Variability of hypericins and hyperforin in *Hypericum* species from the Sicilian flora.

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The data reported in this paper are part of the PhD work of the first Author, dr Silvia Lazzara; no other specific funding was used.

Declarations of interest: none

ABSTRACT

Within Sicilian flora, the genus *Hypericum* (*Guttiferae*) includes 10 native species, the most popular of which is *H. perforatum*. *Hypericum*'s most investigated active compounds belong to naphtodianthrones (hypericin, pseudohypericin) and floroglucynols (hyperforin, adhyperforin), and the commercial value of the drug is graded according to its total hypericins content.

Ethnobotanical sources attribute the therapeutic properties recognized for *H. perforatum,* also to other *Hypericum* species. However, their smaller distribution inside the territory suggests that an industrial use of such species, when collected from the wild, would result in an unacceptable depletion of their natural stands. This study investigated about the potential pharmacological properties of 48 accessions from six native species of *Hypericum*, including *H. perforatum* and five "minor" species, also comparing, when possible, wild and cultivated sources.

The variability in the content of active metabolites was very high, and the differences within the species were often comparable to the differences among species. No difference was enlightened between wild and cultivated plants. A properly planned cultivation of *Hypericum* seems the best option to achieve high and steady biomass yields, but there is a need for phytochemical studies, aimed to identify for multiplication the genotypes with the highest content of the active metabolites.

KEYWORDS: *Hypericum* spp.; traditional and folk medicine; bioactive phytochemicals; cultivation.

Introduction

According to the available literature, 10 *Hypericum* species have been identified in Sicily: *H. aegypticum* L., *H. androsaemum* L., *H. australe* Ten., *H. hircinum* L., *H. perfoliatum* L., *H. perforatum* L., *H. pubescens* Boiss., *H. tetrapterum* Fr., and *H. triquetrifolium* Turra. [1-3] Recently, the species *H. calycinum* L., thought to be native, was added as well. [4] All taxa are distributed across a number of different environments, and information about their traditional uses and chemical composition is available about the majority of them, with the exception of *H. aegypticum* and *H. australe*, that have been mainly addressed to studies concerning their botanical aspects and their naturalistic value. Among the species above, *H. perforatum* is undoubtedly the most famous and the most largely used, and many experiments conducted worldwide have recognized its antioxidant [5-6], antimicrobial [7-10], antifungal [9-11] and antiviral [10][12-15] properties. The Committee on Herbal Medicinal Products (HMPC) of the European Medicine Agency reports three areas for its medical application: the treatment of minor skin diseases, including small wounds, burns and bruises; the symptomatic relief of mild gastrointestinal discomfort; and the relief of temporary mental exhaustion. [16] In Italy, its most famous, widespread and ancient popular way of administration is the oleolite *(Oleum Hyperici)*, that is obtained through a 40-days maceration of flowers in sunflower oil or extra virgin olive oil. [17-18] With an astounding homogeneity of preparation methods and uses across geographical areas, the oleolite of *H. perforatum* is a traditional topical remedy for the treatment of wounds and burns, throughout Mediterranean and European countries from Italy [5], to Spain [15][19], Bulgaria [13], Albania [20], Bosnia-Herzegovina [21], Kosovo [22], and Turkey. [18][23-24]

Interestingly, the same extraction method is occasionally applied also to other *Hypericum* species, with different therapeutic indications according to the geographical location. Hence, the oleolites from *H. perfoliatum* and *H. lydium*, respectively, are used for topical skin application in Sicily [25] and in Turkey. [26] In Turkey, the same preparation from *H. scabrum* finds use to treat peptic ulcer [27] and in England *H. androsaemum* is the basic ingredient of a wound-healing ointment. [28]

The possibility to use other *Hypericum* species as an alternative to *H. perforatum* is not a new issue. [29-30] In traditional use, several *Hypericum* species share the same utilizations, and ethnobotanical sources ascribe well-defined therapeutic actions to almost all of them. For example, significant antioxidant, antifungal and antiviral actions not only are indicated for *H. perforatum*, but also for *H. androsaemum,* [6][8-11][28][31-36] *H. calycinum,* [4][6][9-11][37] *H. hircinum,* [7][9-10][29][38-43] *H. tetrapterum,* [6-7][9-11][30][37][44] and *H. triquetrifolium.* [45-50] An effective radical-scavenging activity, probably consequent to the antioxidant activity demonstrated by many *in vitro* experiments, is claimed for *H. hircinum.* [43] Beneficial effects on CNS due to documented antidepressant, sedative and relaxant properties are attributed also to *H. calycinum,* [4][51] *H. maculatum,* [52] and *H. triquetrifolium.* [47-48] Efficacy for the treatment of minor inflammations of the skin (such as sunburn), and for healing of minor wounds, is reported for *H. hircinum,* [39][53] *H. maculatum,* [52] and *H. pubescens.* [54] Utility for the treatment of stomach and kidneys disorders is declared for *H. androsaemum.* [9][28][31-32][36]

In many cases, however, these actions have not been demonstrated by specific experiments, and there is no actual evidence that the extracts of *Hypericum* species different from *H. perforatum* are effective for the claimed uses. Otherwise, from a survey in the literature, opposite evidences show up, as for example the demonstrated hepatotoxic activity of *H. androsaemum* [55] or the mutagenicity of *H. triquetrifolium* [56]. A proper characterization of all *Hypericum* species, in order to avoid frauds or unintentional misuses, is therefore advocated. [36]

An additional issue comes from environmental concerns. Despite their great commercial importance, *Hypericum*-based market products are mostly derived from plants picked up from the wild, [57] and no information is available about the sustainability of these collection practices. Although the establishment of environmentally friendly gathering practices from natural populations is increasingly encouraged, [58] many countries have expressed a strong concern about the risk of an uncontrollable depletion of this natural resource due to unrestrained collection of wild plants. A number of *Hypericum* species, including *H. perforatum*, are listed among the endangered plants in several areas, from Portugal [31] to Albania [20] and Croatia. [59] Moreover, the world distribution of *Hypericum* species and populations is uneven, spanning from arid and sunny coastal areas to humid riparian and woody mountainous, [60][61] insomuch as in many areas it is claimed to be an invasive weed. [62] Hence, a large variability is expected in phytochemical features and biomass yields, not only among the different species, but also among populations of the same species, and relying upon collection from the wild cannot guarantee a steady supply of raw material. [63]

Presently, specialized cultivations of *Hypericum* in Europe do not involve wide areas, but an increase of its cultivation is expected in the near future. [64] In USA, market indices about pricing of *H. perforatum* herb agree on the conclusion that *Hypericum* field production may allow gaining 2.000 to 3.000 $/acre, provided the harvested biomass is rich in hypericin. [65] Hence, great efforts are addressed to improve field management techniques, with the goal to enhance the yield of those phytochemicals that are thought to be responsible for the therapeutic properties of the plant. [66]

Indeed, in *Hypericum* plants a great metabolic complexity shows up. Saxena *et al.* [67] list about 190 secondary metabolites of *H. perforatum*, belonging to different chemical classes. Although some of them are still undefined, a number of components are thought to be important from the therapeutic point of view. Among these, polyphenols (rutin, hyperoside, isoquercitrin and quercitrin), phenolic acids (chlorogenic acid and caffeic acid), phloroglucinols (hyperforins), naphtodianthrones (hypericins) as non-volatiles, [68-69] and essential oil as volatiles. [9][29] Despite the large number of trials and reviews on this subject, there is no general agreement as far about which chemical compounds are directly responsible for each specific therapeutic property attributed to the plant. [70-74] The most investigated compounds are hypericins (hypericin and pseudohypericin) and hyperforin. Although many Authors claim these compounds to be responsible for the anti-inflammatory action of *H. perforatum,* [18][75] recent findings suggest that such effect should be attributed to the simultaneous action of several different classes of secondary compounds, that have demonstrated additive, synergic or sometimes antagonist effects. Hence, an increasing importance in therapeutic practice is given to the total plant extract, that should be more properly regarded as the active constituent of the plant. [73-74]

The aim of this work was to explore the variability of the content of three major active metabolites (hypericin, pseudohypericin and hyperforin) in six *Hypericum* species native to Sicily, in order to:

1) assess the suitability of five "minor" *Hypericum* species to the same uses that are routinely suggested for *H. perforatum;*
2) compare the levels in the above-mentioned metabolites according to geographical provenances and growth conditions, including wild and cultivated sources and different class of altitudes.

Results and discussion

Differences among species.

The present study concerned 48 *Hypericum* accessions, belonging to six species (**Table 1**).

Table 1 – Codes, provenance, year of collection, specific growth conditions (wild or cultivated), elevation above sea level and GPS coordinates of the collection sites of the 48 studied *Hypericum* accessions.

Species and section ([a])	Sample Code	Herbarium Code ([b])	Provenance	Collection year	Elevation m a.s.l.	GPS coordinates
Wild						
H. perforatum L. (Sect. *Hypericum* L.)	PFR1	SAF100007	Piano Marcato (PA)	2013	1045	37°54'30"N – 14°04'78"E
	PFR2	SAF100006	Piano Ferro (PA) 1	2013	1065	37°54'23"N – 14°04'75"E
	PFR3	SAF100010	Vicaretto (PA)	2013	900	37°53'35"N – 14°05'48"E
	PFR4	SAF100003	Capo Gallo (PA) 1	2013	113	38°12'43"N – 13°17'39"E
	PFR5	SAF100005	M. Petroso (PA)	2013	524	38°05'49"N – 13°15'54"E

	PFR6	SAF100008	Pomieri (PA)	2013	1342	37°51'29"N – 14°04'06"E
	PFR15	SAF100001	Cammarata (AG) 1	2013	420	37°38'03"N – 13°40'56"E
	PFR16	SAF100002	Cammarata (AG) 2	2013	425	37°38'01"N – 13°40'55"E
	PFR26	SAF100004	M. Cammarata (AG) 1	2013	870	37°38'08"N – 13°37'40"E
	PFR7	SAF100013	Contessa Entellina (PA)	2014	830	37°42'60"N – 13°10'93"E
	PFR8	SAF100019	Ucria (ME)	2014	670	38°03'38"N – 14°52'96"E
	PFR9	SAF100018	Polizzi Generosa (PA)	2014	860	37°48'21"N – 14°00'39"E
	PFR10	SAF100017	Piano Ferro (PA) 2	2014	1065	37°54'23"N – 14°04'75"E
	PFR11	SAF100012	Capo Gallo (PA) 2	2014	113	38°12'43"N – 13°17'39"E
	PFR12	SAF100015	Pian dell'Occhio (PA) 1	2014	585	38°06'12"N – 13°13'57"E
	PFR13	SAF100016	Pian dell'Occhio (PA) 2	2014	590	38°06'11"N – 13°14'00"E
	PFR14	SAF100011	Blufi (PA)	2014	710	37°44'51"N – 14°04'55"E
	PFR27	SAF100014	M. Cammarata (AG) 2	2014	870	37°38'08"N – 13°37'40"E
H. perfoliatum L. (Sect. *Adenosepalum* Spach)	PFL1	SAF100020	Cammarata (AG) 1	2013	420	37°38'03"N – 13°40'56"E
	PFL2	SAF100021	Cammarata (AG) 2	2013	425	37°38'01"N – 13°40'55"E
	PFL3	SAF100023	M. Catalfano (PA) 1	2013	150	38°06'37"N – 13°31'20"E
	PFL4	SAF100022	Capo Gallo (PA) 1	2013	85	38°12'37"N – 13°17'29"E
	PFL5	SAF100029	Pian dell'Occhio (PA)	2014	590	38°06'11"N – 13°14'00"E
	PFL6	SAF100032	Ucria (ME)	2014	670	38°03'38"N – 14°52'96"E
	PFL7	SAF100026	Contessa Entellina (PA)	2014	830	37°42'60"N – 13°10'93"E
	PFL8	SAF100031	Polizzi Generosa (PA)	2014	860	37°48'21"N – 14°00'39"E
	PFL9	SAF100027	M. Cammarata (AG)	2014	870	37°38'08"N – 13°37'40"E
	PFL10	SAF100028	M. Catalfano (PA) 2	2014	150	38°06'37"N – 13°31'20"E
	PFL11	SAF100024	Capo Gallo (PA) 1	2014	85	38°12'37"N – 13°17'29"E
	PFL12	SAF100025	Capo Gallo (PA) 2	2014	135	38°12'36"N – 13°17'33"E
	PFL13	SAF100030	Piano Ferro (PA)	2014	1065	37°54'23"N – 14°04'75"E
H. pubescens Boiss. (Sect. *Adenosepalum* Spach)	PUB1	SAF100033	Mazara del Vallo (TP)	2014	260	37°42'09"N – 12°37'28"E

H. tetrapterum Fr. (Sect. *Hypericum* L.)	TRP1	SAF100034	Floresta (ME)	2014	1270	37°58’42”N – 14°56’42”E
H. hircinum subsp. *majus* (Aiton) N. (Sect. *Androsaemum* (Duhamel) Gordon)	HRC1	SAF100035	Sinagra (ME)	2014	280	38°04’37”N – 14°51’32”E
H. calycinum L. (Sect. *Ascyreia* Choisy)	CLC1	SAF100036	Ucria (ME)	2014	700	38°03’26”N – 14°52’12”E
Cultivated						
H. perforatum	PFR17	Cammarata (AG) 1		2014	P [c]	
	PFR18	Cammarata (AG) 2		2014	P	
	PFR19	Cammarata (AG) 3		2014	P	
	PFR20	Cammarata (AG) 4		2014	P	
	PFR21	Cammarata (AG) 5		2014	P	
	PFR22	Cammarata (AG) 6		2014	F	
	PFR23	Cammarata (AG) 6		2014	P	
	PFR28	M. Cammarata (AG) 1		2014	F	
	PFR24	Piano Ferro (PA) 1		2014	P	
	PFR25	Piano Ferro (PA) 3		2014	P	
H. perfoliatum	PFL14	Capo Gallo (PA) 1		2014	P	
H. pubescens	PUB2	Palermo (PA)		2014	P	
H. tetrapterum	TRP2	Palermo (PA)		2014	P	

(a) Taxonomic classification according to Crockett and Robson [76]
(b) Herbarium of Department of Agricultural, Food and Forestry Sciences, University of Palermo, Italy
(c) P: pots; F: open field

The first survey of the overall phytochemical variability of the collected *Hypericum* samples was performed by means of a Cluster Analysis based on the chemical composition of the obtained extracts. The dendrogram obtained by means of the CA is reported in **figure 1**. Only one *H. perforatum* accession (PFR7) was excluded from CA investigations, due to its unusual very large amount of hyperforin (> 30 g kg^{-1}) that did not allow a proper discrimination among the remaining data.

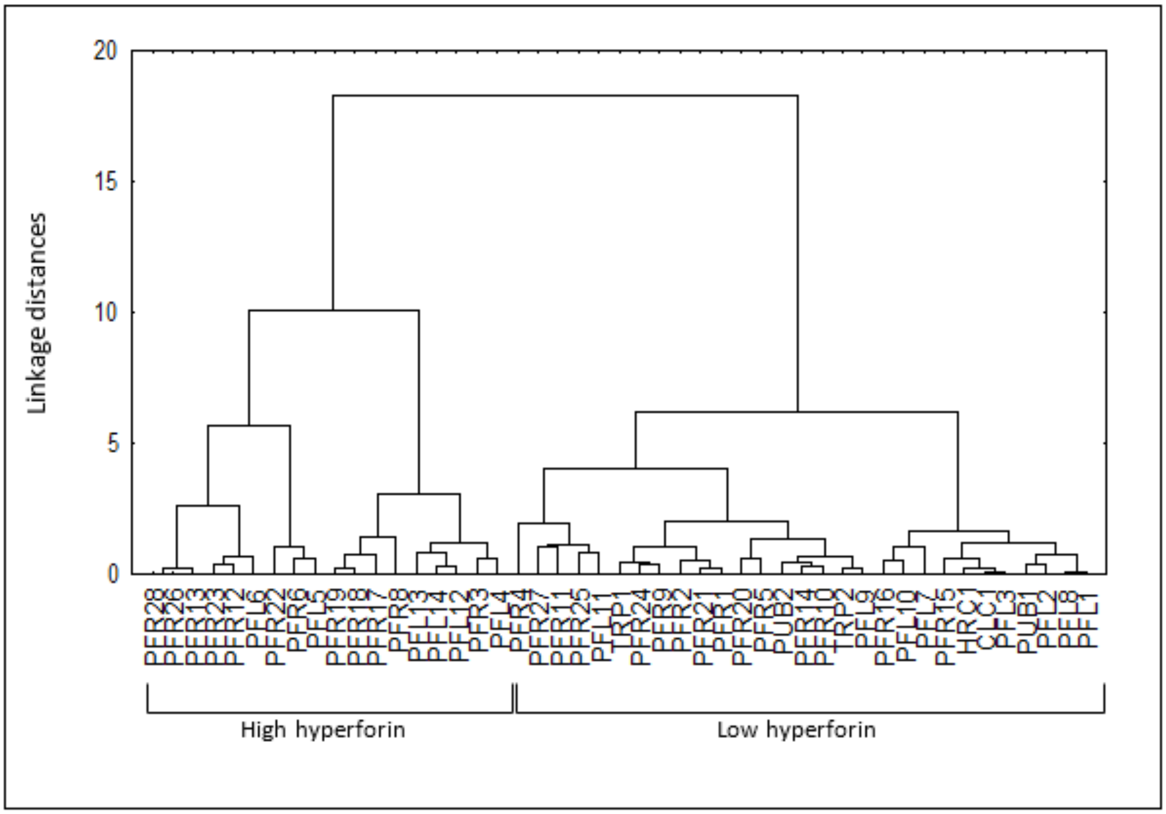

Figure 1 – Dendrogram for all *Hypericum* individuals collected in Sicily in 2013 and 2014 (n=47 pooled data; Complete Linkage method; Euclidean distances metric).

As shown, the CA on pooled data was able to discriminate between two major groups, including 18 and 29 cases, respectively. The ANOVA performed on the two groups (**table 2**) showed that the most significant variable for the partitioning of data was the hyperforin content, that generated a clear distinction between individuals averaging a very high (12.53 g kg^{-1}, cluster 1) and a very low (2.63 g kg^{-1}, cluster 2) hyperforin content. No significant differences showed

Table 2 – Mean values and major statistics of the *Hypericum* groups obtained through cluster analysis.

	Mean	SS between	DF	SS within	DF	*F* ([a])

All pooled data						
Hyperforin g kg^{-1}		1090.293	1	284.004	45	172.76***
group 1 (18 cases)	12.53					
group 2 (29 cases)	2.63					
Pseudohypericin g kg^{-1}		0.046	1	8.326	45	<1 n.s.
group 1 (18 cases)	0.64					
group 2 (29 cases)	0.57					
Hypericin g kg^{-1}		0.035	1	3.283	45	<1 n.s.
group 1 (18 cases)	0.40					
group 2 (29 cases)	0.35					
H. perforatum						
Hyperforin g kg^{-1}		574.535	1	168.825	25	85.08***
group 1 (8 cases)	14.74					
group 2 (19 cases)	4.64					
Pseudohypericin g kg^{-1}		0.026	1	3.691	25	<1 n.s.
group 1 (8 cases)	0.47					
group 2 (19 cases)	0.49					
Hypericin g kg^{-1}		0.001	1	2.457	25	<1 n.s.
group 1 (8 cases)	0.44					
group 2 (19 cases)	0.44					
H. perfoliatum						
Hyperforin g kg^{-1}		391.930	1	60.473	12	77.77***
group 1 (6 cases)	12.13					
group 2 (8 cases)	1.44					
Pseudohypericin g kg^{-1}		0.086	1	2.242	12	<1 n.s.
group 1 (6 cases)	0.96					
group 2 (8 cases)	0.80					
Hypericin g kg^{-1}		0.001	1	0,241	12	<1 n.s.
group 1 (6 cases)	0.29					
group 2 (8 cases)	0,31					
(a) Fisher-Snedecor's F; ***: P≤ 0.001; n.s.: not significant						

up in both hypericins (hypericin and pseudohypericin) levels. All “minor” *Hypericum* species (*H. pubescens*, *H. tetrapterum* and *H. calycinum*) were allocated into the second group, but *H. perforatum* and *H. perfoliatum* were merged into both clusters. Hence, it appears that a clustering only based on chemical composition does not match satisfactorily the species. The CA performed independently on *H. perforatum* (**figure 2**) and *H. perfoliatum* (**figure 3**) allowed partitioning both species into two groups each. Once again, in both species hyperforin content was the most important discriminatory character, allowing to partition between high-hyperforin and low-hyperforin individuals. The pseudohypericin

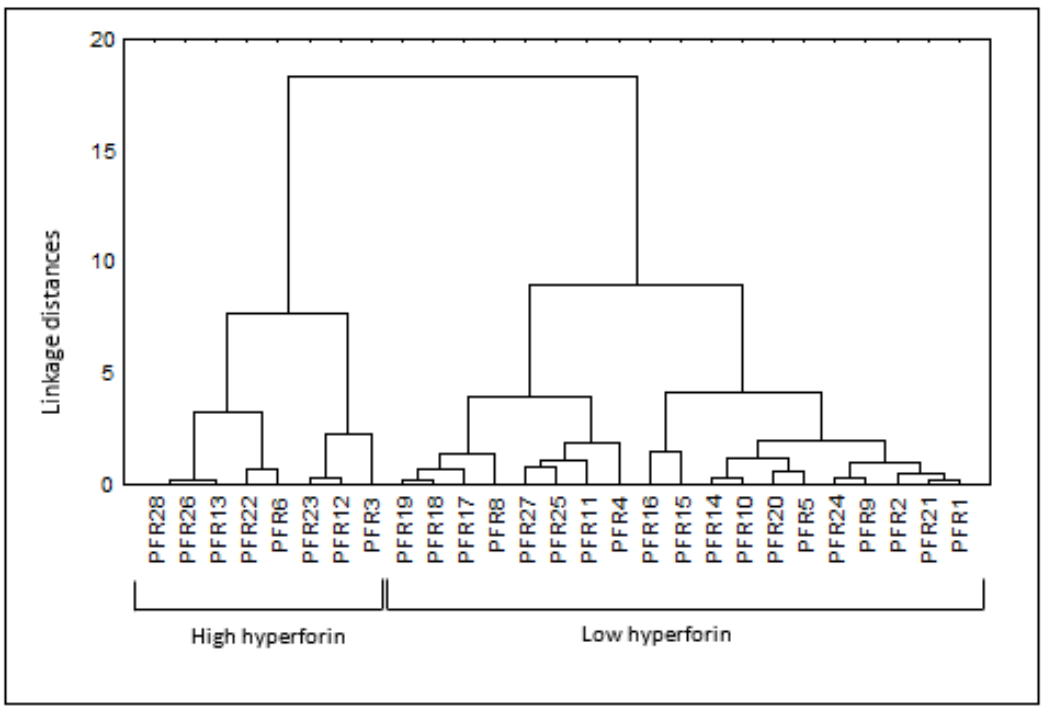

Figure 2 – Dendrogram for the *H. perforatum* individuals collected in Sicily in 2013 and 2014 (n=27; Complete Linkage method; Euclidean distances metric).

and hypericin amounts were instead undifferentiated between groups.

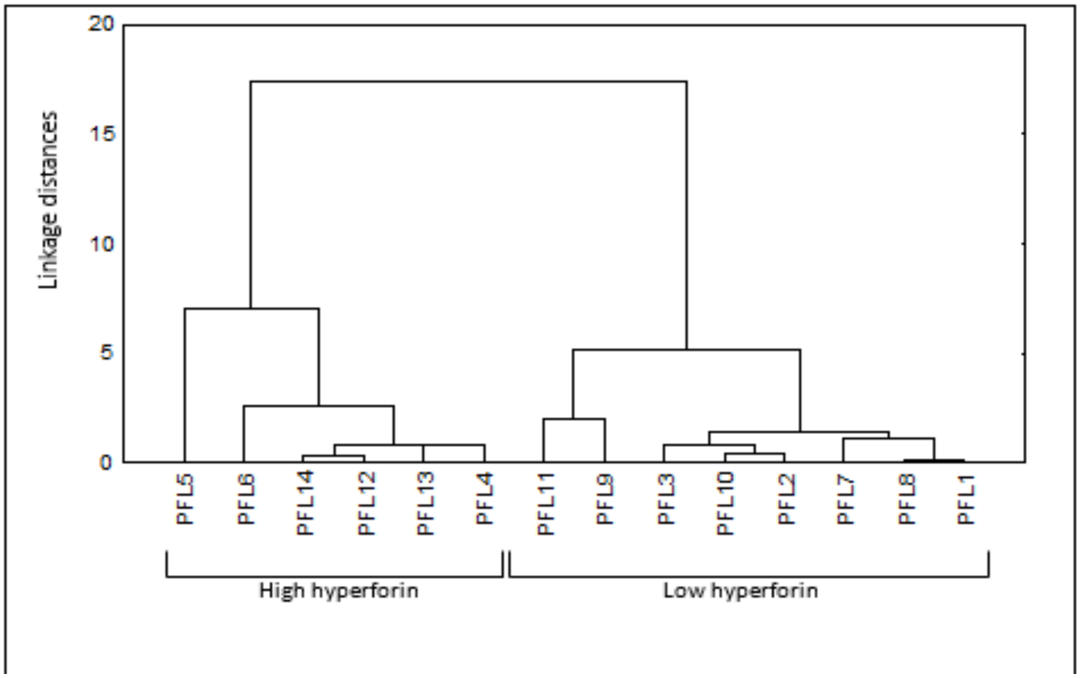

Figure 3 – Dendrogram for the *H. perfoliatum* individuals collected in Sicily in 2013 and 2014 (n=14; Complete Linkage method; Euclidean distances metric).

As shown in **table 3**, the extract yield (% on dry matter) expressed its lower value in *H. hircinum* (18.5%), whereas the highest figure was found in *H. calycinum* (33.8 %). The extract percentage of *H. perforatum* (23.5%) was consistent with the average value of 24.9 % reported

for the same species by Kireeva *et al.* [77], who however found a decrease from vegetative stage (29.9%) to seed capsule formation (16.50%).

Table 3 – Mean values across species and results of the ANOVA of extract yield (%) and active constituents in six *Hypericum* species native to Sicily.

	Hyperforin ($g\ kg^{-1}$)	Pseudohypericin ($g\ kg^{-1}$)	Hypericin ($g\ kg^{-1}$)	Extract (%)
H. perfoliatum	6.02	0.87 a	0.30	21.4
H. perforatum	8.44	0.49 b	0.44	23.5
H. pubescens	1.52	0.80 ab	0.23	29.6
H. hircinum	0.60	0 b	0	18.5
H. calycinum	0.43	0 b	0	33.8
H. tetrapterum	3.64	0.64 ab	0.40	23.8

F value (5, 42) ([a])	1.18 [n.s.]	2.89*	<1 [n.s.]	<1 [n.s.]

(a) Fisher-Snedecor's F; *: P≤ 0.05; n.s.: not significant.
In the pseudohypericin column, values followed by the same letter are not different at P≤0.05 (Tukey's test)

Compared to the results of CA, the univariate ANOVA across species (**table 3**) revealed a different discriminatory importance of chemical compounds. As shown, the hyperforin content, that had evidenced at previous CA the greatest discriminatory power, in this analysis did not overpass the threshold of statistical significance; otherwise, a statistically significant (P≤0.05) differentiation among the species was found based on the pseudohypericin content.

Such a result must surely be attributed to the large intraspecific chemical variability of the examined species. Indeed, although hyperforin values were on average much higher in *H. perforatum* and *H. perfoliatum* than in the other species, the occurrence of low-yielding individuals also inside *H. perforatum* and *H. perfoliatum* reduced the statistical significance of this parameter.

A high level of intraspecific variability in hyperforin content is common in *H. perforatum*, and also other Authors found up to 4-folds differences between minimum and maximum hyperforin amounts in this species. [14][78] There could be many reasons why the hyperforin content may vary as much, including the development stage of plants [79] or the presence in the analyzed samples of stems and leaves, which contain a much lower amount of active compounds. [14] Although big efforts were made to collect homogeneously developed samples, the scarce stability of this parameter suggests the opportunity to pick up only the flowers rather than the flowering tops of plants, a hint that however is quite impossible to follow in the herbal collecting practice.

Unlike *H. perforatum*, information from the literature about the hyperforin content of the other *Hypericum* species is scarce. Some Authors found in *H. tetrapterum* very low hyperforin amounts, [79-80] whereas 3.45 g kg^{-1} hyperforin, a more similar value to those found in our samples, was retrieved by Sagratini *et al.* [42] in *H. tetrapterum* individuals collected in central Italy. In plants of *H. calycinum* this compound was found in limited amounts (0.14 g kg^{-1} according to Sagratini *et al.* [42]) or was not detected at all. [81]

Hypericins (hypericin and pseudohypericin) were absent in *H. hircinum* and *H. calycinum*, whereas in the other species, their relative amounts varied from 0.23 to 0.44 g kg^{-1} (hypericin) and to 0.49 to 0.87 (pseudohypericin). A similar trend was already found in *H. perforatum*, where pseudohypericin content was 2-4 folds higher than hypericin. [82]

It appears that there were not strong differences among species, and a search in the literature corroborates this finding, since a huge variability shows up in most reported phytochemical data. Smelcerović *et al.* [80] found in *H. tetrapterum* 0.10 and 0.09 g kg^{-1} hypericin and pseudohypericin, respectively. Kitanov, [83] in analyzing samples from various *Hypericum* species, obtained average hypericins (hypericin + pseudohypericin) content of 1.25 g kg^{-1} in *H. perforatum*, and 0.52 g kg^{-1} in *H. tetrapterum*. Otherwise, this Author did not detect hypericins in *H. calycinum*, hence deducing that these compounds are not present in the most primitive *Hypericum* taxa, being detectable only in the more phylogenetically advanced taxa.

In our samples, the hypericin content showed a definite, linear and positive association with pseudohypericin, consistent with the hypothesis that they originate from the same precursors. [82] Noticeably, in *H. perforatum* this association proved to follow a different pattern than in the other *Hypericum* species (**figure 4**), as revealed by the different slope of the two regression lines.

In *H. perforatum*, the two compounds showed a sharp direct reciprocal association (R^2=0.728). The bias due to the extreme values of one outlier (PFR11) did not influence substantially the fitting of the regression line, that even after removing the outlier assumed a value not far from the preceding one (R^2= 0.677).

Differences due to the growth site.

A high site-based variability in the chemical composition of *Hypericum* species is acknowledged by many authors, both taking into account the chemical variability due to the provenience, [84] and from the point of view of the cultivation of the same genotype in different environments. [85] Notwithstanding, any attempt to match exactly chemical features with geographical provenience was only partially successful. [86]

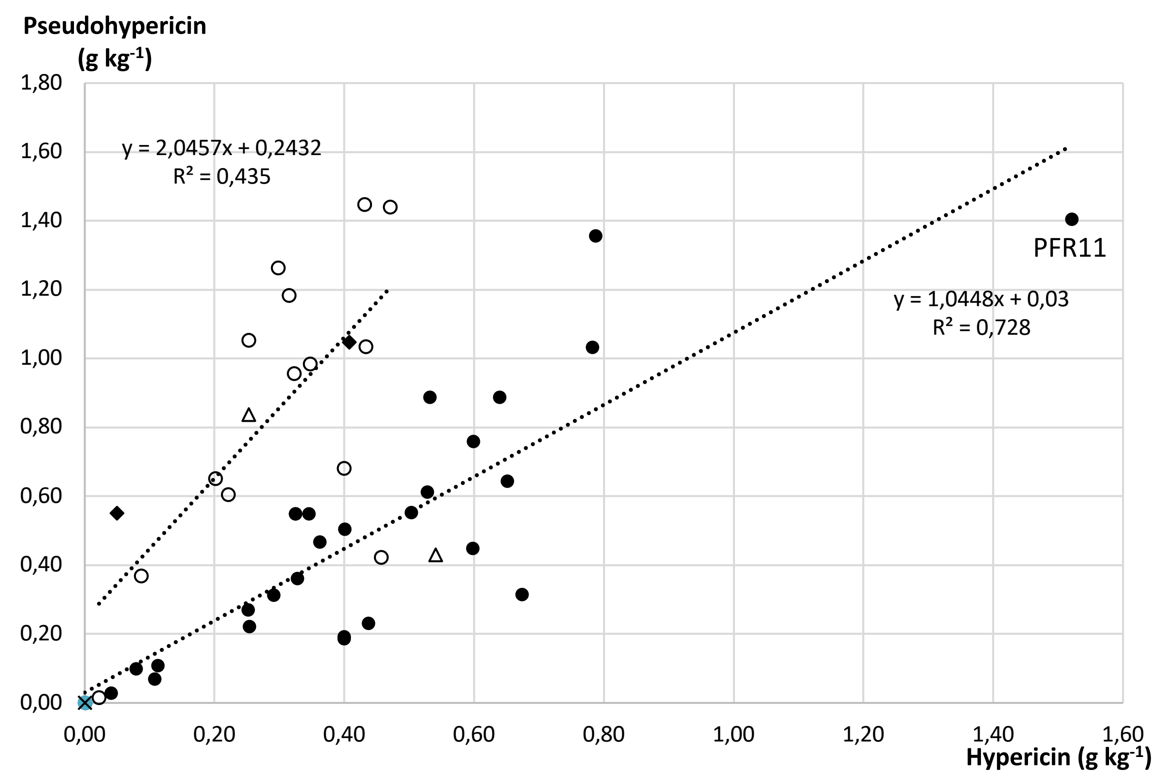

Figure 4. Pseudohypericin *vs* hypericin content in 48 *Hypericum* accessions from Sicily. Legenda: •= *H. perforatum*; o = *H. perfoliatum*; ◊= *H. pubescens*; X= *H. calycinum*; Δ= *H. tetrapterum*; ▫= *H. hircinum*. Regression lines refer to data from samples of *H. perforatum* (lower line) and *H. perfoliatum* (upper

In our analysis as well, the ANOVA on all pooled data **(table 4)** did not highlight significant differences among sites. The pseudohypericin content showed the highest mean value (> 1 g kg^{-1}) in the plants collected from Capo Gallo (PA), including both *H. perforatum* and *H. perfoliatum* individuals.

Additional information may be obtained from the individual analyses, performed separately on both species across sites. *H. perforatum* showed significant differences among sites in the extract yield and hyperforin content, that ranged between maximum values recorded in the plants from Contessa Entellina (35.5 % and 30.31 g kg^{-1} for the two variables, respectively), and minimum values obtained in the samples from Monte Petroso (14.7% extract yield and

2.21 g kg^{-1} hyperforin). In *H. perfoliatum* the variability in hyperforin content was very high: three locations allowed an hyperforin content higher than 10 g kg^{-1}, whereas a very low value (0.1 g kg^{-1}) was found in the accessions from Polizzi Generosa (PA).

Table 4 – Mean values across growth sites of extract yield (%) and active constituents in the extracts from Sicilian *Hypericum* species, and results of the ANOVA for all pooled data, and separately for *H. perforatum* and *H. perfoliatum*.

	All pooled data (n=48; DF: 17;30)				*H. perforatum* (n=28; DF: 12;15)				*H. perfoliatum* (n=14; DF: 7;6)			
	Extract (%)	Hyperforin g kg^{-1}	Pseudohypericin g kg^{-1}	Hypericin g kg^{-1}	Extract (%)	Hyperforin g kg^{-1}	Pseudohypericin g kg^{-1}	Hypericin g kg^{-1}	Extract (%)	Hyperforin g kg^{-1}	Pseudohypericin g kg^{-1}	Hypericin g kg^{-1}
Capo Gallo (PA)	24.2	7.92	1.04	0.51	25.2 ac	5.53 bc	0.76	0.82	23.7	9.11 b	1.18	0.35
Contessa Entellina (PA)	30.9	15.68	0.91	0.32	35.5 a	30.31 a	0.55	0.34	26.3	1.05 c	1.26	0.30
M. Cammarata (AG)	22.3	6.76	0.73	0.40	29.3 ab	12.21 bc	0.71	0.48	15.3	1.31 c	0.76	0.32
Piano dell'Occhio (PA)	26.0	15.11	0.40	0.26	30.6 ab	13.99 bc	0.42	0.34	16.7	17.34 a	0.37	0.09
Piano Ferro (PA)	24.8	5.42	0.64	0.43	23.0 ac	3.99 bc	0.54	0.43	32.0	11.16 ab	1.04	0.43
Polizzi Generosa (PA)	27.5	1.91	0.86	0.46	31.7 ab	3.71 bc	0.76	0.60	23.3	0.10 c	0.96	0.32
Ucria (ME)	28.0	7.45	0.80	0.35	34.5 ab	8.91 bc	1.36	0.79	15.6	13.00 ab	1.05	0.25
Blufi (PA)	33.5	2.94	0.89	0.64	33.5 ab	2.94 c	0.89	0.64				
Cammarata (AG)	17.7	7.21	0.26	0.31	17.7 c	7.21 bc	0.26	0.31				
M. Petroso (PA)	14.7	2.21	0.10	0.08	14.7 c	2.21 c	0.10	0.08				
Piano Marcato (PA)	19.1	3.42	0.22	0.25	19.1 bc	3.42 bc	0.22	0.25				
Pomieri (PA)	20.0	17.68	0.45	0.60	20.0 ac	17.68 ab	0.45	0.60				
Vicaretto (PA)	18.7	10.68	0.32	0.67	18.7 bc	10.68 bc	0.32	0.67				
M. Catalfano (PA)	22.5	0.67	0.22	0.24					22.5	0.67 c	0.22	0.24
Floresta (ME)	18.5	3.99	0.43	0.54								
Mazara d. Vallo (TP)	35.0	0.27	0.55	0.50								
Palermo (PA)	26.6	3.03	0.94	0.33								
Sinagra (ME)	18.5	0.60	0	0								
Mean values	23.3	6.92	0.60	0.37	23.5	8.44	0.49	0.44	21.4	6.02	0.87	0.30
F value ([a])	1.12 [n.s.]	1.26 [n.s.]	1.92 [n.s.]	<1 [n.s.]	3.18*	3.31*	1.50 [n.s.]	<1 [n.s.]	<1 [n.s.]	13.23**	2.57 [n.s.]	<1 [n.s.]

(a) Fisher-Snedecor's F; *: P≤ 0.05; **: P≤ 0.01; n.s.: not significant.

When reported, values in each column followed by the same letter are not different at P≤0.05 (Tukey's test)

Table 5. Frequency distribution and mean values of extract yield (%) and active constituents (g kg^{-1}) in wild Sicilian *Hypericum* species, according to classes of elevation of the collection sites.						
Class interval	n	Frequency (%)	Extract (%)	Hyperforin g kg^{-1}	Pseudohypericin g kg^{-1}	Hypericin g kg^{-1}
	All species (n=35; DF: 4,30)					
<100 m a.s.l.	2	5.7	20.0	7.79	1.05	0.32
101-300 m a.s.l.	7	20.0	24.6	3.38	0.56	0.38
301-600 m a.s.l.	8	22.9	19.0	6.20	0.40	0.22
601-900 m a.s.l.	12	34.3	27.8	7.98	0.78	0.45
>900 m a.s.l.	6	17.1	21.9	7.14	0.46	0.39
Total	**35**	**100**				
Mean (n=35)			**23.7**	**6.50**	**0.61**	**0.37**
F value ([a])			1.96 n.s.	<1 n.s.	1.67 n.s.	<1 n.s.
	H. perforatum (n= 18; DF: 3,14)					
<100 m a.s.l.	0	0				
101-300 m a.s.l.	2	11.1	25.2	5.53	0.76	0.82
301-600 m a.s.l.	5	27.8	22.0	6.33	0.26	0.22
601-900 m a.s.l.	7	38.9	30.6	11.13	0.78	0.59
>900 m a.s.l.	4	22.2	20.2	6.93	0.32	0.34
Total	**18**	**100**				
Mean (n=18)			**25.3**	**8.24**	**0.53**	**0.46**
F value			2.14 n.s.	<1 n.s.	2.65 n.s.	2.21 n.s.
	H. perfoliatum (n=13; DF: 4,8)					
<100 m a.s.l.	2	15.4	20.0	7.79	1.05	0.32
101-300 m a.s.l.	3	23.1	22.7	3.91	0.63	0.32
301-600 m a.s.l.	3	23.1	13.9	5.99	0.65	0.22
601-900 m a.s.l.	4	30.8	21.5	4.36	0.99	0.32
>900 m a.s.l.	1	7.7	32.0	11.16	1.04	0.43
Total	**13**	**100**				

Mean (n=13)		**20.6**	**5.68**	**0.84**	**0.30**
F value		2.35 n.s.	<1 n.s.	<1 n.s.	<1 n.s.
(a) Fisher-Snedecor's F; n.s.: not significant.					

The detection of differences in active metabolites content among different elevation levels was calculated only on the wild accessions. On this topic, literature data are somehow contradictory: some surveys performed in Italian mountain areas did not detect any relationship between elevation and hypericins/hyperforin content, [42][87] whereas an increase of the total hypericins content with increasing altitude from 200 to 600 m a.s.l. was reported in *H. perforatum* flowers collected in Crete. [88] In our sampling, more than 50% of the plants collected from the wild came from sites at an elevation higher than 600 m above sea level. The ANOVA across classes of elevation **(table 5)** did not evidence significant differences in the content of active metabolites, and Pearson's correlation coefficients (r) between altitude values and samples metabolites content, calculated for all pooled data and separately for *H. perforatum* and *H. perfoliatum* (data not shown) always expressed very low values.

Wild or cultivated?

The question whether plants may alter their content in active compounds after moving from wild to cultivated bears a great interest, and the literature offers many contrasting examples about this. In our trial, univariate ANOVA did not evidence significant differences between wild and cultivated sources in the average content of raw extract and active components under study **(table 6).** A definite difference showed up instead between the values of hyperforin content obtained by means of two different methods of cultivation (open field, F, and pots, P), where open field cultivation allowed an overall higher hyperforin yield.

Table 6. Mean values of extract yield (%) and active constituents (g kg^{-1}) in Sicilian *Hypericum* species, and results of the ANOVA according to plant growth conditions (wild and cultivated; open field, F, and pots, P).

	Extract (%)	Hyperforin g kg^{-1}	Pseudohypericin g kg^{-1}	Hypericin g kg^{-1}
Wild	23.7	6.50	.61	.37
Cultivated [(a)]	22.1	8.05	.55	.38
F	23.6	16.8	.53	.36
P	21.8	6.5	.56	.39
F value ([b]) (within cultivated, F *vs.* P; DF: 1, 11)	<1 n.s.	15.84**	<1 n.s.	<1 n.s.
F value (between wild and cultivated; DF: 1, 46)	<1 n.s.	<1 n.s.	<1 n.s.	<1 n.s.

(a) Within cultivated: F=open field; P=pots.
(b) Fisher-Snedecor's F; **: $P \leq 0.01$; n.s.: not significant.

Many arguments support the idea that specialized cultivation is preferable to collection from the wild. By one side, the indiscriminate collection for medicinal purpose of wild species poses a serious hazard to environment and biodiversity. Furthermore, the possibility to modify some special aspect of the growth environment of the plants, with the goal to enhance biosynthesis and storage of some selected compounds, has been demonstrated for many species. [64] Notwithstanding, literature data about the effects of cultivation on *Hypericum* phytochemical features are not many, and mostly restricted to harvest time and conditions. [89]. Kizil *et al.* [90] enlightened the relationship between dry matter yield and hypericin content, on one side, and the development stage of the harvested plants, their age and the height of cutting, on the other side.

Other works have taken into account some aspects of cropping management concerned with the hypericins content of dry herbage. [66][91-93]. However, since so many aspects are involved in hyperforin and hypericins production and storage inside the plants, it appears that further efforts must be addressed to a deeper insight about the best agricultural practices to apply for improving yield and quality aspects of *Hypericum* under cultivation.

Conclusions

In our study, the content of the three studied active compounds (hypericin, pseudohypericin and hyperforin) showed a large variability, both among species and among accessions of the same species. However, measured inter-specific variability was not higher than variability within species. Hence, from the strict point of view of their content in those active metabolites, the studied *Hypericum* species seem almost interchangeable one another. By one hand, this finding enlarge the possibility of use of *Hypericum* species different from *H. perforatum*, and, because of the high number of environments where these species are adapted, the number of agricultural conditions where they may be cultivated is supposed to get higher. By the other hand, the possibility to find low-yielding and high-yielding genotypes in almost all investigated species, stresses the need to pose a great attention on the choice of the individuals to be propagated for commercial purposes.

Open field cultivation seem the best option to obtain high-hyperforin plants; although the cultivation in pots is surely not suitable for industrial purposes, the occurrence of this variability must be taken into account in phytochemical assays for plant grading according to quality.

Our finding no significant difference between wild and cultivated sources encourages the research about suitable and properly tuned cropping techniques. Field cultivation have the sure advantage to allow obtaining higher and steady biomass yields. Hence, cultivation seems the best way to achieve a satisfactory stability in biomass yields as well as a good quality level of the product. [64][94] As far as we know, *H. perforatum* is the only species for which a high number of agronomical trials is available, and for which a concrete possibility exists to fit into high-value cropping systems. Otherwise, the other species have not been addressed to such experiments, and this supports the need for further research. Further phytochemical studies are moreover necessary, to deepen the relationships between the active metabolites content and the growth conditions of plants.

Acknowledgements

The Authors wish to thank Mrs. Tonia Strano (ICB-CNR) for her daily assistance in laboratory skillful technical assistance.

Author Contribution Statement

A.C. was responsible for the study's design development and results treatment, performed the statistical analysis and wrote the first version of the manuscript (draft); S.L. managed the recognition, collection, botanical identification, cultivation and harvest of plants, and helped with the treatment and discussion of results; E.N. was responsible for the preparation of plant extracts and for managing, elaboration, interpretation and discussion of the HPLC/DAD quantitative analyses. All Authors reviewed the first version of the manuscript. Authors are aware and approved the submission of the manuscript.

Experimental section

Plant material.

The plant individuals studied in this trial were collected in Sicily in 2013 and 2014 from May to July, according to the flowering moments of the different species. For wild plants collection, a thorough investigation about the availability of *Hypericum* spp. was performed on an historical basis, by means of a search on the specialized literature. [2][4][60][95-97] In both years, the collection sites were identified by means of their GPS coordinates (Garmin e-trex 30), and site descriptions and photographs were taken. The explored area included different environments of the provinces of Trapani, Palermo, Messina and Agrigento **(Supplementary material, figure S1)**. The botanical identification was performed by the Authors using the available specific literature. [2][4][60][95][97] The collected plants were used to prepare exsiccata in the laboratories of the Council for Agricultural Research and Agricultural Economy Analysis in Bagheria (PA), and specimens from each population were saved in the Herbarium of the Department of Agricultural, Food and Forest Sciences at the University of Palermo (SAF). Registration numbers for each studied population are reported in **table 1**.

From August to October 2013, after seed setting, samples of seeds were collected from all wild identified plant populations. When the seeds amount was high enough, the collected seeds were sown in ordinarily managed 3x2 m plots (F) located in the experimental farm "Sparacia" (Cammarata, AG, Sicily; 37°38'08" N – 13°40'56" E); otherwise, with limited seeds availability, seeds were put in 20-cm diameter pots (P), located in the same area. In both cases, cultivated plants entered the flowering phase in June 2014.

At flowering time, flowering tops (15-20 cm) were picked up from both wild and cultivated plants. The collected samples were stored in paper bags and dried at 20-25 °C in the dark for further analyses. In both years and in all growth conditions, efforts were made to collect the *Hypericum* flowering tops only when plant conditions were optimal, i.e. at full flowering and in presence of an adequate biomass amount. Because of this constraint, from a few wild populations in which, at time of survey, blooming was too late, only seeds samples were collected, and no chemical analysis was carried on.

At the end of the second trial year, a total of 48 plant samples, collected from 18 different sites and obtained both from the wild (35 wild populations) and from cultivated stands (13 plant samples) had been collected and analyzed (**table 1**). Cultivated plants belonged to the species *H. perforatum* (10 accessions), *H. perfoliatum*, *H. pubescens* and *H. tetrapterum* (one accession for each species).

Preparation of plant extracts.

Hypericum air-dried flowered tops (residual moisture content of 8%) were finely ground with a laboratory mill to obtain a homogenous drug powder; 5 g for each sample was extracted in 50 ml of ethanol, at room temperature for 72 hours and under continuous stirring, taking care to avoid light exposure as much as possible, due the photo sensibility of the metabolites of interest. Each extract was filtered and the filter was washed thrice with 10 ml of ethanol. Thereafter, the obtained mixture was dried with a rotary evaporator, in order to measure the dry extract amount of each sample (in percent). The samples for chemical analysis were extracted as mentioned above, then filtered on PTFE 0,45 μ filters (PALL Corporation), put into 2mL amber vials and sent to analytical determinations.

Chemical materials

All solvents used were of HPLC grade and purchased from VWR (Milan, Italy). Pure standards of hyperforin and hypericin were purchased from Labochem science SRL (Catania, Italy).

HPLC/DAD quantitative analyses

Hyperforin and hypericins quantitative analyses were carried out on a Thermofisher Ultimate3000 instrument equipped with a binary high pressure pump and a photodiode array detector. Collected data were processed through a software Agilent OpenLab CDS A.04.05 version. Chromatographic runs were carried out with the following gradient of B (acetonitrile) in

A (ammonium acetate 20 mM in water): 0 min: 50 % B; 25 min: 50% B; 35 min: 10 % B; 45 min: 90 % B; 50 min: 50 % B [98]. The solvent flow rate was 1 mL/min. Quantifications were run at 290 nm for hyperforin with authentic reference substance for the calibration curve ($R^2 = 0.9927$) and at 590 nm for naphthodianthrones using hypericin ($R^2 = 0.9977$) as standards. All analyses were carried out in triplicate by injection of 20 µL of a solution 10mg/mL in methanol "HPLC grade VWR" for each extract.

Statistical analysis.

For a first exploratory survey, all pooled data were first submitted to a Cluster Analysis (CA; complete linkage method; Euclidean distance metric) by means of the software "Statistica 5.2", using as variables the detected levels of each significant chemical compound (hyperforin, pseudohypericin and hypericin). Because of the unbalanced structure of data, that did not allow to perform a pooled ANOVA including all class variables, a univariate ANOVA was separately performed for each given source of variation, namely the species, the provenance, and the growth condition of the plant (i.e. "wild" or "cultivated"). Wild populations were furthermore analyzed based on the elevation (m a.s.l.) of their collection sites. When the ANOVA highlighted the occurrence of statistical differences between the groups, a LSD post-hoc test was performed. [99] In order to have a better insight of data, and to detect any differentiation inside the two major species (*H. perforatum* and *H. perfoliatum*), the analyses were repeated separately for each of them.

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