Flavonoids in *Angiospermatophyta* and *Spermatophyta* Species and their Antioxidant Activity

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The purpose of this study was the quantitative determination of some flavonoids (quercetin, rutin and kaempherol) contained in Angiospermatophyta and Spermatophyta genus plant extracts. Also the antioxidant activity of their hydro-alcoholic extracts was evaluated. The quantitative determination of quercetin, rutin and kaempherol was achieved through high performance liquid chromatography (HPLC) and the antioxidant activity was assessed by the chemiluminescence method. The studied species were: Alchemilla vulgaris - common lady's-mantle (aerial part), Allium ursinum - bear's garlic (leaves), Acorus calamus - common sweet flag (roots), Solidago virga-aurea – goldenrod (aerial part), Agrimonia eupatoria – agrimony (aerial part), Viscum album - European mistletoe (leaves and branches) and Veronica officinalis - common speedwell (aerial part). The hydro-methanolic extracts (98% methanol) of Alchemilla vulgaris and Solidago virga-aurea had the highest antioxidant activity, probably given by the high content in flavonoids of quercetin and rutin type.

Keywords: flavonoids, HPLC, antioxidant activity, chemiluminescence, hydro-alcoholic extracts

The paradox of the aerobe life or the oxygen paradox resides in the fact that the superior organisms cannot exist without it, even if oxygen is dangerous for their existence. This negative part played by the oxygen is related to its external shell structure that contains two un-paired electrons.

The main factor that causes the increase in the intensity of free radicals formation is the oxygen activation. Due to the presence of oxygen not only in the atmosphere but also in most of the substances composing the organisms, the interaction of free radicals with oxygen is inevitable [2].

Flavonoids are antioxidant compounds with the property to intercept and inactivate the free radicals (O_2^{-}•, HO•, SO•, •SH, NO•, etc).

Kaempherol, quercetin and rutin (fig.1) are representative terms for the flavonoid class, having multiple medical applications due to their anti-allergic, anti-carcinogenic, anti-cataract, anti-colitis, anti-diabetic, anti-inflammatory, anti-mutagenic, anti-HIV, anti-ulcer, bactericide and vasodilator properties [3-8].

From a structural point of view quercetin, kaempherol and rutin are phenolic compounds in whose molecule there are heterocycles of the benzopyrane and benzofurane type, where the hetero-atom is the oxygen. To this heterocycle a benzoic nucleus is condensed [9].

The basic structural condition for the efficacy of free radicals' inhibition by flavones is the presence of some o-dihydroxyl groups in B nucleus (3´-OH and 4´-OH) with electron donating properties (fig. 1.b.). The double bond C2-C3 combined with a 4–ketogroup, responsible for the localization of the electron from the B nucleus, continues to intensify the radicals inhibition ability; the saturation of C2-C3 double bound is considered to be the cause for the activity potential loss. Even more, the elimination of free radicals is increased by the presence of 3-OH and 5-OH groups combined with a 4-carbonil group and the double bond C2-C3 [10].

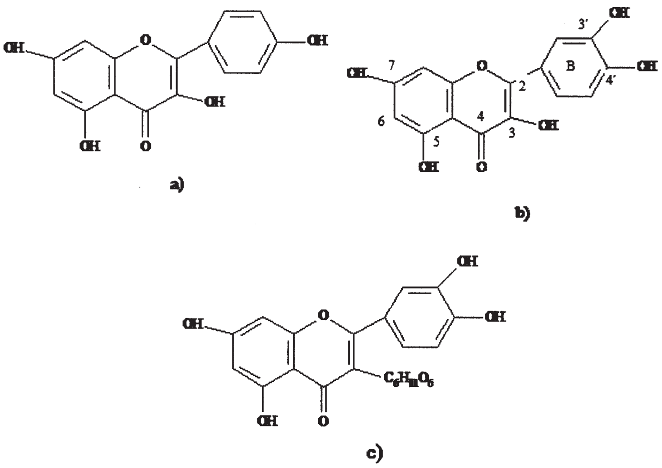

Fig. 1. Structural formulas of kaempherol, quercetin and rutin

Quercetin, rutin and kaempherol meet the basic condition for the efficacy of radical inhibition, which is why we chose them as standards and compared their activity to the antioxidant activities of the investigated plant extracts.

The great efficiency of the antioxidants consists in their synergism, every one of them acting according to different mechanisms and at different levels of the free radical evolution chain in the organism [11].

The specialized literature [11] does not really offer a wide range of information on the quantitative composition of plant extracts belonging to *Angiospermatophyta* and *Spermatophyta* genus, although their therapeutic

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properties are well known [4,5,12]. Therefore, a more thorough study on the composition on the hydro-alcoholic phanerogam plant extracts rich in phenolic compounds and on their antioxidant potential becomes necessary.

The aim of this work consists in the quantitative determination, by HPLC, of quercetin, rutin and kaempherol flavonoids, contained in the extracts obtained from the vegetal material of the following plants: *Alchemilla vulgaris, Allium ursinum, Acorus calamus, Solidago virga-aurea, Agrimonia eupatoria, Vâscum album* and *Veronica officinalis*. In order to determine the antioxidant potential of the investigated hydro-alcoholic extracts (solutions methanol: water with 96% and respectively 80% methanol) the chemiluminometric method was used, a method referred to in literature as providing reproducible results in the study of antioxidant activity of phenolic compounds [13].

Experimental part

The *vegetal material* consisted of *Alchemilla vulgaris* - common lady's-mantle (aerial part), *Allium ursinum* - bear's garlic (leaves), *Acorus calamus* - common sweet flag (roots), *Solidago virga-aurea* – goldenrod (aerial part), *Agrimonia eupatoria* – agrimony (aerial part), *Viscum album* - European mistletoe (leaves and branches) and *Veronica officinalis* - common speedwell (aerial part) from Fares S. A.-Oraştie.

Reagents: luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) – Merck, hydrogen peroxide (30% w/v) - Merck, copper (II) sulfate p.a., sodium hydroxide p.a.., sodium carbonate p.a. and sodium bicarbonate p.a. - Reactivul Bucureşti; methanol p.a., methanol for HPLC and phosphoric acid for HPLC – Merck; standards of quercitine (≥ 99%), rutin (≥ 99,2%), kaempferol (≥ 99%) and myricetin (≥ 99%) - Roth.

The working concentrations were as follows: luminol 2 x 10^{-3} M in Na_2CO_3 (5 x 10^{-2} M) / $NaHCO_3$ (0.3 M) solution; copper sulfate solution 4.79 x 10^{-3} M; hydrogen peroxide solution 8.82 x 10^{-3} M (freshly prepared daily) and sodium hydroxide solution 10^{-2} M.

Hydro-alcoholic extracts were obtained by solid-liquid extraction, using a Soxhlet installation upon a procedure described in the Romanian Farmacopeea [14]. The extraction method was unitary, the operational parameters, respectively the grinding degree, the solvent used, the plant/solvent ratio, the extraction temperature and the extraction type were identical, the only difference being the type of the vegetal material.

Hydro-alcoholic extracts (96% methanol or 80% methanol) with limpid aspect and specific color were obtained: E_1 – methanolic extract of *Alchemilla vulgaris*; E_2 – methanolic extract of *Allium ursinum*; E_3 – methanolic extract of *Acorus calamus*; E_4 – methanolic extract of *Solidago virga-aurea*; E_5 – methanolic extract of *Agrimonia eupatoria*; E_6 – methanolic extract *of Veronica officinalis*; E_7 – methanolic extract of *Viscum album*;

High performance liquid chromatography

A HPLC chromatograph Varian Pro Star type, model 240, with UV-VIS detector (UV-VIS VARIAN model 345) was used.

The chromatographic separation was performed on an Omnispher 5-C18 chromatographic column, 150 x 4.6 mm for kaempherol and quercetin, and on an Inertsil 5-C18 column, 250 x 4.6 mm for rutin.

The mobile phase consisted in a mixture of acetonitrile: phosphate buffer (*p*H 2.5) in a volumetric ratio of 40:60 for kaempherol and quercetin and for rutin was used a mixture of methanol: phosphate buffer in a volumetric ratio of 45:55. The rate of the mobile phase was of 1 mL/min and the injection volume was of 5μL.

Detection was performed at 360 nm for kaempherol and quercetin and at 280 nm for rutin. These wavelength values were determined based on the registered values of UV-VIS spectra for the 3 standards.

In order to determine the calibration curves a number of 6 methanolic solutions of kaempherol, quercetin and rutin were used; their concentrations are given in table 1.

Chemiluminescence method

A SPEKOL 10 spectrophotometer (Carl Zeiss. Jena) (equipped with 30 ml quartz cuvettes) coupled to an acquisition plate Axxi-chrom 727 (computer interface Axxion Chromatography, INC) was used for the chemiluminescence determinations [13].

A mixture containing 5 mL sodium hydroxide solution, 1 mLl copper (II) solution and 3 mL luminol solution was introduced in the quartz vat and submitted to stirring (mixture A).

Separately, 5 mL of hydrogen peroxide solution was mixed with 5 mL of distilled water. Afterwards 0.1 mL of alcoholic plant extract (in 96% methanol) was added thus obtaining *mixture B*.

One minute after the preparation of *mixture B* this was injected with a syringe into *mixture A* and the signal obtained at 420 nm, with the apparatus lamp turned off, was registered for about 5 min.

The antioxidant activity for the methanolic solutions of rutin, quercetin and myricetin standards (1.5 mg/mL) was also measured by the same method.

Table 1

CONCENTRATIONS OF THE METHANOLIC SOLUTIONS OF KAEMPHEROL, QUERCETIN AND RUTIN USED TO DETERMINE THE CALIBRATION CURVES

Concentration [mg/mL]	Kaempherol	Quercetin	Rutin
C_1	$1.5x10^{-1}$	$1.5x10^{-1}$	$1.5x10^{-1}$
C_2	$7.0x10^{-2}$	$8.0x10^{-2}$	$7.5x10^{-2}$
C_3	$4.0x10^{-2}$	$7.5x10^{-2}$	$3.7x10^{-2}$
C_4	$1.8x10^{-2}$	$1.8x10^{-2}$	$1.8x10^{-2}$
C_5	$1.5x10^{-2}$	$1.5x10^{-2}$	$1.5x10^{-2}$
C_6	$1.0x10^{-2}$	$1.0x10^{-2}$	$1.0x10^{-2}$

Table 2
RETENTION TIMES FOR QUERCITIN, KAEMPHEROL AND RUTIN

Standard	Maximum wavelength [nm]	Retention time t_r [min.]
Quercitin	360	3.009 ± 0.244
Kaempherol	360	4.17 ± 0.275
Rutin	280	7.166 ± 0.356

*- five assays have been made

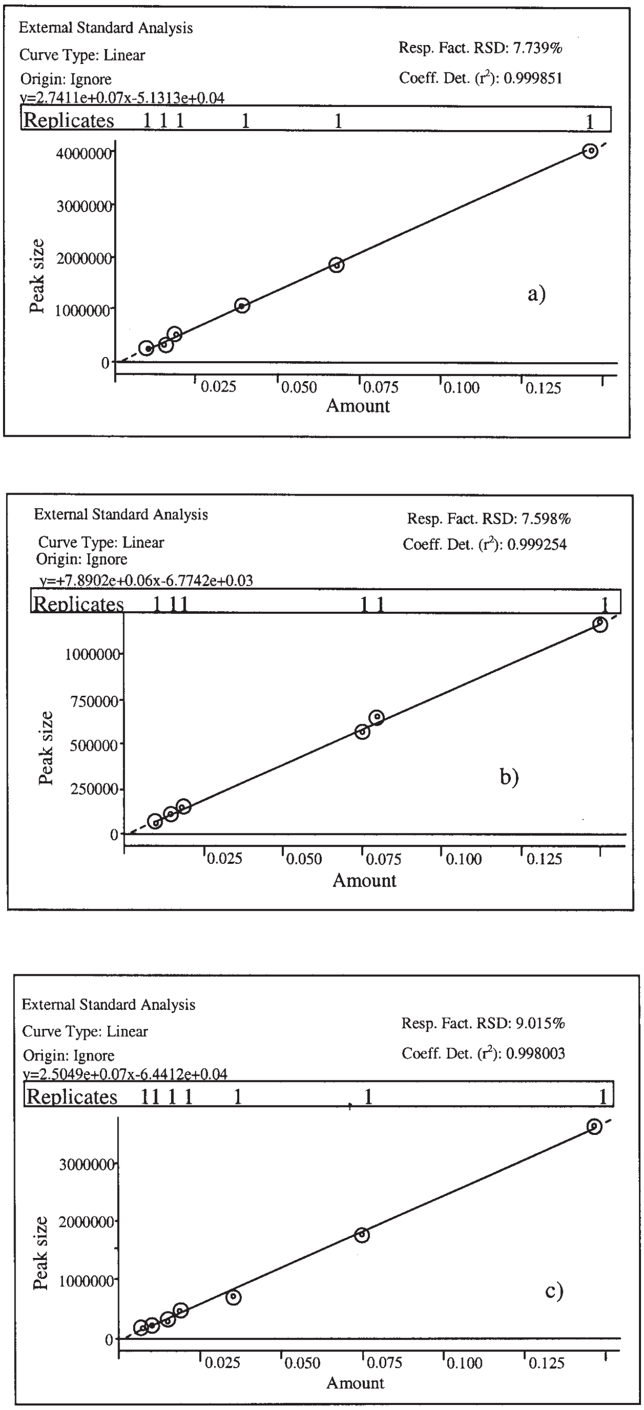

Fig. 2. Calibration curves of the standards:
a)kaempherol, correlation coefficient r^2 = 0.9998;
b)quercetin, correlation coefficient r^2 = 0.9992;
c)rutin, correlation coefficient r^2 = 0.9980

Results and discussions

High performance liquid chromatography

From the chromatograms afferent to the solutions of the three standards, quercetin, kaempherol and rutin were identified based on the retention times presented in table 2.

Once the retention time was established, for each analyzed standard the calibration curves given in figure 2 were determined, using the series of solutions in the concentrations given in table 1.

The equations describing the calibration curves in figure 2 are given by relations (1), (2) and (3):

$$\text{a) } y = 2.7411e^7 x - 5.1313e^4 \text{y} \qquad (1)$$

$$\text{b) } y = 7.8902e^6 x - 6.7742e^3 \qquad (2)$$

$$\text{c) } y = 2.5049e^7 x - 6.4412e^4 \qquad (3)$$

In order to determine the content of kaempherol, quercetin and rutin in the hydro-alcoholic extracts of the investigated plants, the chromatograms at the wavelength afferent to each flavonoid were registered for each extract.

Figure 3 shows the chromatograms for the methanolic extracts of *Alchemilla vulgaris* - common lady's-mantle (aerial part).

Based on the retention time values of the chromatograms afferent to each vegetal extract that was analyzed, the concentrations of kaempherol, quarcetin and rutin were calculated using the equations (1), (2) and (3); the obtained results are given in table 3.

From data presented in table 3 differences regarding the flavonoid content in the studied extracts can be noticed. These differences are given not only by the differences between the chemical composition of the studied plants but also by the type of the vegetal part used to obtain the extracts (leaves, braches, aerial part, roots).

It can be also observed an influence of the extraction mixture composition (ratio methanol: water) on the flavonoid content in the analyzed hydro-alcoholic systems. The increase in the methanol percentage from 80% to 96% leads in most cases to an improvement in the process of flavonoid extraction from the plant material. A similar behaviour has been noticed when the extraction system consisted of ethanol and water [15].

As for the rutin, it can be noticed that, in the hydro-methanolic extracts where it was identified, was in the biggest quantity (as compared to the other two flavonoids). The most widespread flavonoid was quercetin that was identified in all extracts regardless of the alcohol content, except for *Viscum album* (80% methanol), which contained none of the three flavonoids pursued.

Kampherol was not found in *Acorus calamuls* and *Veronica officinalis* (metanol 80%) extracts (80% methanol) and rutin was not found in *Agrimonia eupatori*, *Acorus calamus* (methanol 80%) and *Viscum album* (methanol 96%).

By comparing the results obtained for the investigated plant species with those obtained after the analysis of the flavonoids in *Hydrangea paniculata* and *Zizipus jujuba* species by flash chromatography and high performance liquid chromatography [16] neighboring values were noticed for the 96% methanolic extracts.

For example, the *Solidago virga-aurea* extract (96% methanol) contained 7.1 x 10^{-3} mg/mL quercitin and 9.5 x 10^{-2} mg/mL rutin while the extract of *Hydrangea paniculata* contained 1.0x10^{-2} mg/mL quercitin and 9.3 x 10^{-2} mg/mL rutin.

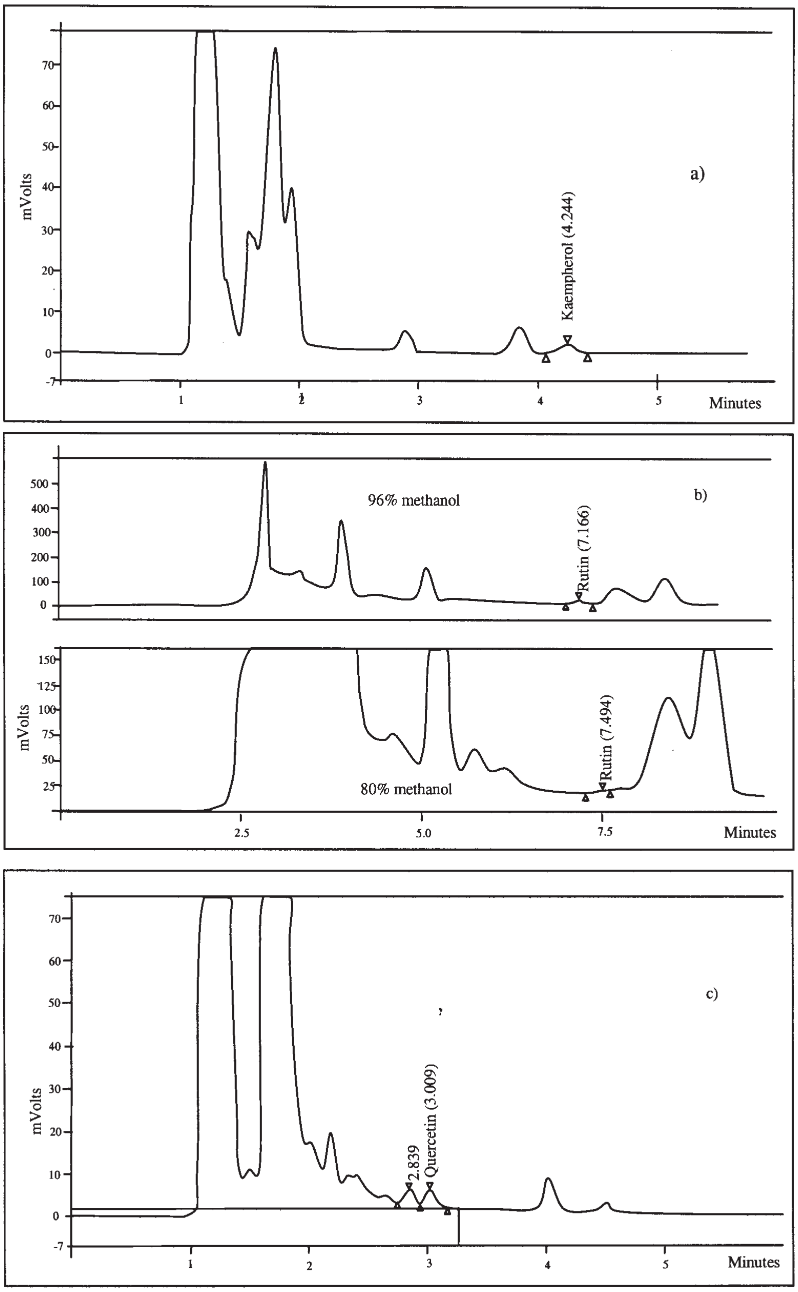

Fig. 3. a)methanolic extract (96% methanol) analyzed at 360 nm for kaempherol
b). methanolic extracts (96% and respectively 80% methanol) analyzed at 280 nm for rutin
c)methanolic extract (96% methanol) analyzed at 360 nm for quercetin

Table 3
QUERCETIN, RUTIN AND KAEMPHEROL CONTENT OF THE HYDRO-METHANOLIC EXTRACTS OF PLANTS FROM *ANGIOSPERMATOPHYTA* AND *SPERMATOPHYTA GENNUS*

VEGETAL EXTRACT	FLAVONOIDS		
	Kaemferol [mg/mL]	Quercitin [mg/mL]	Rutină[mg/mL]
E_1 (96%)	1.9×10^{-3}	13.2×10^{-3}	4.4×10^{-2}
E_1 (80%)	1.2×10^{-3}	3.3×10^{-3}	3.2×10^{-2}
E_2(96%)	2.9×10^{-3}	1.0×10^{-3}	6.8×10^{-2}
E_2 (80%)	1.9×10^{-3}	1.3×10^{-3}	3.8×10^{-2}
E_3 (96%)	—	1.7×10^{-3}	—
E_3 (80%)	—	1.4×10^{-3}	—
E_4 (96%)	1.9×10^{-3}	7.1×10^{-2}	9.5×10^{-2}
E_4 (80%)	1.3×10^{-3}	4.6×10^{-3}	6.6×10^{-2}
E_5 (96%)	2.7×10^{-3}	3.8×10^{-3}	2.2×10^{-2}
E_5 (80%)	2.2×10^{-3}	1.0×10^{-3}	—
E_6 (96%)	2.2×10^{-3}	1.5×10^{-3}	4.7×10^{-3}
E_6 (80%)	—	5.0×10^{-3}	6.6×10^{-3}
E_7 (96%)	2.4×10^{-3}	2.4×10^{-3}	—
E_7 (80%)	—	—	—

E_1 (96%),...., E_7 (96%) – methanolic extracts (96% methanol);

E_1 (80%),...., E_7 (80%) – methanolic extracts (80% methanol);

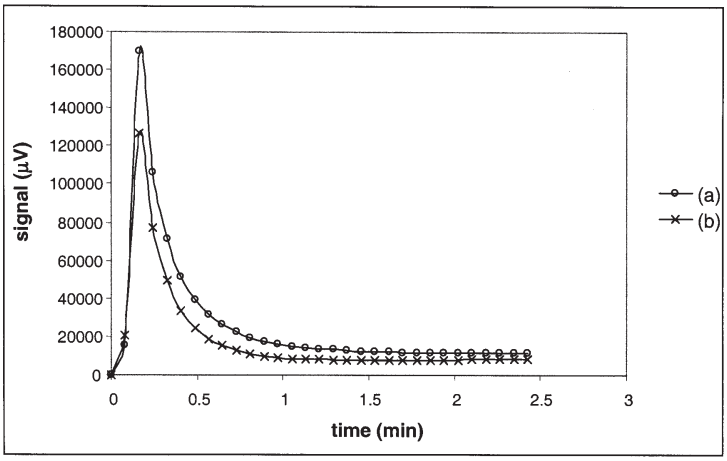

Fig. 4. The shape of the chemiluminescent signal corresponding to the reaction:
a). between luminol and hydrogen peroxide solution without antioxidant added
b). between luminol and hydrogen peroxide solution with antioxidant added (0.1 mL tincture of *Agrimonia eupatoria* – agrimony)

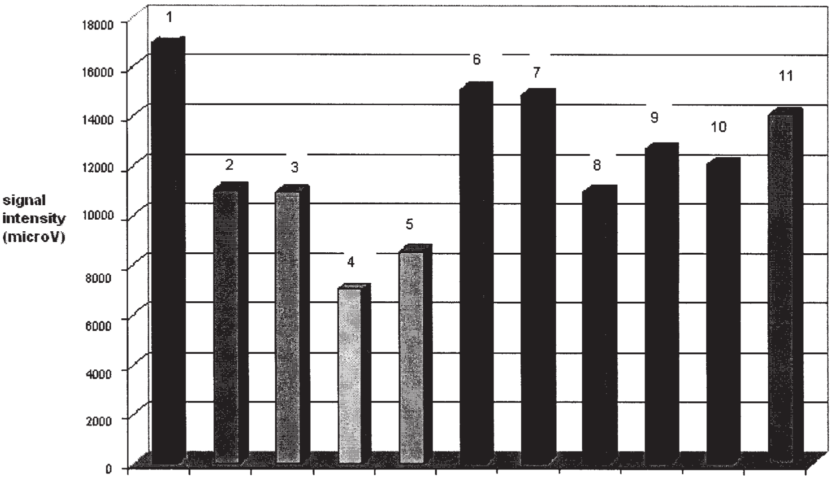

Fig. 5. Comparison of the chemiluminescent signal intensities for:
1- blank assay; 2-kaempherol methanolic solution; 3-quercetin methanolic solution; 4-rutin methanolic solution; 5-methanolic extract (98% methanol) of *Alchemilla vulgaris*; 6-methanolic extract (98% methanol) of *Allium ursinum*; 7- methanolic extract (98% methanol) of *Acorus calamus*; 8- methanolic extract (98% methanol) of *Solidago virga-aurea*; 9- methanolic extract (98% methanol) of *Agrimonia eupatoria*; 10- methanolic extract (98% methanol) of *Veronica officinalis*; 11-methanolic extract (98% methanol) of *Viscum album*

The extract of *Alchemilla vulgaris* contains 4.4 x 10^{-2} mg/mL rutin and the extract *Zizipus jujuba* 3.0 x 10^{-2} mg/mL rutin.

The 80% methanolic extracts make an exception because of the smaller values for the flavonoid content; in some cases the three analyzed flavonoids were not even identified.

Although the data in table 3 suggests a dependency between the content in flavonoids of the extracts and the composition of that hydro-alcoholic extraction systems, respectively the nature of the raw material they are obtained from, these values should not be regarded as absolute, since the content of flavonoids in plants can be influenced by several factors such as climate, soil, cropping period, etc. [17].

The experimental results obtained by chromatographic analysis were in good concordance with those obtained by the use of the external etalon method for the retrieval of known quantities of pure flavonoids [18].

Chemiluminescence method

Luminol chemiluminescence is the result of its reaction in basic environment with powerful oxidation agents such as hydrogen peroxide, leading to the emission of a blue light with a maximum length around 420 nm [19]. The acquisition plate adapted to the spectrophotometer converts the lucent signal in µV.

In order to exemplify the obtained curves, figure 4 shows the shape of the chemiluminescent signal registered for the reaction between luminol and hydrogen peroxide solution compared with the signal registered when over the hzdrogen peroxide solution 0.1 mL methanolic extract (96% methanol) of *Agrimonia eupatoria* – agrimony were added.

Each experiment was repeated four times and for the calculus the averages of the registered values were used.

From figure 4 it can be noticed that immediately after mixing the hydrogen peroxide solution with the luminol solution an intense lucent signal appear, registered by the apparatus as a pick with a maximum around 169604.4 µV (the average of four determinations). The intensity of the lucent signal decreases then rapidly, as the oxygen reactive species (ROS) are consumed. In approximately 2 min the registered signal reaches a minimum (plateau) corresponding to the end of the reaction. If over the hydrogen peroxide solution a certain quantity of antioxidant is added, some of it is consumed, leading to registration of a less intense chemiluminescent signal. The more powerful the antioxidant, the higher the quantity of hydrogen peroxide consumed/minute is (thus remaining a smaller quantity available for the reaction with the luminol solution) and thus the intensity of the chemiluminescent signal is smaller.

In order to evaluate the antioxidant activities of the studied extracts we compared the average of their signal's maximum intensities registered for each case. The obtained results are presented in figure 5 together with those afferent to three standards (rutin, quercetin and myricetin 1.5 mg/mL in methanol).

For quantitative determinations, the inhibition percent (I, %) was determined using relation (4) and the obtained values are given in table 4.

$$I = 100 - \frac{I_{max}^{sample}}{I_{max}^{blank}} \cdot 100 \qquad (4)$$

where:

Table 4
INHIBITION PERCENT VALUES FOR KAEMPHEROL, QUERCETIN, RUTIN AND THE HYDRO-METHANOLIC EXTRACTS OF PLANTS FROM *ANGIOSPERMATOPHYTA* AND *SPERMATOPHYTA* GENNUS

Solution	Kaempherol	Quercetin	Rutin	E1	E2	E3	E4	E5	E6	E7
I (%)	34.86	35.42	58.72	49.75	11.17	12.34	35.32	25.31	28.90	17.02

$I_{\max}^{blank}$ - the average of the signal maximum intensities for the reaction between luminol and hydrogen peroxide solution;

$I_{\max}^{sample}$ - the average of the signal's maximum intensities after adding the antioxidant solution.

The data in table 4 shows that the highest value of the inhibition percentage for the seven analyzed extracts (which means the highest antioxidant activity), comparable with those of the standards, corresponded to common lady's-mantle (49.75%). The lowest antioxidant activity was determined for bear's garlic extract (I = 11.17%).

The inhibition percentage of lady's mantle extract can be explained by the high content of other compounds, besides those studied in this article, with known antioxidant properties, such as ascorbic acid, gallic acid, tannins and phenolic compounds [20].

Trying to correlate the content in quercetin, rutin and kaempherol and the antioxidant activity of the extracts, will not produce satisfying results, which probably suggests that the three flavonoids are not the only ones responsible for it.

Conclusions

The HPLC method has proven to be precise and selective for the identification and cuantification of quercetin, rutin and kaempherol from the vegetal extracts. The flavonoids extraction process from the plant material is influenced both by the nature of the alcohol and its proportion in the extraction mixture. From all the studied extracts, *Alchemilla vulgaris* – common lady's mantle and Solidago virgo-aurea – goldenrod showed the greatest antioxidant activity, probably due to the higher content in phenolic compounds (tannins, antocianidins, flavonoids).

The obtained results may constitute a basis for the evaluation of the preventive role of the studied extracts against the effect of the free radicals and may contribute to the study of their applicability, after purification, in food and pharmaceutical industries.

Antioxidant activity of the flavonoids extracted from the plants had been also discussed in different other studies [21, 22].

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