Secretory structures and essential oil composition in *Stachys officinalis* (L.) Trevisan subsp. *officinalis* (Lamiaceae) from Italy

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Abstract

The secretory structures and the volatile fraction of *Stachys officinalis* (L.) Trevisan subsp. *officinalis* (Lamiaceae) from Italy were studied for the first time. Peltate and small capitate trichomes were observed on the whole plant (leaves and inflorescences). In the peltate trichomes, an unusual polyphenols content was evidenced by the histochemical methods. The volatile fraction was obtained by a solvent extract from the distillation water of leaves and inflorescences and analyzed by GC-MS. Forty-four constituents for leaves, representing 94.1% of the total volatiles, and fifty-seven compounds for flowers, accounting for 90.1% of the total volatiles, were identified. (*E*)-caryophyllene (20.1%), (*E*)-nerolidol (14.3%), caryophyllene oxide (6.1%) and γ-cadinene (5.7%) were recognized as the main constituents for the leaf volatile fraction, while caryophyllene oxide (16.5%), (*E*)-nerolidol (15.4%), humulene epoxide II (9.2%) and α-pinene (7.0%) were the main compounds for the flower volatile fraction.

Keywords: *Betonica officinalis,* Lamiaceae, micro-morphology, glandular trichomes,volatiles, GC-MS analysis.

Introduction

Stachys officinalis (L.) Trevisan (= *Betonica officinalis* L., Lamiaceae), commonly known as wood betony, is a perennial herbaceous plant widely distributed in Europe, Western Asia and Northern Africa (Ball, 1972), preferably in damp meadows and on rocky slopes in forest clearings.

Wood betony was at one time commonly employed as a medicinal plant in the treatment of a wide range of disorders, especially as a nervine and tonic for treating maladies of the head and as an external application to wounds (Grieve, 1984). Wood betony is much less used nowadays (Pignatti, 1982), and more often is part of a mixture of herbs. Morphologically it is a well-defined species; in Italy two different subspecies are recognized (Pignatti, 1982; Goren, 2014).

Despite being a member of a family of numerous aromatic species, the plant does not give off any scent but presents a glandular *indumentum* both on the vegetative and reproductive organs (Giuliani and Maleci Bini, 2012).

In accordance with the phytochemical interest towards other members of the genus *Stachys* (Formisano et al., 2014; Karami and Dehghan-Mashtani, 2015; Leporini et al., 2015), the essential oil composition of this species was the subject of previous studies referring to plants from the Balkan peninsula (Maly, 1985; Chalchat et al., 2001; Grujic-Jovanovic et al., 2004; Bilusic Vundac et al., 2006; Hajdari et al., 2011; Lazarević et al., 2013) and Bulgaria (Dimitrova-Dyulgerova et al,. 2015); in particular, data about Italian *S. officinalis* samples, as well as data on the plant part specification of the volatile fraction lack. In this work we examined the most diffused subspecies: *S. officinalis* subsp. *officinalis*, distributed in the whole peninsula. We investigate in detail the distribution, morphology, histochemistry and ultrastructure of the glandular trichomes as well as the chemical composition of leaf and flower volatile fraction from plants collected near Florence (Italy).

Results and Discussion

Micromorphological investigation

The epidermal surfaces present both non-glandular and glandular trichomes. The non-glandular ones are multicellular and uniseriate, simple or stellate. Two types of glandular trichomes were described in a previous paper (Giuliani and Maleci Bini, 2012): peltate (type A) and small capitate (type B) (Figure 1). A more detailed study allowed the observation of the following features.

Type A trichomes are typical peltate hairs, constituted of one basal epidermal cell, one neck cell and a multicellular head of 8 cells with a large storing chamber in which the secretion is accumulated (Figure 2). Type B hairs are constituted of one basal epidermal cell, one stalk cell and a head of four cells with a thin subcuticular space in which secretion is temporarily stored (Figure 3).

The secretion of type A trichomes proved positive to all the lipophilic stains, particularly to the Nadi reagent (Figure 4), indicating the presence of terpenes. Also an abundant polyphenolic fraction (Figure 5) was detected. In the glandular cells the most striking ultrastructural feature is represented

by plastids associated with endoplasmic reticulum (Figure 6), typical organelles involved in the production of terpenes, and polyphenols as well.

The secretion of type B trichomes proved positive to hydrophilic substances (mainly polysaccharides) (Figure 7); a small lipophilic fraction (terpenes) was also evidenced (Figure 8). The ultrastructural observations evidenced the presence of abundant Golgi bodies, well-developed rough endoplasmic reticulum (RER), and of several electrondense plastids (Figures 9, 10), confirming the presence of abundant polysaccharides and small amounts of terpenes.

Concerning the morphology and distribution, these types of trichomes are consistent with those described in the literature (Giuliani and Maleci Bini, 2008, 2012). However, the secretion of type A trichomes results particularly rich in polyphenols, differently from the literature data referring to this type of trichomes, considered typical essential oil producers (Hallahan, 2000). Type B hairs, as reported also by the literature (Hallahan, 2000), show a secretion mainly composed of polysaccharides with a small fraction of terpenes; however their contribution to the total secretion is poor, as evidenced by the histochemical stains (Figures 7, 8).

Phytochemical investigation

The resulting data on the qualitative and quantitative chemical compositions of the volatile fraction, obtained by a solvent extract from the distillation water of leaves and inflorescences and analyzed by GC-MS are presented in Table 1.

Forty-four and fifty-seven constituents were identified in the leaf and flowers volatiles, accounting for 94.1% and 90.1% of the total oils, respectively. The major constituents of the leaf volatile fraction are (*E*)-caryophyllene (20.1%), (*E*)-nerolidol (14.0%), caryophyllene oxide (6.1%) and γ-cadinene (5.7%). In the flower volatile fraction the major constituents are caryophyllene oxide (16.5%), (*E*)-nerolidol (15.4%), humulene epoxide II (9.2%) and α-pinene (7.0%). The monoterpene and sesquiterpene fractions of the volatiles were comparable in their relative amounts (leaf oil: 3.6% monoterpenes *versus* 86.5% sesquiterpenes; flower oil: 11.0% monoterpenes *versus* 74.0% sesquiterpenes). However, within the sesquiterpenoidic fraction a highly uneven distribution between the hydrocarbons and oxygenated derivatives was observed, with a predominance of non-oxygenated compounds in leaf oil with respect to flower oil where hydrocarbons constituents prevail (Table 1).

Although our samples show, as the previously investigated essential oils (Maly, 1985; Chalchat et al., 2001; Grujic-Jovanovic et al., 2004; Bilusic Vundac et al. 2006; Hajdari et al., 2011; Lazarević et al., 2013), a high amount of sesquiterpenes, in particularly of hydrocarbons derivatives, some quantitative and qualitative differences can be evaluated.

In general, the compounds which characterise the volatiles of Italian samples are absent, or present in very small quantities, in the samples from the Balkan regions (Chalchat et al., 2001; Grujic-Jovanovic et al., 2004; Bilusic Vundac et al. 2006; Hajdari et al., 2011; Lazarević et al., 2013), and vice-versa;

germacrene D, being one of the main component of most previously investigated essential oils (Grujic-Jovanovic et al., 2004; Bilusic Vundac et al., 2006; Hajdari et al., 2011; Lazarević et al., 2013), is present in traces in our samples. Isocaryophyllene and β-caryophyllene, dominating constituents of the essential oil from one population in Montenegro (Chalchat et al., 2001), were totally absent in our samples and in those from Serbia (Grujic-Jovanovic et al., 2004) and Croatia (Bilusic Vundac et al. 2006). (*E*)-caryophyllene and caryophyllene oxide are the only compounds present in comparable percentages in our plants and in plants from Croatia and Kosovo (Bilusic Vundac et al. 2006; Hajdari et al., 2011).

It is noteworthy that, while most of the authors hydrodistilled the plant aerial parts as a whole, Hajdari et al. (2011) and Dimitrova-Dyulgerova et al. (2015) analysed the volatiles separately obtained from leaves and flowers of *Stachys officinalis*. They proved the natural diversity of the chemical composition of the essential oils from separate plant organs. The comparison with our samples evidenced a general variability between the vegetative profiles, as well as between the floral ones; indeed *(i)* among the main components, the only common compounds are (*E*)-caryophyllene in the leaf essential oils and α-pinene in the flower essential oil for the samples from Kosovo (Hajdari et al., 2011), whereas *(ii)* the oils from Bulgaria exhibited great qualitative and quantitative differences (Dimitrova-Dyulgerova et al., 2015).

Conclusions

The micro-morphological investigation on the glandular *indumentum* of *S. officinalis* subsp. *officinalis* allowed to deeply characterize the secretory structures responsible for the synthesis and release of volatiles. In addition the histochemical methods and the ultrastructural observations documented an unusual production of polyphenols in the peltate trichomes.

Our study also revealed significant differences in the volatile compounds profile of Italian *S. officinalis* with respect to plants from the Balkan regions and Bulgaria, indicating the existence of an intra-specific chemical polymorphism, as proved for other *Stachys* species (Goren, 2014). In addition to the different geographic conditions and distillation techniques (Dimitrova-Dyulgerova et al., 2015), the emergence of different essential oil profiles, are related to local abiotic and biotic selective forces that act on the terpene-biosynthetic pathways, which vary in turn according to the different plant tissues and the different plant phenological stages (Barra, 2009).

Experimental

Plant material. Aerial parts of *S. officinalis* subsp. *officinalis* were collected in Vicchio near Florence during the blooming period on June 2014. Samples of the studied material were identified by C. Giuliani according to Pignatti (1982). A voucher specimen has been deposited at the Herbarium of the

Laboratory of Plant Science of the Department of Biology (University of Florence), under the accession number 0045/14.

Micromorphological investigation

This survey was carried out in order to evidence the morphology and distribution of the different types of glandular trichomes by means of light microscopy (LM) and scanning electron microscopy (SEM) and to determine the chemical nature of their secretion by means of different histochemical techniques. To evidence the organelles involved in the secretion production several observations were carried out by *Scanning electron microscopy*. Plant material was first hand-prepared, fixed in 2.5 % glutaraldehyde in phosphate buffer (0.1 M, pH 6.8), dehydrated in an ethanol series up to absolute and then dried using a Critical Point Dryer apparatus. The samples, mounted on aluminium stubs, were coated with gold, and observed with a Philips XL 20 SEM at 10 kV.

Light microscopy. Fresh material was frozen, sectioned and stained with the following techniques: Fluoral Yellow 088 for total lipids (Brundrett et al. 1991), Nile Red for neutral lipids (Greenspan et al, 1985), Nadi reagent for terpenes (David and Carde, 1964), Ruthenium Red (Jensen, 1962) and Alcian Blue (Beccari and Mazzi, 1966) for acid polysaccharides, Ferric Trichloride for polyphenols (Gahan, 1984), and Aluminium Trichloride for flavonoids (Guerin et al., 1981). Control procedures were carried out at the same time. Observations were made with a Leitz DM-RB Fluo optic microscope.

Transmission electron microscopy. 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. Ultrathin sections were stained with uranile acetate and lead citrate. Samples were examined with a Philips EM- 300-TEM. Semi thin sections of this material were also used for LM morphological investigations.

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 extracts were dried over anhydrous sodium sulfate and then stored in sealed vials at - 20°C ready for the GC and GC-MS analyses.

Phytochemical investigation

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 an 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, flow rate 1 ml/min. Split ratio, 1:1.

The identification of the components was made by matching their spectra with those from mass spectral libraries and the identity of each component was confirmed by comparing their retention indices, for both columns, relative to the C8-C26 *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 and floral volatiles was obtained by the normalization method from the GC peak areas, without using correction factors.

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Table 1. Essential oil composition

RI*	Component	L%	F%
800	2-butanone	-	0.3
928	α-thujene	-	0.1
936	α-pinene	3.1	7.0
973	sabinene	-	0.2
977	β-pinene	-	0.3
984	1-octen-3-ol	0.2	0.2
991	myrcene	0.3	0.1
1025	o-cymene	-	0.1
1030	limonene	-	0.7
1095	α-pinene oxide	-	0.4
1101	linalool	0.2	0.3
1107	trans-thujone	-	0.6
1127	α-campholenal	-	0.3
1139	trans-pinocarveol	-	0.2
1146	trans-verbenol	-	0.4
1161	pinocarvone	-	0.1
1191	myrtenal	-	0.1
1203	verbenone	-	0.3
1342	α-cubebene	0.8	0.2
1363	α-ylangene	0.5	0.2
1369	α-copaene	2.0	1.0
1376	β-bourbonene	2.8	2.0
1381	iso-longifolene	0.4	-
1382	β-cubebene	0.2	0.2
1384	β-elemene	0.2	0.2
1411	(*E*)-caryophyllene	20.1	4.0
1421	β-copaene	1.5	0.7
1436	β-gurjunene	0.2	0.2
1447	α-humulene	4.5	2.1
1450	(*E*)-β-farnesene	4.7	0.1
1454	sesquisabinene	-	1.1
1459	germacrene D	0.2	-
1463	pentadecane	-	0.4
1470	γ-cadinene	5.7	3.6
1476	δ-cadinene	3.0	0.4
1480	zonarene	0.3	2.4
1485	(*Z*)-nerolidol	1.8	-
1489	10-epi-cubebol	1.5	2.5
1505	trans-cadina-1(2),4-diene	3.5	-
1508	7-epi-α-selinene	1.2	-
1509	selina-3.7(11)-diene	-	1.3
1512	α-cadinene	4.2	-
1514	cis-calamenene	0.4	0.2
1528	elemol	-	0.6
1543	germacrene B	0.4	2.9
1565	(*E*)-nerolidol	14.3	15.4
1574	caryophyllene oxide	6.1	16.5
1580	β-copaen-4-α-ol	0.7	-
1586	salvial-4(14)-en-1-ene	0.2	0.1
1602	humulene epoxide II	1.1	9.2
1618	1,10-di-epi-cubenol	0.4	-
1634	eremoligenol	0.8	-
1636	1-epi-cubenol	0.7	0.3
1638	epi-α-muurolol	0.3	0.3

1650	α-cadinol	1.4	1.4
1655	intermedeol	0.2	1.2
1659	trans-calamenen-10-ol	-	0.3
1664	valeranone	-	0.3
1691	10-nor-calamenen-10-one	-	0.2
1734	cyclocolorenone	-	0.2
1740	α-oxobisabolene	1.0	2.7
1843	6,10,14-trimethyl-2-pentadecanone	-	1.2
2000	eicosane	-	0.7
2100	heneicosane	-	1.0
2102	phytol	0.1	-
2200	docosane	0.2	0.7
2300	tricosane	0.5	0.4
2400	tetracosane	1.2	-
2500	pentacosane	1.0	-
	Total	**94.1**	**90.1**

Monoterpenes	3.6	11.0
Hydrocarbons	3.4	8.3
Oxygenated	0.2	2.7
Sesquiterpenes	86.5	74.0
Hydrocarbons	56.8	22.8
Oxygenated	29.7	51.2
Others	3.1	4.9

* Experimentally determined retention indices on a HP-5 column (relative to C8-C26); -: the compound was not detected in the essential oil.

Captions to Figures

Figure 1. SEM micrograph showing type A peltate trichome (right) and type B small capitate trichome (left) in *Stachys officinalis* subsp. *officinalis*. **Figures 2-3**. LM semithin sections stained with Toluidine Blue showing type A (2) and type B (3) trichomes. **Figures 4-5**. LM micrographs showing the histochemistry of type A trichome: Nadi reagent (4), and $FeCl_3$ stain (5). **Figure 6**. TEM micrograph of type A trichome. **Figures 7-8**. LM micrographs showing the histochemistry of type B trichome: Ruthenium Red (7), and Nadi reagent (8). **Figures 9-10**. TEM micrographs of type B trichome. (Cw) Cell wall; (g) Golgi bodies; (m) mitocondrion; (n) nucleus; (p) plastid; (rer) RER; (ser) SER; (Ss) Subcuticular space; (v) vacuole.