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Teucrium fruticans L., a Multi-Scale Study: From Trichomes to Essential Oil

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This work represents the first multi-scale study on *Teucrium fruticans* L. cultivated at the Ghirardi Botanic Garden (Lombardy, Northern Italy), combining a micromorphological and a phytochemical survey on the plant's aerial parts. Micromorphological investigations, performed by Light Microscopy, Fluorescence Microscopy and Scanning Electron Microscopy, highlighted the presence of five trichomes morphotypes, distinguished by a different distribution pattern: peltates, short-stalked and ball-like medium-stalked capitates, ubiquitous on the whole plant, medium-stalked and long-stalked capitates, exclusive to the floral whorls. Both peltates and medium-stalked capitates were recognized as the main terpene production sites. Phytochemical characterization focused on the essential oils (EOs), obtained by Clevenger-type hydrodistillation in February and April 2022 and characterized by Gas Chromatography-Mass Spectrometry (GC/MS), which resulted mainly formed by sesquiterpene hydrocarbons. The February EO profile was characterized by β-caryophyllene (28.30%) and germacrene D (19.16%) as main compounds, while in April β-myrcene was detected at high percentage (13.77%), in addition to the previous two components (15.72% and 11.55%, respectively). Literature data, dealing with the biological activities of the main oil constituents, highlighted an anti-microbial, anti-inflammatory, and anti-tumor potential, due to the high content in sesquiterpenes and, particularly, of β-caryophyllene and germacrene D.

Keywords: micromorphology, histochemistry, phytochemistry, hydrodistillation, sesquiterpenes.

Introduction

Teucrium fruticans L. (Lamiaceae, sect. *Teucrium* Benth.)[1] is a perennial shrub mostly widespread in the Mediterranean region, with a western distribution and habitat preferences in rocky slopes, escarpments, fields and coastline calcareous rocks.[2,3] The plant shows erect ascending and quadrangular stems, which can reach up to 120 cm high; lanceolate-ovate leaves, with entire margins, grey-tomentose in the lower lamina, hairless and dark green in the upper side, with opposite insertion on the stem; blue-violet flowers assembled in terminal clusters in summer.[4]

Teucrium derives from the mythological Teucer, a son of the king of Salamis, who was the first to take advantages of the medicinal properties of these plants.[1] In Italy, the traditional uses of *T. fruticans* still

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survive. In Tuscany, depurative and diuretic effects are ascribed to the leaf infusions,[3,5] while in Sicily, where the plant is commonly known as 'erba ricottara', the direct application of the leaves is a useful remedy in the case of haemorrhoids.[2]

Micromorphological investigations were developed for several members of the genus *Teucrium*[6–10] mainly from the Mediterranean region,[6,8–11,13,14] Iran,[12] and South Africa,[7] but no studies about the secreting structures of *T. fruticans* were reported. Previous Italian, Turkish, and Iranian works[11,12,14] highlighted the importance of the *indumentum* features on different plant parts as distinctive taxonomic characters at section level or for species classification.[11,12] Other morphological surveys were focused on the link of trichome morphotypes, distribution pattern, and mechanism of secretion release with phytochemical data: *T. chamaedrys* from Italy,[6] *T. polium* from Greece,[8] *T. capitatum* and *T. salviastrum* from Portugal,[9,10] and three endemic South African species[7] in which this type of investigation was linked to the local ethnopharmacognostic importance of the *taxa*.[7] Concerning the histochemical information, no contributions on *T. fruticans* were found, whereas only two works reported evidence about the chemical nature of the secretory products of different trichomes types of the Italian *T. chamaedrys*.[6,13] In addition, two works reported a generic evaluation of the lipophilic secretion of the Portuguese *T. capitatum* and *T. salviastrum*.[9,10]

As it pertains to the phytochemical data, a recent review offered an overview of the chemical composition and biological activities of the essential oils (EOs) isolated from several *Teucrium* species from all over the world.[1] Regarding the Italian territory, a survey analysed the chemical composition and the quali-quantitative variability of the essential oil of three Sicilian congeneric species,[15] whereas only two works focused on the EO characterization of *T. fruticans* aerial parts, with samples coming from Tuscany, Sicily, and Malta, respectively.[3,16] Moreover, the total phenol content of the EtOH/H_2O extracts of *T. fruticans* inflorescences from Sicily was investigated, together with its *in vitro* antioxidant and antimicrobial activities against pathogenic bacteria.[5]

The antioxidant, antimicrobial, antitumor, anti-inflammatory, and antinociceptive activities, together with the bacterial antimutagenic potentiality and the inhibition capacity on the acetylcholinesterase enzyme, are reported for the EO of *Teucrium* species,[1] but no particular evidence emerged referring to *T. fruticans* oil derivatives. Finally, many species of the genus have been proved to have antioxidant, antimicrobial, and antifungal activities potentially useful in preservative processes,[5] as well as antiphytoviral, insecticidal, and antiprotozoal activity, with a final view to using EOs as valid and eco-friendly alternatives to traditional chemical insecticides and pesticides.[1]

Considering this, in this work we proposed for the first time a combined survey on the micromorphology and phytochemistry of *T. fruticans* cultivated in Northern Italy (Ghirardi Botanic Garden, University of Milan, Toscolano Maderno, BS, Lombardy – Northern Italy). This study is part of a wider project entitled 'Botanic Garden, factory of molecules…work in progress' (Lombardy Region Call for the Enhancement of Museums Lr. 19/2016, year 2021) and is intended to: 1) investigate the micromorphological features of the vegetative and reproductive organs by means of Light Microscopy, Fluorescence Microscopy, and Scanning Electron Microscopy, with the aim to outline the glandular and non-glandular trichome morphotypes and their distribution pattern; 2) present an in-depth histochemical investigation useful to identify the main lipophilic and hydrophilic compound classes; 3) characterize the chemical composition of the EOs from the plant aerial parts; 4) correlate the EO profile to the potential biological activity ascribed to the dominant compounds by the literature.

Results

Micromorphological Survey

Trichome Morphotypes

The glandular *indumenta* of the vegetative and reproductive organs consisted of peltate and capitate trichomes (*Figure 1a–h*).

The peltate hairs were composed of one basal epidermal cell, one neck cell, and four secretory cells arranged in a circle (*Figure 1a*, *b*, *e*). Mature trichomes were about 40–60 μm in height and 50–60 μm in diameter at the head. The secretory head was surrounded by a broad subcuticular space, giving to each trichome a spherical shape. Cuticular rupture was often observed in SEM micrographs, in the form of a detached cap (*Figure 1b*) or following a horizontal line of apparent fragility in the diametrical region of the head (*Figure 1e*).

The capitate trichomes were composed of one to three stalk cells and a one(two)-celled secretory head (*Figure 1b–i*). As the number of stalk cells and the features of the head were quite variable, capitates can

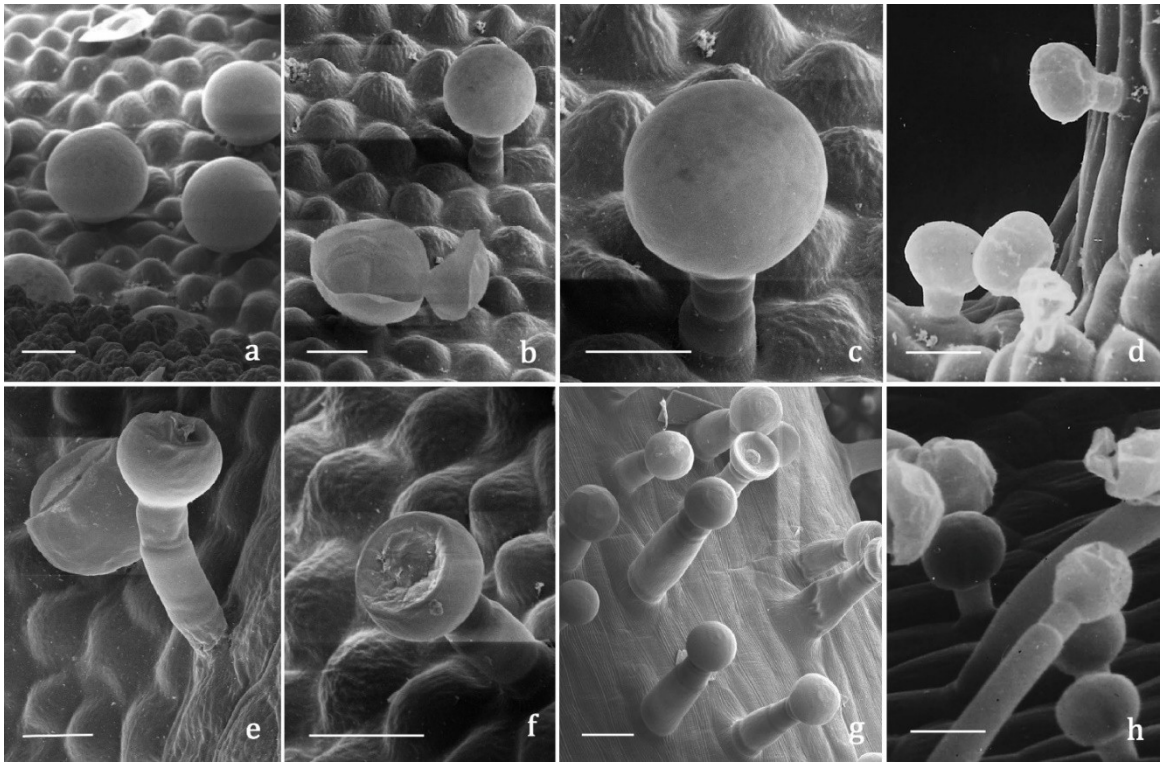

Figure 1. SEM micrographs showing the trichome morphotypes in *Teucrium fruticans* L. (a) peltate trichomes on corolla; (b) peltate and ball-like medium-stalked capitate trichomes on corolla; (c) ball-like medium-stalked capitate trichome on corolla; (d) short-stalked capitate trichomes on leaf; (e) peltate and medium-stalked capitate trichomes on calyx; (f, g) medium-stalked capitate trichome on corolla; (h) short-stalked (asterisk) and long-stalked (arrowhead) capitate trichomes on corolla. Scale bars = 20 μm.

be divided into four subtypes: short-stalked, medium stalked, ball-like medium stalked, and long-stalked. The short capitates consisted of one basal cell, one stalk cell and one(two) head cells (*Figure 1d*, *h*); they were about 20–30 μm in height and 20–30 μm in diameter at the head. The medium capitates possessed one basal cell, one(two)-celled stalk slightly enlarged at the base and a globose one-celled head (50–60 μm high and 20–30 μm wide in diameter at the head). The ball-like medium capitates exhibited features consistent with the stalk, while the head diameter was higher, ranging from 50 to 60 μm. The long capitates were composed of one basal cell, two(three) stalk cells, and one(two) head cells. At maturity, they were about 120–140 μm in height and the head diameter was about 15–20 μm. In the capitates, the secretion accumulated in the small subcuticular space was shown in SEM images by a slight cuticular elevation; on the contrary, the ball-like medium capitate morphotype showed a broad subcuticular space like that of the peltates.

The non-glandular trichomes belonged to two main types, both multicellular and uniseriate. We distinguished thin, filamentous, and sharply pointed hairs, with an average length of 300–400 μm. They were straight or variously twisted, forming a felt-like covering that partially or totally obscured the epidermis of the organs bearing them, so that the observation of the glandular trichomes was not easily allowed. Shorter pointed hairs were also observed, with an acute apex and an average length of 130–160 μm; the cell diameter was progressively smaller moving from the base to the tip. Although erect, the most distal cells were leaning towards the apex of the organs. Some non-glandular trichomes appeared to have a relatively smooth surface; others had cuticular outgrowths towards the basal cells.

Trichome Distribution

The data on trichome distribution on stems, leaves, bracts, calyces, and corollas of *T. fruticans* are reported in *Table 1* and *Figures 2*(*a*–*h*) and *3*(*a*–*k*). The dense covering of the long, thin, filamentous hairs prevented the direct observation of the glandular *indumenta*, however the cutting procedure to obtain the cross

Table 1. Distribution pattern of the glandular and non-glandular trichomes in *Teucrium fruticans* L.

Trichome type	Stem	Leaf		Bract		Calyx		Corolla	
		adax	abax	adax	abax	adax	abax	adax	abax
peltate	±	±	+	±	+	−	++	−	++
short capitate	+	+	+	±	+	−	+	−	±
ball-like medium capitate	−	±	±	±	±	−	−	−	±
medium capitate	−	−	−	−	−	−	−	+	±
long capitate	−	−	−	−	−	−	−	+	−
filamentous simple non-glandular	++	+	++	+	++	+	++	+	+
pointed simple non-glandular	±	±	±	±	±	+	±	++	+

Symbols: (−) missing, (±) sporadic, (+) present, (+ +) abundant.

sections for the histochemical survey allowed us to assess the evaluation of the trichome distribution.

The leaves mainly exhibited peltates and short capitates, with sporadic ball-like medium capitates; the peltates seemed to be mainly on the interveinal regions, while the short capitates occurred along the veins, especially on the adaxial lamina (*Figure 2d*, *f*). The two morphotypes of non-glandular hairs were on both sides: the filamentous hairs formed a dense felt-like covering, whereas the shorter ones were distributed mainly on the veins and margins (*Figure 2c–h*). The stems and bracts displayed features consistent with the leaves concerning the hair morphotypes, however they were characterized by a lower density. On flowers, the five glandular trichome morphotypes displayed a diverse distribution pattern on the different whorls. Calyces and corollas exhibited peltates and short capitates on the abaxial sides, the latter being more abundant on calyces (*Figure 3d*, *f–h*). On the petal abaxial side also occasional ball-like capitates and medium capitates occurred, whereas medium and long capitates were on the adaxial side. Therefore, the long capitates resulted exclusive of the corolla adaxial surface, prevailing at the tube level in comparison to the lower lip. They were also abundant at the proximal portion of the staminal filaments (*Figure 3e*, *i–k*), together with pointed non-glandular hairs.

Glandular Trichome Histochemistry

The results of the histochemical investigation are shown in *Table 2* and *Figure 4*(*a–i*). The peltate trichomes produced exclusively terpenic substances copiously stored within the subcuticular space, which appeared filled with flocculent material (*Figure 4a*, *b*). The short- and long-stalked capitates produced and released exclusively a hydrophilic secretion (*Figure 4 c*, *i*).

The medium capitates massively synthetized phenolic compounds, together with a lower terpenic fraction (*Figure 4e*, *f*); the hydrophilic dyes invariably exhibited negative responses (*Figure 4d*). The ball-like medium capitates showed only faintly positive responses to muco-polysaccharides (staining few secretion droplets within the storing chamber) and terpenes (staining only the cytoplasm of the secretory cells) (*Figure 4g*, *h*).

Phytochemical InvestigationEssential Oils Characterization

The GC/MS characterization of the EOs obtained from February (vegetative stage) and April (anthesis stage) 2022 samplings are reported in *Table 3*. February EO profiles were characterized by the presence of 51 compounds, accounting for 91.88% of the total.

Table 2. Results of the histochemical tests on the glandular trichomes of *Teucrium fruticans* L.

Stainings	Target-compounds	peltate	short capitate	ball-like capitate	medium capitate	long capitate
Fluoral Yellow-088	Total lipids	++	−	±	+	−
Nile Red	Neutral lipids	++	−	−	+	−
Nadi reagent	Terpenoids	++	−	+	+	−
Ruthenium Red	Acid polysaccharides	−	+	−	−	+
Alcian Blue	Muco-polysaccharides	−	+	+	−	+
Ferric Trichloride	Polyphenols	−	−	−	++	−

Symbols: (−) negative response; (±) sporadically positive response; (+) positive response; (+ +) intensely positive response.

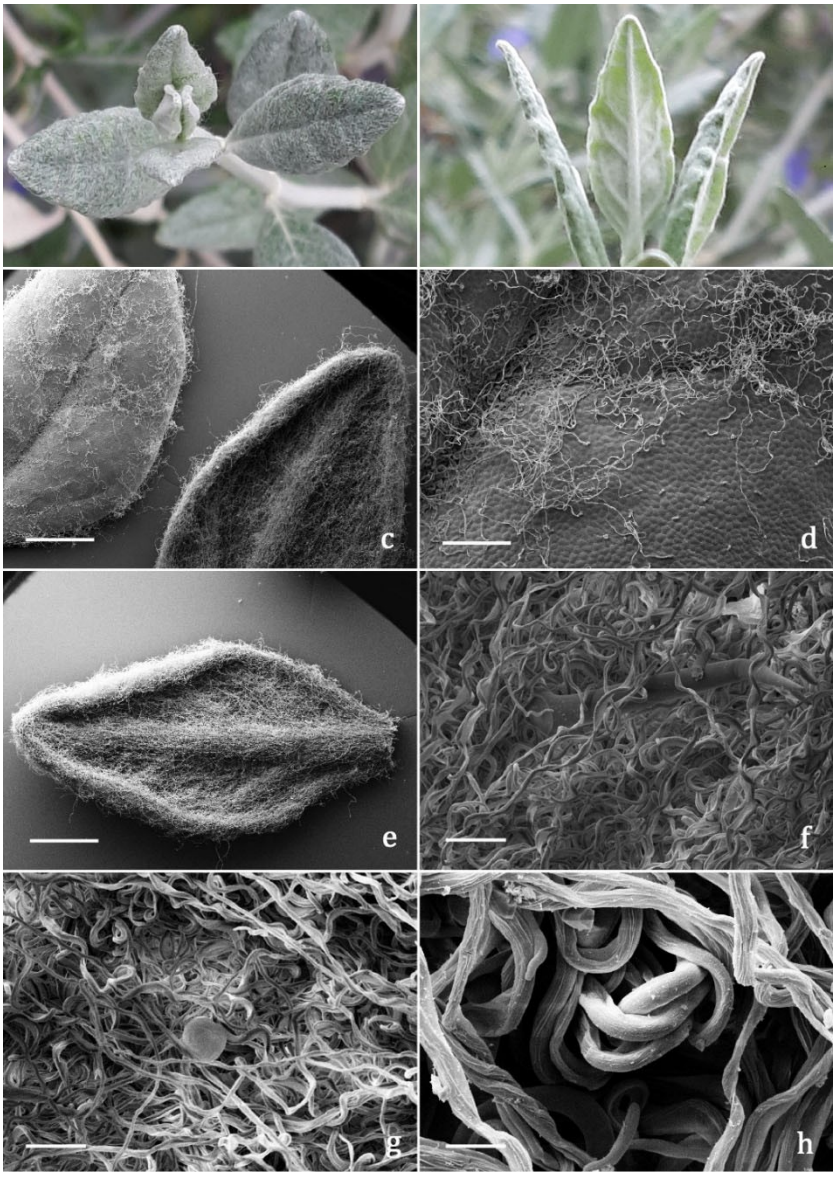

Figure 2. (a, b) Macrographs showing a leaf of *Teucrium fruticans* L. (c–h) SEM micrographs showing trichome distribution pattern on the leaves of *Teucrium fruticans* L. (c) General view of the leaves, adaxial (left) and abaxial (right) sides. (d) Detail of the leaf adaxial side. (e) General view of the leaf abaxial side. (f–g) Details of the leaf abaxial side: interveinal region with a pointed non-glandular hair (f) and a peltate trichome (g), along with abundant filamentous non-glandular trichomes. (h) Detail of the cuticular surface of the filamentous non-glandular trichomes. Scale bars: c, e = 1 mm; d = 200 μm; g = 50 μm; f = 20 μm; h = 10 μm.

Sesquiterpene hydrocarbons dominated the profiles, with 17 derivatives and a relative percentage of 69.30%. Oxygenated sesquiterpenes were the second main represented class of compounds (21 compounds, 11.81%), followed by non-terpene derivatives (5 compounds, 4.13%), fatty acids and their derivatives (4 compounds, 3.99%), monoterpene hydrocarbons (3 compounds, 2.86%), and oxygenated monoterpenes (2 derivatives, 0.65%).

The main components were β-caryophyllene (**14**, 28.30%), germacrene D (**20**, 19.16%), α-humulene (**18**, 7.27%), and β-selinene (**21**, 6.98%); four compounds were found with percentage greater than 2.2%: linalool (**3**, 2.47%:), β-caryophyllene oxide (**33**, 2.30%), α-cadinol (**44**, 2.88%) and hexahydrofarnesylacetone (**49**, 2.88%); all the remaining compounds were detected with values lower than 2.2%.

Concerning April EO profiles, 33 compounds were identified, with a total percentage of 93.19%. Sesqui-

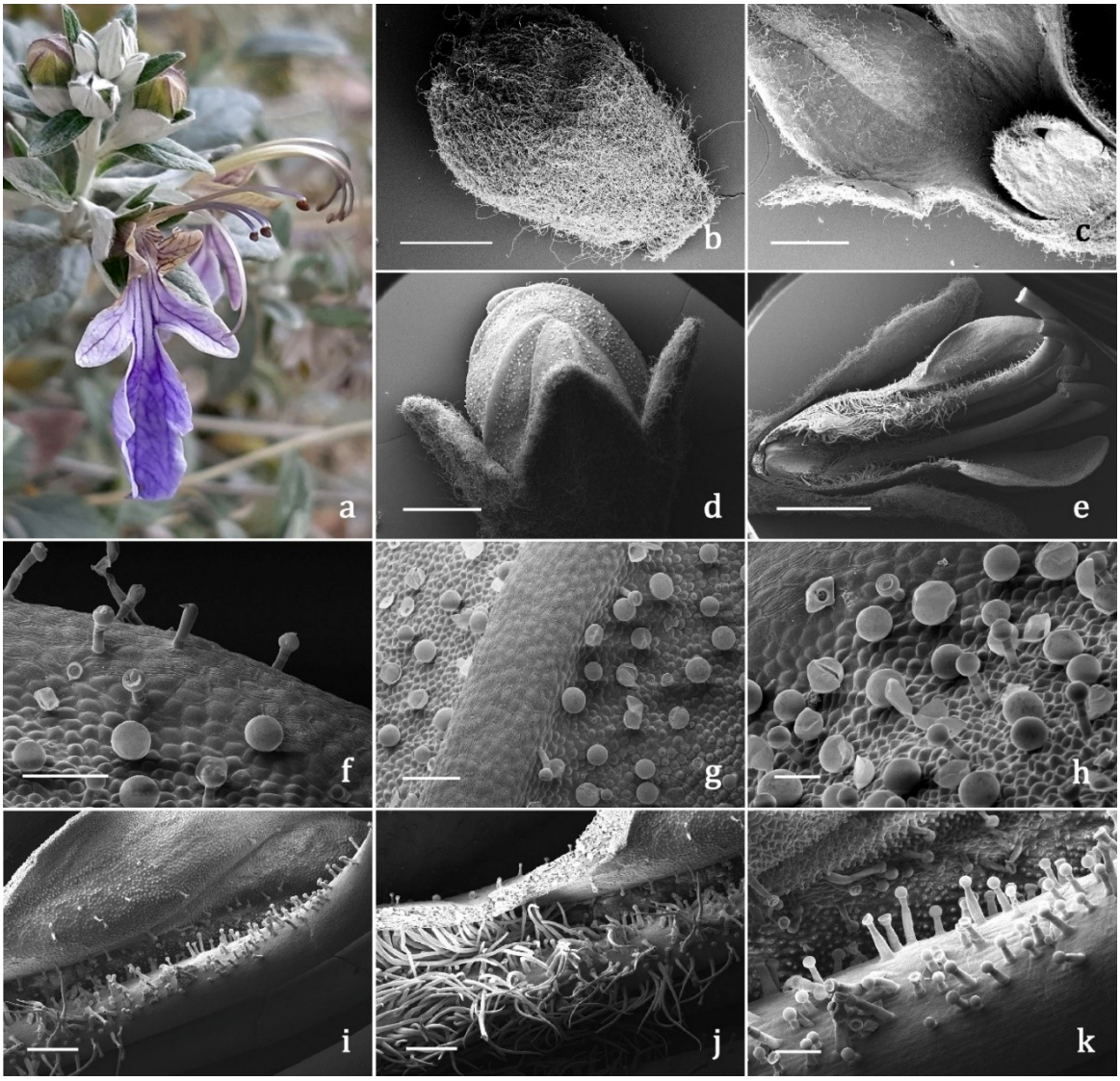

Figure 3. (a) Macrograph showing a flower of *Teucrium fruticans* L. at anthesis. (b–k) SEM micrographs showing trichome distribution pattern on the flower of *Teucrium fruticans* L. (b) Floral bud fully covered by filamentous non-glandular trichomes. (c) Calyx adaxial surface. (d) Distal portion of the calyx and corolla abaxial surfaces. (e) Corolla adaxial surfaces and staminal filaments. (f–h) Corolla abaxial side: margin of the lower lip (f), veinal region (g), interveinal region (h) with peltates, short-stalked, medium-stalked, and ball-like capitates. (i–k) Corolla adaxial surface at the proximal portion and staminal filament with abundant long-stalked capitates and pointed non-glandular trichomes. Scale bars: b–e = 1 mm; i, j = 200 μm; f, g, k = 100 μm; h = 50 μm.

terpenes were still the main class of derivatives, with 17 compounds and a percentage of 53.02%, while the second main class was represented by monoterpene derivatives (3 compounds, 20.65%), due to a high percentage of β-myrcene (**1**, 13.77%), followed by β-pinene (**2**, 3.68%), and linalool (**3**, 3.20%). Oxygenated sesquiterpenes (10 compounds) accounted for 7.94%, while non terpenes (3 compounds) and fatty acid derivatives (1 compound) were found in percentages of 5.30% and 5.82%, respectively.

The main component of April 2022 EO was β-caryophyllene (**14**, 15.72%), followed by β-myrcene (**1**, 13.77), and germacrene D (**20**, 11.55%); α-humulene (**18**, 6.49%), β-selinene (**21**, 6.49%), β-caryophyllene oxide (**33**, 1.82%), and hexahydrofarnesylacetone (**49**, 1.55%) were still among the main derivatives, but were slightly reduced, while α-cadinol (**44**, 2.96%) and linalool (**3**, 3.20%) were slightly increased. Although the number of total identified components in April EO was lower than in February EO, the presence of seven exclusive compounds (**1**, **2**, **11**, **15**, **17**, **19**, **22**) was

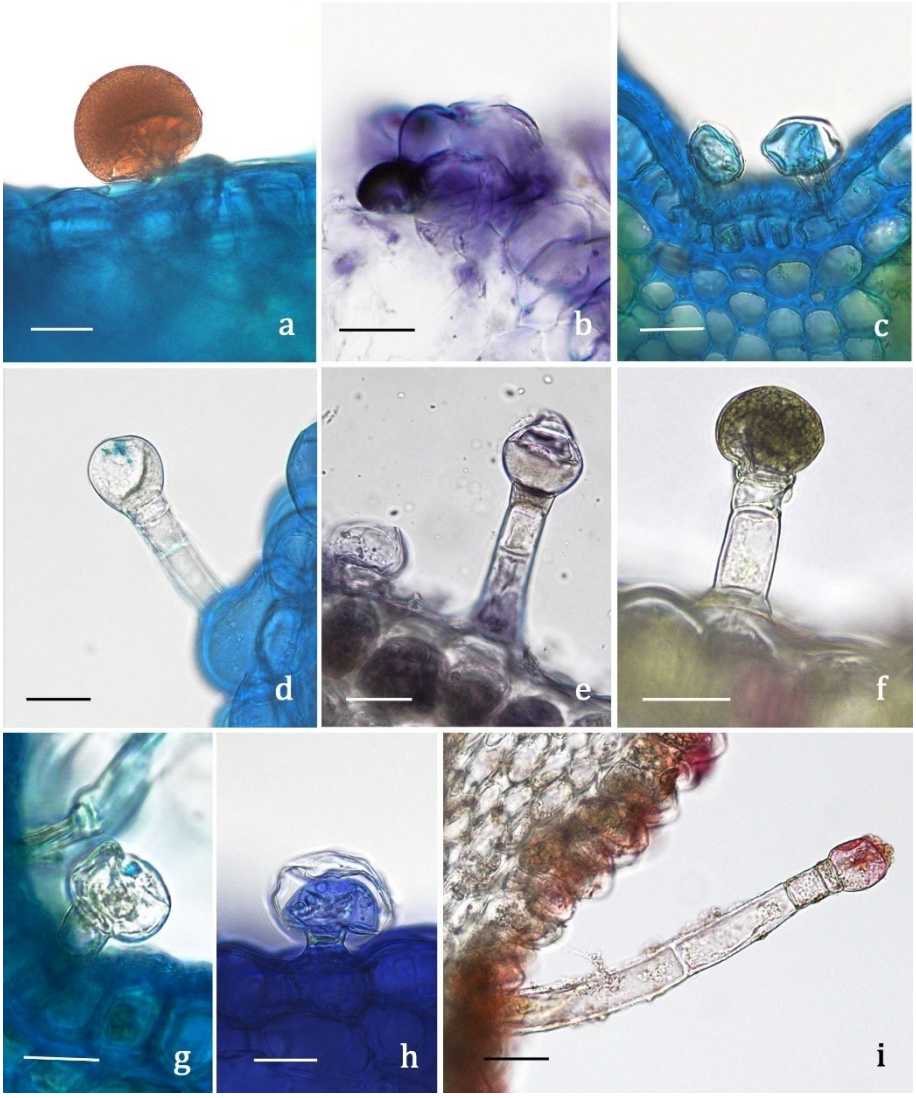

Figure 4. LM micrographs showing the results of the histochemical investigation on the glandular trichomes in *Teucrium fruticans* L. (a–b) peltate: Alcian Blue (a), Nadi reagent (b). (c) Short-stalked capitate: Alcian Blue. (d–f) Medium-stalked capitate: Alcian Blue (d), Nadi reagent (e), Ferric Tricloride (f). (g–h) Ball-like medium-stalked capitate: Alcian Blue (g) and Nadi reagent (h). (i) Long-stalked capitate: Ruthenium Red. Scale bars = 20 μm.

highlighted; among them, β-myrcene (**1**, 13.77%) was the most abundant, followed by β-pinene (**2**, 3.68%).

Therefore, the variability between the two profiles was primarily linked to quantitative data, with the exception of the occurrence of β-myrcene (**1**) and β-pinene (**2**) at the flowering stage.

Discussion

Micromorphological Survey

Previous research into micromorphological features of *T. fruticans* mentioned nutlet morphology, pollen, and vegetative anatomy, with only general references to trichomes. The target species was studied in association with congeneric species from the same geographical areas or in relation to habitat-related adaptation.[17] Altogether the above-mentioned micromorphological traits proved to have taxonomic value for sectional and interspecific classification in the genus.[18–20] However, the *indumentum* features of the target species were never thoroughly investigated, and this work represents the first in-depth report about its secretory structures.

Our micromorphological survey on the vegetative and reproductive organs evidenced the presence of

Table 3. Chemical composition of EOs obtained from *Teucrium fruticans* L. in February (vegetative stage) and April (anthesis stage) 2022.

		Constituents	LRI[a]	Feb22[b] %	Apr22[b] %
1	MH	β-myrcene	964		13.77
2	MH	β-pinene	1002		3.68
3	MH	**linalool**	1078	**2.47**	**3.20**
4	MH	terpinolene	1299	tr	
5	SH	α-terpinyl acetate	1311	0.13	0.16
6	MO	terpineol	1314	0.23	0.45
7	SH	α-Copaene	1338	0.12	0.35
8	SH	β-bourbonene	1346	0.26	0.72
9	SH	β-*cis*-caryophyllene	1360	tr	tr
10	SH	Tetrahydrogeranylacetone	1366	tr	tr
11	NH	Dihydrodeidro-b-ionone	1372		0.89
12	MO	Methyl eugenol	1375	0.42	tr
13	MO	Isospathulenol	1376		tr
14	SH	**β-caryophyllene**	1380	**28.30**	**15.72**
15	SH	β-Copaene	1386		1.06
16	SH	β-cubenene	1388	0.56	
17	SH	Isogermacrene D	1403		0.76
18	SH	**α-humulene**	1419	**7.27**	**6.49**
19	SH	epi-β-Caryophyllene	1422		1.56
20	SH	**Germacrene D**	1442	**19.16**	**11.55**
21	SH	**β-selinene**	1449	**6.98**	**6.49**
22	SH	γ-gurjunene	1454		2.05
23	SH	α-selinene	1455	1.79	
24	SH	β-bisabolene	1461	1.21	1.74
25	SH	δ-cadinene	1471	0.78	1.36
26	SH	β-sesquiphellandrene	1475	1.08	1.49
27	SH	α-ylangene	1484	tr	
28	SO	α-copaen-11-ol	1511	tr	
29	SO	Caryophyllene peroxide I	1516	tr	
30	SO	Nerolidol	1524	tr	0.31
31	SO	Farnesol	1527	0.36	
32	SO	Spathulenol	1541	0.27	0.11
33	SO	**β-caryophyllene oxide**	1545	**2.30**	**1.82**
34	SH	β-elemene	1549	tr	
35	SO	Epiglobulol	1557	0.19	0.16
36	SO	Viridiflorol	1565	0.21	0.20
37	SO	Humulene epoxide II	1569	0.37	0.52
38	SO	Ledene oxide (II)	1575	0.11	0.10
39	SO	1,10-diepicubenol	1585	0.21	
40	SO	Isoaromadendrene oxide	1605	tr	
41	SO	Cubenol	1612	0.22	0.20
42	SO	T-muurolol	1615	0.30	
43	SO	δ-cadinol	1618	0.15	
44	SO	**α-cadinol**	1627	**2.88**	**2.96**
45	SO	Aromadendrene oxide II	1631	0.10	
46	SO	Valeranone	1645	0.59	
47	SO	Bisabolene epoxide	1658	0.22	
48	SO	(1R,7S)-Germacra-4(15),5,10(14)-trien-1β-ol	1663	0.17	
49	SO	**Hexahydrofarnesyl acetone** (**6,10,14-trimethyl-2-pentadecanone**)	1802	**2.88**	**1.55**
50	NH	Dodecanoic acid derivative	1829	0.93	
51	FA	Palmitic acid	1913	1.11	5.82
52	MH	Geranyl-p-cymene	1977	0.31	
53	FA	Myristic acid derivative	1981	0.57	
54	FA	γ-palmitolactone	2006	0.33	
55	SH	Zizaene (Khusimene)	2079	1.36	1.53

Table 3. (cont.)

		Constituents	LRI[a]	Feb22[b] %	Apr22[b] %
56	FA	Palmitic acid derivative	2104	1.97	
57	NH	Eicosane	2299	0.55	2.33
58	NH	Heneicosane	2371	0.97	2.07
59	NH	Octacosane	2435	0.82	
		Monoterpene hydrocarbons		**2.86**	**20.65**
		Oxygenated monoterpenes		**0.65**	**0.45**
		Sesquiterpene hydrocarbons		**69.30**	**53.02**
		Oxygenated sesquiterpenes		**11.81**	**7.94**
		Non terpenes		**3.27**	**5.30**
		Fatty acids and derivatives		**3.99**	**5.82**
		Identified compounds		**91.88**	**93.19**

The main common compounds are highlighted in bold characters. [a]LRI=Linear Retention Index, experimentally obtained on a VF-5 ms column using a C6–C36 mixture of n-alkanes. [b]Data reported were obtained as average of three replicates.

glandular and non-glandular *indumenta*. The glandular trichomes belonged to the peltate and capitate types, widespread in all the representatives of the Lamiaceae family.[21–25]

The structure of peltates matched the results of previous works performed on congeneric species. As a matter of fact, they exhibited a tetracellular secreting head.[6,14,18] With regards to the capitates, the short- and long-stalked subtypes were consistent with literature information,[6] matching the morphotypes already described: various subforms of types B capitates in Navarro and El Qualidi,[18] type B, and C capitates in Bini Maleci and Servettaz.[14]

The occurrence of the medium-stalked and ball-like morphotypes was never observed in the *Teucrium* species examined so far. However, it should be noted that some authors did not report a detailed partition of capitates, whereas others recognized various subforms adopting discordant terminologies. Therefore, performing an accurate comparison with all the previously described morphotypes resulted difficult to do. A redefinition of trichome nomenclature would be highly desirable.

Concerning the non-glandular *indumentum*, the simple, uniseriate hairs were observed in all the species examined so far, generally defined as unbranched[11,12] or aciculate.[14] The filamentous hairs were detected in all the member of *T.* sect. *Teucrium* from Iran and Turkey;[11,12] they matched the type H hairs in Navarro and El Qualidi[18] and the coating flagelliform trichomes in Grubešić et al.[19] They partially or totally covered the epidermis of the vegetative and reproductive organs, making the observation of the glandular hairs tough.

The distribution pattern of the different trichomes types proved consistent with literature information, especially with reference to the peltate and short-stalked capitates, being widespread on the whole plant epidermises of all the investigated congeneric species.[6,11,12,17–20] Concerning the long-stalked morphotype, it was peculiar of the reproductive organs, specifically to the corolla, whereas in *T. chamaedrys* it exclusively occurred on the calyx.[6,14] The medium stalked morphotypes were exclusive of the corolla, while the ball-like capitates resulted sporadic on leaves, bracts, and corollas. However, the existence of discordant terminologies in previous contributions made the comparison with literature information for the two latter types of hairs unreliable.

Concerning the histochemistry, this work represents the first contribution in which different histochemical dyes were employed to characterize the chemical nature of the secretory products in *T. fruticans*. The glandular activity led to a heterogeneous secretion, with lipophilic and hydrophilic components, as documented in our previous study on *T. chamaedrys*.[6]

In the peltates, the secreting activity was fully oriented toward the synthesis and storage of materials of terpenoidic nature, as occurred in several congeneric species[7–10] and in other members of Lamiaceae, such as *Scutellaria altissima*, *S. caucasica*, *Lavandula dentata*, and *Ballota acetabulosa*.[21–24] In *T. chamaedrys*, on the contrary, the peltate secretion resulted to be more complex, with a low terpenoidic content and major hydrophilic fractions, as it was also observed in *Scutellaria brevibracteata*.[25]

The short capitates showed an exclusive hydrophilic secretion, consisting of mucopolysaccharides, as widely documented in most of the studied members of Lamiaceae.[6,26]

The medium and the ball-like capitates displayed heterogeneous secretion, due to the co-occurrence of terpenoids, along with polyphenolic or polysaccharidic fractions, respectively. The long capitates were exclusive mucopolysaccharides producers, whereas they synthetized only terpenes in *T. chamaedrys*.[6] They produced, on the contrary, a complex secretion in *S. brevibracteata*, *S. altissima*, *S. caucasica*, and *B. acetabulosa*.[21,22,24,25]

Previous works on congeneric species of *Teucrium* highlighted the general lipophilic secretion of the capitate morphotype by recording the positive response to Sudan Black, Nile Blue, and Osmium tetroxide reagents,[9,10] but no partition on capitate subforms was indicated.

In the attempt at drawing a link between the histochemical results and the trichome distribution pattern, we can assume that the EO production of *T. fruticans* could be correlated mainly with the presence of peltate trichomes, both on leaves and flowers, as well as of ball-like and medium capitates. On the other hand, it is plausible to think that non-terpene derivatives could be linked to an abundance of capitate trichomes, mainly the short and long-stalked ones, on the vegetative and the reproductive organs, respectively.

In this work, we highlighted the well-known importance of the non-glandular trichomes as distinctive taxonomic characters for the recognition of *Teucrium* sect. *Teucrium*, confirming the presence of the simple filamentous coating hairs on the whole plant surface.[6,14]

Phytochemical Investigation

Focusing on phytochemistry, data highlighted for both samples a volatile profile with high complexity, with respectively 51 and 33 compounds identified in February and April 2022 EOs. In both profiles a clear predominance of sesquiterpenes over monoterpenes was detected. This result was consistent with data reported for *T. fruticans* grown in Sicily, Malta, and Tuscany, though a limited total number of compounds were found in the Sicilian and Maltese populations. The EOs investigated herein, as those obtained from *T. fruticans* growing in the Mediterranean region,[3,16] were characterized by the production of β-caryophyllene (**14**) and germacrene D (**20**) as main sesquiterpene constituents. Moreover, the presence of β-myrcene (**1**) and β-pinene (**2**) in April EOs suggested that their production may be related to the flowering phase. This hypothesis is supported by the results from Flamini et al.[3] following the comparison of the EOs profiles obtained at anthesis and fruiting stages. As a matter of fact, these authors highlighted the presence of these compounds only at the flowering phase.

These two molecules were recognized among the twelve most common volatile compounds spontaneously emitted in floral scents.[27] These could explain their production in the target species during the flowering period, with the primary purpose of attracting and guiding pollinators. Additionally, myrcene is also involved in the synthesis of pheromones of some insects, including moths.[28] However, alternative and synergistic roles, including plant defense, may be hypothesized. Indeed, myrcene (**1**) is involved in tritrophic protective mechanisms and has been shown to have allelopathic defense functions,[29] in addition to inhibit fungal proliferation together with β-pinene (**2**).[30] A synergistic defense strategy was also documented in conifers as a mechanism against the attack of pests.[31]

Nevertheless, the EO profiles investigated herein differed for some of the other main compounds from Sicilian, Maltese, and Tuscan samples. In detail, our samples seemed to be unique in the production of α-cadinol (**44**) and hexahydrofarnesyl acetone (**49**), while the Sicilian and Maltese species in that of 1-octen-3-ol,[16] and the Tuscan one in that of β-phellandrene.[3] By comparing the most abundant compounds of other congeneric species belonging to the *Teucrium* section, it was found that β-caryophyllene and germacrene D were already detected.[1] In addition, β-caryophyllene (**14**) was registered among the main compounds in species of different origins belonging to *Polium* and *Chamaedrys* sections, such as *T. montanum*, *T. capitatum* and T. *folium*, also those coming from Sicily.[15]

Given that there is no evidence referring to the potential biological activity of *T. fruticans* EO derivatives, some evaluations could be proposed, by taking into consideration the bioactivity ascribed by the literature to the main detected compounds. Focusing on the main components, several works reported the potential biological actions of EOs with β-caryophyllene (**14**) as main constituent.[1,32,33] This bicyclic sesquiterpene was shown to be responsible for antioxidant, antimicrobial, anti-inflammatory, analgesic, anti-neurodegenerative, and antitumor effects.[1,32,34] Furthermore, a synergistic action be-

tween all the major sesquiterpenes recognized in *T. fruticans* EO should be considered: for example, β-caryophyllene (**14**) and germacrene D (**20**), in association to α-cadinol (**44**), are recognized as major compounds in plant essential oils with anti-tumor potentials[32,35–37] and could induce a marked reduction of IL-1 and TNF-α levels in mice treated with oils rich in them. Moreover, the mixture of β-caryophyllene and α-humulene (**18**) could activate anti-inflammatory pathways, by inhibiting both COX2 and iNOS,[38,39] and even the cannabinoid system.[40] Specifically, *in vivo* studies demonstrated that the cannabinoid type 2 receptor agonism of β-caryophyllene mediated effects in different pathological conditions of various apparatuses.[33] Antifungal, antibacterial, and cytotoxic actions were identified as other activities of these sesquiterpenes, some of which are shared by β-selinene (**21**),[32] which was shown to have antioxidant, antibacterial, anti-inflammatory, analgesic, and antipyretic *in vitro* effects.[32,41] Finally, hexahydrofarnesyl acetone (**49**) is noteworthy, as it resulted to be one of the most abundant compounds in Nigerian *Ficus* spp. essential oils showing, among others, antibacterial, antioxidant, anti-inflammatory, and antifungal properties.[42] The overall information on the EO compounds from *T. fruticans* aerial parts and their potential biological activities could become useful to drive specific *in vitro* bioactivity assays in future studies.

As final consideration, it is interesting to note that all the molecules discussed so far could also explain properties unrelated to human pharmacology, such as antiphytoviral, insecticidal, larvicidal, antiprotozoal, repellent, and phytotoxic,[1,32,43] worthy of further study. Finally, many of these compounds conferred a typical woody odor to the essential oil, a feature that could find potential usefulness in the food and cosmetic fields.[43]

Conclusions

This multidisciplinary investigation allowed us for the presentation of a new approach to study the target species, by combining the micromorphological characterization of the glandular *indumentum* with the production of essential oil. Five trichomes morphotypes were detected, together with their distribution pattern on the vegetative and reproductive organs, by means of light and scanning microscopy techniques. Both peltates and medium-stalked capitates were the main trichomes involved in the release of terpenes, to a lesser degree supported by ball-like capitate morphotype, as confirmed by the first histochemical survey presented herein. In addition, for the first time, the essential oil's profile of a species coming from Northern Italy was characterized through GC/MS, with β-caryophyllene, germacrene D, α-humulene and β-selinene as dominant compounds. The correlation of the phytochemical results with the biological activities ascribed by the literature to the most abundant compounds, allowed us for a better understanding of the potentiality of this plant's derivative and the importance of future bioactivity assays.

Finally, the overall information derived from the scientific research will be made available to the visitors of the Ghirardi Botanic Garden through the development of original interpretative apparatuses in an Open science perspective, according to the policies of the University Third Mission.

Experimental Section

Plant Material

Teucrium fruticans L. was cultivated at the Ghirardi Botanic Garden (Toscolano Maderno, BS, Lombardy, Italy) of the Department of Pharmaceutical Sciences of the University of Milan. The sampling procedures for the micromorphological and phytochemical investigations were performed concurrently in February and April 2022. Voucher specimens were labelled GBG2021/051 and GBG2021/052 and deposited in the *Herbarium* of the Ghirardi Botanic Garden. Prof. G. Fico identified the species according to Pignatti et al.[4]

Micromorphological Survey

The morphotypes, distribution pattern, and histochemistry of the trichomes on stems, leaves, bracts, calyces, and corollas were studied by means of Light Microscopy (LM), Fluorescence Microscopy (FM) and Scanning Electron Microscopy (SEM). At least ten replicates per each plant part were examined to evaluate the variability level of the micromorphological features. The distribution pattern of trichomes was qualitatively assessed using the following symbols: (−) missing, not observed in any of the replicates; (±) sporadic, present in no more than four replicates; (+) present in all the replicates; (++) abundant and covering the whole organ surface in all the replicates.

Light Microscopy (LM) and Fluorescence Microscopy (FM)

Cross sections of the fresh examined plant parts were obtained with a razor blade and were analysed by LM and FM.

The main classes of the secondary metabolites produced in the glandular trichomes were investigated using the following histochemical tests: Toluidine Blue as a general dye,[44] Fluoral Yellow-088 for total lipids,[45] Nile Red for neutral lipids,[46] Nadi reagent for terpenes,[47] Ruthenium Red for acid polysaccharides,[48] Alcian Blue for muco-polysaccharides,[44] and Ferric Trichloride for polyphenols.[49] Control staining procedures were concurrently performed.

Observations were carried out under a Leitz DM-RB Fluo optical microscope equipped with a Nikon® digital camera.

Scanning Electron Microscopy (SEM)

For SEM observations, small segments of the examined vegetative and reproductive organs were FAA-fixed for 7 days, dehydrated with ascending ethanol series up to absolute, critical-point dried, and carbon gold-coated.

Observations were performed under a Zeiss® EVO MA15 SEM operating at 10 kV at the Interdepartmental Center for Electron Microscopy and Microanalysis Services (M.E.M.A.) of the University of Florence (Florence, Italy).

Phytochemical Survey

Essential Oils Isolation

Plant aerial parts were air-dried and stored at room temperature, in the dark. For the hydrodistillation, 110 g of plant aerial parts were coarsely grounded, transferred in a 4 L flask containing 2.0 L of water and subjected to distillation in a standard Clevenger apparatus for 3 h.

Distillation was repeated three times for each sample and oils obtained were analyzed by GC/MS separately.

GC/MS Characterization

The GC/MS analyses were performed at the Department of Chemistry, University of Milan, using a TRACE ISQ QD Single Quadrupole GC/MS. Separation of EO was performed with a capillary column VF-5 ms (5% phenyl-methyl-polysiloxane, length 30m, 0.25 mm i.d., 0.1 μm film thickness). The temperature gradient was: 8 min at 50 °C, then 4 °C min^{-1} to 60 °C, then 6 °C min^{-1} from 60 °C to 160 °C, and finally 20 °C min^{-1} from 160 °C to 280 °C. Injector and detector temperatures were set to 280 °C; carrier gas Helium, flux 1 ml min^{-1}. Mass range detected was 50–500 *m/z*. The EOs were analyzed pure or diluted 1:100 with hexane; injection volume was 1 μl. Mass spectra were analyzed using the Wiley Mass spectra Library, NIST Mass Spectral Search Program and 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). Relative percentages reported were obtained as average of measures obtained for each sample, with a standard deviation always below 5.0% of each single value.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contribution Statement

C. Giuliani, M. Bottoni, F. Milani collected plant material for investigations. C. Giuliani, M. Bottoni performed micromorphological and histochemical analyses. M. Bottoni, F. Milani, L. Santagostini isolated and analyzed essential oils. C. Giuliani, M. Bottoni wrote the draft manuscript. All the authors revised and edited the manuscript. The research design was elaborated, supervised, and administrated by G. Fico, also responsible for funding acquisition. All authors approved the published version of the manuscript.

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