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Morphological, histochemical and phytochemical investigation of the genus *Hypericum* of the Central Italy

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Abstract

Eight entities of the genus *Hypericum* that spontaneously grow on the Central Italy (Appennino Umbro-Marchigiano) have been studied under the morphological, histochemical and phytochemical aspects. From the morphological standpoint, they differ in the shape and size of flowers and leaves and in the dimension and distribution of the secretory structures through the various parts of the plant. It has been possible, with the histochemical and phytochemical studies, to localize and identify some secondary metabolites inside the secretory structures.

Keywords: *Hypericum*; Morphology; Histochemistry; Phytochemistry; Appennino Umbro-Marchigiano

1. Introduction

The use of *Hypericum* in the popular medicine as antiinflammatory and in the healing of sores and burns of various origin has been and is currently very diffuse, although the

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Fig. 1. (a) *H. perforatum* var. *perforatum*; (b) *H. perforatum* var. *angustifolium*; (c) *H. tetrapterum*; (d and e) *H. hirsutum*; (f) *H. hyssopifolium*; (g) *H. richeri*; (h) *H. androsaemum*; and (i) *H. montanum.*

increased interest for this herb is because of its new therapeutic applications [1–5]. Previous investigations on the morphology and histochemistry regarded in particular the *Hypericum perforatum* [6–10].

In the present work, eight species (Fig. 1) of the genus *Hypericum* (Guttiferae) that spontaneously grow on the Appennino Umbro-Marchigiano [11,12] have been studied under the morphological, histochemical and phytochemical aspects: *H. perforatum* L sp. *perforatum*, *H. perforatum* L. sp. *angustifolium* DC, *H. hirsutum* L., *H. tetrapterum* Fries, *H. montanum* L., *H. hyssopifolium* Chaix, *H. richeri* Vill., *H. androsaemum* L.

The aim was the outstanding of the main differences of the genus *Hypericum* under the qualitative and quantitative standpoint for possible therapeutic applications.

2. Experimental

2.1. Plant material

The samples of the eight species examined have been collected during flowering in various localities of the Appennino Umbro-Marchigiano, as reported in Table 1.

2.2. Methods

The morphological study has been performed on fresh material observed with a photostereoscopy Wild 308700, a photomicroscope Olympus BH2, and a lucid chamber Leitz SM-LUX.

The histochemical study has been carried out to evidence the presence of hypericin, flavonoids and of essential oil in the various parts of the plant. The flavonoids and hypericin locations have been determined with the fluorescence through the photomicroscope Olympus BH-2 RFCA with a 455-nm dicroic mirror, a 475-nm arrest filter and

Table 1
Collection localities and habitat of the genus *Hypericum* of the Appennino Umbro-Marchigiano*

Species	Collection locality	Height (m above the sea level)	Habitat
H. perforatum ssp. *angustifolium*	Meadows of Ragnolo	1320	Arid pasturelands
	Piangrande	1300	
H. perforatum ssp. *perforatum*	Piangrande	1300	Pasturelands
H. richeri	Piangrande	1300	Mat-grass pastures
H. tetrapterum	Montelago	920	Wet environment
H. hyssopifolium	Meadows of Ragnolo	1320	Cliffy environment
H. montanum	Montelago	920	Woods
H. hirsutum	Montelago	920	Woods and hedges
H. androsaemum	Paganico	670	Chestnut woods

* The voucher specimens were deposited in the Herbarium Camerinensis (CAME), Department of Botany and Ecology of UNICAM, Camerino.

a 420- to 440-nm field of excitation. The presence of the essential oil has been evidenced by histochemical reaction with Sudan III and glacial acetic acid as a control [6].

A phytochemical analysis was performed to confirm the presence of the compounds mentioned above. The top of the flowers have been collected around 20 cm from the flower apex; the material, dried at r.t. has been ground with a blender MFC model DCFH 48 IKA-WERK using filters of 1 mm in diameter. Samples, 5 g each, were Soxhlet extracted with 400 ml of MeOH–acetone, 1:1. The evaporated extracts were dissolved in 50 ml of MeOH, filtered, evaporated in vacuo, then taken up with MeOH [13]. Each extract was chromatographed in reverse phase by an HPLC HP 1090 equipped with an autosampler HP series 1100, binary pump and DAD detector. The column used was a Jupiter 5 μ (Phenomenex) C18 300 Å 250×4.6 mm, equipped with a column guard Cartridge widepore C18 4×3-mm protection system Cartridge. The monitored wavelengths were 270 and 590 nm that allowed the determination of flavonoids and naphthodianthrones; reference wavelength was 470 nm. The adopted chromatographic method was the one of Brolis et al. [14], with some modifications; the ternary pumping system was replaced with a binary one. Eluent A was water (0.02% phosphoric acid pH 2,7), eluent B acetonitrile/methanol (90:10) linear gradient (elution: 0–10 min isocratic 85% A, 10–30 min linear gradient from 85% A to 65% A), using the following chromatographic conditions: sample concentration 3 mg/ml in methanol, injections volume of 1 μl, column temperature 30 °C, flow of 1 ml/min, λ of acquisition 270 and 590 nm (passing bandwidth 4 nm), reference λ 470 nm (passing bandwidth 80 nm). The method adopted for the components identification was the comparison of their retention times with respect to those of standards chromatographed under the same conditions. In addition, UV spectra and those of the standards were compared using the DAD. The obtained chromatograms have been compared with those reported in the literature [14, 15]. Concentrations have been determined using the method of the direct calibration [16].

3. Results and discussion

3.1. Morphology

From the morphological and anatomical standpoint, three kinds of secretory structures have been evidenced: black nodules, translucent glands and secretory canals (Figs. 2–7).

The black nodules are present in the petals of all the studied species, with the exception of *H. montanum*; in the sepals of all the species; in the leaves of all the species with the exception of *H. hyssopifolium* and *H. hirsutum*; in the stems except for *H. hyssopifolium*, *H. montanum* and *H. hirsutum*; in the stamens except for *H. hirsutum*. Their shape is spheroidal, are numerous along the margins of the lamina and rarely found inside. The black nodules in the petals of *H. perforatum* var. *perforatum* are present inside, with a longitudinal and spheroidal shape.

The secretory canals of lengthened shape are located in the petals and sepals of all the species; they lack on stems, leaves and stamens.

The translucent glands, generally small, numerous and spherical, are present only in all species leaves.

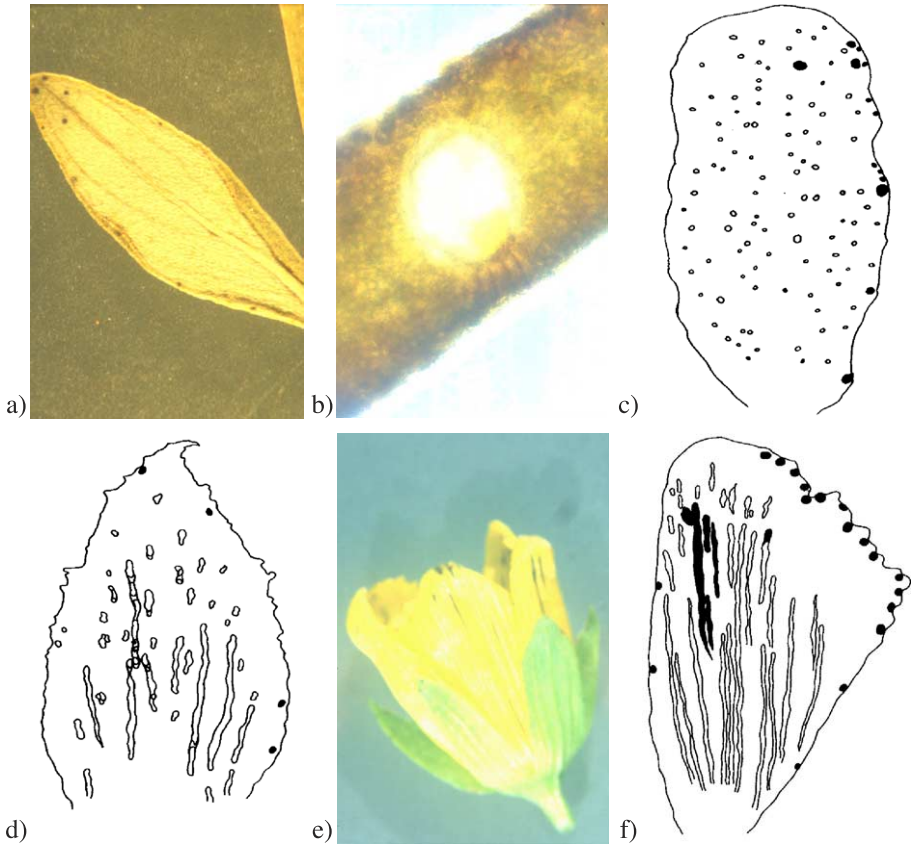

Fig. 2. *H. perforatum* var. *perforatum* (a) leaf with nodules along the border and apex; (b) transversal section of the leaf with the translucent gland; (c) leaf with nodules and translucent gland; (d) sepal with nodules and canals; (e) flower; and (f) petal with nodules stretch and canals.

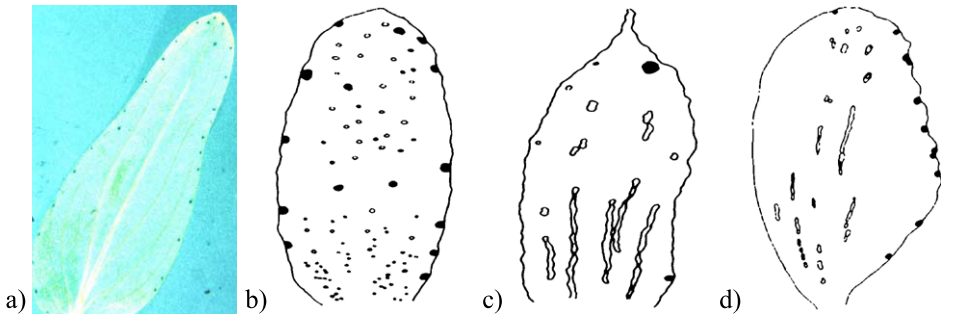

Fig. 3. *H. perforatum* var. *angustifolium* (a) leaf with nodules along the border and inside the lamina ; (b) leaf with nodules and translucent glands; (c) sepal with nodules and canals; and (d) petal with nodules and canals.

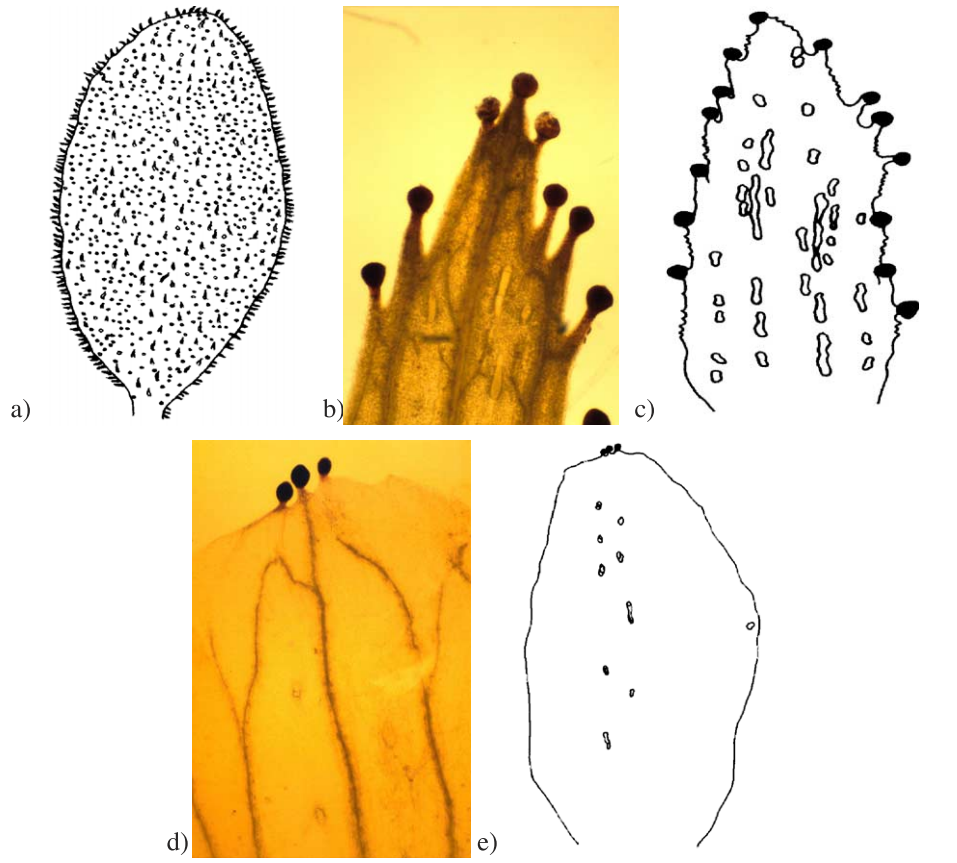

Fig. 4. *H. hirsutum* (a) leaf with the translucent gland; (b and c) sepal with nodules and canals; and (d and e) petal with nodules and canals.

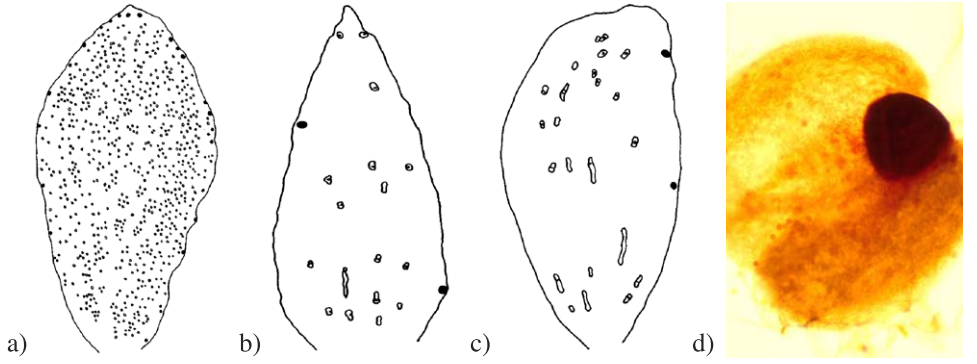

Fig. 5. *H. tetrapterum* (a) leaf with nodules and translucent glands; (b) sepal with nodules and canals; (c) petal with nodules and canals; and (d) stamen with nodule.

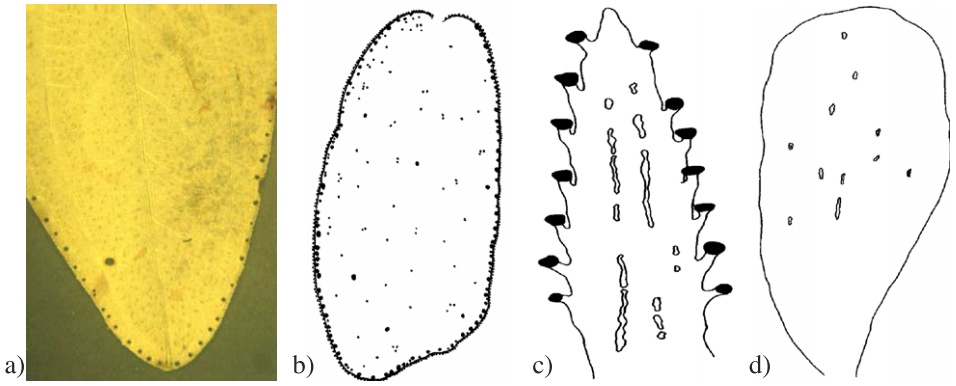

Fig. 6. *H. montanum* (a and b) leaf with nodules and translucent glands; (c) sepal with nodules and canals; and (d) petal with canals.

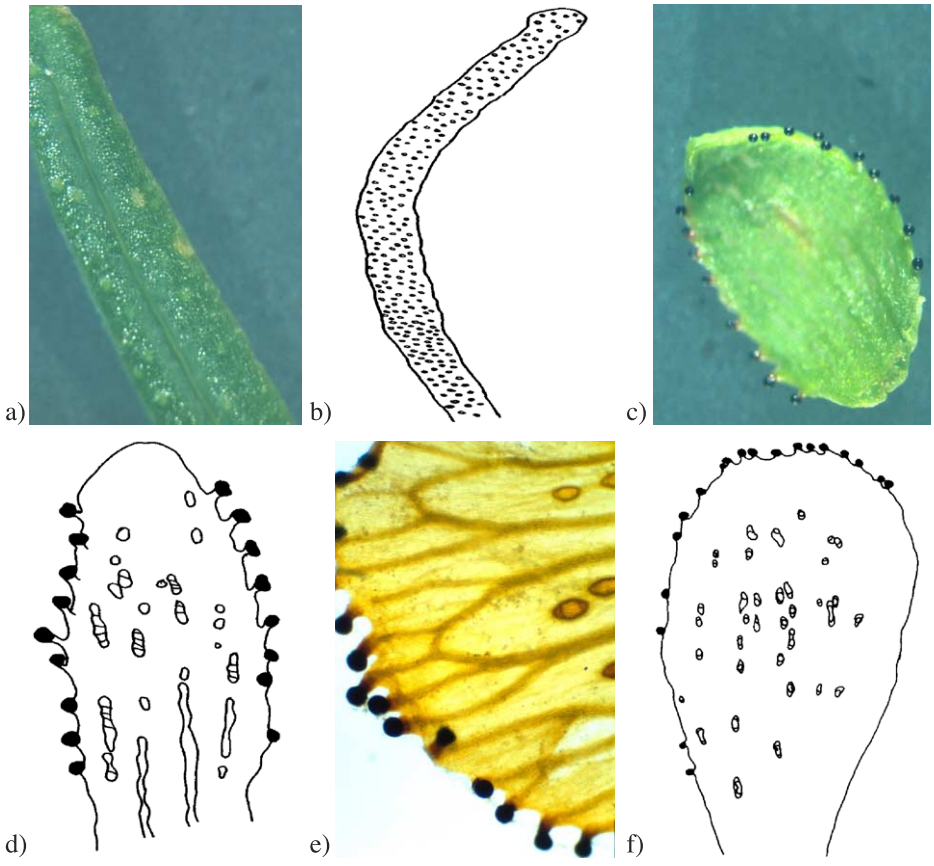

Fig. 7. *H. hyssopifolium* (a and b) leaf with translucent glands; (c and d) sepal with nodules and canals; and (e and f) petal with nodules and canals.

3.2. Histochemistry

The fluorescence study (Fig. 8) pointed out the presence of hypericin (black arrows) in the black nodules and secretory canals. Inside the secretory canals, flavonoids have been identified too (white arrows). The Sudan III reaction allowed the identification of the essential oil that was found in the translucent glands and secretory canals (dot-filled arrows).

3.3. Phytochemistry

From the sample analysis performed with the HPLC have been identified qualitatively and quantitatively eight secondary metabolites. Table 2 reports for each species and

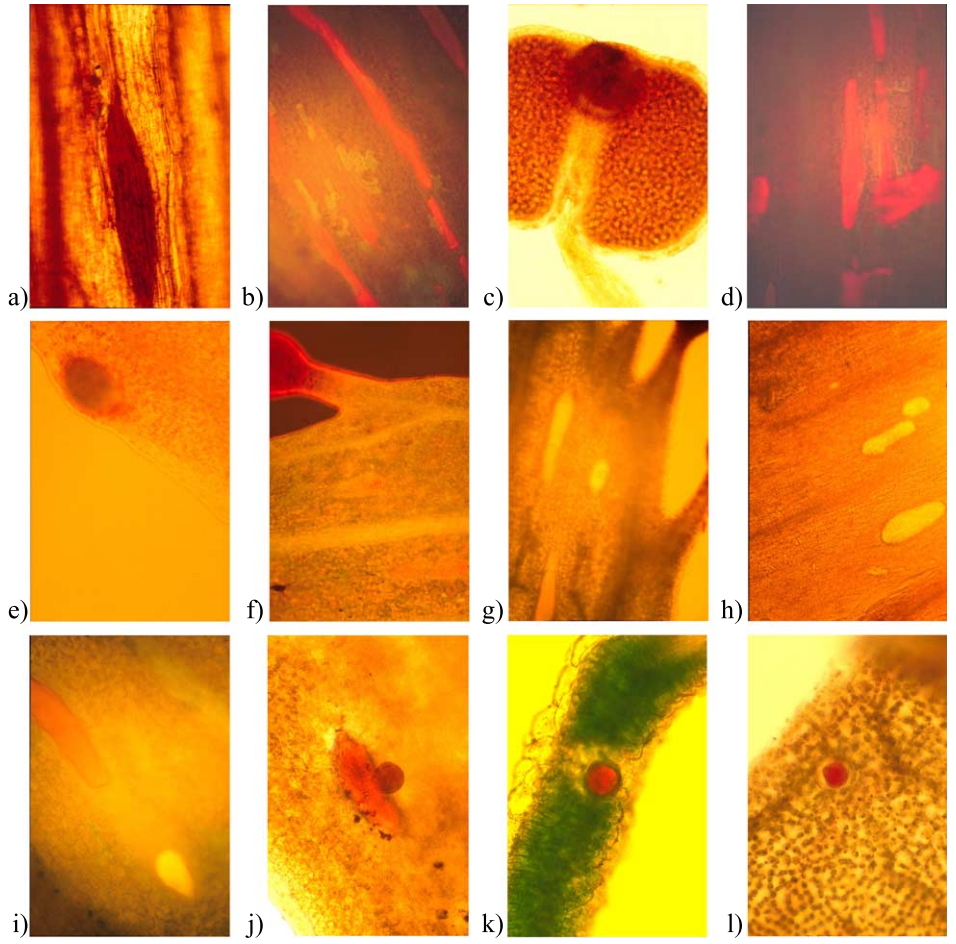

Fig. 8. Hypericin in (a–c) *H. perforatum* var. *perforatum*; (d) *H. perforatum* var. *angustifolium*; (e) *H. tetrapterum*; (f) *H. montanum*. Flavonoids in (g) *H. hirsutum*. Flavonoids and hypericin in (h) *H. perforatum* var. *angustifolium*; (i) *H. hyssopifolium*. Essential oil in (j and k) *H. perforatum* var. *angustifolium*; (l) *H. hirsutum*.

Table 2
Secondary metabolites identified in *Hypericum* of the Appennino Umbro-Marchigiano*

Species	Chlorogenic acid	Rutin	Hyperoside	Isoquercitrin	Quercitrin	Quercetin	Hypericin	Hyperforin
H. perforatum ssp. *angustifolium* (meadows of Ragnolo)	2.191	27.308	7.685	1.294	2.733	0.490	9.346	31.816
H. perforatum ssp. *angustifolium* (Piangrande)	1.018	6.417	7.310	1.700	0.766	0.088	14.792	8.351
H. perforatum ssp. *perforatum* (Piangrande)	1.114	17.656	2.068	7.767	1.619	0.900	8.339	27.884
H. richeri (Piangrande)	4.922	5.526	2.917	1.896	0.861	0.170	9.008	0.188
H. tetrapterum (Montelago)	5.019	12.521	3.277	3.734	1.523	0.623	7.351	0.403
H. hyssopifolium (Prati di Ragnolo)	1.786	0.400	3.242	1.609	0.592	0.148	10.296	0.130
H. montanum (Montelago)	0.416	0.692	3.688	1.677	0.655	0.603	8.336	0.402
H. hirsutum (Montelago)	8.896	1.000	0.203	1.904	0.276	0.526	11.410	0
H. androsaemum (Paganico)	1.623	0.516	2.010	1.686	0.118	0.547	6.296	0

* Dry weight (μg/g).

locality the mean values obtained from the analysis carried out at different periods of flowering (Table 2).

From the above, the following considerations arise: hypericin, present in all the samples analysed, reaches the maximal concentration in *H. perforatum* var. *angustifolium* (14.792 μg/g); hyperforin showed high values (31.816 and 27.884 μg/g) in *H. perforatum* var. *angustifolium* and var. *perforatum*, respectively; in *H. richeri*, *H. tetrapterum*, *H. montanum* and *H. hyssopifolium*, it was present only at low concentrations; in *H. hirsutum* and *H. androsaemum*, it was not detected. Chlorogenic acid and flavonoids were present in all the samples analysed, and their concentrations varied depending on the species and collection locality.

4. Conclusions

From the morphological standpoint, there are some differences between the eight entities studied: size and shape of leaves and flowers, shape and distribution of the secretive structures on the herb's parts. The main differences are in agreement with the Italian flora [12] and European flora [11] reports, while the others may be new elements for the identification and/or characterization of the various species.

The histochemical study allowed the localization of the accumulation tasks of some secondary metabolites localized inside the secretive structures; hypericin is stored in all the petals, sepals, leaves, stems and stamens nodules that are sticked on those. It is also found in the secretory canals of petals and sepals of all the species.

Flavonoids are in the same secretory canals that contain hypericin and the essential oil, so they are in sepals and petals of all the species, while they are absent in the stems, stamens and leaves. Not always that it was possible to keep in evidence the presence of flavonoids because their yellow color was hidden by the color of hypericin.

The essential oil is then present in the secretory canals of petals, sepals and in the translucent glands of all species' leaves.

From the phytochemical study, we deduced that chlorogenic acid, flavonoids and hypericin are present in all of the species examined, while hyperforin (that reaches the highest concentration in *H. perforatum*) is absent in *H. hirsutum* and *H. androsaemum*. Furthermore, without considering this last exception, from the qualitative standpoint between the species of the genus *Hypericum* of the Appennino Umbro-Marchigiano, there are no marked differences. Differently, looking at the quantitative aspects, there are some important variations that surely depend on the collection locality, ambient and ecological factors. It is noteworthy to observe that the two varieties of *H. perforatum* are showing the highest values of hypericin and hyperforin, objects of interest because of their antidepressant activity and used as markers of the commercial products. High values of hypericin and flavonoids are detected also in the other species studied, hence, they may find important application although they are not yet considered as officinal.

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