Structure-Radical Scavenging Activity Relationships of Flavonoids from *Ziziphus* and *Hydrangea* Extracts

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Methanol extracts prepared from Ziziphus and Hydrangea fruits and leaves were examined for their antioxidant activity. Antioxidative activities were studied in sunflower oil at 98^{0}C, by measuring peroxide value, and with the spectrophotometric method, which uses 1, 1 diphenyl-2-picrylhydrazyl (DPPH) free radicals. The relationship between the structural characteristics of pure four flavonoids and their antioxidant activity was also studied. The obtained results suggest that the free radical scavenger potential of these polyphenolic compounds closely depends on the particular substitution pattern of free hydroxyl groups on the flavonoid skeleton.

Keywords: flavonoids, antioxidant activity, peroxide value, structure-radical scavenging activity relationships, HPLC

The inhibition of the oxidative degradation processes caused by the highly reactive oxygen species supposes the action of a control factor namely antioxidants. An antioxidant may be defined as „any substance that when present at low concentrations, compared with those of the oxidizable substrate, significantly delays or inhibits oxidation of that substrate".

Antioxidants were used as additives in fats, oils and manufactured food for preventing or delaying the oxidative degradation of food. For this reason, in the latest years the interest of changing the synthetic antioxidants with natural ones has increased. Antioxidant compounds in food play an important role as a health-protecting factor. The main characteristic of an antioxidant is its ability to trap free radicals. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radical such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanism [1,2].

Radical scavenging action is dependent on both reactivity and concentration of the antioxidant. The efficiency of a vegetable extract is due to the action of all the active principles, not only to the main active principle. For this reason, in the experimental part we studied the antioxidant activity of some vegetable extracts with known flavonoids content on sunflower oil termically treated.

Flavonoids possess antioxidant and free radical scavenging activity in foods and this is the reason that they can be applied in preventing or treating human diseses. Until now, the flavonoid action mechanism was not elucidated, so we tried to obtain a structure-antioxidant activity correlation of the studied flavonoids.

According to data literature, as for the relationship between flavonoid structure and their radical-scavenging activity, the radical-scavenging activity of flavonoids depends strongly on the molecular structure, the number and the substitution pattern of hydroxyl groups [3].

The presence of a 3',4'-dihydroxy in the B ring, in combination with a 4-carbonyl function and C_2-C_3 double bound increases the radical scavenging activity [4].

Phenolic compounds detected in vegetable extracts analized are a group of natural 2-phenil-benzopyran derivatives with two benzene rings (A and B) and a pyran ring C. Transformation of the pyran ring cause variation of the antioxidant properties.

Relationship between flavonoid structure and their radical scavenging activity was used for correlation of their antioxidant activity with their molecular structure; this study was realized with Hyperchem program [5].

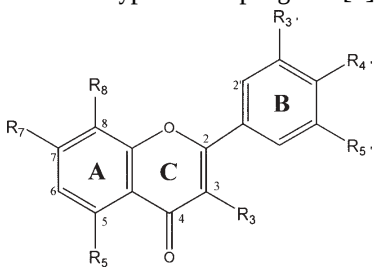

Fig. 1. General structure of the tested flavonoids

Table 1
MOLECULAR STRUCTURES OF FLAVONOIDS AND LIST OF SUBSTITUENTS ON THE CHROMONE (R_3-R_8) AND PHENYL RING ($R_{3'}$-$R_{5'}$)

No.	Name	R_3	R_5	R_7	R_8	$R_{3'}$	$R_{4'}$	$R_{5'}$
1	Kaempferol	OH	OH	OH	H	H	OH	H
2	Myricetin	OH	OH	OH	H	OH	OH	OH
3	Quercetin	OH	OH	OH	H	OH	OH	H
4	Rutin	O-$C_{12}H_{21}O_9$	OH	OH	H	OH	OH	H

Experimental part

Apparatus:

- agilent 1100 HPLC with binary pump and vacuum degasser, Nucleosil C_{18} column, particle diameter 5μm, column length/diameter 4x125 mm, UV detector with variable wavelength, soft HPChemStation;
- kern analytical balance;
-LABORTA rotaevaporator control 4002.

Materials and methods

Chemicals:

-acetonitrile (HPLC grade), phosphoric acid 85%, Merck; methanol p.a., Merck, 1,1-diphenyl-2-picrylhydrazil (DPPH), Merck;

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-standard flavonoids solutions 0,001 g/L: myricetin (90%), quercetin (98%), kaempferol (90%), rutin (95%) from Sigma Aldrich;

-acetic acid: chloroform solution 3:2 (v/v), 0,01N sodium thiosulfate solution, 1% amidon solution, Chimopar Bucharest;

-virgin sunflower oil obtained by cold compression without antioxidants added (Ufs);

-virgin sunflower oil with synthetic antioxidants added: standard flavonoids solution: quercetin (Q), rutin (R), kaempferol (K), myricetin (My);

-virgin sunflower oil with natural antioxidants added: alcoholic extract on:

Ziziphus jujuba leafs (E1), *Ziziphus jujuba* fruits (E2), *Ziziphus lotus* leafs (E3), *Ziziphus lotus* fruits (E4), *Hydrangea paniculata* leafs (E5), *Hydrangea aspera* leafs (E6).

The flavonoid compounds content in sample was:

- $142.25 \cdot 10^{-5}$ mol rutin /100 mL oil (Ufs + E1);
- $4.24 \cdot 10^{-5}$ mol myricetin /100 mL oil; $40.42 \cdot 10^{-5}$ molkaempferol /100 mL oil; $7.56 \cdot 10^{-5}$ mol rutin /100 mL oil (Ufs + E2);
- $130.73 \cdot 10^{-5}$ mol rutin/100 mL oil (Ufs + E3);
- $4.31 \cdot 10^{-5}$ mol myricetin /100 mL oil; $40.86 \cdot 10^{-5}$ mol kaempferol /100 mL oil; $7,98 \cdot 10^{-5}$ mol rutin /100 mL oil (Ufs + E4);
- $64.69 \cdot 10^{-5}$ mol rutin /100 mL oil; $261.05 \cdot 10^{-5}$ mol quercetin /100 mL oil; $17.11 \cdot 10^{-5}$ mol luteolin /100 mL oil (Ufs + E5);
- $5.81 \cdot 10^{-5}$ mol rutin /100 mL oil; $10.42 \cdot 10^{-5}$ mol quercetin /100 mL oil; $4.36 \cdot 10^{-5}$ mol luteolin/100 mL oil (Ufs + E6).

Preparation of the extracts

1 g of vegetable material was extracted with 40 mL methanol for 10 min at reflux. The solution was cooled and filtered and then was concentrated at 1/3 from initially volume [6].

Oil samples preparation

0.1 mL vegetable extracts respectively standard solution were dissolved in 100 mL virgin sunflower oil obtained by cold compression. The emulsion obtained was kept at 98^0C for 12 h. The peroxide value (PVs) of samples were analyzed at every 60 min [7]. The PVs was determined by making at the same time a control sample. The determinations were made triple for each sample.

Analysis of flavonoids in extracts

The flavonoids content of vegetable extracts were determined by using the Hasler and Stichler assay [6]. HPLC separation conditions were as follows: a Nucleosil C_{18} column, particle diameter 5μm, column length/ diameter 4x125 mm, solvent systems were deionised water : H_3PO_4 = 98 : 2 (*p*H=2,5) (solvent A) and acetonitrile (solvent B). Flavonoids were separated by a linear gradient elution of solvent A : B = 50 : 50 for 12 min, 20 : 80, 15 min, 50 : 50 for 20 min, at 35^0C with a flow rate of 1.5 mL/min, monitoring at 280 nm (for rutin and kaempferol) and 370 nm (for myricetin and quercetin) with a UV detector. Injection volume was 20 μL.

Antioxidant activity determination

In order to determine the antioxidative activity of pure flavonoids and that of vegetable extract two methods were applied:

- the spectrophotometric method, which uses 1, 1 diphenyl-2-picrylhydrazyl (DPPH) free radicals, when the extinction was followed for 60 min at λ=517 nm at room temperature. The antioxidant activity commensurate with DPPH percentage was calculated with the next formula [8]:

$$\%\ Antioxidant\ activity = \frac{A_{(t=o)} - A_{(t=60)}}{A_{(t=o)}} \cdot 100 \qquad (1)$$

where:

$A_{(t=o)}$ = sample absorbance at t=0 min.

$A_{(t=60)}$ = sample absorbance at t=60 min.

- peroxide values (PV) of sunflower oil were measured to determine the antioxidative activity of pure flavonoids and vegetable extracts. Peroxide values were determined periodically, using AOAC (1995).

Antioxidant activity of pure flavonoids and vegetable extracts in the sunflower oil was calculated as percent variation of PV of the sample (s) versus that control sample (c) [7]:

$$\%\ Antioxidant\ \ activity = 100 \cdot \left[1 - \frac{IP_s}{IP_c}\right] \qquad (2)$$

where:

IP_s = peroxid value variation of the sample;

IP_c = peroxid value variation of the control sample.

Study structure-radical scavenging activity relationships in flavonoids series studied

The study of relationship between flavonoid structure and their radical scavenging activity was realized with Hyperchem program. The starting point for the following calculations is represented by the flavonoid structures that exist in the program's data base. The minimum energy geometrical configuration was determined through semi-empirical (molecular orbital) calculations PM3, RHF, *in vacuo* using the Polak-Ribiere minimization algorythm with RMS gradient 0,01 Kcal/Å·mol [5].

Rutin is one of the most common, wide speaded natural flavonoids [5] and also represents the active principle detected in all the analyzed vegetable extracts. For this reason, we realised a correlation [11] between antioxidant activity [%] of vegetable extracts and the amount of rutin existing in 1 mL of extract.

Results and discussion

HPLC analysis

The active principle concentration from vegetable extracts used, determined by HPLC analysis are presented in table 2.

Antioxidant activity

Antioxidant activity was determined for standard flavonoids solution (10^{-3} g/L) and for vegetable extracts, where the flavonoids compounds detected by HPLC analysis (table 2). The antioxidant activity of vegetable extract was evaluated comparatively with antioxidant activity of the main compound detected in each extract.

DPPH radical-scavenging capacity (antioxidant activity) of standard flavonoid respectively of vegetable extracts are presented in table 3.

From the value of antioxidant activity we can see that vegetable extracts E4, E3 and E2 developed a lower antioxidant activity, less than 20%. Vegetable extracts E5 and E6 have a comparative antioxidant activity with the one of rutin etalon.

Structure – antioxidant activity relationships

The corresponding datas of the calculated properties, such as: van der Waals surface (A), moleculare volume

Table 2
CONCENTRATION OF ACTIVE PRINCIPLE DETECTED IN VEGETABLE EXTRACTS ANALYZED

Vegetable material	Compound	Flavonoids, mg / 100g dried mass	Flavonoid, mg /mL extract
Ziziphus jujuba leafs (E1)	Rutin	86,810	0,0868
Ziziphus jujuba fruits (E2)	Myricetin	13,894	0,0138
	Kaempferol	57,640	0,0576
	Rutin	30,710	0,0307
Ziziphus lotus leafs (E3)	Rutin	52,101	0,0521
Ziziphus lotus fruits (E4)	Myricetin	24,301	0,0243
	Kaempferol	72,101	0,0721
	Rutin	41,201	0,0412
Hydrangea paniculata leafs (E5)	Quercetin	108,010	0,1080
	Rutin	193,900	0,1939
Hydrangea aspera leafs (E6)	Rutin	95,010	0,0950
	Luteolin	40,590	0,04059

(V), partition coefficient (log P), refraction (R), polarizability (α), heat of formation (ΔH_f), hydration energy (E_h), dipole moment (μ) are presented in table 4.

Although the kaempferol and the rutin have the same number of hydroxyl groups, they present different antioxidant activity. The presence of another hydroxyl group in the myricetin molecule doesn't involve a raise of the antioxidant activity value, as it was expected. A possible explanation according to the literature datas [3] is that the presence of a hydroxyl group in the 5' position has a negative effect on the antioxidant activity, while the presence of the hydroxyl groups in 3 and 3' positions improves the antioxidant activity.

The presence of a glicozide in position 3 of the C quercetin heterocycle considerably diminish the antioxidant activity of the rutin.

As it can be observed (fig. 2), in the first hour of termical treatment the calculated antioxidant activity with respect to the control sample oxidation is the lowest for the kaempferol and quercetin, the remaining flavonoids having a comparable activity.

As the termical treatment continues, the antioxidant activity is lower for all the compounds, and after 5 h appears a pro-oxidant activity in the case of kaempferol. The pro-oxidant activity appears after 6 h also for the myricetin, and, after 8 h , all the compounds show pro-oxidant activity. The antioxidant activity of pure flavonoids in sunflower oil is very low.

In case of sunflower oil samples treated with vegetable extract (E1-E6) after 1 h heating at 98^{0}C, antioxidant activity have only *Ziziphus jujuba* fruits extract (E2). This protector effect can't be explained by his content in active principle. All the other extracts showed pro-oxidant activity in sunflower oil.

The problem observed in performing experiments with sunflower oil was the low solubility of flavonoids solution and especially plant extracts in the oil. The reason is certainly the variation of solubility of individual flavonoids in the oil and their hydrophobicity/hydrophilicity.

Table 3
ANTIOXIDANT ACTIVITY OF STANDARD FLAVONOID AND VEGETABLE EXTRACTS

Vegetable extract	Antioxidant activity (%)	Standard solution	Antioxidant activity (%)
E1	24.16	Quercetin	92.38
E2	14.04	Rutin	40.94
E3	11.88	Kaempferol	93.69
E4	7.87	Myricetin	90.08
E5	44.22	-	-
E6	34.01	-	-

Table 4
CALCULATED MOLECULAR DESCRIPTORS FOR THE STUDIED FLAVONOIDS

No.	A [Å^2]	V [Å^3]	Log P	R [Å^3]	α [Å^3]	ΔH_f [kcal/mol]	E_h [kcal/mol]	μ [D]
1	359.65	737.21	2.99	81.56	27.9	-181.7012	-27.01	4.12
2	375.09	785.02	-5.04	84.77	29.18	-269.153	-39.55	4.38
3	369.28	769.88	-4.01	83.17	28.54	-217.734	-32.64	1.52
4	569.75	1392.1	-5.91	146.47	54.75	-598.683	49.52	2.16

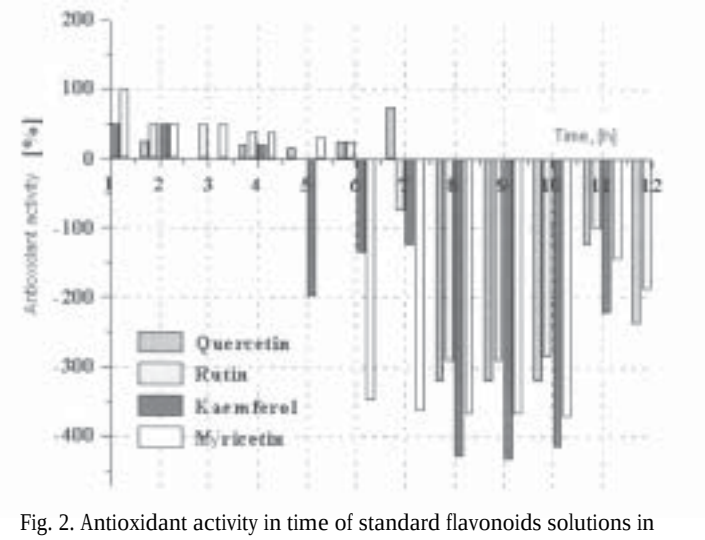

Fig. 2. Antioxidant activity in time of standard flavonoids solutions in sunflower oil at 98°C

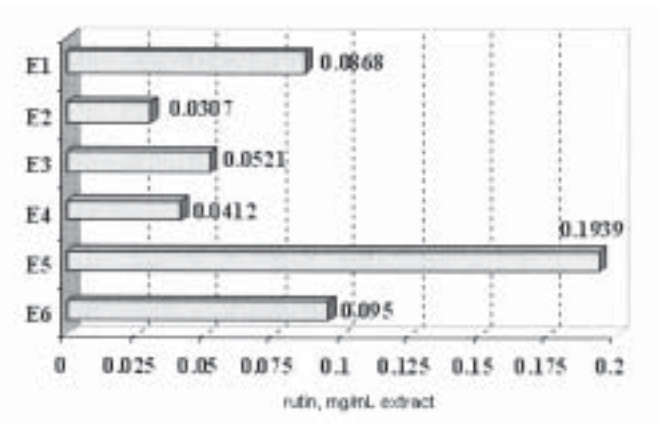

Fig. 4. Rutin content in *Ziziphus* and *Hydrangea* extracts

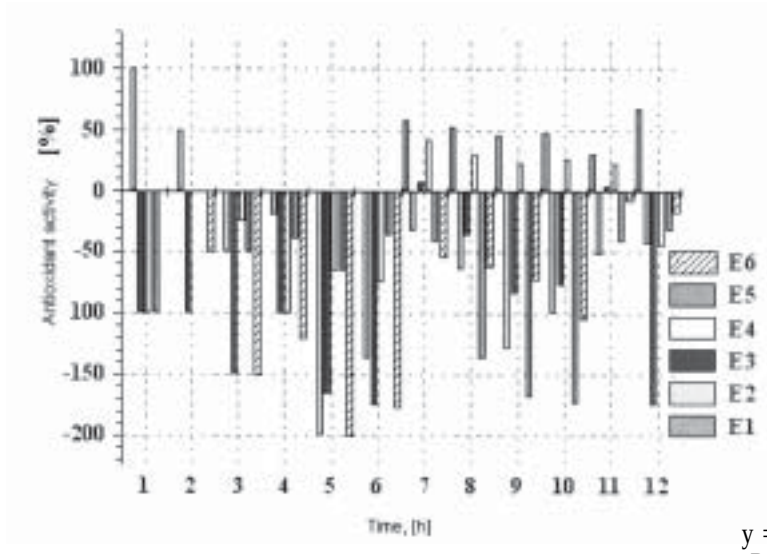

Fig. 3. Antioxidant activity in time of vegetable extracts in sunflower oil at 98°C

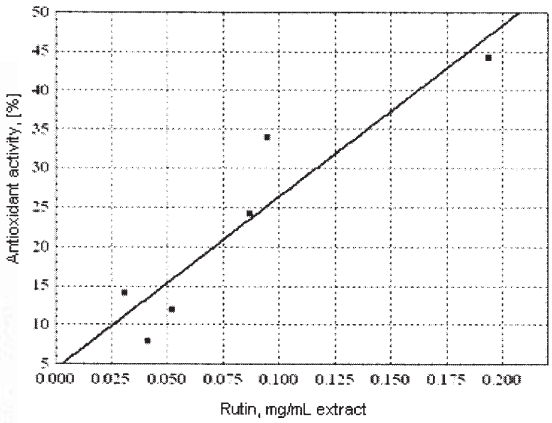

Fig. 5. Linear dependence between antioxidant activity of vegetable extracts and amount of rutin existing in extracts

$$y = 4{,}3823 + 219{,}9043 \times x \quad (3)$$
$$R = 0{,}9278,\ p = 0{,}0076$$

Further, it is generally believed, that the lipid oxidation begins and is most intense in the membrane or interphases of an emulsion.

The rutin content in vegetable extract are presented in figure 4, and correlation between antioxidant activity of vegetable extracts [%] and the amount of rutin existing in 1 mL of extract can also be seen in figure 5.

The value of the correlation coefficient is acceptable, indicating a suitable correlation by linear regression which denote the rutin contribution as active principle at antioxidant activity of vegetable extracts studied.

Conclusions

As polyphenolic compounds, flavonoids have the ability to act as antioxidants by a free radical scavenging mechanism with the formation of less reactive flavonoid phenoxyl radicals. The hight potential of flavonoid compounds to scavenge free radicals may be explained by their ability to donate a hydrogen atom from their hydroxyl group and thereby scavenge the free radicals.

The results show that, in sunflower oil, the antioxidant activity of the pure flavonoids investigated depends on the number and location of hydroxyl groups on the aromatic

ring. All tested flavonoids have hydroxyl groups in positions R5, R7 and 4' and differ in substituents located in positions R3, 3' and 5'.

The most effective radical scavengers are flavonoids with hydroxyl group at the C-3 posotion (Q, K, My). This flavonoids have a strongly antioxidant activity and a high ability to discolour DPPH solution. 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.

The results show that only *Ziziphus jujuba* fruits extract (E2) have antioxidative activity.

Linear regression analysis confirmed that variations in the OH substitution pattern were responsabile for variation of the radical scavenging activity of the flavonoids studied.

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