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# The Role of Secreting Structures Position on the Leaf Volatile Organic Compounds of *Hypericum androsaemum*

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*Hypericum androsaemum* L. presents typical translucent, essential oil producing glands, which are distributed on the leaf along both margins (margin glands) and on the lamina (lamina glands). The gland secretion was studied by histochemical and chemical analysis; the gland content was sampled directly from the secretory glands, and the volatile organic compounds (VOC) of the margin and lamina glands were separately analyzed. The lipophilic fraction of the lamina glands had as main components: (*E*)-2-hexenal (15.5%), hexadecanoic acid (14.7%), β-caryophyllene (11.2%), germacrene B (11.0%) and γ-himachalene (9.8%). The lipophilic fraction of the margin glands had as its main components: β-pinene (22.0%), limonene (17.6%), (*E*)-β-ocimene (6.1%), methyl linoleate (5.7%), terpinolene (5.4 %), (*E*)-2-hexenal (4.9%) and α-pinene (4.1%).

**Keywords:** *Hypericum androsaemum* L., Hypericaceae, Translucent glands, Histochemistry, Volatile organic compounds, GC-MS analysis.

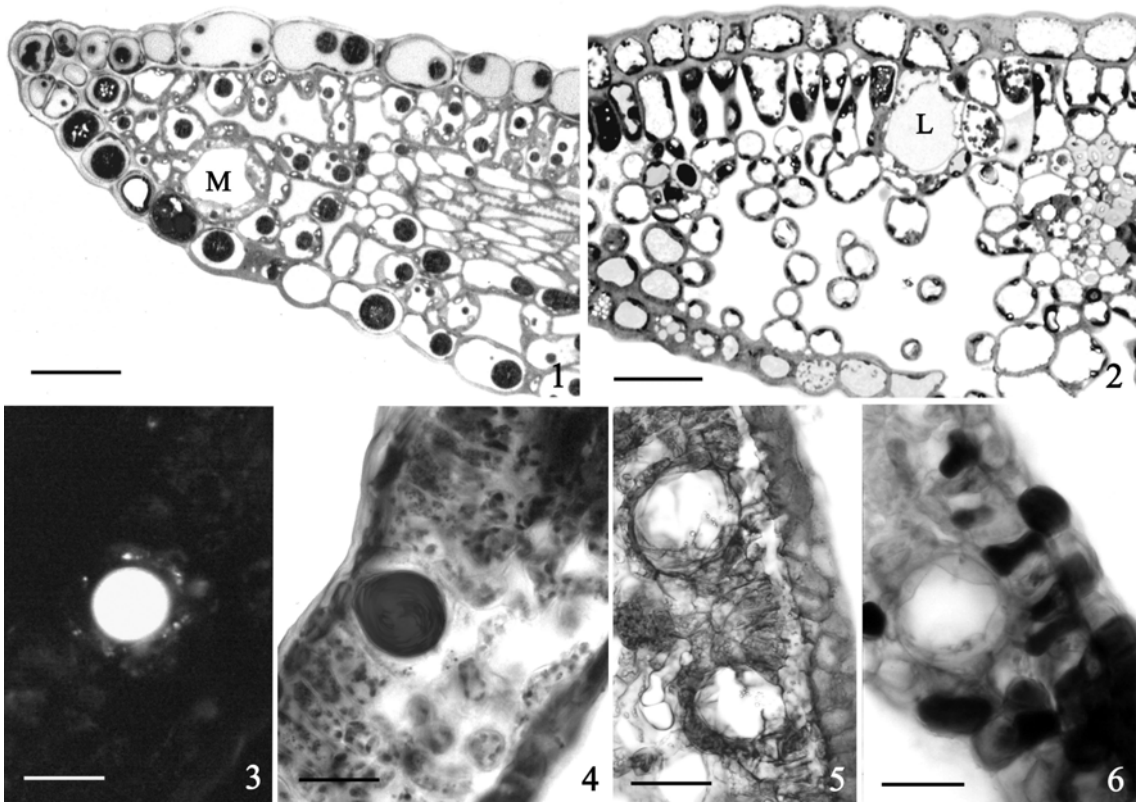
*Hypericum androsaemum* L. (Hypericaceae) is a shrub with yellow flowers, widely distributed in western Europe, growing preferably in damp and shady places [1]; in Italy, it is present in the whole peninsula [2,3]. In folk medicine *H. androsaemum* is employed to treat skin diseases, in particular burns, for gastro-intestinal problems [4,5], and for its diuretic and anti-hepatotoxic activities [6]. It is often used as a substitute for the better known *H. perforatum* L., with the same applications.

On the leaves of *H. androsaemum* only translucent glands, containing lipophilic substances, are present; black nodules, typical secreting structures of *H. perforatum*, are lacking.

Most of the recent phytochemical reports about *H. androsaemum* concern its phenolic components, including phenolic acids, flavonoids, and xanthones [7,8 and literature therein]. The essential oil composition has been studied only in Portuguese and Iranian samples [9-12]. The biological activity of the species has been recently reported [6,13-15]. In this current work we investigate the distribution, morphology and histochemistry of the glands and we report for the first time the composition of the leaf volatile organic compounds (VOC), directly sampled with microneedle shuttle analysis [16].

The numerous translucent glands are densely distributed along the margins (margin glands) and scattered on the lamina (lamina glands) (Figures 1, 2). The lamina presents small depressions corresponding to the glands; the margin glands protrude on the abaxial side of the lamina. These structures are constituted of a layer of flattened cells that delimit a large cavity where the secretion is accumulated (Figures 1, 2). The secretion proved positive to lipophilic stains (Figure 3), particularly to the Nadi reagent (Figure 4), but gave a negative response to the stains specific for polysaccharides (Figure 5) and polyphenols (Figure 6). These results indicate that the gland content is mainly constituted of volatile compounds.

Analysis of the secretion, directly sampled from the glands, was carried out separating the margin glands from those of the lamina. The VOC composition of the margin and lamina glands contents is shown in Table 1.




**Figures 1**, **2**: Sections of *H. androsaemum* leaf stained with Toluidine Blue. Note the localization of a margin gland (M) (1) and of a lamina gland (L) (2). Bars = 25 μm. **Figures 3-6**: Histochemistry of a translucent gland - Positive response to lipophilic stains: Fluoral Yellow-088 (3) and Nadi reagent (4). Negative response to hydrophilic stains: Ruthenium Red (5) and $FeCl_3$ (6). Bars = 25 μm.

Forty-two and thirty compounds were identified in the margin glands and lamina glands, respectively, representing 98.9% of the total in both. The lamina glands presented, as main components, (*E*)-2-hexenal (15.5%), hexadecanoic acid (14.7%), β-caryophyllene (11.2%), germacrene B (11.0%), and γ-himachalene (9.8%). The main components of the margin glands were: β-pinene (22.0%), limonene (17.6%), (*E*)-β-ocimene (6.1%), methyl linoleate (5.7%), terpinolene (5.4%), (*E*)-2-hexenal (4.9%), and α-pinene (4.1%).

Monoterpene hydrocarbons made up 61.4% of the margin glands VOC, but only 5.2 % of those of the lamina glands. Significant amounts of sesquiterpene hydrocarbons (41.8%) were found in the lamina glands and much less in the margin glands (12.4%). However, the oxygenated sesquiterpene (3.7% and 3.3%) and oxygenated monoterpene (1.3% and 1.7%) proportions were similar in the two (Table 1). Of the fifty-one different compounds identified, twenty-one were common to both the margin and lamina glands.

Although the morphology of the margin and the lamina glands is very similar, their VOC compositions are quite different, probably because some metabolic pathways are not activated. Particularly we may postulate that the 1,3 hydride shift of the menthyl cation and the thujyl cation were not activated in the lamina glands (Table 1), whereas the formation of bisabolyl and humulyl cations via himachalene cation - longibornane transposition were not activated in the margin glands, owing to the lack of related compounds (Table 1).

The VOC composition of the glands, here reported, is not directly comparable with the literature data, which relates to the composition of essential oils obtained by hydrodistillation [10-12]. However we can note several affinities with respect to the plants from Portugal [10, 11]. Indeed several compounds, such as (*E*)-2-hexenal, limonene, caryophyllene derivatives, germacrene D and germacrene B are present both in our samples and the Portuguese [10, 11]. However, the essential oil of the plants from Iran [12] showed a more different composition having only few compounds, particularly caryophyllene derivatives, in common with our samples.

## Experimental

***Plant material:*** Plants of *H. androsaemum* were collected from the Euganean Hills (Padua) in June 2002 and cultivated in pots at the Botanical Garden of Florence. Fresh leaves were collected, before blossom,

**Table 1**: Percentage composition and retention indices of leaf VOC from the margin (M) and lamina (L) glands of *H. androsaemum*. The compounds are listed in order of their elution from a HP-5 column.

| RI | Compounds | M % | L % |
|---|---|---|---|
| 802 | Hexanal | - | 4.0 |
| 854 | (*E*)-2-Hexenal | 4.9 | 15.5 |
| 939 | α-Thujene | 1.5 | - |
| 948 | α-Pinene | 4.1 | 0.5 |
| 988 | Sabinene | 1.3 | - |
| 995 | β-Pinene | 22.0 | 2.4 |
| 1000 | *n*-Decane | 1.0 | 3.4 |
| 1002 | Myrcene | 1.8 | 1.0 |
| 1008 | Mesitylene | 0.4 | 1.3 |
| 1038 | *p*-Cymene | 0.2 | - |
| 1042 | Limonene | 17.6 | 1.3 |
| 1057 | (*E*)-β-Ocimene | 6.1 | - |
| 1067 | γ-Terpinene | 1.2 | - |
| 1091 | Terpinolene | 5.4 | - |
| 1095 | *p*-Cymenene | 0.2 | - |
| 1100 | *n*-Undecane | 1.2 | - |
| 1154 | Camphor | 0.6 | 1.7 |
| 1188 | Naphthalene | 2.0 | 2.4 |
| 1192 | *p*-Cymen-8-ol | 0.7 | - |
| 1200 | *n*-Dodecane | 0.3 | - |
| 1300 | *n*-Tridecane | 0.3 | 0.9 |
| 1354 | α-Terpinyl acetate | 1.0 | - |
| 1394 | β-Elemene | 1.0 | 0.7 |
| 1400 | n-Tetradecane | 0.3 | - |
| 1424 | β-Caryophyllene | 2.5 | 11.2 |
| 1435 | γ-Elemene | 0.5 | 1.3 |
| 1460 | α-Humulene | 0.5 | 2.2 |
| 1485 | γ-Himachalene | - | 9.8 |
| 1487 | Germacrene D | 2.4 | - |
| 1492 | β-Selinene | 0.7 | - |
| 1499 | α-Selinene | 0.9 | 3.1 |
| 1510 | Germacrene A | 0.5 | - |
| 1523 | δ-Cadinene | 0.2 | 1.9 |
| 1541 | α-Cadinene | 0.6 | - |
| 1546 | Selina-3,7(11)-diene | 0.5 | 0.6 |
| 1562 | Germacrene B | 2.1 | 11.0 |
| 1600 | *n*-Hexadecane | 0.5 | 1.4 |
| 1628 | *trans*-Isolongifolanone | - | 0.6 |
| 1635 | *epi*-α-Cadinol | 3.2 | - |
| 1649 | Selina-3,11-dien-6α-ol | - | 1.2 |
| 1659 | Selin-11-en-4α-ol | 0.4 | - |
| 1660 | *neo*-Intermedeol | - | 1.0 |
| 1669 | (*E*)-Bisabol-11-ol | - | 0.5 |
| 1695 | Eudesm-7(11)-en-4-ol | 0.1 | - |
| 1700 | *n*-Heptadecane | 0.4 | 1.1 |
| 1800 | *n*-Octadecane | 0.3 | - |
| 1900 | *n*-Nonadecane | - | 0.4 |
| 1972 | Hexadecanoic acid | 1.8 | 14.7 |
| 2047 | Methyl linoleate | 5.7 | - |
| 2100 | *n*-Heneicosane | - | 0.8 |
| 2300 | *n*-Tricosane | - | 1.0 |
| | **Total %** | **98.9** | **98.9** |

for morphological and chemical analyses. Voucher specimens have been deposited in the Herbarium Centrale Italicum of Florence (FI), labelled "Maleci L. 15/06/2002 Colli Euganei (Pd)".

Micromorphological analyses were performed on fresh material by using Scanning Electron Microscopy (SEM) and Light Microscopy (LM).

***SEM observations:*** Small pieces of plant material were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 6.8, dehydrated in ethanol in ascending grades up to absolute, and then subjected to critical point drying and carbon/gold coating.

***LM observations:*** Frozen fresh material was sectioned and stained with Sudan Black B [17], Fluoral Yellow-088 [18] and Nile Red [19] for lipids, Nadi reagent for terpenes [20], Ruthenium Red [21] for polysaccharides and Ferric Trichloride for polyphenols [22].

***Sampling of the translucent glands secretion:*** The sampling was carried out by means of special microneedles (MNs) [16]. One hundred margin glands and 100 lamina glands were perforated and their secretions were accumulated in microvials containing dichloromethane.

***Gas chromatography (GC) and gas chromatography-mass spectrometry (GCMS):*** The GC analyses were carried out using a Varian 3300 instrument equipped with a FID and a HP-InnoWax capillary column (30 m x 0.25 mm, film thickness 0.17 μm), working from 60°C (3 min) to 210°C (15 min) at 4°C/min or an HP-5 capillary column (30 m x 0.25 mm, film thickness 0.25 μm) working from 60°C (3 min) to 300°C (15 min) at 4°C/min; injector and detector temperatures, 250°C; carrier gas, helium (1 mL/min); split ratio, 1 : 10.

GC-MS analyses were carried out using a Hewlett Packard 5890 GC-MS system operating in the EI mode at 70 eV, using the two above mentioned columns. The operating conditions were analogous to those reported in the GC analyses section. Injector and transfer line temperatures were 220°C and 280°C, respectively. Helium was used as the carrier gas at a flow rate of 1 mL/min; Split ratio, 1:1.

Identification of the components was made by matching their spectra with those from MS libraries and the identity of each component was confirmed by comparing their RIs, for both columns, relative to C6-C22 *n*-alkanes with those from the literature. When reported, co-elution gas chromatography with reference compounds was used for an additional confirmation of the compound identity.

The percentage composition of the leaf VOC was obtained by the normalization method from the GC peak areas, without using correction factors.

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