0.18	-0.01	0.00	-0.03	-0.32	-0.22	**0.75**		
i	0.15	0.17	-0.04	0.19	0.16	0.03	0.01	0.28	0.17	-0.00	

x- van der Waals surface
y- molar volume
z- partition coefficient
q- refractivity
r- polarizability
s-forming heat
v-hydration energy
w- dipole moment
j- antioxidant activity
k- no. of –OH group
i- presence/absence C_2=C_3

$$j = 3.8536 + 16.71271 \cdot k \quad (2)$$

$r^2 = 0.5678;\ r = 0.7536,\ p = 0.000002;\ F = 35.4779$

Correlative analysis based on equation (2) shows that there are appreciable correlations between the antioxidant activity of the studied flavonoids and the number of phenolic -OH groups.

Conclusions

Computational chemistry is of real use in the field of flavonoids research, helping considerably to find new objectives and links to characterize their properties. A number of 29 flavonoid compounds have been studied in terms of structural characteristics. The methods used for molecular modeling were semi-empirical molecular orbital calculations (AM1, RHF), in vacuo, Polack-Ribier algorithm with a gradient RMS of 0.01 kcal/Å·mol. The theory aims to solve Schrodinger's equation for a molecular system and to find its energy as well as other molecular properties that result from it.

Regarding the structure-activity relationship of flavonoids, the study confirms the correlation of antioxidant activity with the number of phenolic –OH groups. A significant correlation was found between the antioxidant activity of flavonoids and their number of –OH groups. So, a mathematical statistical model that describes this correlation was developped. The values of the model indicators ($r^2 = 0.5678$; $r = 0.7536$) show that it describes properly the variation of antioxidant activity with the number of phenolic –OH groups.

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