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Hypericum perforatum L., *H. maculatum* Crantz., *H. calycinum* L. and *H. pulchrum* L.: Phytochemical and Morphological Studies

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Dedicated to the memory of Professor Ivano Morelli.

Four species of *Hypericum* growing in Italy were characterized morphologically and chemically: *Hypericum perforatum* L., *H. maculatum* Crantz., *H. calycinum* L. and *H. pulchrum* L. The composition of secondary metabolites (phloroglucinols, naphthodianthrones, flavonoids) in the aerial parts of plants collected in different habitats was analysed. The four species show different compositions of phloroglucinols and naphthodianthrones, but there was no qualitative difference in flavonoid content of the species analysed. Study of main-constituent variation during the ontogenetic cycle showed that hypericin decreases and hyperforin increases during the reproductive phase. In St. John's Wort, hypericin and hyperforin are thought to be localised in black nodules. Our investigation shows no clear correlation between either the presence or absence of nodules and hypericin or hyperforin content.

Keywords: flavonoids, *Hypericum*, naphtodianthrones, ontogenetic cycle, phloroglucinols.

The genus *Hypericum* (Guttiferae) comprises herbs and shrubs, distributed all over the world, with long, opposite leaves and flowers usually organised in a terminal inflorescence.

Many ancient writers wrote about the medical properties of this genus and in particular of St. John's Wort, noting its use as a vulnerary and as a balm for wounds, burns, ulcers, and bites [1-2]. In recent years *Hypericum perforatum* has received increasing attention for the treatment of mild and moderate depression [3-4].

The great interest on *H. perforatum* and its potential for human health have encouraged us to investigate the productivity of some *Hypericum* species growing in Italy. In this work four species were studied: *H. perforatum* L., *H. maculatum* Crantz., *H. calycinum* L. and *H. pulchrum* L. All these species are herbaceous plants. *H. perforatum* is characterized by a two – winged stem and black nodules over the whole plant; *H. maculatum* is different only for the four – edged stem. *H. pulchrum* is characterized by sessile leaves, small flowers, hirsute sepals with black nodules, and petals, stems and sepals with translucent glands. *H. calycinum* has typical inflorescences, but the black nodules and translucent glands are absent [5]. We have characterized each species chemically and morphologically. The chemical study has been concerned with the composition of flavonoids, phloroglucinols and naphtodianthrones; the morphological analysis has regarded the presence and distribution of secretory structures.

In this study we have analysed the secondary metabolites with interesting and demonstrated biological activity (**a**, chlorogenic acid; **b,** rutin; **c**, hyperoside; **d**, isoquercitrin; **e**, quercitrin; **f**, quercetin; **g**, hypericin; **h,** hyperforin) [3-4,6-8]. The MeOH extracts were analysed by RP-HPLC. The identification of peaks was effected on the basis of

the comparison of retention times and the use of a spectral library based on pure compounds previously described.

The qualitative analysis has shown four different profiles. The first profile, belonging to *H. perforatum* is characterized by the presence of all compounds under study: **a** (λ = 270 nm; Rt = 13.5), **b** (λ = 270 nm, Rt = 25.2), **c** (λ = 270 nm, Rt = 25.8), **d** (λ = 270 nm, Rt =26.3), **e** (λ = 270 nm, Rt = 30.2), **f** (λ = 270 nm, Rt = 37.4), **g** (λ = 590 nm, Rt = 42.9), **h** (λ = 270 nm, Rt = 51.3)**.** The second profile, typical of *H. maculatum* is characterized by the absence of hyperforin. The third profile is characterized by the absence of hypericin (*H. calycinum*). The fourth profile, where hypericin and hyperforin are absent, characterises *H. pulchrum*.

For quantitative analysis we produced eight calibration curves, as described in "experimental". For all compounds, a linear relationship between peak area and concentration was observed, with a correlation coefficient always better than $r = 0.997$. Analysis of the four species under study was performed during the flowering phase (Table 1). In all species, the content of **a** was highly variable from 0.16 to 4.98%. Rutin (**b**) and hyperoside (**c**) (not always detectable separately) were the more abundant flavonoids, except in *H. calycinum* where quercitrin (**e**) and quercetin (**f**) were more important. *H. perforatum* was characterized by the presence of hypericin (**g**) (0.13-0.18 %) and hyperforin (**h**) (up to 10%). A considerable amount of **g** was also detected in *H. maculatum* and of **h** in *H. calycinum* (0.54%). The analysis of samples of *H. perforatum* collected at different altitudes showed that the contents of chlorogenic acid, flavonoids and hypericin seem not to be affected by the altitude; on the contrary very significant decreases were found in the **h** content in the sites at higher altitudes (from 10% to 3%) (Table 1).

Furthermore in *H. perforatum*, the analysis of metabolite content was performed during the reproductive phase (pre-flowering I, flowering II and fruiting phase III) with the following results. **a**: the content was variable in the different populations studied; **b, c, d, e**: there was a gradual decrease from the I to III phase; **f**: the content of this compound reached the maximum level during the II phase; **g**: the content decreased with values in a range of less than 10%; **h**: the content was low in the I phase and

Table 1: Secondary metabolites content during the flowering phase (% dry wt.).

Samples (Altitude)	a	b	c	d	e	f	g	h
				H. perforatum				
4 (176 m)	3.45	6.65		2.27	2.97	0.14	0.13	10.05
8 (180 m)	4.98	7.24		7.10	1.50	0.47	0.13	10.76
9 (186 m)	4.70	6.12		3.20	4.10	0.10	0.13	10.10
5 (200 m)	4.62	9.35	2.08	1.30	5.44	0.10	0.15	10.65
12 (200 m)	0.16	7.24		5.89	3.04	0.45	0.14	10.20
7 (470 m)	2.27	8.24		3.70	2.18	0.23	0.13	7.50
11 (500m)	1.27	7.98	2.98	2.59	0.86	0.58	0.18	8.25
6 (800 m)	0.99	5.16	2.88	2.29	0.94	0.23	0.16	5.10
10 (900 m)	3.26	11.78	2.09	2.35	1.50	0.10	0.17	5.40
1 (1090 m)	0.63	4.08	1.89	1.71	1.72	0.57	0.14	3.54
3 (1400 m)	1.73	8.42	1.92	1.36	0.36	1.55	0.15	3.09
2 (1600 m)	0.47	11.13	2.07	1.56	0.47	0.70	0.13	3.74
				H. maculatum				
13 (560 m)	0.64	10.72		3.16	0.19	2.13	0.12	-
				H. calycinum				
14 (180 m)	-	0.30	0.63	0.37	2.28	1.54	-	0.54
				H. pulchrum				
15 (560 m)	0.24	4.30	0.61	2.55	3.52	0.30	-	-

(**a**) chlorogenic acid, (**b**) rutin, (**c**) hyperoside, (**d**) isoquercitrin, (**e**) quercitrin, (**f**) quercetin, (**g**) hypericin and (**h**) hyperforin.

Table 2: Secondary metabolite content (%, dry wt.) during the reproductive phase of *H. perforatum* collected in Comabbio.

	a	b	c	d	e	f	g	h
Phase I	3.69	12.95	3.56	3.48	2.09	0.12	0.13	4.80
Phase II	2.27	8.24		3.70	2.18	0.23	0.13	7.50
Phase III	0.56	3.75	1.26	1.07	1.51	0.15	0.11	8.00

Table 3: Localities and identification numbers of samples.

Samples	Locality	Herbarium No.
H. perforatum		
	Valle d'Aosta	
1	Anthey-St. Andrè (AO - 1090 m)	Hy-101
2	Colle de Joux (AO - 1600 m)	Hy-102
3	Crest (AO - 1400 m)	Hy-103
	Lombardia	
4	Canegrate (MI - 176 m)	Hy-104
5	Collebeato (BS - 200 m)	Hy-105
6	Colle Brianza (LC – 800 m)	Hy-106
7	Comabbio (VA – 470 m)	Hy-107
8	Parabiago Canale Villoresi (MI - 180 m)	Hy-108
9	Parabiago Santa Maria (MI - 186 m)	Hy-109
10	Pezzaze (BS - 900 m)	Hy-110
	Friuli Venezia Giulia	
11	Costa (UD – 500 m)	Hy-111
12	Monte Spaccato (TS – 200 m)	Hy-112
H. maculatum		
	Friuli Venezia Giulia	
13	Ampezzo Carnico (UD - 560 m)	Hm-101
H. calycinum		
	Lombardia	
14	Brescia (BS - 180 m)	Hc-101
H. pulchrum		
	Piemonte	
15	Mondovì (CN - 560 m)	Hp-101

reached the maximum in the III phase, with an increase of more than 50%. Table 2 shows an example (sample collected in Comabbio).

Morphological characteristics

H. perforatum: Leaves: presence, distribution and density of black nodules (b.n.) is variable among populations: sometimes only on the upper side of the lamina, and sometimes on both sides. Along with b.n., it is possible to find translucent glands, which confer the typical aspect at the leaves. Stems: b.n. are always present; there are only differences in the density of these structures. Petals and sepals: b.n. are always present on the borders. In some populations there are also secretory canals on all surfaces. Ovary: the surface is rich in translucent glands. B.n. are only present on the placenta. Stamen: one b.n. is always present between the thecae of anthers.

H. maculatum: This species shows, in particular, red glands on the stems.

H. calycinum: B.n. are completely absent.

H. pulchrum: B.n. are only on the sepals. From the above results it appears that *H. maculatum* and *H. calycinum* may be considered a good source of phloroglucinols and naphthodianthrones, respectively. It is worthwhile to notice that the production of **g** in *H. maculatum* is comparable, in quantity, to that of the well-known *H. perforatum.* It is also important to note the significant influence of altitude on the productivity of **h** in *H. perforatum.* Finally, even if many authors report that **g** and **h** are localised in b.n. [9-10], our investigation shows no clear correlation between the presence/absence of nodules and either hypericin or hyperforin content.

Experimental

Plant material: Fifteen populations of *Hypericum* belonging to the species *H. perforatum*, *H. maculatum*, *H. calycinum* and *H. pulchrum* were collected in different localities of Valle d'Aosta, Piemonte, Lombardia and Friuli Venezia Giulia, in Northern Italy during the summer of 2000 and determined according to Pignatti [5]. Voucher specimens are deposited in the Dipartimento di Biologia, Università di Milano. Table 3 shows localities, altitude and identification numbers of samples.

***Extraction and separation*:** Dried powdered aerial parts (1 g), taken 20-25 cm from the apex, as described in the Italian F.U., were extracted in a Soxhlet apparatus with 200 mL of MeOH for six hours. From the extract solution, 4 mL was diluted to 10 mL and submitted to RP-HPLC on a Merck LiChrospher 100 RP-18 column (5 μm, 250 x 4 mm, flow rate 1mL min^{-1}) with ternary gradient elution [A: H_2O (acidified at 0.3% with H_3PO_4); B: ACN; C: MeOH; gradient: 0 min 100% A; 10 min 85% A, 15% B; 30 min 70% A, 20% B; 40 min 10% A, 75% B; 55 min 5% A, 80% B; minimum re-equilibration time between two injections: 10 min]. The detection range was 270-590 nm.

Chlorogenic acid (**a**), rutin (**b**), hyperoside (**c**), isoquercitrin (**d**), quercitrin (**e**), quercetin (**f**), hypericin (**g**) and hyperforin (**h**) were obtained commercially, **a-g** from Extrasynthese, Genay, France, and **h** from PhytoLab GmbH e Co. KG, Labor Addipharma, Hamburg, Germany. These compounds were used to produce a spectral library in order to identify chromatographic peaks. The concentration of pure compounds was 0.4 mg mL^{-1} and the injection volume was 15 μL. The analytical chromatographic analyses were performed with a Merck-Hitachi L 6200 system with a Hewlett Packard 1040 photo diode array detector, controlled by HP-Chemstation (Hewlett Packard) software. Calibration curves for **a**-**h** were realized with solutions of known concentrations (0.4, 0.2, 0.1, 0.05, 0.025 mg/mL).

Morphological analyses: The morphological analyses were performed using a stereomicroscope, model MZ 6, Leica Mycrosystems S.p.A, Milano, Italy.

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