TÜBİTAK

Turkish Journal of Botany

http://journals.tubitak.gov.tr/botany/

Research Article

Turk J Bot
(2013) 37: 847-858
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doi:10.3906/bot-1206-22

A further analysis of secretory structures of some taxa belonging to the genus *Hypericum* (Clusiaceae) in relation to the leaf vascular pattern

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Received: 15.06.2012 • **Accepted:** 04.04.2013 • **Published Online:** 06.09.2013 • **Printed:** 30.09.2013

Abstract: This work extends knowledge about the distribution of secretory structures (black nodules, translucent glands, and type A and B secretory canals) to other species of the genus *Hypericum* L., as only *H. perforatum* L. appears to have been widely investigated. Moreover, the current study was extended to include leaf vascular patterns. In the species studied, a possible correlation between the presence of black nodules and a particular biological form, that of hemicryptophytes scapose perennials such as *H. perfoliatum* L., *H. perforatum* L., *H. pubescens* Boiss., *H. tetrapterum* Fr., and *H. triquetrifolium* Turra, was noted. These black nodules are not present in *H. androsaemum* L. and *H. hircinum* L., which are bushy nanophanerophytes, or in *H. aegypticum* L., a xero-halophyte. The xylem pattern results are camptodromous brochidodromous in *H. androsaemum*, *H. hircinum*, *H. perforatum*, and *H. pubescens*; basal acrodromous in *H. perfoliatum*, *H. tetrapterum*, and *H. triquetrifolium*; and camptodromous cladodromous in *H. aegypticum*.

Key words: *Hypericum*, leaf vascular pattern, black nodules, secretory canals, translucent glands

1. Introduction

Hypericum L. is a genus of the family Clusiaceae, belonging to the subfamily Hypericoideae (Cronquist, 1981). It includes more than 460 species that are spread throughout the world, and many are well represented in the Mediterranean and the near Middle East (Campbell & Delfosse, 1984). Recently, interest in this genus has increased because it is the source of a wide variety of compounds with biological activity (Nahrsted & Butterweck, 1997). The active ingredients derived from some species belonging to the *Hypericum*, particularly hypericin, hyperforin, and essential oils, are located in several unusual secretory structures known as black nodules, translucent glands, and secretory canals (Blenk, 1884; Green, 1884; Weill, 1903; Siersch, 1927; Metcalfe & Chalk, 1950; Curtis & Lersten, 1990; Baroni Fornasiero et al., 1998; Bottega et al., 1999; Baroni Fornasiero et al., 2000; Ciccarelli et al., 2001a, 2001b; Onelli et al., 2002; Ciccarelli et al., 2007; Yamaner et al., 2013), features that are above all used for taxonomic purposes (Robson, 1977, 1981; Pignatti, 1982; Keller, 1985). More recently Lotocka and Osinska (2010) have studied the anatomy and ultrastructure of internodes, leaves, and petals, comparing some of the taxa belonging to the genus *Hypericum* with 2 *H. perforatum* L. genotypes. Nürk and Crockett (2011) have investigated and described 36 taxonomic sections by combining morphologic characteristics with the biogeographical distribution and always quoting *H. perforatum* and *H. androsaemum* L. marginally but without considering the merits of either its leaf vascular pattern or its secretory structures. Finally, Gitea et al. (2011) have studied the secretory structures of several species belonging to the genus *Hypericum*, including *H. perforatum* and *H. tetrapterum* Fr., with particular reference to the anatomy of the stem and the translucent glands present in the lamina of the latter, for analysis considerations. *H. perforatum* has been, therefore, extensively investigated with regard to type and location of secretory structures, and other species have been only marginally investigated.

The aim of this work is to increase our understanding of these structures through a comparative screening of the leaves of other species such as *H. perfoliatum* L., *H. pubescens* Boiss., *H. tetrapterum* Fr., *H. triquetrifolium* Turra, *H. androsaemum* L., *H. hircinum* L., and *H. aegypticum* L., chosen with the intent of understanding how heterogeneous environments can influence the presence and distribution of such structures. Moreover, the study has been framed in relation to leaf vascular pattern, as this aspect is little studied.

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2. Materials and methods

2.1. Plant sampling

The material examined was taken directly from the stations mentioned in the literature (Pignatti, 1982; Giardina et al., 2007) during the flowering period. Eight *Hypericum* species with diverse biological forms and ecological categories were examined, as follows:

Five perennial hemicryptophytes scapose (H scap): *H. perforatum* and *H. perfoliatum*, 2 mesophytes found for the first time in Europe, with the exception of the far north, and for the second time in the Mediterranean region, respectively. Both are present throughout the territory of Italy to a height of 1400 m above sea level and were collected in the Quacella zone at Madonie Park (North Sicily-Palermo). *H. pubescens*, a mesophyte that colonises humid environments, subsalsi at times, present in Portugal, southern Spain, Sicily, and Malta. Collected in the Torretta Granitola zone in the municipality of Campobello di Mazara (Trapani). *H. tetrapterum*, a meso-hygrophyte that colonises marshes and reeds, present in south-central Europe, and extends into Sweden, grows throughout the territory of Italy in Sicily. Collected near Cesarò in Nebrodi Park (Messina). *H. triquetrifolium*, a xerophyte that grows in the Eastern Mediterranean. Present only in extreme southern Italian regions, in Sicily it is found in arid and uncultivated areas near Dingoli, Piana degli Albanesi (Palermo).

Two nanophanerophytes (NP): *H. androsaemum*, a mesophyte present in western Europe, locally in southern Europe, and found throughout the territory of Italy to a height of 1400 m above sea level. Collected in Nebrodi Park, in oak forests, and among holly along the path to Maulazzo, above Militello Rosmarino (Messina). *H. hircinum*, a mesophyte found in moist and shady localities. Present in the Mediterranean region and found throughout central and southern Italy. Collected at Nebrodi Park near Floresta (Messina).

One Chamaephyte fruticose (Ch frut): *H. aegypticum*, a xero-halophyte that colonises sea cliffs. Present in central Eastern Mediterranean islands, from Sardinia to the island of Crete, and the island of Lampedusa in Sicily (Tutin et al., 1972).

Overall, plant materials were collected from 7 locations. Voucher specimens allowed us to enrich the collection maintained at the herbarium of Palermo (PAL).

2.2. Tissue analysis

After several observations and preliminary fresh staining to determine the consistency of the material and primitive distribution of the tissues and glands within the various organs (Johansen, 1940; Sass, 1958; Jensen, 1962), some of the material was set in formalin aceto-alcohol (FAA), and part was dehydrated with ethanol at increasing concentrations, stained with 1% alcoholic safranin (Catalano, 1925; Beccari & Mazzi, 1966; Colombo, 2003), further dehydrated with absolute alcohol and xylol, and embedded in balsam for the prepared permanent mounts. Furthermore, the vegetative apex, mature leaves, sepals, petals, and flower buds were cleared with the Fuchs method (Fuchs, 1963), modified by the authors in order to obtain a study material that would not be macerated but intact throughout the xylem pattern that develops in the leaf parenchyma. A light counterstain with 1% safranin in an alcoholic solution allowed us to better highlight the degree of lignification of the vascular elements, sclereids, and idioblasts and enhance variations in contrast between black nodules and translucent glands. The permanent preparations were photographed with a LM Leica DMLS, while the digital images were obtained with a NIKON DS camera (Head DS-Fi1).

2.3. Data analysis

The measures relating to the dimensions and relative distances of black nodules, translucent glands, and secretory canals were obtained through an image analyzer program integrated with a DS camera (Head DS-Fi1). The venation pattern was graded according to the protocol of Hickey (1979) and Ash et al. (1999).

3. Results

3.1. Leaf vascular pattern

3.1.1. *Hypericum perforatum* L.

Leaves generally lanceolate with an apical orientation and simple organisation (Figure 1a). Symmetrical linear lanceolate lamina. Acute apex. Acute base. Whole foliar margin with a paper texture. On the foliar blade, along the margins, black nodules are distinguished and signs

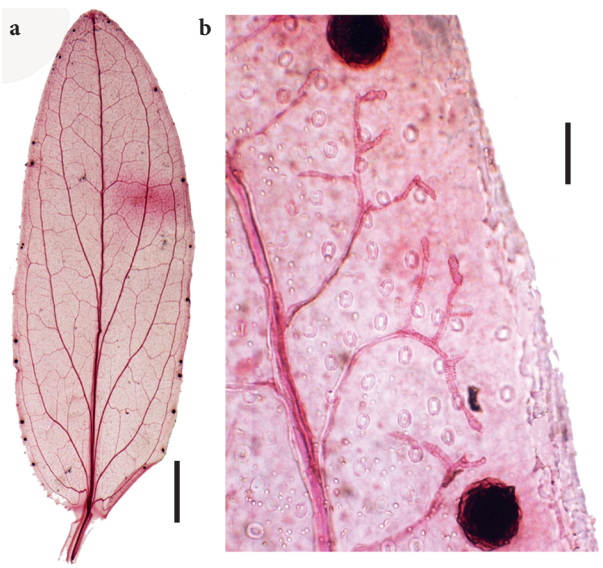

Figure 1. *Hypericum perforatum*: a- cleared leaf, b- areoles and veinlet terminations. Scale bars: a = 1 mm, b = 100 µm.

of depressions over the entire surface (perforations) underlying translucent glands that develop in the foliar mesophyll. The petiole is absent. Leaf pinnate, semicraspedodrome. Primary veins of robust size. Secondary veins with an angle of acute and narrow divergence, uniform, moderate, with a straight course. There are random cross-linked tertiary veins of order III. There are veins of a higher order than III forming a network in which the order is not easily distinguishable. There are incomplete marginal veins. The areoles are irregular with medium-sized cuts, randomly oriented within which they develop branched veins from one to several times (Figure 1b).

3.1.2. *Hypericum perfoliatum* L.

Leaf commonly stringed amplexicaul with an apical orientation and simple organisation (Figure 2a). Symmetrical oblong lamina (2:1). Elliptical apex. Obtuse base. Whole foliar margin with a paper texture. Black nodules are distinguished in the proximity of the margin and translucent glands over the entire lamina but develop in the mesophyll. The petiole is absent. Pinnate leaf, acrodromous baseline. Primary veins of robust size. Secondary veins with an angle of acute and narrow divergence, with smooth arc wrinkles. Inter-secondary veins absent. Random cross-linked tertiary veins. There are veins of a higher order than III forming a network in which the order is not easily distinguishable. There are incomplete marginal veins, simple free branches, curved. The areolae, with incomplete closed mesh randomly oriented, are polygonal and large (Figure 2b).

3.1.3. *Hypericum pubescens* Boiss.

Leaves usually elliptic to ovate or almost sub-round with an apical orientation and simple organisation (Figure 3a). Symmetrical lamina with an ovate to elliptical shape. Obtuse apex. Roped base. Whole margin with a paper texture. On the foliar lamina, along the margins of the leaf, there are a few large black nodules and translucent glands over the entire surface of the foliar lamina. Normal marginal petiole. Pinnate leaf, camptodromous, brochidodromous. Primary veins of a robust size. Secondary veins with an angle of moderate acute divergence, uniform with a slightly sinuous pattern. Intercostal areas present. Tertiary veins orthogonally cross-linked. There are also slender veins of order IV and V. Incomplete marginal veins. Areolae forming unclosed meshes of a general pentagonal shape and small size and free branches branched only once (Figure 3b).

3.1.4. *Hypericum tetrapterum* Fr.

Dimorphic leaves, mostly elliptical, others ovate-lanceolate, with an apical orientation and simple organisation (Figure 4a). Symmetrical elliptical lamina. Acute apex. Obtuse base. Whole margin with a paper texture. Present on the foliar lamina are black nodules along the margins and translucent glands all over the lamina. Petiole absent. Pinnate leaf, acrodromous baseline. Primary veins of a moderate size. Secondary veins with an acute narrow angle of divergence with a straight course. There are veins of orders III, IV, and V present, scarcely identifiable and forming a grid. Incomplete marginal veins. Polygonal areolae, small, within which free branches

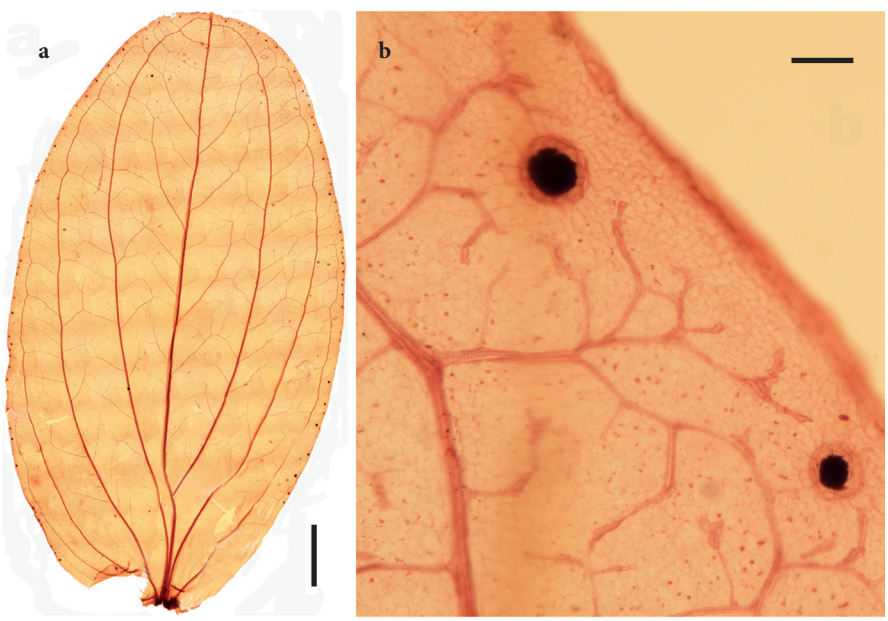

Figure 2. *Hypericum perfoliatum*: a- cleared leaf, b- areoles and veinlet terminations. Scale bars: a = 2 mm, b = 100 µm.

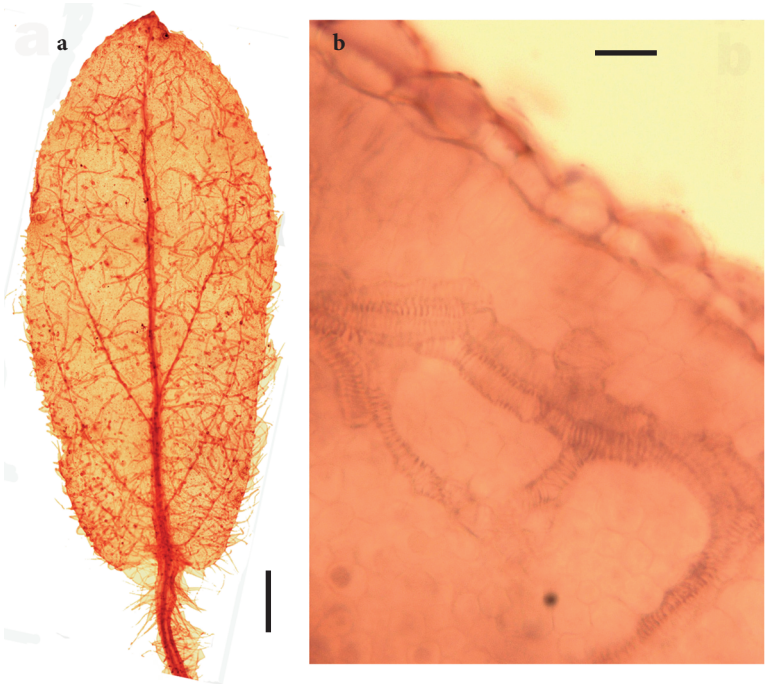

Figure 3. *Hypericum pubescens*: a- cleared leaf, b- areoles and veinlet terminations. Scale bars: a = 1 mm, b = 25 µm.

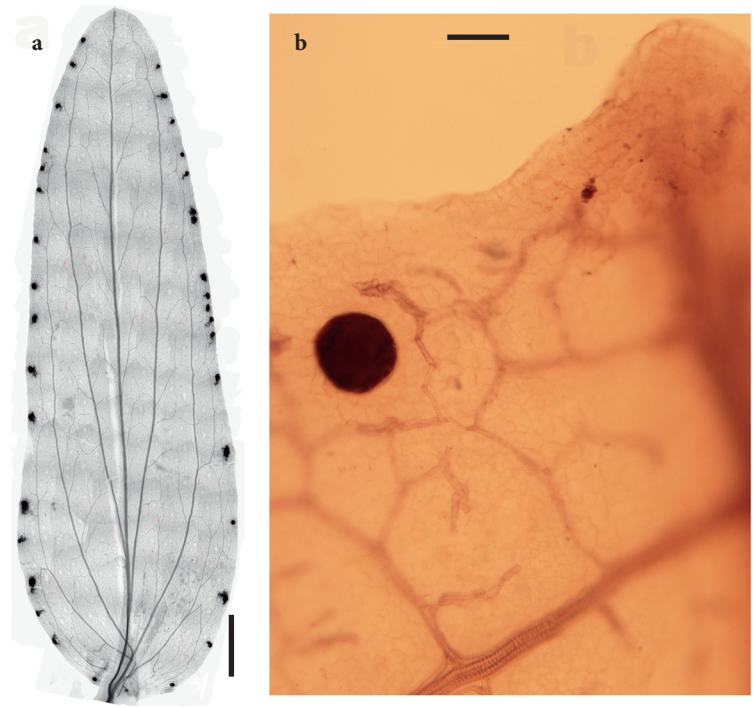

Figure 4. *Hypericum tetrapterum*: a- cleared leaf, b- areoles and veinlet terminations. Scale bars: a = 2 mm, b = 100 µm.

are distinguished, from simple to branched, with terminal tracheids, short and stubby, accompanied by scleroid idioblasts (Figure 4b).

3.1.5. *Hypericum triquetrifolium* Turra

Leaves are generally lanceolate-amplexicaul with an apical orientation and simple organisation (Figure 5a).

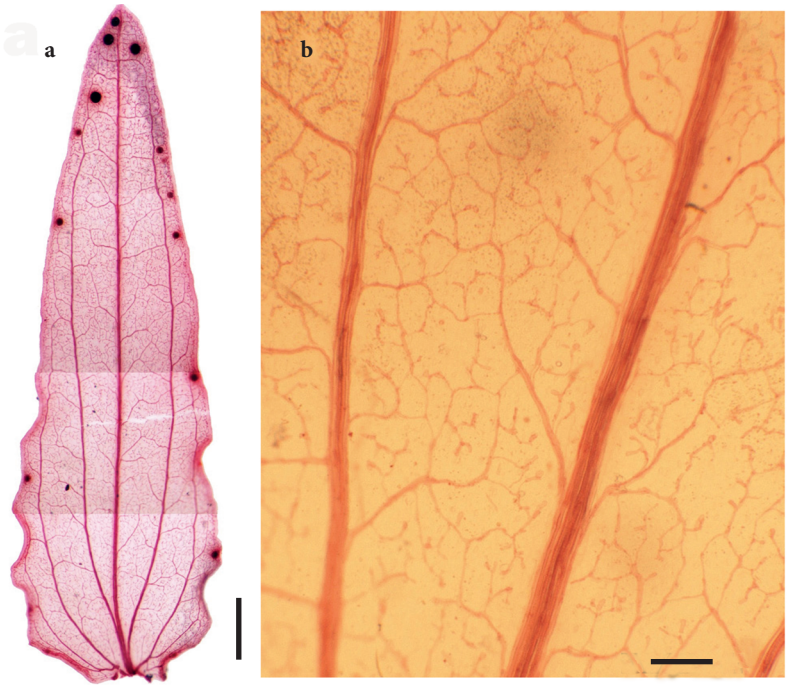

Figure 5. *Hypericum triquetrifolium*: a- cleared leaf, b- areoles and veinlet terminations. Scale bars: a = 1 mm, b = 250 µm.

Symmetrical lamina with wavy margins, ovate lanceolate. Acute apex. Truncated base. Whole margin with a paper texture. Black nodules are found along the margin, and throughout the lamina perforations in the shape of depressions underlying the translucent glands that open in the foliar mesophyll. Petiole absent. Pinnate leaf, perfect acrodromous base. Primary veins of robust size. Secondary veins with an angle of acute and narrow divergence with a straight course. Random cross-linked tertiary veins. There are veins of orders IV and V present, not easily distinguishable. Incomplete marginal veins rather lignified. The areoles are irregular, polygonal, and veinlets with greatly lignified terminal tracheids inside (Figure 5b). These branches are usually one- or bi-branched with a straight or bent course. The terminal tracheids are composed of spiral tracheids with spheroidal idioblasts.

3.1.6. *Hypericum androsaemum* L.

Ovate leaves with an apical orientation and simple organisation. Symmetrical ovate lamina. Acute apex. Roped base. Whole margin with a paper texture. There are no black nodules, and on the lamina there are characteristics of depressions that indicate the presence of translucent glands in the mesophyll. Petiole is almost absent. The leaf is semi-amplexicaul. Pinnate leaf, camptodromous brochidodromous. Primary veins of a slender size. Secondary veins with an angle of moderate acute divergence, uniform with a straight course. Random cross-linked tertiary veins. There are also veins of orders IV and V forming a grid. Marginal veins with loops within which there are free simple linear curved branches, branched once. Slightly lignified terminal tracheids (Figure 6a). There were also sepals and petals clarified, where there were significant differences because these whole fruits are widely inserted on the thalamus. We did not find, as we did with nomofilos, the median primary veins from which secondary veins depart. We discovered primary veins flanked by 2 thinner commissurals, flanked in turn by 2 other commissurals, thinner and less lignified where the overall development is always of the camptodromous type, brochidodromous with marginal veins with loops within which free long branches, slightly curved, uni-branched, or bi-branched, greatly lignified by the presence of long rows of annular spirals and terminal tracheids flanked by filiform sclereids (Ananda Rao, 1991). In the petals (Figure 6b) there are also principle veins and 2 commissurals from which depart pairs of secondary veins of order II that delimit wide irregular walls, within which we identify irregular areolae with well-marked free branches; all the orders of veins, especially the marginal ones, have well lignified tracheids and are also flanked by filiform sclereids.

3.1.7. *Hypericum hircinum* L.

Sessile leaf from lanceolate to ovate-lanceolate with an apical orientation and simple organisation (Figure 7a). Symmetrical oblong ovate lamina. Apex acute. Rope based. Whole foliar margin with a paper texture. Black nodules are absent, but there are numerous translucent glands in

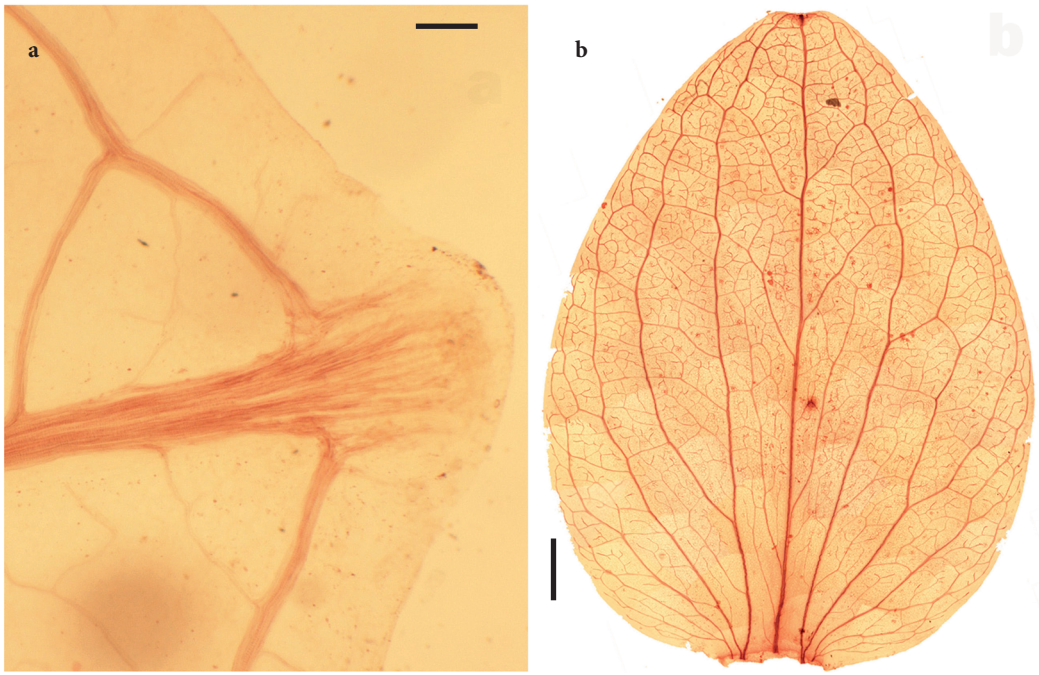

Figure 6. *Hypericum androsaemum*: a- areoles and veinlet terminations, b- cleared petal. Scale bars: a = 100 µm, b = 2 mm.

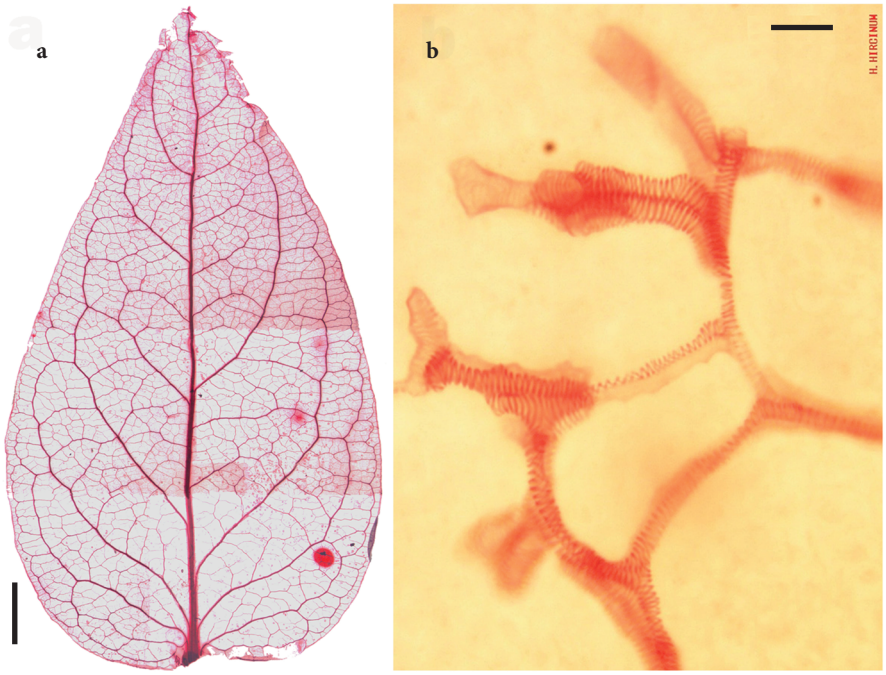

Figure 7. *Hypericum hircinum*: a- cleared leaf, b- areoles and veinlet terminations. Scale bars: a = 5 mm, b = 100 µm.

the mesophyll surmounted by rounded depressions on the foliar lamina. The petiole is absent. Pinnate leaves, semicraspedodromous. Primary veins of robust size. Secondary veins with an angle of broad acute divergence. Their thickness is moderate with a curved pattern. Random reticular tertiary veins are present. There are veins of a higher order than III, with resolution not easily distinguishable, and marginal veins with loops. Simple free branches. Areolae, with incomplete growth and a random arrangement, irregularly shaped (Figure 7b).

3.1.8. *Hypericum aegypticum* L.

Sub-spatulate and elliptical leaf with an apical orientation and simple organisation (Figure 8a). Symmetrical lamina with an elliptical shape. Acute apex. Acute base. Whole margin with a thick and stiff leathery texture. There are no black nodules but rather translucent glands throughout the foliar surface. Short and stocky petiole present. Pinnate leaves, camptodromous cladodromous. Primary veins of a massive size, highly lignified and a straight course. Secondary veins with an angle of acute and wide divergence. The secondary veins present variations in the angle of divergence, as those found above the middle part of the leaf have an obtuse angle, while those that are below present an acute and wide angle. Tertiary veins with a branched pattern are present. Veins of orders IV and V are found forming a grid. There are incomplete marginal veins. The areoles are polygonal, small, imperfect, randomly oriented, with very simple and generally free branches, straight or curved, with robust and lignified terminal tracheids inside (Figure 8b).

3.2. Secretory structures

The entire *Hypericum* genus is characterised by the presence of black nodules, translucent glands (Curtis & Lersten, 1990), and secretory canals (Ciccarelli et al., 2001b); the nodules and glands are located everywhere in the epigean apparatus, particularly on the leaves. Type A secretory channels of small lumen are present within the vascular bundles in the outer part of the phloem and are particularly visible in the veins of orders I and II, which are better evidenced thanks to the large dimensions of these structures. Other secretory channels (type B) of large lumen are highlighted within the foliar mesophyll and mixed with translucent glands with which they share structure and ontogeny. According to Ciccarelli et al. (2001b), there is a third type of secretory channel (type C) that differs from type B solely by the mode of differentiation during ontogeny, and it is found only in the ovary and stylus. The black nodules, glands internally filled, accumulate a "black content" that is granular but not resin (Curtis & Lersten, 1990), also run through the mesophyll externally also very evident in that they are located along the margin edge of the leaves, sepals, and petals. These nodules are also surrounded by a sheath of 2–3 layers of flattened cells and are present in all the taxa examined (Figure 9), with the exception of *H. hircinum, H. aegypticum*, and *H. androsaemum*. The translucent glands (cavity of oil) (Curtis & Lersten, 1990) are typically schizogenic and optically empty. Many are identified by the naked eye, by transparency on the foliar lamina, and by replicas, indirectly by depressions of various sizes and structures using an optical microscope. Even when the leaves are clarified at low magnification they are found at various depths in the mesophyll, and when they are contiguous to the 2 surfaces (adaxial and abaxial) they present characteristics of depressions in these glands, because the epidermal cells are flattened while those surrounding are very convex; it gives the impression of observing a hole. They are consistently present in all taxa

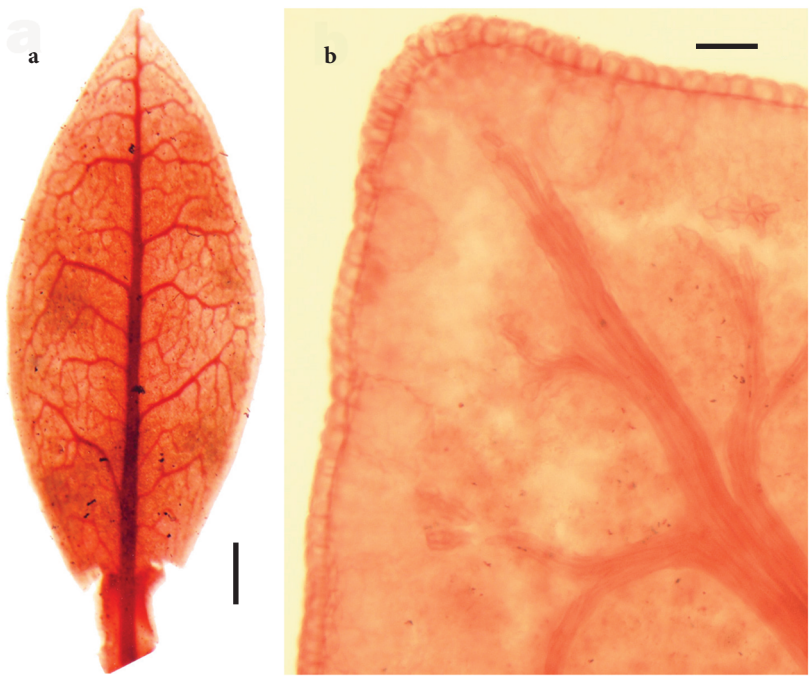

Figure 8. *Hypericum aegypticum*: a- cleared leaf, b- areoles and veinlet terminations. Scale bars: a = 1 mm, b = 100 µm.

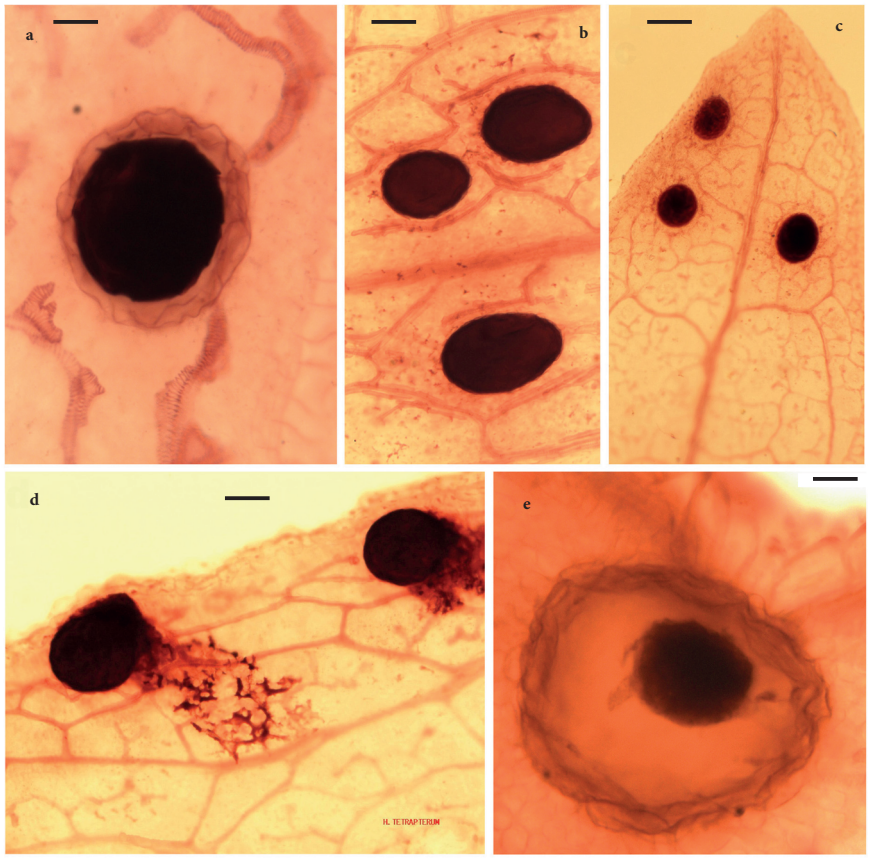

Figure 9. Black nodules: a- *Hypericum perfoliatum*, b- *Hypericum perforatum*, c- *Hypericum triquetrifolium*, d- *Hypericum tetrapterum*, e- *Hypericum pubescens*. Scale bars: a and e = 25 µm, b and d = 100 µm, c = 250 µm.

and develop in the foliar mesophyll. Further data relevant to the typology, location, dimensions, and possible periodism of the secretory structures are presented in the Table.

4. Discussion

Our comparative secretory foliar structures study shows that, within the species studied, there is possible correlation between the presence of black nodules and a particular biological form, that of hemicryptophytes scapose perennials, such as *H. perfoliatum, H. perforatum*, *H. pubescens*, *H. tetrapterum*, and *H. triquetrifolium*. These nodules are not present in *H. androsaemum* and *H. hircinum*, which are bushy nanophanerophytes, or in *H. aegypticum,* a xero-halophyte that grows in rupicola habitats. The black nodules, if any, occur with medium and small individual morphological characteristics and with certain periodism in *H. perfoliatum* and *H. perforatum*, and are large (from 100 to 150 µm) in *H. tetrapterum*, *H. triquetrifolium*, and *H. pubescens* but without periodism. The translucent glands are a constant feature of all the taxa researched, and 3 types were identified: large glands (≅ 107 × 53 µm at 93 × 72 µm) in *H. perfoliatum* (Figure 10a) and *H. perforatum* (Figure 10b), which occupy all the mesophyll and are tangent to the 2 leaf surfaces; medium sizes (≅ 77 × 60 µm) in *H. tetrapterum* (Figure 10c) and *H. triquetrifolium* (Figure 10d), which are mainly located immediately below the adaxial surface and completely shut down the palisade and partially shut the spongy tissue; and small glands (from 40 µm to 61 × 54 µm) in *H. aegypticum* (Figure 11a), *H. hircinum* (Figure 11b), and *H. pubescens* (Figure 11c), which occupy all the spongy tissues running tangent to the abaxial surface. The sizes of the translucent glands are not significant, in fact most types can coexist within the same species as in *H. androsaemum* where they are identified as translucent glands of medium and small sizes, slightly offset in a part of mesophyll (Figure 11d). In this case there is no correlation between type of translucent gland and biological form. The same is also valid for the secretory canals that are present in all the taxa examined, in the A and B forms, with the exception of *H. androsaemum*, which has only the A form. The presence and position of these canals, associated with the phloem (Figure 11e) or

Table. Secretory structures.

Species	Black nodules				Translucent glands			Secretory canals	
	Present / absent	Middle diameter µm	Middle distance µm	Periodism	Present / absent	Middle diameter µm	Location	Type A Ø µm	Type B Ø µm
Hypericum perforatum	+	70 × 71	450	2:1	+	93 × 72	mesophyll	6 × 7	107 × 90
H. perfoliatum	+	50	440	3:1	+	107 × 73	mesophyll	5 × 5	69 × 67
H. pubescens	+	120 × 102	2014	-	+	40 × 40	mesophyll	4 × 5	32 × 35
H. tetrapterum	+	210 × 144	1000	-	+	77 × 60	mesophyll	5 × 5	55 × 58
H. triquetrifolium	+	154 × 128	1230	-	+	77 × 66	mesophyll	6 × 6	127 × 103
H. androsaemum	-	-	-	-	+	82 × 55	mesophyll	6 × 6	-
H. hircinum	-	-	-	-	+	61 × 54	mesophyll	5 × 5	52 × 55
H. aegypticum	-	-	-	-	+	54 × 45	mesophyll	6 × 6	60 × 60

Symbols: + = present, - = absent.

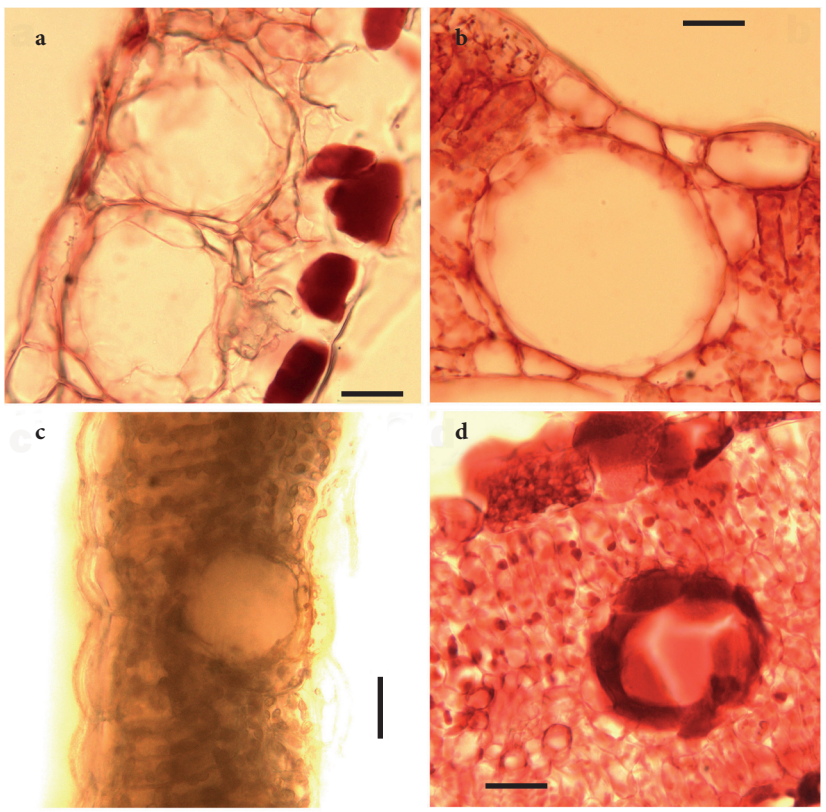

Figure 10. Translucent glands: a- *Hypericum perfoliatum*, b- *Hypericum perforatum*, c- *Hypericum tetrapterum*, d- *Hypericum triquetrifolium*. Scale bars: a, b, and d = 25 µm; c = 100 µm.

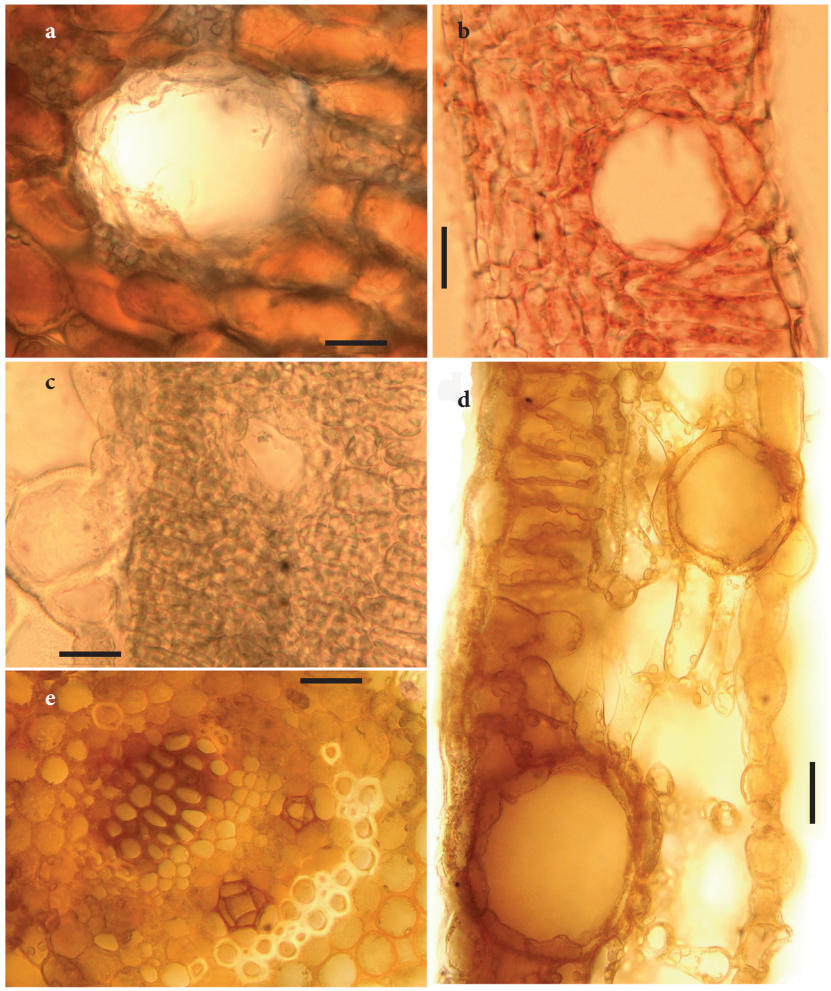

Figure 11. Translucent glands: a- *Hypericum aegypticum*, b- *H. hircinum*, c- *H. pubescens*, d- *H. androsaemum*, e- *H. androsaemum* secretory canals associated with the phloem. Scale bars: a, b, c, and e = 25 µm; d = 100 µm.

inserted in the mesophyll, are all that can provide protection from herbivores, because other secretory structures present in whole fruit have a time-limited efficacy, being ephemeral and limited to the period of anthesis. Another consideration is that the phloem secretory canals do not contain essential oils, as the foliar secretory tissues do, but alkaloids, lipids, resins, and tannins. Finally, the presence of these secondary metabolites contributes to the swelling of the tissues, protecting them from limited environmental stress (Lotocka & Osinska, 2010). We think that the presence of these structures is the type of compensatory strategy that the plant has adopted to protect the buds and insure its survival. The leaves of the taxa studied have also been clarified. The analysis of the xylem pattern clarified the adaptation of the species to different environments, as they are highly vascularised leaf organs that respond to edaphic-environmental changes. *H. androsaemum* and *H. pubescens* have pinnate camptodromous brochidodromous leaves, and in this case the veins of orders I, II, and III are predominantly arranged in the middle of the lamina and far from the margin because these species vegetate in humid areas, and therefore do not require timely vascularisation. In *H. perfoliatum*, *H. tetrapterum*, and *H. triquetrifolium* the leaf vascular pattern is the acrodromous basal pinnate type with a peripheral vascularisation with veins of orders I and II, which are rather sturdy because they vegetate in uncultivated and barren areas, hedges, and woods, and undergo increased edaphic stress; the xylem pattern, however, is more lignified overall, with small areolas where they develop thicker and shorter terminal tracheids, usually bi-branched. *H. perforatum* and *H. hircinum* have a xylem pattern of a semicraspedodromous pinnate type; short and stubby marginal veins branch off from veins of order II, which arrive at the margin. Vascularisation is therefore timely because the environment is generally more arid and harsh. Finally, *H. aegypticum* shows a pattern of

camptodrome cladodrome pinnate organisation; the veins of order I are very robust and lignified, and veins of order II branch off from them as a pinnate leaf. However, in the middle part of the intercostal panel many branches are formed that arrive at the margin. The areolae are small with stubby and lignified free branches. In the taxa, the terminal tracheids of the leaf xylem pattern terminating in the proximity of secretory structures (blacks nodules and translucent glands) are arranged in a "basket" and are very numerous and intertwined, apparently making no contact with these, even if the clarifications show 2 or more random tracheids arranged in a suspected contiguity.

Acknowledgements
The authors would like to thank Professors Francesco Maria Raimondo and Vincenzo Ilardi for their assistance in the field. We would also like to acknowledge the 2 anonymous reviewers for their suggestions.

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