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Composition of Essential Oils from Leaves and Flowers of *Stachys germanica* subsp. *salviifolia* (Ten.) Gams (Labiatae) and Related Secretory Structures

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The essential oils from both leaves (L) and flowers (F) of *Stachys germanica* subsp. *salviifolia* (Ten.) Gams (Labiatae/Lamiaceae) from Italy were analyzed by GC-MS for the first time. The distribution, morphology and histochemistry of the different types of glandular trichomes present on the epidermal surface were also determined. Twenty-nine constituents, representing 89.4% of the total leaf oil, and forty-one compounds, forming 97.8% of the total flower oil, were identified. Germacrene D (39.4%), phytol (10.2%), β-bourbonene (3.5%) and β-ylangene (3.3%) were recognized as the main constituents of the leaf essential oil, while limonene (24.1%), β-pinene (18.7%), germacrene D (12.8%) and (*E*)-nerolidol (6.6%) were the main compounds of the flower essential oil.

Keywords: *Stachys germanica* subsp. *salviifolia*, Labiatae/Lamiaceae, essential oil, GC-MS analysis, glandular trichomes, micromorphology; histochemistry.

Stachys germanica, widely distributed in Europe, particularly in the Mediterranean basin [1,2], is a very polymorphous species showing considerable morphological and ecological variability. Indeed, several subspecific taxa have been recognised; in Italy, three subspecies have been identified [3]. In this work we examined *S. germanica* subsp. *salviifolia* (Ten.) Gams, present throughout central and southern Italy in dry and sunny places, prevalently on calcareous soil and in ruderal areas that have undergone anthropical influences.

Plants of the genus *Stachys* have been widely applied in folk medicine and phytotherapy; in particular, *S. germanica* L. is used in gastrodynia and painful menstruation in folk medicine in Iran [4]. It is also employed in folk veterinary medicine in Italy to treat problems of the skin and wounds in asses and horses [5]. The essential oils of this taxon have been tested for antibacterial activities [6-10].

The essential oils both from leaves (L) and flowers (F) were obtained by hydrodistillation in a Clevenger-type apparatus. GC/MS analysis enabled the identification of 53 compounds in all, 29 for leaves and 41 for flowers, representing 89.4% and 97.8% of the total essential oils, respectively. The relative concentrations of the volatile components identified are presented in Table 1.

In the leaf essential oil the most abundant compounds were germacrene D (39.4%), phytol (10.2%), β-bourbonene (3.5%) and β-ylangene (3.3%). The essential oil was constituted mainly of sesquiterpenes (52.9 %).

In the flower essential oil, the major constituents were limonene (24.1%), β-pinene (18.7%), germacrene D (12.8%) and (*E*)-nerolidol (6.6%). The main fractions were constituted of monoterpenes (47.5 %) and sesquiterpenes (40.6%).

The total amount of the distilled essential oils was < 0.01%. Germacrene D was the main constituent of the leaf essential oil, but only the third major constituent of the flower essential oil.

Table 1: Percentage composition of the essential oil from leaves (L) and flowers (F) of *S. germanica* subsp. *salviifolia*.

RI	Compounds	L %	F %
859	(*Z*)-3-Hexenol	-	1.2
938	α-Pinene	-	1.0
957	Allylbenzene	-	1.3
979	β-Pinene	-	18.7
981	1-Octen-3-ol	-	1.4
992	Myrcene	-	1.6
997	*n*-Octanal	-	0.2
1002	(*E*)-3-Hexenyl acetate	-	0.2
1004	δ-2-Carene	-	0.2
1030	Limonene	-	24.1
1060	γ-Terpinene	-	0.3
1096	Linalool	0.5	0.3
1103	*n*-Nonanal	-	0.8
1179	Terpinen-4-ol	-	0.4
1186	β-Fenchyl alcohol	-	0.3
1201	*n*-Decanal	-	0.4
1346	α-Terpinyl acetate		0.8
1375	α-Copaene	0.3	0.6
1385	β-Bourbonene	3.5	1.9
1387	(*E*)-β-Damascenone	0.4	-
1389	β-Cubebene	0.3	0.2
1392	β-Elemene	2.4	3.0
1419	β-Ylangene	3.3	4.4
1433	β-Copaene	2.1	2.4
1434	β-Gurjunene	1.4	1.0
1458	allo-Aromadendrene	-	0.8
1480	Germacrene D	39.4	12.8
1497	Bicyclogermacrene	-	3.2
1500	Pentadecane	1.6	-
1503	Germacrene A	-	0.2
1508	β-Bisabolene	-	0.3
1511	γ-Cadinene	-	0.3
1523	δ-Cadinene	0.4	1.3
1570	(*E*)-Nerolidol	2.3	6.6
1576	Germacrene D-4-ol	-	0.5
1600	Hexadecane	0.3	-
1611	Tetradecanal	0.4	-
1638	*epi*-α-Muurolol	-	1.3
1700	Heptadecane	0.8	-
1715	Pentadecanal	2.2	-
1726	Mint sulfide	0.8	0.3
1815	Hexadecanal	0.8	-
1839	Neophytadiene	0.4	-
1847	6,10,14-Trimethyl-2-pentadecanone	2.6	0.6
1900	Nonadecane	0.9	-
1977	Hexadecanoic acid	3.2	0.6
2000	Eicosane	0.3	-
2100	Heneicosane	2.4	0.2
2103	Octadecanal	2.1	-
2106	Phytol	10.2	0.8
2192	Octadecanol acetate	1.9	-
2200	Docosane	2.2	0.5
2300	Tricosane	-	0.8
	Total %	**89.4**	**97.8**

Phytochemical investigations of this subspecies from Croatia [11] and from different subspecies of *S. germanica* from the Balkan regions [12-14] and from Hungary [15] have been reported. Germacrene D represents the main compound in all the investigated samples, while the other major constituents of our samples are either lacking or are present in very small amounts in the plants of other origins. If these observations are confirmed also for the other taxa of the *S. germanica* group, germacrene D could be considered a taxonomic marker for this group. Nevertheless, the differences in the other major compounds indicate the existence of a chemical polymorphism, that could be related to different microclimatic factors or to genetic differentiations.

In the genus *Stachys* various types of glandular trichomes were described in a previous paper [16]. A more detailed study of *S. germanica* subsp. *salviifolia* allowed observation of the following types of trichomes:

Type **A** (Figure 1), present on leaves and inflorescences, is a peltate hair with an elongated basal epidermal cell, so that a well developed stalk is formed [17]; this kind of hair has also been observed in other Labiatae species [17]. The secretion stored in the subcuticular space consists only of lipophilic components (Figure 2).

Type **B** (Figure 3), localised on the whole plant, is a small capitate hair with a thin subcuticular space. The histochemical stains indicate a secretion composed both of hydrophilic and lipophilic substances.

Type **C** (Figure 4), present only on the inflorescences, is a large capitate trichome with a multicellular head; each secretory cell bears on the apex its own small subcuticular space (Figure 5). The secretion is mainly polysaccharidic (Figure 6), but with polyphenolic (Figure 7) and lipophilic fractions.

Therefore, the essential oil is mainly produced and secreted by type A trichomes (evidenced by Nadi reagent). The other two types of trichomes present a complex secretion of both hydrophilic and lipophilic substances, in which the essential oil represents a minor fraction.

The different composition between leaf and flower oils could be related to the different types of trichomes observed on the vegetative and reproductive organs, as already reported for other Labiatae species [18,19]. Indeed, in *S. recta*, bearing a single type of trichome, both on leaves and inflorescences, a similar essential oil composition was found [20].

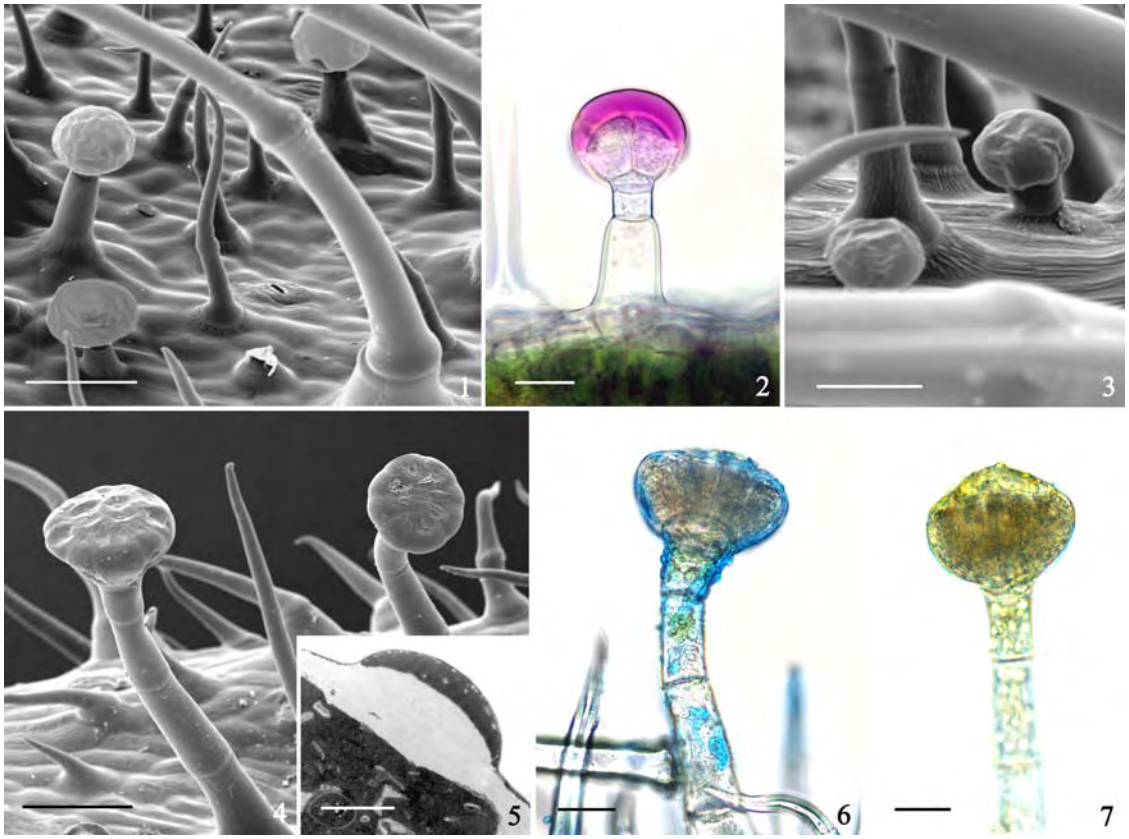

Figure 1: Type A peltate trichomes on the abaxial side of the corolla. Bar = 50 μm. **Figure 2**: Histochemistry of type A trichome: Nadi reagent. Bar = 20 μm. **Figure 3**: Type B small capitate trichomes on the abaxial side of the calyx. Bar = 20 μm. **Figure 4**: Type C large capitate trichomes on the abaxial side of the calyx. Bar = 50 μm. **Figure 5**: Type C trichome: particular of the subcuticular space on the apex of a secreting cell. Bar = 0.2 μm. **Figures 6**, **7**: Histochemistry of type C trichome: Alcian Blue (5) and $FeCl_3$ (6). Bars = 25 μm.

Experimental

Plant material: Aerial parts of *S. germanica* subsp. *salviifolia* (Ten.) Gams were collected at Mt Morello near Florence on July 2007. Samples of the studied material were identified according to Pignatti [21]. Voucher specimen was deposited in FI (Herbarium Centrale Italicum), labelled "Giuliani C. 10/07/2007 Mt Morello".

Isolation of the essential oil: The fresh leaves and inflorescences were separately subjected to hydrodistillation in a Clevenger type apparatus for 3 h using *n*-hexane as solvent. The concentrations of the identified volatile components are presented in Table 1 according to their elution order from a DB-5 column. The extracts were dried over anhydrous sodium sulfate and then stored in sealed vials at -20°C ready for the GC and GC-MS analyses.

Gas chromatography (GC) and gas chromatography-mass spectrometry (GCMS): The GC analyses were carried out using an Agilent 6890N instrument equipped with HP-WAX and HP-5 capillary columns (30 m x 0.25 mm, 0.25 μm film thickness), working with the following temperature programme: 10 min at 60°C, and subsequently up to 220°C at 5°C/min; injector and detector temperatures, 250°C; carrier gas, helium (1 mL/min); split ratio, 1:20. GC/MS analyses were carried out using an Agilent 5970 GC-MS system operating in the EI mode at 70 eV, using the same columns.

The identification of the components was made for both columns by comparison of their retention time with respect to *n*-paraffin (C6-C22) internal standards. The mass spectra and Kovats Indices (KI) were compared with those of commercial (NIST 98 and WILEY) and home-made library mass spectra built up from pure compounds and MS literature data. Area percentages were obtained electronically from the GC-FID response without the use of an internal standard or correction factors.

Micromorphological analysis: These were performed on fresh material (stems, leaves, bracts, calyces and corollas) 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 coated.

LM observations: Frozen fresh material was stained with Nadi reagent for terpenes [22], Alcian Blue [23] for acid polysaccharides and ferric trichloride for polyphenols [24].

TEM observations: Small pieces of plant material were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 6.8 and post fixed in 2% OsO_4, dehydrated in ethanol in ascending grades up to absolute and embedded in Spurr's resin. Ultra thin sections were stained with uranile acetate and lead citrate. Samples were examined with a Philips EM-300-TEM.

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