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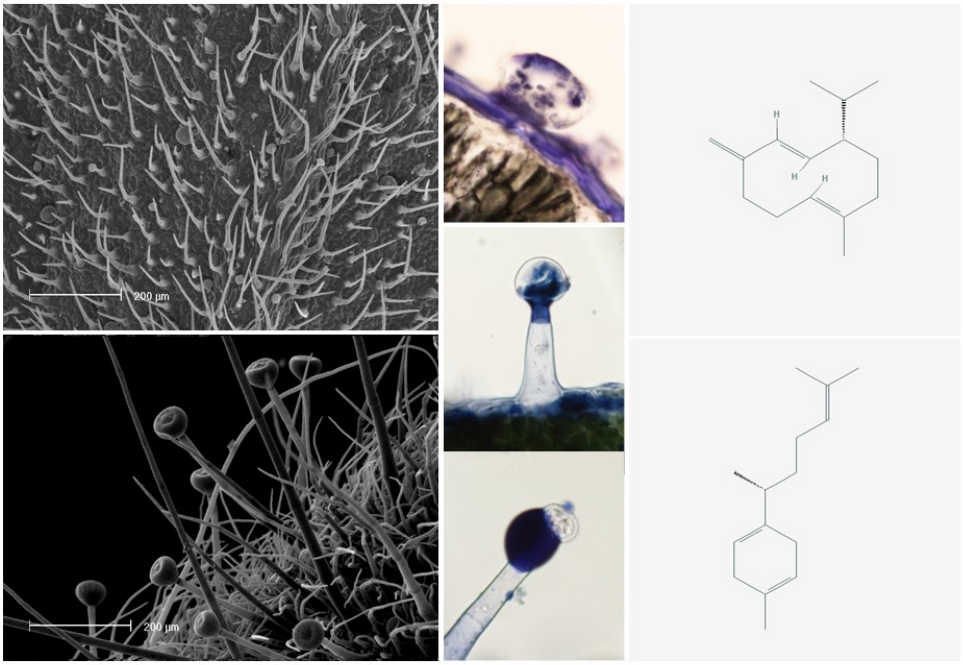

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Scutellaria brevibracteata subsp. *subvelutina* (Rech.f.) Greuter & Burdet: morphological and phytochemical characterization.

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Abstract

A micromorphological and phytochemical survey was performed on *Scutellaria brevibracteata* subsp. *subvelutina* (Lamiaceae) cultivated in Italy. The *indumentum* of the vegetative and reproductive organs was investigated: peltate, short-, medium- and long-stalked capitates were described. Histochemistry evidenced similar results for the peltates and the long-stalked capitates, differences for short and medium capitates. For the first time, this work reported the leaf and flower VOC characterization, together with the first analysis of the EO obtained from the aerial parts of Italian samples. The floral profile resulted more complex than the foliar one, due to the higher number of the total compounds (40 vs 27) and of the exclusive constituents (24 vs 11). 16 common compounds were detected, with β-caryophyllene as the most abundant one. The EO was characterized by 23 compounds, among which β-caryophyllene dominated. The peltates, the medium and the long-stalked capitates resulted the producers of the investigated compounds.

Keywords

Scutellaria brevibracteata subsp. *subvelutina*, Micro-morphology, HS-SPME, Hydrodistillation, GC/MS, VOC profile, Essential oil

1. Introduction

Scutellaria L., commonly known as skullcaps (Formisano et al. 2013; Sripathi and Ravi 2017), is one of the largest genera of the Lamiaceae family and includes 471 species (Yilmaz et al. 2019). The genus has a subcosmopolitan distribution with the main centers of diversity distributed in the Andes, in the Iran-Turanian region and in the Eastern Mediterranean (Paton 1990). In particular, in Turkey, *Scutellaria* consists of 39 *taxa*, 17 of which are endemic (Yilmaz et al. 2019).

Scutellaria brevibracteata Stapf. is widespread in the Mediterranean region and includes four subspecies: *S. brevibracteata* subsp. *brevibracteata*, *S. brevibracteata* subsp. *icarica* (Rech.f.) Greuter & Burdet, *S. brevibracteata* subsp. *subvelutina* (Rech.f.) Greuter & Burdet and *S. brevibracteata* subsp. *pannosula* (Rech.f.) Greuter & Burdet (Tutin et al. 1972). Among them, the subsp. *icarica* is located in the Greek islands Ikaria and Samos in the Northern Aegean Sea, nearby the Turkish coasts (Edmondson 1982). The subsp. *brevibracteata* and subsp. *pannosula* are endemic of Turkey and subsp. *subvelutina* is distributed in Southern Turkey and in Western Syria (Yilmaz et al. 2019). The four subspecies differ for the presence of erect or prostrate stems, for the colour and pubescence degree of the leaves, for the corolla length and for the bracts size (Edmondson 1982).

S. brevibracteata subsp. *subvelutina* is a perennial herb with erect or ascending stems, mostly pubescent at the distal portion and with pubescent leaves, green or violet in colour; the bract length is equal or slightly higher than those of the calyx and the corolla is 14-21 mm long. A peculiar feature is the presence of velutinose hairs (Edmondson 1982).

Ethnobotanically, little is known about the traditional uses of the species belonging to *Scutellaria*. In Eastern Anatolia, the infusion of the dried leaves is used for wound healing, as haemostatic and tonic (Özçelik et al. 1990; Baytop 1999). Moreover, its infusion and decoction are considered remedies against tumor and haemorrhoids and show astringent properties (Cakilicioğlu and Türkoğlu 2010). Other uses are described for *Scutellaria* species from many other places in the world (Kokakowska 2017; Irvin et al. 2019).

In the Lamiaceae family, glandular hairs represent important sites for the synthesis of natural bioactive compounds, which display a crucial role in the seduction towards pollinators or seed dispersers or in the repulsion strategies against phytophagous insects or pests (Maffei 2010; Giuliani et al. 2017).

The literature proposes few morphological studies on the structure of the glandular trichomes in *Scutellaria* species (Giuliani and Maleci Bini 2008; Dereboylu et al. 2012; De Oliveira et al. 2013; Naghiloo et al. 2014; Cali 2017a; 2017b), none of them referring to *S. brevibracteata*.

Concerning the phytochemical state of the art, works on the VOC emission profile are lacking, whereas works related to the characterization of the essential oil of *Scutellaria* species are few (Skaltsa et al. 2000; Yu et al. 2004; Skaltsa et al. 2005; Rosselli et al. 2007). In particular, there are only 2 contributions about S. *brevibracteata* (Formisano et al. 2013; Yilmaz et al. 2019). The former authors also investigated the ecological role of the essential oil (Formisano et al. 2013).

Regarding the subsp. *subvelutina*, the literature proposes only one contribution on the analysis of the essential oil composition of plants form Turkey (Yilmaz et al. 2019).

Only one contribution refers about the biological activity of the subspecies, reporting the inhibitory effects on the tyrosinase caused by the methanol extract of the aerial parts (Şenol et al. 2010).

This work is part of the project "Botanic Garden, factory of molecules", with the financial support from the Lombardy Region (Italy). In this context, the goals of this work were: **1.** to describe the morphology and the distribution pattern of the glandular trichomes on the vegetative and reproductive organs of *S. brevibracteata* subsp. *subvelutina*, by means of light microscopy and scanning electron microscopy; **2.** to characterize the secreted substances through histochemical analysis; **3.** to correlate the micro-morphological investigation of the secreting structures with the productivity of secondary metabolites through the phytochemical characterization of the volatile organic compounds (VOCs) spontaneously emitted by leaves and flowers and the essential oil (EO) obtained from the aerial parts.

2. Results

2.1 Micromorphological investigation

2.1.1 Trichome morphotypes and distribution pattern

The micromorphological survey revealed the occurrence of both glandular and non-glandular trichomes in the *indumentum* of the investigated organs.

The glandular trichomes belong to two main types, peltate and capitate. The latter may be distinguished into three subtypes: short-, medium-, and long-stalked (**Fig. S1, B-D**).

The peltate (**Fig. S1A**) is composed by one basal epidermal cell, one neck cell and 4-8 head cells surrounded by a large storing chamber (30-40 μm in diameter). It is observed on the interveinal regions of the abaxial leaf surface and on the reproductive organs (**Fig. S2, B, E, F**).

The short-stalked capitate (**Fig. S1B**, 30-35 μm in length) is made up by one basal cell, one neck cell and 2 secreting cells with a thin subcuticular space (15-25 μm in diameter). It is generally placed in epidermal depressions so that it slightly protrudes from the surface and is ubiquitous to all the investigated organs, with a preferential distribution pattern on the veinal system. The medium-stalked capitate (**Fig. S1C**, 45-70 μm in length) consists of one protruding basal epidermal cell, one stalk cell and of a 2-4-celled head (30-40 μm in diameter). It is sporadically observed on leaves, whereas it is characterized by a higher density on calyces and corollas. The long-stalked capitate (**Fig. S1D**, 250-400 μm in length) is composed by one basal cell, 3-4 stalk cells and by a multicellular head (30-50 μm in diameter) characterized by a small subcuticular space located in the centre. This hair is exclusively observed on the calyx, and it is absent at the skullcap. The distribution pattern of the glandular trichomes is reported in **Table S1**. The non-glandular hairs are uniseriate with a smooth cuticle and display a different length depending on the organ bearing them (**Fig. S2, A-G**). Indeed, they are shorter (70-90 μm in length) on leaves, where they appear erect or curved with a sharp tip facing towards the leaf apex (**Fig. S2, A-B**); they are longer (150- 400 μm) and thinner on calices and corollas without any preferential orientation.

2.1.2 Histochemistry

The results of the histochemical survey on the glandular trichomes are reported in **Table S2** and **Fig S3**. In the peltate trichomes the responses to all the lipophilic stainings were positive, as well as to $AlCl_3$, indicating the presence of terpenes and of major flavonoid derivatives (**Fig. S3, A-C**). The secreted material of the short capitate proved positive only to the hydrophilic dyes, indicating the exclusive production of polysaccharides (**Fig. S3D**). As regards to the medium capitate, the secretion proved positive to all the lipophilic stainings, particularly to the Nadi reagent, indicating that they are exclusive terpene producers (**Fig. S3E**). The long capitate showed intense positive responses to all the lipophilic dyes, proving the production of terpene derivatives as well as of major polyphenol and flavonoid fractions (**Fig. S3, F-H**).

2.2 Phytochemical investigation

2.2.1 VOC emission

The VOC emission profiles revealed a total of 51 different compounds. 27 and 40 compounds were identified in the leaf and in the flower profiles, respectively (**Table 1**). In the leaf, the most abundant chemical class was represented by sesquiterpene hydrocarbons (66.27%), followed by non-terpene substances (21.4%). Oxygenated monoterpenes and hydrocarbons derivatives were present in comparable amounts, 4.82% and 2.46%, respectively. The main compound was β-caryophyllene (*28*; 44.05%), followed by decanal (*13*; 10.37%), β-bourbonene (*20*; 5.01%), γ-muurolene (38; 4.33%) and nonanal (*8*; 4.04%). 11 exclusive compounds were identified, among which germacrene D (*39*; 3.96%) dominated. The other exclusive compounds occurred in amounts lower than 2.0%. The floral profile was dominated by sesquiterpene hydrocarbons (88.65%), followed by non-terpene substances (4.85%) and by comparable relative percentages of oxygenated monoterpenes (1.3%) and hydrocarbon derivatives (0.57%). We also detected oxygenated sesquiterpenes (2.78%), totally absent in the leaf profile. The most abundant compound was the sesquiterpene hydrocarbon β-caryophyllene (*28*; 26.56%), followed by β-curcumene (*45*; 23.43%), α-cedrene (*26*; 8.71%), β-bisabolene (*44*; 6.28%) and γ-muurolene (*38*; 5.72%). 24 exclusive compounds were identified, among which β-curcumene (*45*; 23.43%) was the most abundant one, followed by α-cedrene (*26*) and β-bisabolene (*44*).

The comparison between the two profiles evidenced the occurrence of 16 common compounds, among which the most abundant was β-caryophyllene (*28*), present in different relative abundances: it occurred in leaves (44.05%) in a percentage value almost double compared to flowers (26.56%). Similarly, decanal (*13*) was more abundant in the leaf profile (10.37%), compared to the floral one (0.2%). Most of the common compounds, β-pinene (*2*), 1,8-cineole (*5*), nonanal (*8*) and β-bourbonene (*20*) occurred in lower amounts in the flowers (0.33%; 1.03%; 0.11%; 0.16%), in comparison to the leaves (2.46%; 3.82%; 4.04%; 5.01%). α-Humulene (*35*) (2.06% leaves; 1.94% flowers) and γ-muurolene (*38*) (4.33% leaves; 5.72% flowers) were present in comparable percentages, as well as the minor compounds with amounts lower than 1.0%.

2.2.2 EO profile

The EO composition is reported in **Table 2**: a total of 23 compounds were identified, accounting for 96.55% of the total oil. The extraction yield was 0.06%. Sesquiterpene hydrocarbons represented the most abundant fraction (54.59%). Oxygenated sesquiterpenes and non-terpene derivatives followed in similar percentage (14.60% and 17.45%, respectively), while monoterpenes showed the lower relative abundance. The dominant compound was β-caryophyllene (*15*; 42.60%), followed by hexahydrofarnesylacetone (*23*; 9.96%) and linalool (*8*; 7.80%). Nonanal (*9*; 5.57%), humulene (*16*; 5.20%) and caryophyllene oxide (*18*; 4.82%) occurred at similar amounts. Except for other three sesquiterpenes (*17*; *20*; *22*), the remaining compounds were present at relative abundances lower than 2.0%.

3. Discussion

The glandular trichomes observed on *S. brevibracteata* subsp. *subvelutina* corresponded to the two main types widespread in the family Lamiaceae: peltate and capitate (Werker 2000; Giuliani et al. 2017; Najar et al. 2018). The peltates were present both on the vegetative and the reproductive component, as documented in other *Scutellaria* species (Giuliani and Maleci Bini 2008; Dereboylu et al. 2012; De Oliveira et al. 2013; Cali 2017a; 2017b). In addition, three subtypes of capitates were distinguished: short-, medium- and long-stalked. The capitates were widespread on the whole epidermal surfaces of the plant, with a different distribution *pattern* for each subtype. The short capitates were ubiquitous, while the medium capitates mostly occurred on calyces and corollas, rarely on the leaves, and the long capitates were exclusively distributed on the calyx. The short-stalked subtypes were described in all Lamiaceae species examined so far (Dereboylu et al. 2012). On the contrary, the medium-stalked capitates had never been observed before in the *Scutellaria* genus. The long-stalked capitates, with a multicellular head, occurred in *S. galericulata* (Giuliani and Maleci Bini 2008; Dereboylu et al. 2012;) and in several species of *Stachys*, representing diagnostic microcharacters for the intra-genus classification. The histochemical analysis revealed a similar complex chemical composition for the peltates and the long-stalked capitates, due to the presence of terpenes and polyphenols, in particular flavonoids. Different secretion profiles were obtained for the short capitates, for the exclusive production of polysaccharides, and for the medium capitates, for the exclusive secretion of terpenes. Although the peltates are considered typical EO producers in the Lamiaceae (Hallahan 2000; Werker 2000), they may display a secretion having a much more complex chemical composition, as it was documented in *S. galericulata* (Giuliani and Maleci Bini 2008) and in other members of the family. Therefore, our results highlighted that the secretion of terpenes was due to three different trichomes morphotypes: peltates, medium- and long-stalked capitates. However, based on the different distribution pattern, it is possible to hypothesize that this productivity is exclusively due to the peltates on the leaves, while all the trichomes types contribute to the production of these substances on the flowers. Based on the different trichome abundances and on the presence of a broader subcuticular space in the peltates compared to the capitates involved in the same secretion type, it is possible to conclude that peltates represent the main terpenes producers.

Concerning the phytochemical investigation, the analysis of the VOC emission represents an element of novelty. The main chemical class was represented by sesquiterpene hydrocarbons in both profiles, (66.27% leaves; 88.65% flowers), followed by non-terpene derivatives (21.40% leaves; 4.85% flowers), while the oxygenated sesquiterpenes were totally absent in the leaves, similarly to the apocarotenoids in the flowers. Overall, 16 common compounds were identified, dominated by β-caryophyllene (*28*), accounting for different percentages: its relative abundance

was almost double in the leaves (44.05%) than in the flowers (26.56%). Similarly, decanal (*13*) was very abundant in the leaves (10.37%) compared to the flowers (0.2%), while α-humulene (*35*) (2.06% leaves; 1.94% flowers) and γ-muurolene (*38*) (4.33% leaves; 5.72% flowers) occurred in similar percentages. However, a high level of variability emerged between the leaf and the flower profiles. Indeed, the floral profile was more complex than the leaves one, because of the presence of a higher number of compounds, 40 and 27 respectively. Moreover, the floral profile was characterized by 24 exclusive compounds, dominated by β-curcumene (*45*; 23.43%), while the leaf profile presented 11 exclusive compounds, among which germacrene D dominated (*39*; 3.96%). As regard to the ecological role of the common compounds, previous studies documented that β-caryophyllene (*28*) is involved both in the attraction strategies towards pollinators (Zhang 2018), sometimes in association with α-humulene (*35*) (Abraham et al. 2018), as well as in defence mechanisms against pests, parasites and herbivores (Jürgens et al. 2003; Degenhardt et al. 2009; Dória et al. 2010; Dunkić et al. 2011; Curtois et al. 2012; Smith et al. 2012; Köllner et al. 2013; Feng et al. 2017). This last activity is typical of the sesquiterpene hydrocarbons, especially β-bourbonene (*20*) (Birkett et al. 2008), whose biosynthesis may be induced by herbivory (Köllner et al. 2013). Specific studies for the other above-mentioned common compounds are lacking.

Concerning the ecological role of the flower exclusive compounds, β-curcumene (*45*; 23.43%) and α-cedrene (*26*; 8.71%) were very abundant:an attracting function towards pollinators is ascribed to them as part of volatile floral phytocomplex (Paulo et al. 2001). All the leaf exclusive compounds were present in relative amounts close to 1%, except for germacrene D (*39*), to which a defensive role is ascribed (Birkett et al. 2008). *(E)*-Geranyl acetone (*34*), α-farnesene (43) and n-tetradecane (*24*) are involved in different VOC-mediated tritrophic interactions (Pinto-Zevallos et al. 2018; Morawo et al. 2016), while linalool (*7*) shows both attractive and repulsive actions (Stevenson 2019). On these bases, the protective role should mostly be performed by leaves, also thanks to the higher amount of the common compound with this specific function in comparison to their abundance at flower level. On the contrary, the attractive function is primarily expressed by the flower exclusive *bouquet*. Nevertheless, because of the presence of double-action compounds in both profiles, especially β-caryophyllene, a synergy leaf-flower emerges in relation to a protective action.

This work represents the first contribution about the EO characterization of the *S. brevibracteata* subsp. *subvelutina* from Italy. The obtained oil yield was very low (0.06%), as documented for other congeneric species (Rosselli et al. 2007; Formisano et al. 2013). From a quantitative point of view, the Italian subspecies EO showed a lower number of total compounds, 23, compared to the same subspecies from Turkey, 44 (Yilmaz et al. 2019). Nevertheless, in both cases, sesquiterpene hydrocarbons were the main compound class and the most abundant compound was β-caryophyllene, as resulted in the Turkish subspecies (Yilmaz et al. 2019). Concerning the other compounds, several differences emerged: in the plants from Turkey, linalool (12.4%) and hexadecanoic acid (10.8%) occurred in considerable amounts, whereas, for the Italian subspecies, only linalool (7.8%) was present, associated with hexahydrofarnesylacetone (9.96%), accounting for 2.3% in the Turkish sample. In their work referred to the species *S. brevibracteata*, Formisano et al. (2013) reported that the EO of samples from Lebanon was dominated by β-caryophyllene (14.4%), followed by hexadecanoic acid (12.6%), phytol (10.7%) and 4-vinylguaiacol (10.2%). In addition, germacrene D was one of the main compounds in most of the EO of congeneric species (Cicek et al. 2010; Cicek et al. 2011) whereas, in our samples, this molecule is present in lower amount (3.88%). However, the comparison with literature data resulted problematic due to the different geographical origin of the samples and to the fact that the subspecies taxonomic level is not indicated by Formisano et al. (2013).

Concerning the EO biological activity, antifeedant properties towards *Spodoptera littoralis*, a serious crop pest, was reported (Formisano et al. 2013; Ribeiro et al. 2015;), as well as an action in the modulation of the insect feeding behaviour (Formisano et al. 2013).

This work represents a further step in the characterization of the species collected at the G. E. Ghirardi Botanic Garden (Toscolano Maderno, Lombardy, Italy), which is totally dedicated to medicinal plants. This multidisciplinary approach will help to present scientific insight on the species to the visitors and convey a new awareness of the plant organism.

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Conflicts of interest
The authors declare no conflicts of interest.

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Table 1. HS-SPME profiles of the leaves and flowers of *Scutellaria brevibracteata* subsp. *subvelutina*

	I.r.i.	Compounds	Relative Abundance (%)	
			Leaves	Flowers
1	941	α-pinene	-	tr
2	982	β-pinene	2,46	0.33
3	1009	*(Z)*-3-hexenol acetate	-	0.21
4	1032	limonene	tr	0.12
5	1034	1,8-cineole (=eucalyptol)	3.82	1.03
6	1062	γ-terpinene	-	0.12
7	1101	linalool	tr	-
8	1102	nonanal	4.04	0.11
9	1143	camphor	1.00	0.27
10	1172	1-nonanol	0.77	-
11	1189	α-terpineol	-	tr
12	1192	methyl salicylate	tr	0.17
13	1204	decanal	10.37	0.20
14	1227	methyl nonanoate	-	tr
15	1306	undecanal	1.24	-
16	1328	methyl decanoate	-	3.94
17	1345	7-*epi*-silphiperfol-5-ene	-	0.46
18	1351	α-cubebene	1.33	1.01
19	1376	α-copaene	1.76	0.49
20	1384	β-bourbonene	5.01	0.16
21	1388	α-duprezianene	-	1.89
22	1390	β-cubebene	0.44	1.56
23	1398	1,7-di-*epi*-α-cedrene	-	0.28
24	1399	*n*-tetradecane	1.02	-
25	1402	italicene	-	0.72
26	1409	α-cedrene	-	8.71
27	1418	β-cedrene	-	tr
28	1420	β-caryophyllene	44.05	26.56
29	1429	β-copaene	0.73	0.58
30	1430	methyl undecanoate	-	0.22
31	1432	β-gurjunene	1.16	-
32	1441	aromadendrene	tr	tr
33	1447	*cis*-muurola-3,5-diene	-	0.23
34	1453	*(E)*-geranyl acetone	1.30	-
35	1456	α-humulene	2.06	1.94
36	1460	*(E)*-β-farnesene	-	1.29
37	1463	α-acoradiene	-	0.79
38	1477	γ-muurolene	4.33	5.72
39	1482	germacrene D	3.96	-
40	1483	*ar*-curcumene	-	2.77
41	1495	α-zingiberene	-	3.64
42	1500	*n*-pentadecane	1.22	-
43	1507	*(E,E)*-α-farnesene	1.44	-
44	1509	β-bisabolene	-	6.28
45	1512	β-curcumene	-	23.43
46	1524	δ-cadinene	-	0.14
47	1549	elemol	-	1.92
48	1581	caryophyllene oxide	-	0.11
49	1600	*n*-hexadecane	1.95	-
50	1603	5,7-di-*epi*-α-eudesmol	-	0.75
51	1700	*n*-heptadecane	0.79	-
		Monoterpene hydrocarbons	**2.46**	**0.57**
		Oxygenated monoterpenes	**4.82**	**1.30**
		Sesquiterpene hydrocarbons	**66.27**	**88.65**
		Oxygenated sesquiterpenes	**-**	**2.78**
		Apocarotenoids	**1.30**	**-**
		Non-terpene derivatives	**21.4**	**4.85**
		Total	**96.25%**	**98.15%**

Table 2. Composition of the essential oil obtained from the aerial parts of *Scutellaria brevibracteata* subsp. *subvelutina*

	I.r.i.	**Compounds**	**Relative Abundance %**
1	998	octanal	0.38
2	1011	δ-2-carene	0.09
3	1026	limonene	0.17
4	1031	*(E)*- β-ocimene	0.13
5	1043	*trans*-α-ocimene	0.28
6	1055	γ-terpinene	0.16
7	1060	*(E)*-2-octenal	0.40
8	1094	linalool	7.80
9	1098	nonanal	5.57
10	1193	terpineol	1.29
11	1261	*(Z)*-2-decenal	1.15
12	1339	β-longipinene	0.84
13	1369	α-copaene	0.68
14	1377	β-bourbonene	1.38
15	1415	β-caryophillene	42.60
16	1451	α-humulene	5.20
17	1477	germacrene D	3.88
18	1581	caryophyllene oxide	4.82
19	1622	humulene oxide II	1.58
20	1635	*trans*-α-bisabolene epoxide	3.42
21	1659	β-acaredienol	1.23
22	1686	α-bisabolol	3.54
23	1834	hexahydrofarnesyl acetone	9.96
		Monoterpene hydrocarbons	**0.83**
		Oxygenated monoterpenes	**9.09**
		Sesquiterpene hydrocarbons	**54.59**
		Oxygenated sesquiterpenes	**14.60**
		Apocarotenoids	**9.96**
		Non-terpene derivatives	**7.49**
		Total	**96.55**

Scutellaria brevibracteata subsp. *subvelutina* (Rech.f.) Greuter & Burdet: morphological and phytochemical characterization.

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1. Materials and methods

1.1 Plant material

Scutellaria brevibracteata subsp. *subvelutina* is cultivated at the Ghirardi Botanic Garden (Toscolano Maderno, BS, Italy) of the Department of Pharmaceutical Sciences of the University of Milan. The sampling for the micro-morphological analysis was carried out on plants in full-bloom in summer 2019. Collection of plant material for the phytochemical investigation (VOCs and essential oil) was performed simultaneously.

1.2 Micromorphological analysis

The *indumentum* was examined on both the vegetative and the reproductive organs (leaves, calyces and corollas) by means of light microscopy, as well as scanning electron microscopy. At least ten replicates of each plant part were collected and examined to assess the micromorphological variability of the *indumentum*.

1.2.1 Light microscopy (LM)

Histochemical investigation was performed to evidence the main chemical classes of metabolites in the trichome secretory products. For each replicate of all the fresh plant parts, hand-made sections (40-50 µm thick) and semi-thin sections (20-25 µm thick) obtained by means of a cryostat, were stained with the following dyes: 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 for acid polysaccharides (Jensen 1962), Alcian Blue for muco-polysaccharides (Beccari and Mazzi 1966), Ferric Trichloride for polyphenols (Gahan 1984) and alluminium trichloride for flavonoids (Guerin et al. 1971). Control procedures were also performed for all the histochemical dyes. Primary fluorescence of the trichome secretory products were also evaluated under UV and Blue lights. Observations were performed under a Leitz DM-RB Fluo Optic microscope equipped with a digital camera Nikon DS-L1.

1.2.2 Scanning electron microscopy (SEM)

For each of the investigated plant parts, small segments were fixed in FAA (formaldehyde: glacial acetic acid: 70% ethanol 5:5:90 by volume) at 4°C for 7 days. Therefore, the samples were dehydrated in ascending ethanol series up to absolute with 20-min intervals, transferred to acetone, critical point-dried in a Balzer Union CPD 020 apparatus and sputter coated with gold. Observations were made under a Philips scanning electron microscope at an accelerating voltage of 20 Kv.

1.3 Phytochemical investigation

1.3.1 VOCs

Three leaves and three flowers were cut and immediately inserted into separate glass vials of suitable volume for the sampling.

HS-SPME Sample analysis – Supelco SPME (Solid Phase Micro-Extraction) devices coated with polydimethylsiloxane (PDMS, 100 µm) were used to sampling the headspace. SPME sampling was performed using the same new fibre, preconditioned according to the manufacturer instructions, for all the analysis. Sampling was accomplished in an air-conditioned room (22 ± 1°C) to guarantee a stable temperature. The sampling temperature was chosen to avoid the thermal damage of the superficial glandular hairs of the leaves and, thus, any artificial-induced volatiles release. After 30 min of equilibration time, the fibre was exposed to the headspace for 30 min. The equilibration time was chosen as the ideal one after several trials at different intervals. The sampling time was experimentally determined to obtain an optimal adsorption of the volatiles, to avoid both under- and over-saturation of the fibre and of the mass spectrometer ion trap. Once sampling was finished, the fibre was withdrawn into the needle and transferred to the injection port of the GC-MS system. All the SPME sampling and desorption conditions were identical for all the samples. Furthermore, blanks were performed before each first SPME extraction and randomly repeated during each series. Quantitative comparisons of relative peaks areas were performed between the same chemicals in the different samples.

GC/MS Analysis – The analyses of the headspace compositions were performed at the Department of Pharmacy of the University of Pisa. The GC/EI-MS analyses were performed with a Varian CP-3800 apparatus equipped with a DB-5 capillary column (30 m X 0.25 mm i.d., film thickness 0.25 µm) and a Varian Saturn 2000 ion-trap mass detector. The oven temperature was programmed rising from 60° C to 240° C at 3° C/min; injector temperature, 220°C; transfer-line temperature, 240° C; carrier gas, He (1 mL/min). The acquisition parameters were as follows: full scan; scan range: 35-300 m/z; scan time: 1.0 sec; threshold: 1 count.
The identification of the constituents was based on the comparison of their retention times (t_R) with those of pure reference samples and their linear retention indices (LRIs) determined relatively to the t_R of a series of *n*-alkanes. The mass spectra were compared with those listed in the commercial libraries NIST 14 and ADAMS and in a home-made mass-spectral library, built up from pure substances and commercial essential oils of known composition, and MS literature data (Adams, 1995).

1.3.2 EO

Plant aerial parts at blooming were dried at room temperature in the dark and stored under the same conditions until the hydrodistillation process. The EO hydrodistillation was performed in a standard Clevenger apparatus for 2 h.

OE - EO was obtained by hydrodistillation with a Clevenger apparatus.

GC/MS Analysis – GC-MS analyses were performed at the Department of Chemistry, University of Milan, using a TRACE ISQ QD Single Quadrupole GC-MS.
EO separation was performed by a capillary column VF-5ms (5% phenyl-methyl-polisiloxane, length 30 m, 0,25 mm i.d., 0.1 µm film thickness); the temperature gradient was: 8 min at 50 °C, then 4 °C/min till 60 °C, then 6 °C/min from 60 °C to 160 °C and finally 20 °C/min from 160 °C to 280 °C. Injector and detector temperatures were set to 280°C; carrier gas He, flux 1 ml/min: the mass range detected was 50-500 m/z. EO were analyzed pure or diluted 1:100 with n-hexane, with injection volume of 1 µl.
Mass spectra were analyzed by Wiley Mass spectra Library, NIST Mass Spectral Search Program e NIST Tandem Mass Spectral library 2.3; compounds were identified by mass fragmentation and retention index, compared with data stored in mass databases (WILEY, NIST18).

Tables

Table S1. Distribution pattern of the glandular trichomes.

Trichome type	Leaf abaxial surface	Leaf adaxial surface	Calyx abaxial surface	Calyx adaxial surface	Corolla abaxial surface	Corolla adaxial surface
Peltate	++	-	-	++	-	++
Short-capitate	++	++	-	+	-	+
Medium-capitate	+	-	-	+	-	+
Long-capitate	-	-	-	++	-	-

(−) absent; (+) present; (++) abundant

Table S2. Results of the histochemical tests on the glandular trichomes.

Stainings	Target-compounds	peltate	short capitate	medium capitate	long capitate
Fluoral yellow-088	Total lipids	++	−	++	++
Nile Red	Neutral lipids	+	−	+	+
Nadi reagent	Terpenoids	++	−	++	++
Ruthenium Red	Acid polysaccharides	−	+	−	−
Alcian Blue	Mucopolysaccharides	−	+	−	−
Ferric Tricholoride	Polyphenols	+	−	−	+
Aluminium Trichloride	Flavonoids	+	−	−	+

(−) negative; (+) positive; (++) strongly positive

Figure S1 a-f. Glandular trichome morphotypes in *S. brevibracteata* subsp. *subvelutina*. LM. A. Peltate. B. short-stalked capitate. C. Medium-stalked capitate. D. Long-stalked capitate.

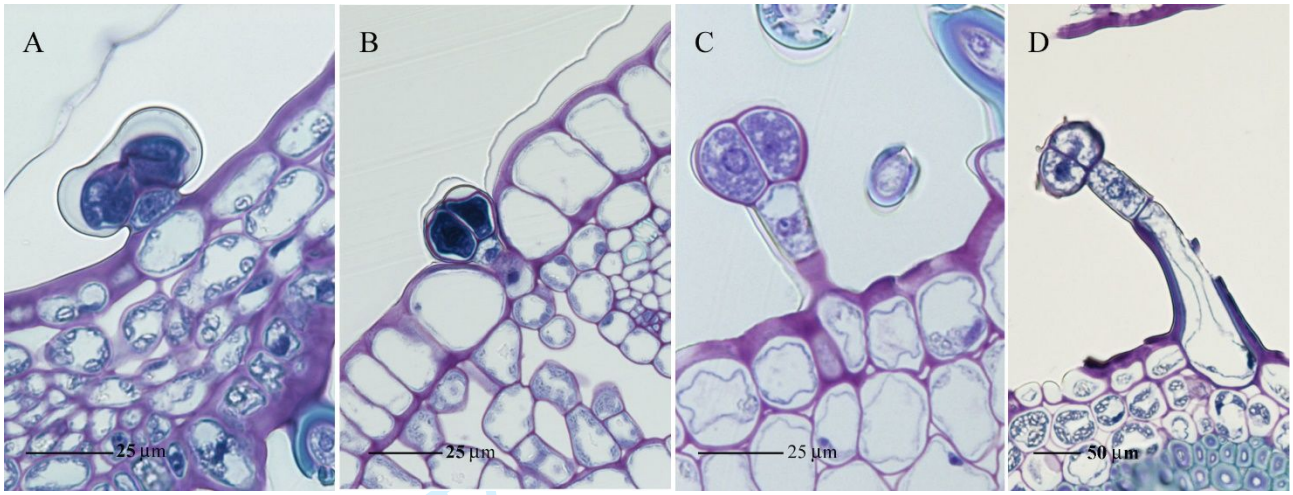

Figure S2. Trichomes distribution pattern in *S. brevibracteata* subsp. *subvelutina*. SEM. A. Leaf adaxial surface with peltates, short and medium capitates and non-glandular trichomes. B. Leaf abaxial surface with short and medium capitate and non-glandular hairs. C. General view of a flower with evident abundant non-glandular hairs and long capitates. D. Calyx abaxial surface below the skullcup with long capitates. E. Calyx abaxial side above the skullcup with medium and long capitates. F. Corolla abaxial surface with peltates and short capitates. G, H. Long capitate trichomes; note the particular of the secreting head.

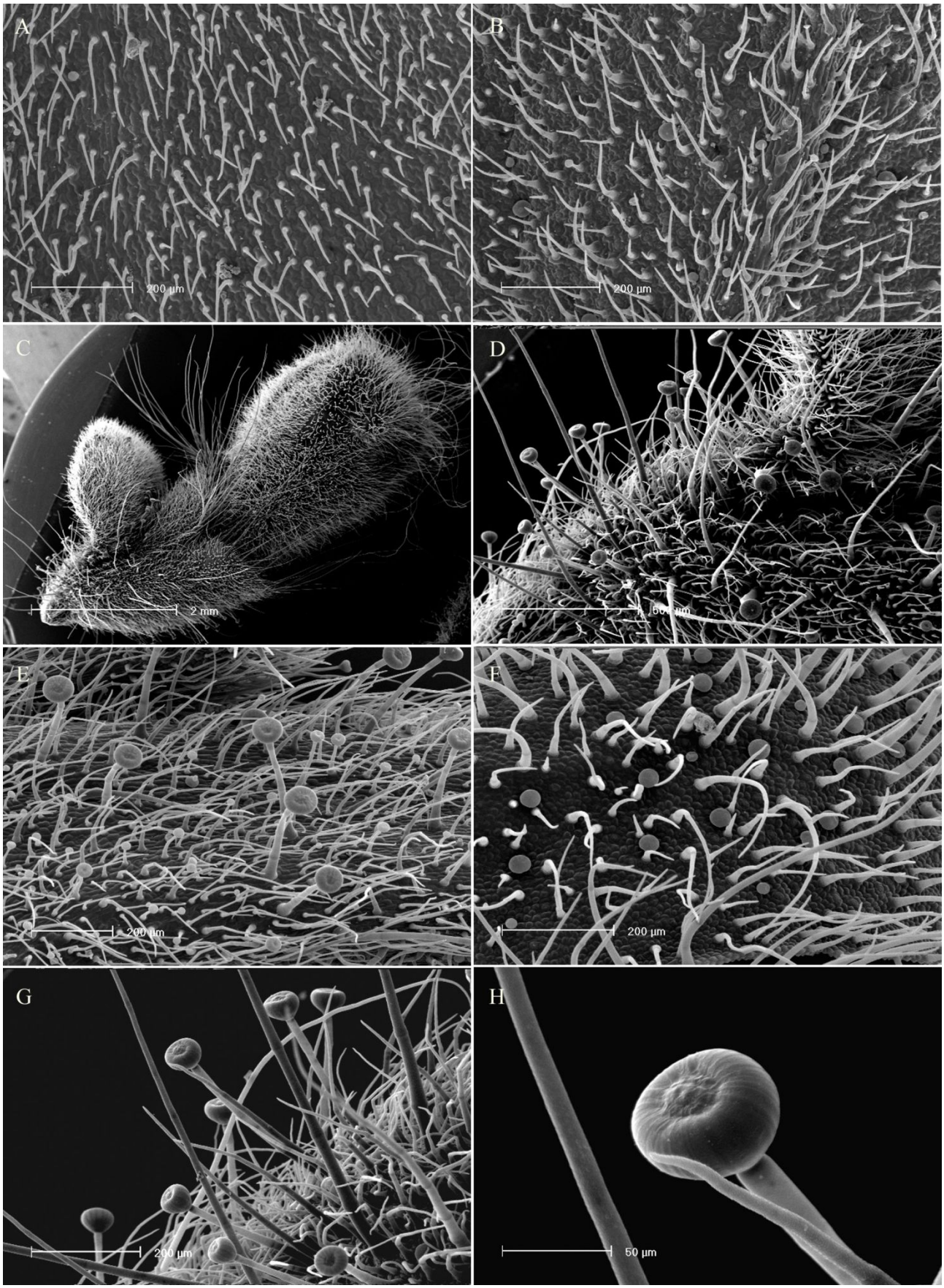
A
200 µm
B
200 µm
C
2 mm
D
E
200 µm
F
200 µm
G
200 µm
H
50 µm

Figure S3. Histochemistry of the glandular trichomes *S. brevibracteata* subsp. *subvelutina*, LM. A-C. Peltate trichome: Nadi reagent (A); FeCl3 (B); AlCl3 (C). D. Short capitate trichome: Alcian Blue. E. Medium capitate trichome: Nadi reagent. F-H. Long capitate trichome: Fluoral Yellow-088 (F); Nadi reagent (G); FeCl3 (H).