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ANTIOXIDANT FLAVONOL GLYCOSIDES FROM *Dorycnium hirsutum**

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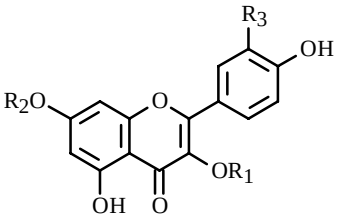
Seven flavonol glycosides were isolated and identified from the aerial parts of Dorycnium hirsutum, *together with catechin, D-pinitol, β-sitosterol, and stearic acid. The extracts and the isolated flavonol glycosides were evaluated for their antioxidant activity, using the DPPH test (radical scavenging) and the lipoxygenase assay (lipid peroxidation).*

Key words: *Dorycnium hirsutum*, Leguminosae, antioxidant, activity, DPPH, lipid peroxidation, flavonol glycoside.

The genus *Dorycnium* includes twelve species distributed all over the world, characterized by the presence of the aminoacid canavanin, which represents the only constituent previously isolated from *D. hirsutum*. From the literature several mono and di-glycosides of kaempferol, quercetin, and myricetin were identified in *D. pentaphyllum* (known also as *D. suffruticosum*) [1], while kaempferol 3,7-di-*O*-α-L-rhamnopyranoside, and kaempferol 3-*O*-β-D-glucopyranoside-7-*O*-α-L-rhamnopyranoside were isolated from aerial parts of *D. rectum* [2].

During our systematic studies on flavonoids of the *Leguminosae* family we have investigated *Dorycnium hirsutum* Ser. in DC. (*Leguminosae*) [3], a perennial herb with white-pink flowers in axillary heads, widely distributed in the Mediterranean area and known in Italy as "Erba veglia" or "Stringiamore" [4].

The phytochemical investigation on *D. hirsutum* aerial parts led to the isolation and characterization of seven flavonol glycosides (**1–7**), sorted as kaempferol or quercetin derivatives with the sugar moiety linked at position 3 and/or 7.



1 - 7

1: R_1 = α-L-Rha*f*, R_2 = H, R_3 = H
2: R_1 = α-L-Rha*f*, R_2 = α-L-Rha*f*, R_3 = H
3: R_1 = (4″-OAc)-α-L-Rha*f*, R_2 = α-L-Rha*f*, R_3 = H
4: R_1 = β-D-Glc*p*, R_2 = α-L-Rha*f*, R_3 = H
5: R_1 = α-L-Ara, R_2 = α-L-Rha*f*, R_3 = H
6: R_1 = α-L-Rha*f*, R_2 = H, R_3 = OH
7: R_1 = (4″-OAc)-α-L-Rha*f*, R_2 = α-L-Rha*f*, R_3 = OH

The determination of the total flavonoid content of dried aerial parts and of the methanolic, EtOAc, *n*-BuOH residues, according to the method described in the Italian Pharmacopoeia, showed that the amount of flavonoids in the EtOAc extract was considerably higher than in the other extracts, as reported in Table 1.

*Dedicated to the memory of Prof. I. Morelli.

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TABLE 1. Total Flavonoid Content in Extracts and Dried Aerial Parts of *D. hirsutum*

Plant material	Weight, mg	Flavonoid weight, mg	% (w/w)
Dried aerial parts	600	8.16	1.36
MeOH extract	135.1	5.92	4.38
EtOAc extract	134.7	24.07	17.87
n-BuOH extract	144.1	1.66	1.15

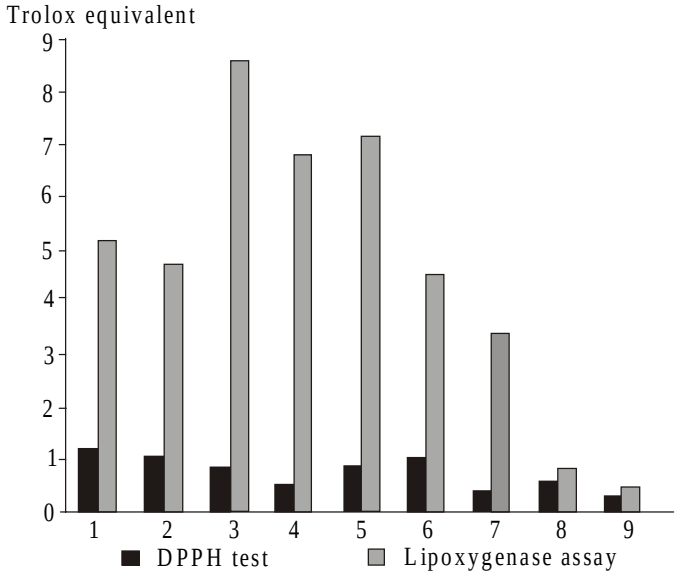

Fig. 1. Antioxidant activity of two extracts (8, EtOAc; 9, *n*-BuOH) and seven flavonoids (**1–7**) isolated from *D. hirsutum*: in the DPPH test, in the lipoxygenase assay.

The antioxidant activity of the EtOAc and *n*-BuOH residues along with that of seven flavonoids isolated from the plant was determined using the DPPH test and the lipoxygenase assay. The resulting activity was reported as Trolox equivalents (1 μM TE) (Fig. 1).

In the DPPH test kaempferol 3-*O*-α-L-rhamnopyranoside (**1**) showed the best antioxidant activity (1.226 TE), while in the lipoxygenase assay kaempferol 3-(4″-*O*-acetyl)-*O*-α-L-rhamnopyranoside-7-*O*-α-L-rhamnopyranoside (**3**) showed the best activity in the lipid peroxidation.

The antioxidant activity of compound **1** and kaempferol 3,7-di-*O*-α-L-rhamnopyranoside (**2**) was very similar ($p>0.05$) in both tests. These results are in agreement with Heim [5] in which the A-ring substitution (7-*O*-glycosilation) does not affect the tested activity. Glycosides are usually weaker antioxidants than their corresponding aglycones, but the presence of two glycosyl substituents instead of one does not consistently decrease antioxidant ability. The acetylation at 4″ position of rhamnose in compound **3**, in comparison with **2**, induces no significant variations in both test ($p<0.05$). Between the acylated flavonoids [compounds (**3**) and quercetin 3-(4″-*O*-acetyl)-*O*-α-L-rhamnopyranoside-7-*O*-α-L-rhamnopyranoside (**7**)], compound **3** revealed a higher antioxidant activity, statistically significant ($p<0.001$) in both tests.

The presence in compound kaempferol 3-*O*-β-D-glucopyranoside-7-*O*-α-L-rhamnopyranoside (**4**) of a glucose moiety instead of a rhamnose, as in **2**, did not affect the antioxidant activity in the lipoxigenase test ($p>0.05$), while a significant reduction was observed in DPPH test ($p<0.001$). The substitution with arabinose, as in kaempferol 3-*O*-α-L-arabinopyranoside-7-*O*-α-L-rhamnopyranoside (**5**), did not affect the antioxidant activity in both test.

The butanolic residue is always less active than the EtOAc residue, in agreement with the higher flavonoid content detected in the latter extract. In the DPPH test the difference between the two extracts is statistically significant ($p<0.01$); on the contrary the lipoxygenase test revealed differences that are not very significant. In both assays the antioxidant activity of the two crude extracts is generally lower compared to the antioxidant activity of each pure compound, as shown in Table 2 and Fig. 1.

TABLE 2. Antioxidant Activity of Butanolic Extract, Ethyl Acetate Extract and of Isolated Compounds

Compound	mol TE/g*	
	DPPH test	lipoxygenase assay
1	1.23±0.19	5.24±0.6
2	1.08±0.09	4.71±0.99
3	0.83±0.11	8.58±1.79
4	0.52±0.11	6.83±1.45
5	0.89±0.11	7.23±1.19
6	1.04±0.16	4.56±0.88
7	0.39±0.1	3.40±0.73
Ethyl acetate extract	0.60±0.09	0.81±0.16
Butanolic extract	0.29±0.03	0.47±0.13

*The values are the average of three determinations (± S.D.).

Differences in antioxidant behaviour of the isolated flavonoids can be ascribed to the different methods used for the determination: in fact the DPPH test is not discriminative with respect to the radical species [6], while the protection of lipid peroxidation is more complex.

Antioxidants can exercise their protective properties at different stages of the oxidation process and by different mechanisms that can include free radical scavenging activity and also deactivation of metals, inhibition of breakdown of lipid hydroperoxides to unwanted volatile products, regeneration of "primary" antioxidant, singlet oxygen quenching, etc. [7], [5]. The TE values obtained with the lipoxygenase assay are significantly ($p>0.001$) higher compared to values obtained with the DPPH test, because the lipoxygenase assay (enzymatic assay) has better sensitivity in comparison with the DPPH assay (colorimetric assay).

EXPERIMENTAL

Plant material. *Dorycnium hirsutum* Ser. in DC. aerial parts were collected on the coasts near Livorno (Italy) in April 2000 and identified by Dr. S. Maccioni. A voucher specimen is deposited in the Herbarium Botanici Pisani PI-Nuove Acquisizioni (no. 3697-*Dorycnium hirsutum*/22).

Extraction and Isolation. Dried and powdered aerial parts (1450 g) were extracted in a Soxhlet apparatus with *n*-hexane, $CHCl_3$, and MeOH in sequence to obtain the corresponding extracts E (28.9 g), C (77.6 g) and M (68.4 g). The methanolic extract was partitioned first with EtOAc (A residue, 15.4 g) and then with *n*-BuOH (B residue, 35.3 g). The chloroform extract, submitted to gel filtration on Sephadex LH-20 eluted with MeOH–$CHCl_3$ (1:1), afforded pure D-pinitol (47 mg), β-sitosterol (20 mg), and stearic acid (52 mg). Residue A, fractionated by gel filtration on a Sephadex LH-20 column (eluting with MeOH), yielded **3** (6 mg), **7** (10 mg), **1** (14 mg), quercetin 3-*O*-α-L-rhamnopyranoside (**6**) (14 mg), and catechin (141 mg). From the B residue, after submission to gel filtration on Sephadex LH-20 eluting with MeOH–H_2O, 8:2, **2** (11 mg), **4** (16 mg) and **5** (6 mg) were obtained. The identification of all the isolated compounds was deduced by analysis of their ^1H and ^{13}C NMR spectra in comparison with those of published data [8–15].

Total Flavonoid Determination. The total flavonoid content in the dried aerial parts (600 mg) and in the methanolic (135.1 mg), EtOAc (134.7 mg), and *n*-BuOH (144.1 mg) extracts of *D. hirsutum* was determined according to the method described in the Italian Pharmacopoeia [16], measuring the absorbance at 425 nm in a Perkin–Elmer UV-Vis spectrometer Lambda 11 and using quercetin dihydrate as standard. The determination coefficient was above 0.99 (five points; three assays).

Determination of Free Radical Scavenging Action. The antioxidant activity of the extracts and isolated compounds was examined using the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH). The free radical scavenging activity was measured using the method of Mellors and Tappel [17] appropriately modified. The extract B was dissolved in methanol, while the other samples were dissolved in ethanol. The solutions of samples were added to 1.5 mL of 100 μM DPPH ethanolic solution. The absorbance's decrease at 517 nm was recorded after 20 min and the antioxidant activity was expressed as percent decrease in absorbance

(corrected for the control, without antioxidant agents added). The values obtained were used to determine Trolox equivalents (TE).

Lipoxygenase Inhibitory Assay. Lipoxygenase are iron-containing enzymes that catalyze the dioxygenation by molecular oxygen of *cis,cis*-1,4-pentadiene fatty acids. The activity of the enzyme was assayed spectrophotometrically according to Holman. This method was modified by Sud'ina et al. [18]. The assay mixture (1 mL) consisted of 10 mM linoleic acid, the sample (or the same quantity of ethanol/methanol as reference), and 50 mM sodium phosphate, pH 6.8. This mixture was maintained at 23°C for 20 min. Subsequently, 0.18 μg /mL commercial 5-lipoxygenase was added to the mixture and the formation of hydroperoxides from linoleic acid was observed spectrophotometrically at 235 nm at 23°C. The results were expressed as percent of lypoxygenase activity decrease and the values obtained were utilized to determine Trolox equivalents (TE).

Statistical Analysis. All experiments were performed at least three times. The results were expressed as mean ± standard deviation (S.D.). The Student *t*-test was used to determine the statistical significance of the results obtained analyzing each sample with two different assays; $p<0.05$ was considered statistically significant.

Statistically significant differences between median of groups were measured using the nonparametric Kruskal-Wallis test followed by the Dunn multiple comparison test, considering statistically significant $p<0.05$.

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