



Stachys italica Mill.: synecology, functional compounds and potential use of an Italian endemic taxon

E. V. Perrino^{1,3} · R. P. Wagensommer² · G. N. Mezzapesa³ · A. Trani³

Received: 15 August 2024 / Accepted: 5 November 2024 / Published online: 15 November 2024
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Abstract

Main conclusion The metabolomic of the Italian endemic species *Stachys italica* was investigated and potential positive metabolites for human's health were detected, quantified and discussed in relation to its synecology.

Abstract *Stachys italica* is a species endemic to central-southern Italy, traditionally used for human consumption. The present research reports the results of a phytosociological study of this species in two southern regions of Italy (Apulia and Calabria). The collected plant material was used to make two types of extracts: hot water infusion to evaluate the use of this plant as tea and hydroalcoholic extraction to evaluate the use of it in herbal liqueur preparation. The extracts obtained by the hot water infusion had a values of total polyphenols and antioxidant capacity similar to values found in black tea. The analysis of non-volatiles compounds revealed the presence of biologically active substances like lavandulifolioside, verbascoside, and methoxyflavones. The analysis of volatiles fraction of metabolites demonstrated fifty-three volatiles compounds in the plant aerial part, 19 of which belonging to monoterpenoids, and 17 to the class of sesquiterpenoids. Among them, α -pinene, β -pinene, cis-ocimene, limonene and t-caryophyllene, were the most abundant compounds. The results were compared with already published results and referred to other similar species (such as *Sideritis syriaca*) which represent herbal mixtures usually collected and used as “mountain tea” in the Balkans and Eastern European countries.

Keywords Antioxidant · GC/MS · LC/MS · Metabolites · Monoterpenoids · Mountain tea · Phytosociology · Polyphenols · Sesquiterpenoids · Terpenes · Vegetation

Abbreviations

SGR San Giovanni Rotondo [Foggia province, Apulia, Italy]
SMM Santa Maria del Monte [Acquaformosa, Cosenza province, Calabria, Italy]

Communicated by Dorothea Bartels.

✉ E. V. Perrino
enrico.perrino@unifg.it

¹ Department of Agriculture, Food, Natural Resources and Engineering (DAFNE), University of Foggia, 71100 Foggia, Italy

² Faculty of Education, Free University of Bozen-Bolzano, Viale Ratisbona 16, Bressanone, 39042 Bolzano, Italy

³ CIHEAM, Mediterranean Agronomic Institute of Bari, Via Ceglie 9, Valenzano, 70010 Bari, Italy

Introduction

There is an increasing interest in the properties and potential use of wild species which foster the research activity in this field (Margiotta et al. 2022; Accogli et al. 2023). The reasons fall in the continuous research of more natural and healthier food (Borelli et al. 2020; Perrino et al. 2023), but also in the valorization of marginal areas (Ben Mahmoud et al. 2024), and conservation of agroecosystems biodiversity (Calabrese et al. 2015). The Mediterranean basin, one of the major “hot spot” centers of plant biodiversity in the world (Perrino et al. 2013; Galewski et al. 2021; Turco et al. 2023), is a candidate to be one of the most suitable areas for the discovery of wild edible plant species as beneficial for human health (Perrino et al. 2023).

The valorization of wild species, incorrectly defined as weeds in agricultural systems (Perrino and Calabrese 2018; Aviron et al. 2023), is of great importance to prevent the loss of biodiversity and promote sustainable agriculture. Wild species plants are useful for several reasons, such as the use

of their extracts as a source of natural herbicidal compounds (Casella et al. 2023) or in the food sector as source of flavorings and antioxidants (Pawera et al. 2020; Benedec et al. 2023). Among wild species, aromatic and medicinal plants draw a great deal of interest in the scientific community because they are recently rediscovered as great potential source of biological active compounds (Perrino et al. 2021; Valerio et al. 2021).

The genus *Stachys* L., described by Linnaeus (1753), is one of the largest genera of the Lamiaceae family and comprises more than 300 species (Harley et al. 2004), classified in 19 sections. These species are widespread in temperate and tropical regions of Mediterranean, Asia, America and southern Africa (Tundis et al. 2014; Salmaki et al. 2019). The genus is categorized into two subgenera, *S.* subgen. *Betonica* (L.) R. Bhattacharjee and *S.* subgen. *Stachys*, because of important botanical and phytochemical differences (Koeva-Todorovska 1979; Marin et al. 2004). In recent years, some authors consider them as two distinct genera, *Stachys* and *Betonica* (Bartolucci et al. 2024).

The name of the genus derives from the Greek term «stachys (=στάχυς)», referring to the inflorescence shape which resemble the “ear of corn” and the inflorescences of the genus *Triticum* L. (Poaceae).

The genus includes several species used in traditional medicine of several countries, with different names. Herbal preparations of *Stachys* spp. are widely used in folk medicine to treat a wide range of disorders and diseases, including stress, skin inflammation, stomach disorders, and genital cancers (Karioti et al. 2010). In particular, herbal teas from these plants, known as “mountain tea”, in the Balkan region, are used as tonics, carminatives, diuretics, and digestives (Özcan et al. 2001; Delazar et al. 2011); in addition, they are used as culinary herbs and as source of flavoring substances (Palá-Paúl et al. 2006). For instance, in Italy, the species *S. recta* L., already listed in the European Pharmacopoeia (Gören 2014), is known as yellow woundwort, and is locally called “erba della paura”, referring to the anxiolytic properties of its herbal tea, while *S. lavandulifolia* Vahl is called “Chaaye Koochi” in Iran (Delazar et al. 2011; Delnavazi et al. 2018). Referring to the Italian country, the leaves infusion of *S. annua* (L.) L. and *S. recta* are used for face washing and headaches relieve (Lucchetti et al. 2019). The decoction of the aerial parts of *S. recta* is also consumed as a purifier (Cornara et al. 2014). Furthermore, in an area of central Italy, *S. officinalis* (L.) Trevis., mentioned in the anthroposophical pharmaceutical code (APC) (Gören 2014), is used to produce an oily extract effective to heal wounds and useful as yellow paint for wood (Cornara et al. 2014; Lee et al. 2018).

Stachys italica Mill. [syn.: *Sideritis italica* (Mill.) Greuter & Burdet] was validated as a distinct species in relatively recent times (Greuter 1986) (Fig. 1).

Previously, the Italian populations were ascribed to *Sideritis sicula* Ucria (Fiori 1926), and afterwards included in *S. syriaca* L. (Pignatti 1982). *S. italica* is an endemic species of central-southern Italy (Bartolucci et al. 2024), that grows in garrigues and dry meadows on limestone. The Umbro-Marchigiano Apennine (central Italy) represents its northern limit distribution (Giuliani et al. 2011; Bartolucci et al. 2024) (Fig. 2). It is a white-wooly shrub, 20–60-cm tall, with oblanceolate-spathulate leaves and yellow flowers with wooly calyx gathered in verticillasters, emanating a sage scent.

In Sicily, this Italian endemic taxon, as indicated for other species of *Stachys* of the Balkan Region, is used to prepare herbal medicines and traditional teas known as “mountain teas”, while dried leaves are put over cuts and wounds to make them heal rapidly, and shepherds of the Madonie Mountains used to keep some leaves in their pockets or wallets to have them ready when needed (Lentini 2000).

Tomou et al. (2020) presented an extensive review about the traditional uses, phytochemistry and bioactivity of extracts obtained from plants belonging to the *Stachys* genus. More than 25 species were commonly consumed as herbal teas for the treatment of infections, common cold, gastrointestinal disorders, inflammation, etc. In most traditional uses, the dry aerial part of the plants is infused in hot water or used to make hydro-alcoholic extracts. Unfortunately, many of the papers reported chemical characterization of the extracts obtained with toxic solvents (Napolitano et al. 2022), or the extract with ethanol and or water were manipulated before the analysis (Goulas et al. 2014), or the analytical procedures followed were not scientifically sound. All these aspects are responsible of a lack in the scientific literature of trustworthy data about the characteristics of the extracts, according to traditional uses, from this plant material in the real conditions as they are used for human’s consumption.

Taxonomy, nomenclature and morphology

The genus *Stachys* is widely distributed in the Mediterranean region and south-western Asia, and is less concentrated in North and South America; it extends into southern Africa but is entirely absent from Australia and New Zealand (Bhattacharjee 1974; Güner and Ferrer-Gallego 2021). The majority of *Stachys* species grow in forests, rocky places, and on limestone. The genus is composed of annual and perennial herbs and also small shrubs (Bhattacharjee 1980).

Taxonomy

Originally, the genus described by Linnaeus (1753) reported only eight species, occurring mostly in



Fig. 1 Herbarium samples from Apulia (BI 43939) (A) and Basilicata (BI 57628) (B) regions

Europe. Subsequently, some botanists (e.g., Bentham 1832; Boissier 1879) made crucial contributions to the subgeneric classification of *Stachys* considering their annual or perennial plants behavior, and especially on morphological characters (number of flowers in verticillasters, calyx shape, corolla color, size of bracteoles and stem type herbaceous or woody). A century later a taxonomic revision with new assignments was presented (Bhattacharjee 1980; Krestovskaya 2007), and several studies were carried out in different territories, such as North America (Nelson 1981), Mexico and Central America (Turner 1994), Southern Africa (e.g., Basset and Munro 1986; Mulligan and Munro 1989), tropical and Eastern Africa (e.g., Demissew 1993; Harvey 1996), Balkan Peninsula (Bilušić Vundać 2019), Turkey (Akçicek 2020), and the Iranian plateau (Rechinger 1982). Scientific papers with phylogenetic approach (Bendiksby et al. 2011; Dündar et al. 2013) proposed different taxonomic results than the previous ones, showing that *Betonica* L. is not closely related to *Stachys* L. and that the monophyly of several sections (Bhattacharjee 1980; Rechinger 1982) must be questioned, although they confirmed the micromorphological characteristics (Karaismailoğlu and Güner 2021; Güner 2022). Further studies on a reasonable number of taxa through nuclear ribosomal DNA markers

and phylogenomic show that both delimitation of genera in Stachydeae and the subgeneric classification of *Stachys* need substantial revisions (Salmaki et al. 2012; Kharazian et al. 2015).

Chromosome number

Stachys italica: $2n=32$ (Contandriopoulos 1978; Baltisberger et al. 1996)

Nomenclature

Lamiales Nakai (1838)
Lamiaceae Martinov (1820)
Stachys L. (1753)
Stachys italica Mill. (1768)

Synonyms

Stachys germanica subsp. *italica* (Mill.) Briq. in Lab. Alp. Mar.: 222 (1893); *Sideritis italica* (Mill.) Greuter & Burdet in Willdenowia 15: 78 (1985).

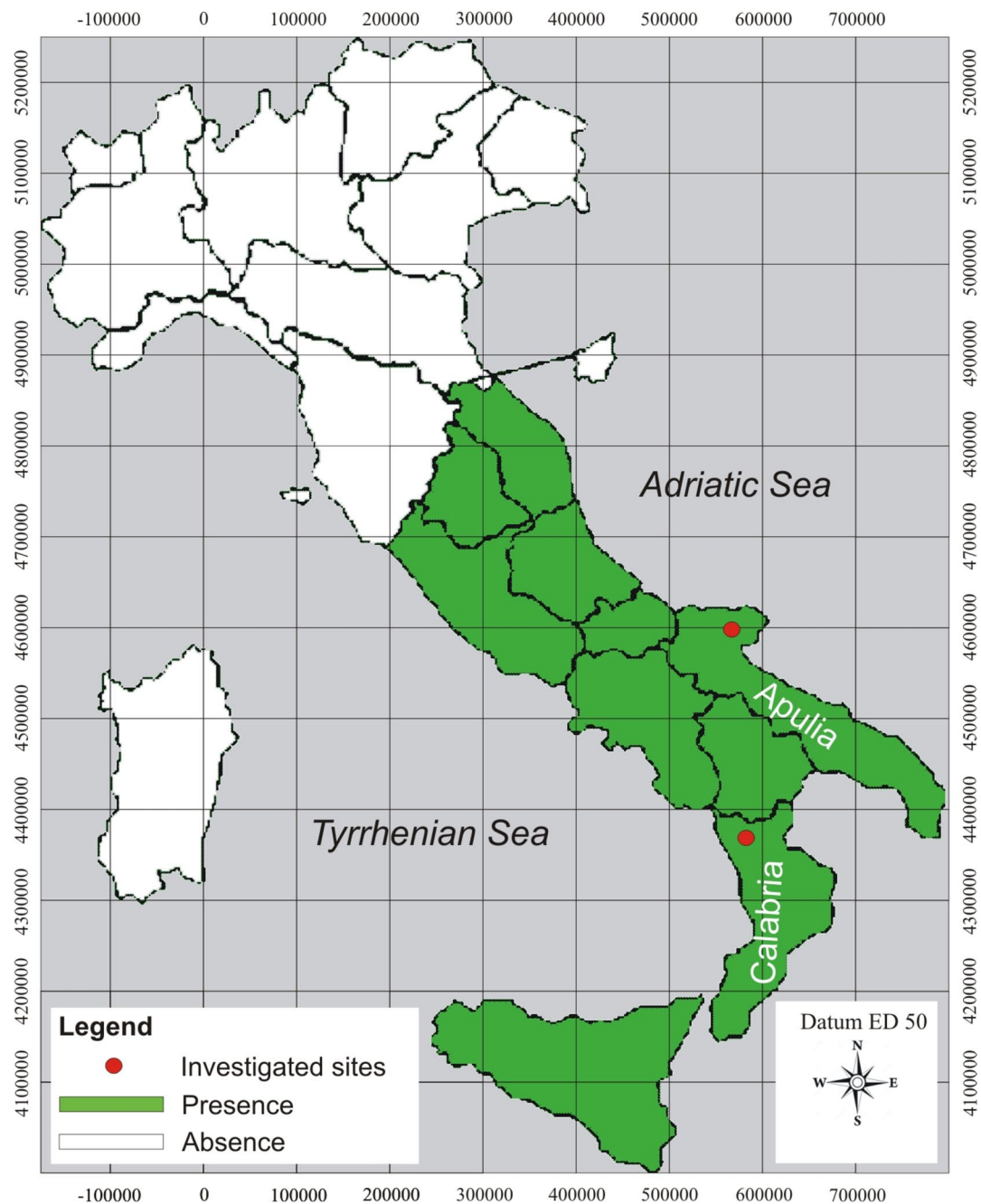


Fig. 2 Geographic distribution at regional level of *S. italica* (Italian endemism), with investigated sites

Habitat

Garrigues, dry calcareous stony perennial grasslands (Fig. 3). In Sicily and in the Gargano, it is usually linked to the Mediterranean belt, while in other Italian territories it also widely occurs in the Sub-Mediterranean belt (Biondi et al. 2014), from 500 to 1950 m a.s.l.

Morphology

Suffruticose perennial plant, 10–50 cm high, white-woolly with dense and felty tomentum; erect stems, branched and lignified at the base. Lower leaves oblanceolate-spatulate, 10–60 × (5)6–20 mm, petiolate, with entire or crenulate margin, the upper leaves 80 × 18 mm, opposite, entire,

Fig. 3 *S. italica*. Environment (A), habit (B), inflorescences (C) and leaves (D) at San Giovanni Rotondo (Foggia), 12 June 2023 (A, B and C) and at Acquaformosa (Cosenza), 26 June 2023 (D). Pictures by E.V. Perrino



linear-lanceolate and sessile. Spiciform inflorescence, with 6–12 flowers in $\pm$ spaced verticillasters, surrounded by large floral bracts (9–12 $\times$ 7–10 mm), suborbicular, entire, with a pointed appendix of 2–3 mm. The plant is very variable due to the hairiness of the bracts which can be very tomentose-felty or with scattered hairs or subglabrous. Calyx bell-shaped, almost actinomorphic, woolly, with a 6–7 mm tube with 10 ribs and with five equal teeth, sharp, of about 2 mm. Corolla (9–12 mm) zygomorph, bilabiate, pale yellow, with bifid upper lip and trilobed lower lip with $\pm$ equal lobes. Stamens 4, included in the corolla. 4-locular upper ovary. The fruit is composed of four nucules (tetrachenium) rounded at the apex (Tutin et al. 1968; Pignatti et al. 2017).

The main objectives of the present work are to investigate the ecological context in which *S. italica* grows in Italy, to identify and characterize the metabolites extractable from the aerial part by traditional method preparation, and to evaluate their biological activity.

Materials and methods

The study was conducted in the 2023 with surveys on two populations, in Santa Maria del Monte (municipality of Acquaformosa, Cosenza province, Calabria, later on indicated with SMM) and in Posta Padovano (municipality of San Giovanni Rotondo, Foggia province, Apulia, later on indicated with SGR). Surveys were carried out in June, and ecology, vegetation and populations health were investigated. During the surveys, some specimens were collected and used for laboratory analysis.

Vegetation and ecological characterization

Phytosociological characterization was performed through the phytosociological method of the Zurich – Montpellier School (Braun-Blanquet 1932) with information on physiographic data (relevé identification code, geographic coordinates in WGS84, altitude (m a.s.l.), aspect, slope ($^{\circ}$),

relevé area (m²), stoniness (%), rockiness (%), total cover (%), average height of herbaceous layer (cm), number of individuals in the population (estimate), habitat of Directive 92/43/EEC. The plant species cover was visually assessed based on the following abundance scores: (r) < 1% and rare; (+) < 1%; (i) 1–5%; (ii) 6–25%; (iii) 26–50%; (iv) 51–75%; and (v) 76–100%. For the identification of taxa, Flora Europaea (Tutin et al. 1968) and Flora d'Italia (Pignatti et al. 2017) were used; for nomenclature Bartolucci et al. (2024) and Galasso et al. (2024) were followed, and for syntaxonomic nomenclature Mucina et al. (2016) was consulted. The collected plant material will be stored at the Herbarium Horti Botanici Barensis of the University of Bari (BI) (*legit and determinavit*: Perrino E.V.).

Soil samples were collected from the first 20 cm of depth. For each studied area, 5–9 subsamples were taken, then mixed together to obtain a homogeneous mass, finally divided in three main samples for each area. After collection, soil samples were sieved (< 2 mm) and stored for physical and chemical analysis. Particle size distribution was determined by the pipette method, whereas textural class was categorized according to the USDA classification. Soil pH was measured both in water and CaCl₂ solution. Electrical conductivity (EC) was measured on a soil to distilled water ratio (1:2; w/v). Soil organic carbon (OC) was determined according to the Walkley and Black method. Total nitrogen (N) was determined with the Kjeldahl method. The available P was measured in sodium bicarbonate alkaline extracts and determined colorimetrically (Olsen and Sommers 1982).

Metabolites extraction

Plant samples collected during botanical surveys were air dried at room temperature and in the dark. All aerial parts were used (leaves, stem and flowers). The drying phase was considered accomplished when a constant weight was reached. After the drying phase, the plant material was grinded using a coffee grinder for 15 s. The obtained powder was weighted in amber glass bottles and added of hot water or hydroalcoholic solution using 1:25 (w/v) ratio, according with the indication of monograph of the European Medicines Agency (EMA) (2016) concerning different species and subspecies of the genus *Sideritis*. The water decoction was performed using distilled water heated at 90 °C and 15 min time infusion. Then the extracts were cooled down and filtered using 0.45 µm cellulose recycled filters and stored at –20 °C until the analysis. For hydro-alcoholic extraction, dried vegetable material was added of 70% (v/v) ethanol, and, after mixing, the bottle was kept in the dark for 3 days and mixed every 12 h. The extract was then filtered using 0.45 µm cellulose recycled filters and stored at –20 °C until the analysis.

Total polyphenol content

Total polyphenols were determined by a spectrophotometric Folin assay according to Wrolstad et al. (2005). Plastic cuvettes of 4 mL capacity were filled with 1.58 mL HPLC grade water, 20 µL extract and 100 µL Folin reagent. The cuvettes were covered with parafilm, mixed and left for 5 min, then 300 µL of freshly prepared Na₂CO₃ (20%) were added and mixed. After 90 min the absorbance was read at 765 nm against a blank made at the same way of sample but using clean extraction solution (water or hydro-alcoholic solution) instead of sample extract. Calibration was done using gallic acid standard in the range of 10–800 mg/L. The results were expressed as mg of gallic acid equivalent on 100 g of plant material dry weight or per mL of extract.

Total antioxidant activity

Total antioxidant activity was determined using the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay calibrated with Trolox (Sigma Aldrich). The radical form of ABTS was obtained by mixing 10 mL of ABTS (7 mM) with an equal volume of persulphate (4.95 mM). The mixture was left at room temperature in the dark for 12 h, then stored refrigerated for a maximum of ays. Using the stock solution of ABTS radical, a dilution was made obtaining a final absorbance at λ 730 nm of 0.7 units. The calibration range was 25–800 nmol/mL of Trolox equivalent. The assay was carried out as follow. In plastic cuvettes of 1 cm optical length, to 980 µL of ABTS diluted radical solution 20 µL of sample or standard were added. The cuvettes were closed using parafilm, mixed and left for 25 min. A blank was also prepared in the same manner but using the extraction solution instead of sample. After 25 min the absorbance was read at 730 nm against a cuvette with water. The difference between the sample and the blank containing only the extraction solution was determined and used in the calculation. The results were expressed as µmol/g on dry weight plant material or mM of Trolox Equivalent Antioxidant Capacity (TEAC) referring to the extracts.

Non-volatiles metabolites identification by liquid chromatography coupled with mass spectrometry (LC–MS)

Three microliters of the extract were injected in the UHPLC Ultimate 3000 system (Dionex, Thermo Fisher Scientific) equipped with LPG-3400RS pump, WPS-3000 autosampler, TCC-3000 column oven, and a Photodiode Array Detector PDA 3000. Chromatographic separation was obtained by the column Zorbax Eclipse XDB C₁₈, 10 cm of length, 2.1 mm of internal diameter, 1.8 µm of particles size (Agilent) using a binary gradient with formic acid 0.1% in water (solvent

A), methanol/acetonitrile/formic acid (50/50/0.1, by vol.) (solvent B). The solvent B gradient program was 5% initial, isocratic for 1 min, increased to 28% in 4 min, to 55% in 20 min, to 90% in 2 min, isocratic for 3 min, equilibration to the initial conditions for 5 min. The column temperature was set at 30 °C constant, and the mobile phase flow rate at 0.25 mL/min. The identification of compounds was performed by using a TSQ Quantum™ Access MAX Triple Quadrupole mass spectrometer equipped with a HESI interface. The MS conditions were capillary temperature 330 °C; source heater temperature 280 °C; nebulizer gas N₂; sheath gas flow 35 psi; auxiliary gas flow 10 arbitrary units; capillary voltage −2.8 kV. Data were acquired in negative ionization mode using a data-dependent method. The data-dependent settings were: full scan from 250 to 850 m/z, activation level 500 counts, isolation width 1 Da, default charge state 2, collision induced dissociation energy (CID) 35 eV, collision gas pressure 1.5 mTorr of Argon bip. All data were acquired and processed using Xcalibur v.2 (Thermo Fischer Scientific). The identification of compounds was achieved by comparing λ_{max} , [M−H][−] and MS/MS fragmentation patterns with literature data and MS/MS library Nist search algorithm. Quantitative data were obtained by the addition of syringic acid standard methanolic solution (70 µg/mL final concentration) to the sample before the injection and comparing the area of each compound peak with that of syringic the chromatogram acquired at 280 nm.

Solid phase micro extraction (SPME) followed by gas chromatography mass spectrometry (GC–MS) analysis of volatile metabolites

The extraction of volatiles compounds was obtained using the solid phase micro extraction technique with a three-phase fiber, divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) 50/30 µm, 1 cm length (Supelco). The samples (10 mg of flowers and leaves or 100 µL of extracts) were placed in 20 mL dedicated SPME vials, then equilibrated at 50 °C per 2 min, and finally the fiber was exposed in the headspace of vials for 5 min for volatiles absorption. The volatiles were desorbed by exposing the fiber in the injector port of the GC system heated at 230 °C. All the process of equilibration, extraction and injection was performed by robotic autosampler Combi-PAL tx. The GC coupled with mass spectrometry (GC-MS) was composed of a Clarus 680 GC equipped with an Elite-5 MS fused silica capillary column (30 m × 0.25 mm and 0.25 µm film thickness) and interfaced with a single quadrupole mass spectrometer Clarus SQ8C (Perkin Elmer). Mass spectra of target compounds were obtained by electron impact ionization system with standardized ionization energy of 70 eV. Helium 5.5 was used as a carrier gas at a constant

flow rate of 1 mL/min. The injection was performed in splitless (closed split valve for 1 min) at 230 °C. The oven temperature was programmed from 50 °C to 110 °C at 3 °C/min, then raised to 230 °C at 5 °C/min, hold at the final temperature for 3 min. Transfer line and source temperatures were set at 250 °C. Data were collected in full scan mode in the range 33–300 m/z. The qualitative results include compound identification and area percentage of related peak in the total ions chromatogram. Compounds identification was performed by both retention indexes (RI) and mass spectra (MS) search in NIST and Wiley databases and bibliography (Babushok et al. 2011).

Statistical analysis

The statistical analysis, mean values, two ways-ANOVA, and graphical were elaborated with SPSS v. 22 (IBM).

Results

Vegetation and ecological characterization

The context in which *S. italica* grows is very similar in both investigated areas with small differences linked to microvariations in the environment and bioclimatic factors. The phytosociological survey (Table 1) (relevés codes: 12.06.23.01 and 26.06.23.01) showed that *S. italica* is one of the characteristic species of *Sideritido italicae-Stipetum austroitalicae* Fanelli et al. 2001 corr. Terzi, Di Pietro & D'Amico 2010 association, of *Hippocrepido glaucae-Stipienion austroitalicae* Biondi & Galdenzi 2012 suballiance and *Hippocrepido glaucae-Stipion austroitalicae* Forte & Terzi in Forte, Perrino & Terzi 2005 alliance (Mucina et al. 2016), very rich in endemic species [*Stachys italica* Mill., *Stipa austroitalica* Martinovský, *Iris pseudopumila* Tineo, *Dianthus brutius* Brullo, Scelsi & Spamp. subsp. *brutius*, *Dianthus tarentinus* Lacaita, *Alyssum diffusum* Ten. subsp. *garganicum* Španiel, Marhold, N.G.Passal. & Lihová, *Gelasia villosa* (Scop.) Cass. subsp. *columnae* (Guss.) Bartolucci, Galasso & F.Conti, *Thymus spinulosus* Ten.], and with a high coverage percentage for many taxa in both sites.

From syntaxonomical point of view, this analysis does not confirm the alliance to the *Scorzoneretalia villosae* Horvatić 1975 order, but rather to the *Scorzonero villosae-Chrysopogonetalia grylli* Horvatić & Horvat in Horvatić 1963 order as already provisionally suggested by Di Pietro and Wagensommer (2014), clarifying the request for further syntaxonomic investigations outside the Apulia region of the Sub-Mediterranean xeric pastures on rocky calcareous soils of Southern Italy (Mucina et al. 2016).

Table 1 Phytosociological data related to the two sampling areas

Locality	SGR	SMM
Relevé code	12.06.23.01	26.06.23.01
Latitude (WGS84)	41°42.787'N	39° 44.994'N
Longitude (WGS84)	15°45.943'E	16° 4.441'E
Altitude (m.a.s.l.)	626	1148
Aspect	SE	E
Slope (°)	12	20
Relevé area (m ²)	80	100
Stoniness (%)	3	10
Rockiness (%)	40	25
Cover total (%)	85	90
Average height of herbaceous layer (cm)	15	12
Number of individuals collected	60	40
Soil deep (cm)	20	25
Habitat Directive 92/43/EEC	62A0	62A0
Charact. Ass. <i>Sideritido italicae-Stipetum austroitalicae</i> Fanelli et al. 2001 corr. Terzi et al. 2010		
* <i>Stachys italica</i> Mill	2	2
Charact. Suball. <i>Hippocrepido glaucae-Stipienion austroitalicae</i> Biondi and Galdenzi 2012, All. <i>Hippocrepido glaucae-Stipion austroitalicae</i> Forte et al. 2005		
* <i>Stipa austroitalica</i> Martinovský	3	3
<i>Petrorhagia saxifraga</i> (L.) Link subsp. <i>gasparrinii</i> (Guss.) Pignatti ex Greuter & Burdet	1	+
<i>Hippocrepis glauca</i> Ten.	+	—
* <i>Iris pseudopumila</i> Tineo	+	—
* <i>Alyssum diffusum</i> Ten. subsp. <i>garganicum</i> Španiel, Marhold, N.G.Passal. & Lihová	+	—
* <i>Dianthus brutius</i> Brullo & Spamp. subsp. <i>brutius</i>	—	1
* <i>Dianthus tarentinus</i> Lacaita	—	+
Charact. Ord. <i>Scorzonero villosae-Chrysopogonetalia grylli</i> Horvatić & Horvat in Horvatic 1963		
* <i>Gelasia villosa</i> (Scop.) Cass. subsp. <i>columnae</i> (Guss.) Bartolucci, Galasso & F.Conti	2	1
<i>Phleum hirsutum</i> Honck. subsp. <i>ambiguum</i> (Ten.) Cif. & Giacom	2	—
<i>Teucrium capitatum</i> L. subsp. <i>capitatum</i>	1	1
<i>Eryngium amethystinum</i> L.	1	+
<i>Anthyllis vulneraria</i> L. subsp. <i>rubriflora</i> (DC.) Arcang	+	1
<i>Convolvulus cantabrica</i> L.	+	+
<i>Koeleria splendens</i> C.Presl	1	—
<i>Satureja montana</i> L. subsp. <i>montana</i>	+	—
Charact. Cl. <i>Festuco valesiaceae-Brometea erecti</i> Br.-Bl. & Tüxen ex Br.-Bl. 1949		
<i>Festuca circummediterranea</i> Patzke	+	2
<i>Potentilla pedata</i> Willd. ex Hornem	+	1
<i>Centaurea deusta</i> Ten. subsp. <i>deusta</i>	+	+
<i>Trifolium campestre</i> Schreb	+	+
<i>Euphorbia spinosa</i> L. subsp. <i>spinosa</i>	1	—
<i>Helianthemum oelandicum</i> (L.) Dum.Cours. subsp. <i>incanum</i> (Willk.) G.López	1	—
<i>Poterium sanguisorba</i> L.	1	—
* <i>Thymus spinulosus</i> Ten.	1	—
<i>Melica ciliata</i> L. [subsp. <i>magnolii</i> (Godr. & Gren.) K.Richt.]	+	—
<i>Trifolium arvense</i> L. subsp. <i>arvense</i>	+	—
<i>Hypericum perforatum</i> L.	—	1
<i>Plantago maritima</i> L. subsp. <i>serpentina</i> (All.) Arcang	—	1
<i>Centaureum erythraea</i> Rafn subsp. <i>erythraea</i>	—	+
<i>Echinops ritro</i> L.	—	+

Table 1 (continued)

Locality	SGR	SMM
Transg. <i>Tuberarietea guttatae</i> (Br.-Bl. in Br.-Bl., Roussine and Nègre 1952) Rivas Goday and Rivas-Martínez 1963 nom. mut. propos. Rivas-Martínez, Diaz, Fernández-González, Izco, Loidi, Lousa & Penas		
<i>Brachypodium distachyon</i> (L.) P.Beauv	+	+
<i>Filago pyramidata</i> L.	+	+
<i>Triticum vagans</i> (Jord. & Fourr.) Greuter	+	+
<i>Hippocrepis ciliata</i> Willd	1	–
<i>Lagurus ovatus</i> L. [subsp. <i>ovatus</i>]	1	–
<i>Macrobriza maxima</i> (L.) Tzvelev	1	–
<i>Hypochaeris achyrophorus</i> L.	+	–
<i>Avena barbata</i> Pott ex Link	+	–
<i>Stachys romana</i> (L.) E.H.L.Krause	+	–
<i>Trifolium stellatum</i> L.	+	–
<i>Xeranthemum inapertum</i> (L.) Mill	–	+
<i>Trifolium strictum</i> L.	–	+
Transg. <i>Lygeo sparti-Stipetea tenacissimae</i> Rivas-Martínez 1978 nom. conserv. propos. Rivas-Martínez, Diaz, Fernández-González, Izco, Loidi, Lousa & Penas 2002		
<i>Carlina corymbosa</i> L.	+	1
<i>Phlomis herba-venti</i> L. subsp. <i>herba-venti</i>	+	1
<i>Convolvulus elegantissimus</i> Mill	+	+
<i>Petrosedum ochroleucum</i> (Chaix) Niederle subsp. <i>mediterraneum</i> (L.Gallo) Niederle	+	+
<i>Asphodelus ramosus</i> L. subsp. <i>ramosus</i>	2	–
<i>Daucus carota</i> L. subsp. <i>carota</i>	+	–
<i>Reichardia picroides</i> (L.) Roth	+	–
<i>Dactylis glomerata</i> L. subsp. <i>hispanica</i> (Roth) Nyman	+	–
<i>Pallenis spinosa</i> (L.) Cass. subsp. <i>spinosa</i>	+	–
Other species	24	19

*Endemic taxon

The comparison of the two sites revealed two slightly different conditions within the same class. The most significant difference between the two plant communities was observed in the transgressive classes (*Lygeo sparti-Stipetea tenacissimae* and *Tuberarietea guttatae*), which can be explained by the different exposition and overgrazing in relief 1 (SGR) than in relief 2 (SMM). Specifically, a high coverage of *A. ramosus* L. subsp. *ramosus* suggests overgrazing (especially by cow) in the Gargano site (SGR).

From topographic and ecological points of view, there are differences between the vegetation recorded in the two investigated areas. SGR has an elevation of 626 m a.s.l., whereas SMM reaches 1148 m a.s.l. The exposition in the two areas is comparable (SE in SGR and E in SMM), but with different slope (12° in SGR and 20° in SMM). The stoniness in the two area is different too (3% in SGR and 10% in SMM), whereas the rockiness is higher in SGR (40%) than in SMM (25%). The soil in both sites is a silty soil, characterized by poor total carbonate, low phosphorus availability and rich in total nitrogen and organic carbon with a well-balanced C/N ratio (Table 2).

Table 2 Soil parameters in the two sites (SGR, San Giovanni Rotondo; SMM, Santa Maria del Monte)

Soil parameters	Unit	SGR	SMM
Sand (2–0.05 mm)	g/kg	161	154
Silt (0.05–0.002 mm)	g/kg	787	807
Clay (<0.002 mm)	g/kg	51	38
Total carbonate	g/kg	<0.1	<0.1
pH H ₂ O		6.9	7.3
pH CaCl ₂		6.4	6.6
Electrical conductivity (1:2)	dS/m	0.14	0.12
Total nitrogen	g/kg	6.7	7.4
Available P ₂ O ₅	mg/kg	24	20
Organic carbon	g/kg	69.0	73.8
Organic matter	g/kg	119.0	127.2
C/N		10.3	9.9

Antioxidant activity and total polyphenol content

The results of total polyphenols and antioxidant activity are shown in Table 3. The 2-way ANOVA revealed

Table 3 Total polyphenols (TP) in mg of gallic ac. equivalent (GAE) and antioxidant activity in Trolox equivalent antioxidant capacity (TEAC) per g dry weight of plant aerial parts, or per volume of extract, obtained from *S. italica* samples collected in the two targets areas (SGR, San Giovanni Rotondo; SMM, Santa Maria del Monte)

Extraction	Area	TP GAE		TEAC	
		mg/g dw	mg/100 mL	μmol/g dw	mM
Ethanol	SGR	5.7	26.0	23	1.0
	SMM	6	27.2	25	1.1
Water	SGR	35.8	162.5	158	7.2
	SMM	13.6	62.0	74	3.4
Standard error of the mean ($n = 12$)		0.29	1.34	2.27	0.13
Two ways ANOVA significance					
Extraction		<0.01		<0.01	
Area		<0.01		<0.01	
Extraction* Area interaction		<0.01		<0.01	

significant differences for all comparisons and for the interaction between extraction method and collection area. Surprisingly the water infusion extract reported a higher concentration of total polyphenols and a higher antioxidant activity than the ethanol extract ($P < 0.01$). Comparing the two areas, samples collected in SGR reported a higher value of antioxidant activity and total polyphenols only in the water infusion extracts, thus explaining the significance of the interaction.

There was a significant interaction effect between the extraction method and the sampling area. This significance was illustrated in Fig. 4. The high significant difference between the two areas could be highlighted only by splitting the results in function of the extraction method and sampling area.

It could be hypothesized that a higher concentration of some polar constituents with anti-scavenging activity occurred in SGR samples compared to SMM samples and that these compounds could be glycosylated flavonoids.

Metabolites identification and quantification by chromatography techniques coupled to mass spectrometry (LC/MS–MS and GC–MS)

Non-volatiles metabolites present in the extracts, obtained by hot water or hydro-alcoholic infusion, were identified and quantified by ultra-high performance liquid chromatography coupled with triple quadrupoles detectors. Fifteen compounds were tentatively identified considering the already published papers and searching in the Nist library (Table 4). The compound at retention time 9.11 min was tentatively

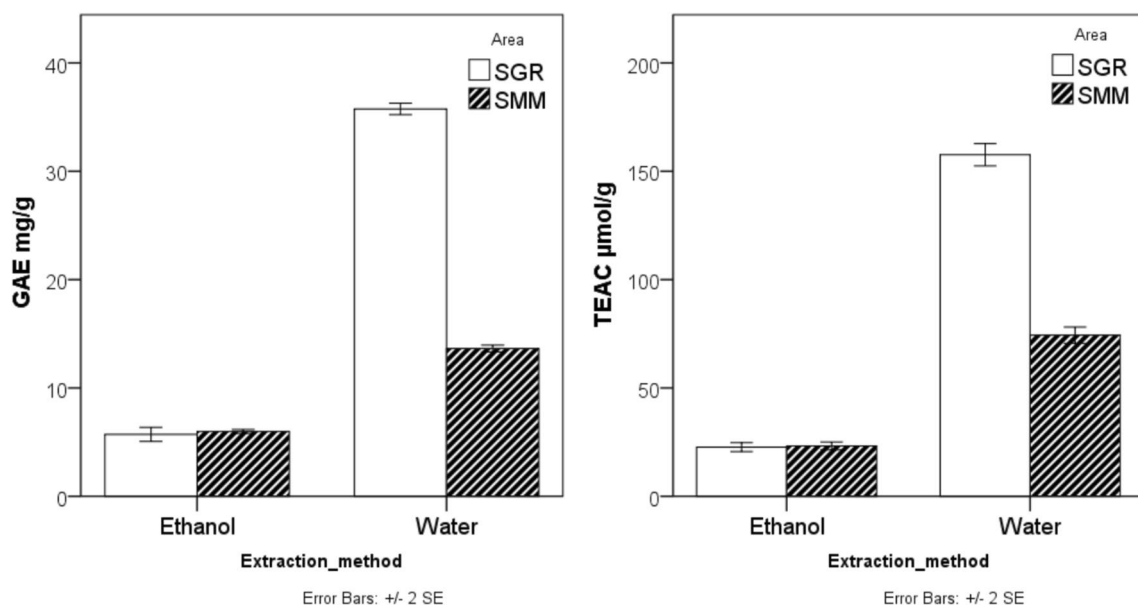


Fig. 4 Results of the total polyphenols (GAE, gallic acid equivalent) and total antioxidant activity (TEAC) of *S. italica* comparing the type of extraction and the site of collection. Error bars represent the standard error of the means, $n = 12$

Table 4 Identification data obtained by LC/MS–MS of metabolites extracted from *S. italica* samples.

RT	[M–H] [–]	λ _{max} (nm)	MS ²	Identification	References ^a
7.28	353	302	191 (100)	3-caffeoylquinic acid	1,2,3
9.11	711	280, 310	163 (100), 144 (50), 186 (40), 205 (40), 367 (20)	Coumaric glucoside derivate	4
9.92	755	322, 290	593 (80), 161 (50), 461 (21)	Lavandulifolioside	1,2,3
10.24	623	330, 290	161 (100), 461 (40)	Verbascoside	1,2,3
11.67	667	278, 340, 299	301 (100)	Hypolaetin 7-O-[6'-O-acetyl]-allosyl(1–2) glucoside	1,3
12.04	637	330, 290, 270	175 (100), 461 (90), 161 (70), 193 (50), 315 (10)	Leucoseptoside A	1,3
13.6	651	306, 278, 326	285 (100)	Isoscutellarein 7-O-[6'-O-acetyl]-allosyl (1–2) Glu	1,3
14.33	681	278, 340	314 (100), 299 (50)	3-O-methylhypolaetin 7-O-[6'-O-acetyl]-allosyl (1–2) Glu	1
15.61	623	278, 306	299 (100), 284 (5), 461 (3)	4'-O-methylisoscuteallarein diglycoside	1
17.89	709	296, 310	300 (100), 667 (20), 648 (10), 487 (10)	Hypolaetin-acetylated derivate	3
19.36	665	306, 278, 330	298 (100), 284 (10)	4-O-Methylisoscuteallarein 7-O-[6-O-acetyl]-allosyl 1–2) Glu	1,2
21.45	577	318, 272	268 (100), 145 (40), 413 (10), 307 (8)	Apigenin 7-(6-p-coumaroyl)Glu	2
21.79	577	318, 272	268 (100), 145 (20), 413 (30), 307 (5)	Apigenin 7-(4-p-coumaroyl)Glu	2
24.56	313	248, 340	283 (100), 255 (60)	5,7-Dihydroxy-3',4'-dimethoxyflavone	5
27.5	343	278, 334	297 (100), 270 (80), 194 (40)	5,3'-Dihydroxy-6,7,4'-trimethoxyflavanone	2,5

^a1-Petreska et al. (2011), 2-Napolitano (2022), 3-Tomou et al. (2023), 4-Tentatively identified, 5-Nist ms/ms library

RT retention time; [M–H][–] negative charged molecular mass m/z; MS² m/z signals pattern, relative intensity in brackets, obtained by collision induced dissociation from the [M–H][–] parent, λ_{max} nm wavelength of maximum absorbance at peak apex

identified considering the UV absorbance and the presence of characteristics signals at m/z 163 (coumaric acid), and 205 or 367 (saccharides). All compounds belonged to two main classes, flavones or caffeoyl-hydroxytyrosol derivatives or, more generally, phenylpropanoids and phenylethanoids derivatives.

The concentrations of the compounds identified in the extracts of *S. italica* are reported in Table 5. Considering the type of extraction, it is worth mentioning that the higher concentration in the hot water infusion samples of all compounds eluted before the two apigenin glucosides in respect to the concentration found in the ethanol extracts. At the same time the concentration of the last compounds, less polar and all glycosylated flavones, was higher in the ethanolic extracts. The comparison between the two sampling areas highlighted also a higher concentration of almost all compounds in the SGR than in SMM.

The investigation of volatile compounds in the *S. italica* samples was performed by SPME extraction followed by GC–MS analysis. A total of 53 volatiles' compounds were identified in the head space of the analyzed samples (Table 6). Terpenoids are the most representative class of compounds identified with 19 monoterpenes and 17 sesquiterpenes. Even if there is a strict relation between

the volatiles present and their concentration in the raw material and in the extracts, not all volatiles identified in these later were found in the aerial part. In fact, aldehydes, organic acids and esters, can be considered involved in chemical reactions (oxidation and hydration) producing new compounds during the extraction process. The most concentrated compounds found in the aerial parts of the plant were α and β pinene (from 28 to more than 50% of total area), limonene, the *cis* and *trans* isomers of ocimene, linalool and caryophyllene. The ethanolic extracts showed a similar profile but with the presence of phellandrene and farnesene, which were not found in the aerial part. The hot water extracts were characterized by an important increase of the relative area of aldehydes, alcohols, acids and esters probably because of the nucleophilic addition of H₂O and oxidation reactions. The water extracts were also characterized by an increased percentage of linalool, α-terpineol and the two isomers of bisabolol in respect to the values found in the raw material.

Focusing the attention to the comparison of terpenoids in the samples collected in the two areas, there were significative difference for α and β pinene which showed a higher relative area value in SMM samples.

Table 5 Concentration of the identified compounds in the extracts of *S. italica*

Identification	Apex RT	Ethanol		Water		SE	Sig.	
		SGR	SMM	SGR	SMM		Extract	Area
3-Caffeoylquinic acid	7.28	5.2	3.5	29.0	19.5	1.5	*	*
Coumaric glucoside derivate	9.11	4.0	0.7	9.7	1.9	0.9		*
Lavandulifolioside	9.92	31.8	19.4	76.7	31.2	2.6	*	*
Verbascoside	10.24	12.8	10.5	41.8	15.1	3.2	*	*
Hypolaetin 7-O-[6'-O-acetyl]-allosyl(1-2)glucoside	11.67	13.0	13.6	42.8	29.0	1.8	*	*
Leucoseptoside A	12.04	3.9	1.6	6.4	0.7	2.0		
Isoscutellarein 7-O-[6'-O-acetyl]-allosyl (1-2)Glu	13.6	17.5	19.5	48.0	36.1	2.8	*	*
3-O-methylhypolaetin 7-O-[6'-O-acetyl]-allosyl (1-2) Glu	14.33	13.2	20.5	58.9	43.1	1.5	*	*
4'-O-methylisoscuteallarein diglycoside	15.61	3.3	1.0	6.8	2.7	1.3		*
Hypolaetin-acetylated derivate	17.89	18.1	7.2	0.1	3.5	2.2	*	*
4-O-Methylisoscuteallarein 7-O-[6-O-acetyl]-allosyl 1-2) Glu	19.36	27.6	6.3	91.9	27.9	1.8	*	*
Apigenin 7-(6-p-coumaroylGlu)	21.45	4.7	4.7	1.2	1.3	0.5	*	
Apigenin 7-(4-p-coumaroylGlu)	21.79	2.6	2.5	1.1	1.1	0.7	*	
5,7-Dihydroxy-3',4'-dimethoxyflavone	24.56	1.5	0.9	0.0	0.0	0.5	*	
5,3'-Dihydroxy-6,7,4'-trimethoxyflavanone	27.5	2.5	1.0	0.9	0.9	0.5	*	

Concentrations are the mean value of three determinations expressed in mg per L of syringic acid equivalent

SE standard error of the means, Sig.* significance 2 ways ANOVA at $P < 0.05$

For the identification refer to Table 4 and retention time

Discussion

Environmental and vegetation issue

The environments in which *S. italica* was found, even if they have different pedo-climatic characteristics, can be referred to the same perennial grassland association *Sideritido italicae-Stipetum austroitalicae* Fanelli, Lucchese et Paura 2001 corr. Terzi, Di Pietro et D'Amico 2010. From a conservation and management point of view, the community aspects of this association fall within the community habitat of Directive 92/43/EEC “Eastern sub-Mediterranean dry grasslands (*Scorzonetalia villosae*)” (code 62A0).

The grasslands vegetation of habitat 62A0 is very rich in plant species at Mediterranean level, hosting up to 80 plant species/m², many of which are rare and endangered or endemic, including *Stipa austroitalica*, listed in Annex II of Habitats Directive (Fanelli et al. 2001; Forte et al. 2005). This habitat is characterized by a wide variety of grasses and aromatic herbs and is found on thin, well-drained, calcareous soils and is strongly influenced by climatic factors, topographic characteristics, soil conditions, management practices, grazing intensity (Ellenberg 1986) and in general human activities. The main causes of the decline of these calcareous grasslands in the survey sites are irrational grazing, reforestation, succession, and land use changes. Grazing by domestic farm animals remains one of the key factors in maintaining the right drought

soil, so changes in livestock load size will always affect this habitat (Rodwell 1992; Celaya et al. 2022) and are the main reason for its unfavorable condition. Furthermore, the remained grasslands, thanks to the presence of livestock, are often not properly managed from an ecological point of view, with high pressure of grazing in such area and other areas with no pressure at all. Overgrazing, especially during the summer, implies excessive nibbling, trampling and poaching, causing both soil erosion and a decrease in species-richness and structural diversity, with a loss of tall herbs and an increase of invasive thorny species of little appeal to livestock [e.g., *Crataegus monogyna* Jacq., *Prunus spinosa* L., *Cytisus infestus* (C.Presl) Guss.].

Transitions to shrubby and woody vegetation, which develop as management loosens, are also part of the 62A0 habitat, as observed in some areas of the SGR and SMM sites (Figs. 5 and 6).

A rational and well balanced system management of these areas should consider sheep or goat livestock (better if sheep) with a pressure of grazing never exceeding 0.1 livestock unit ha⁻¹yr⁻¹, and continuous grazing with peaks in spring and autumn. Browse from shrub or forest formations and agricultural sub-products (e.g., stubble) are complementary sources of food for livestock. Their conservation or restoration is, therefore, an advisable measure for this habitat type and *S. italica*.

Table 6 Data related to the volatiles metabolites in *S. italica* samples obtained by SPME–GC–MS

ID	RI	RI r. ¹	Plant		Ethanol		Water		SE	Sig.	
			SGR	SMM	SGR	SMM	SGR	SMM		Extr	Area
Aldehydes (7)											
Hexanal	799	799	0.39	0.23	–	–	–	–	0.1		
Hexenal	846	853	1.87	0.35	1.45	2.28	0.10	0.12	0.1	*	
Benzaldehyde	957	962	–	–	–	–	14.15	20.85	3.3	*	
Octanal	1001	1003	0.31	0.16	–	–	2.28	3.97	0.5	*	
Nonanal	1103	1103	1.40	1.16	–	–	1.88	1.07	0.2	*	
Decanal	1204	1205	–	–	–	–	0.66	0.56	0.2	*	
Decenal	1260	1263	–	–	–	–	1.31	0.37	0.2	*	
Alcohols (6)											
3-Hexen-1ol	851	853	–	–	0.62	0.64	0.45	6.11	0.5		*
2-Hexen-1ol	863	864	–	–	0.91	0.29	3.25	2.94	0.3	*	
1-Octen-3-ol	977	980	–	–	–	–	18.39	20.08	1.5	*	
3-Octanol	996	993	–	–	–	–	0.40	1.06	0.2	*	
1-Octanol	1067	1071	0.34	0.27	–	–	–	–	0.1		
1-Nonanol	1169	1173	–	–	–	–	0.96	1.74	0.4		
Acids and esters (2)											
Nonanoic ac	1273	1275	–	–	–	–	7.85	4.10	0.6	*	
Benzyl benzoate	1765	1761	0.17	0.07	0.44	0.73	7.24	2.09	0.8	*	
Monoterpenes (19)											
α-Pinene	930	936	11.66	18.50	13.72	23.60	–	–	1.2	*	*
Sabinene	968	973	1.23	1.81	0.25	1.09	–	–	0.3	*	
β-Pinene	975	977	17.26	32.64	13.68	23.31	–	–	0.9	*	*
α-Myrcene	987	989	2.97	3.18	–	–	–	–	0.2		
α-Phellandrene	1004	1004	2.83	0.76	0.72	1.05	–	–	0.3	*	
δ-3-Carene	1007	1011	3.31	0.60	1.23	2.07	–	–	0.2	*	
α-Terpinene	1014	1017	0.23	0.19	–	–	0.18	0.34	0.1	*	
m-Cymene	1021	1022	1.22	0.15	0.78	0.14	0.01	0.01	0.2	*	*
Limonene	1027	1029	11.47	10.22	2.74	3.41	–	–	0.5	*	
β-Phellandrene	1028	1030	–	–	1.07	2.00	–	–	0.3	*	
cis-Ocimene	1035	1038	16.95	12.44	1.20	1.54	–	–	1.1	*	
trans-Ocimene	1043	1047	3.72	3.00	0.62	0.94	–	–	0.2	*	
γ-Terpinene	1055	1060	1.20	0.77	0.78	1.84	0.54	1.08	0.6		
Terpinolene	1082	1087	0.78	0.86	–	–	–	–	0.3		
Linalool	1099	1099	5.29	6.39	–	–	16.52	18.16	1.5	*	
Pinocarveol,trans-	1137	1140	–	–	–	–	0.04	0.75	0.1		
Pinocarpone	1157	1160	0.31	0.22	–	–	0.58	0.86	0.1	*	
Terpinen-4-ol	1176	1177	–	–	–	–	0.10	1.63	0.5	*	
α-Terpineol	1190	1195	0.72	0.73	–	–	6.31	5.87	0.3	*	
Sesquiterpenes (17)											
α-Cubebene	1345	1351	0.34	0.05	–	–	1.56	0.97	0.2	*	
α-Copaene	1373	1376	1.14	0.09	0.42	0.36	–	–	0.2	*	
α-Gurjunene	1402	1409	0.24	0.07	–	–	–	–	0.1		
t-Caryophyllene	1420	1420	4.11	0.86	1.65	0.91	0.26	0.43	0.3	*	*
β-Farnesene, cis-	1445	1446	–	–	0.13	0.06	–	–	0.1		
β-Farnesene	1451	1456	0.25	0.20	54.79	29.97	–	–	1.0	*	
γ-Murolene	1472	1476	0.83	0.45	–	–	–	–	0.2		
Germacrene D	1477	1480	1.01	0.23	0.29	0.27	–	–	0.1	*	
α-Farnesene, (Z,E)-	1492	1491	–	–	0.37	0.35	–	–	0.1	*	
β-Bisabolene	1505	1506	1.80	1.35	0.64	0.58	–	–	0.2	*	

Table 6 (continued)

ID	RI	RI r. ¹	Plant		Ethanol		Water		SE	Sig.	
			SGR	SMM	SGR	SMM	SGR	SMM		Extr	Area
γ -Cadinene	1510	1513	0.23	0.08	–	–	–	–	0.1		
δ -Cadinene	1515	1519	1.03	0.16	–	–	–	–	0.2		*
β -Sesquiphellandrene	1521	1523	0.16	0.06	–	–	–	–	0.1		
α -Bisabolene	1538	1540	0.23	0.14	–	–	–	–	0.1		
α -Acorenol	1628	1630	–	–	–	–	2.43	0.87	1.1	*	
β -Bisabolol	1673	1672	0.63	0.35	–	–	3.59	1.44	0.8	*	
α -Bisabolol	1683	1681	1.15	0.13	–	–	8.96	2.55	0.3	*	
Terpenes derivate (2)											
Bornyl acetate	1281	1283	0.37	0.81	–	–	–	–	0.1		
Geranyl-p-cymene	1950	1953	0.86	0.28	1.50	2.57	–	–	0.2	*	

Values reported are area percentage of the peaks in the total ions chromatogram

RI retention index, RI r. retention index reference, SE standard error of the means, Sig.* 2 ways ANOVA significance at $P < 0.05$



Fig. 5 Plant communities on survey day. Posta Padovano in the municipality of San Giovanni Rotondo (Province of Foggia) (SGR) (12 June 2023). Picture by E.V. Perrino



Fig. 6 Plant communities on survey day. Santa Maria del Monte in the municipality of Acquaformosa (Province of Cosenza) (SMM) (26 June 2023). Picture by E.V. Perrino

Antioxidant activity and total polyphenols content

As already mentioned in previous sections, few research papers report results for the antioxidant activity and total polyphenols in the extract of plant material belonging to *Stachys* or *Sideritis* with comparable methods of extraction to that proposed in the present paper. Nevertheless, the results reported in Table 3 showed that the more effective method of extraction of biological active compounds from the aerial part of *S. italica* is the hot water infusion. The maximum value of total polyphenols compounds concentration in these extracts was 36 mg of GAE per g of dry weight plant material or 162 mg of GAE per 100 mL of extract. Goulas et al. (2014) studied the phytochemicals of the mountain tea obtained using a *Sideritis syriaca* decoction in hot water and found a concentration of total polyphenols of about 1860 mg of GAE/100 g DW. Our results, converted in comparable units, is around 3600 mg GAE/100 g. Sarikurcu et al. (2020) investigated the biological active substances present in *Sideritis perfoliata*, using a continuous water extraction process and reporting a total polyphenols content of 52 mg GAE per g DW, very close to the value found in the present report. The same authors reported also 150 mg of Trolox equivalent per g dw of total antioxidant capacity, tested with the ABTS assay. This value is more than three time higher than the maximum value found in the presented results (40 mg/g). Finally, Pljevljakušić et al. (2011) studied the chemical properties of *Sideritis raeseri* water extract with comparable method of analysis and extraction, reporting value of total polyphenols very close the presented results (15–34 mg GAE/g DW).

Comparing these results with the value of total polyphenols and antioxidant capacity found in the most known and widely used black tea, it is possible to highlight

that the hot water extract of *S. italica* showed a content of total polyphenols similar to that reported for black tea (9.6 mM GAE in the present results vs 10–15 mM GAE in black tea by Carloni et al. 2013), but a lower value for the antioxidant capacity (7 vs 25 mM TEAC).

LC/MS–MS and GC–MS analysis

The LC/MS–MS analysis showed the presence of interesting compounds, both in ethanolic and water extracts of *S. italica*. Fifteen compounds were identified, eight of them belong to the class of flavone whereas the others can be classified as phenylpropanoids and phenylethanoids. Among these compounds it is noteworthy to mention the presence of lavandulifolioside, a trisaccharide containing one molecule of hydroxytyrosol and one of caffeic acid (Fig. 7). There is scientific evidence about the pharmacological properties of this compounds, able to produce negative chronotropism and decrease of blood pressure in rats, at a dose concentration higher than 250 µg per kg of body weight (Milkowska-Leyck et al. 2002). Considering the highest concentration of lavandulifolioside found in the water infusion extract (76 mg/L), and 100 mL as serving cup, we obtain an amount of 7600 µg which divided per 76 kg body weight (adult man average weight) produce a dose of about 100 µg/kg body weight, enough to exert a significative effect on consumers. Most of the effects attributed to the consumption of mountain tea obtained by hot infusion of aerial part of plant belonging to the *Stachys* or *Sideritis* genus are described as anxiolytic and sedative, and a key role in explaining these pharmacological

effects surely can be assumed to the lavandulifolioside molecule. Other compounds found were the two methoxyflavones, considered antitumoral and antiviral (Zhao et al. 2022), and verbascoside, which have anti-inflammatory effects on skin, endothelium, intestine and lungs (Alipieva et al. 2014), thus, explaining the beneficial effect on cold, and stomach-ache of this tea consumption.

The composition of essential oil of *S. italica* was previously studied by Basile et al. (2006), Formisano et al. (2010) and Giuliani et al. (2011). All of them used hydrodistillation for the extraction of the essential oil and collected the plant material in the south of Italy (in Sicily the first two and Marche region the last one). It should be highlighted that the present outcomes were obtained with a different technique of extraction which can be itself responsible of differences in the analytical results. Considering only the terpenes fraction of volatiles and comparing the obtained results with the above mentioned published papers, there is a good overlapping with the volatiles identified in samples of *S. italica* collected in the Marche region (Giuliani et al. 2011), with 27 terpenes out of 36 in common. The most concentrated terpenes resulted to be the same, the two isomers of pinene and ocimene among the monoterpenes and t-caryophyllene among the sesquiterpenes. There is only one important difference regarding the absence of limonene and, at the same time, the presence of sylvestrene. These two compounds have a very close retention index, so it is hypnotizable that they are the same compound. The results presented by the other two papers are related to *S. italica* collected in the Sicily region and seem to be of low quality, with a low number of identified compounds, but they listed among the most concentrated compounds limonene, thus supporting our results. The value of area percentage could not be comparable for the already explained reason about the different techniques of extraction.

Considering other similar species used as “mountain tea” in other European countries, there are interesting similarities in term of volatiles composition. In Albania and Macedonia, *Sideritis scardica* Griseb. and *S. raeseri* Boiss. & Heldr., are characterized by the presence of high percentage of α-pinene, β-pinene, β-phellandrene, α-cubebene, α-copaene, and β-bisabolene (Karapandzova et al. 2013). In Turkey, *Sideritis argyrea* P.H.Davis and *S. hololeuca* Boiss. & Heldr. have even more affinity in quantitative terms with *S. italica* for the high concentrations of α-pinene (from 17 to 30%) and β-pinene (from 24 to 33%), but also for sharing 12 other volatiles (Maral et al. 2022). These affinities with the Turkish species are also confirmed for *Sideritis bilgeriana* P.H.Davis, *S. tmolea* P. H. Davis and *Sideritis congesta* P.H.Davis & Hub.-Mor. (Özcan et al. 2001). *Sideritis montana* L. in Croatia, is very similar also in qualitative composition with more than 20 compounds in common with *S. italica*, even

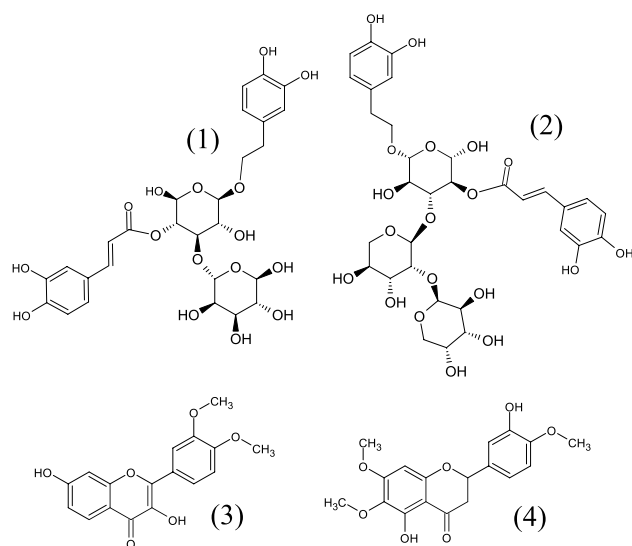


Fig. 7 Chemical structure of key compounds identified and quantified in the extracts of *S. italica* by LC/MS–MS. (1) Verbascoside, (2) lavandulifolioside, (3) 3,7-dihydroxy-3',4'-dimethoxyflavone, (4) 5,3'-dihydroxy-6,7,4'-trimethoxyflavanone

if the prevalent compound is germacrene D (23%) instead of β -pinene (Marić et al. 2021). Finally, the Greek endemic *Sideritis clandestina* Hayek subsp. *peloponnesiaca* (Boiss. & Heldr.) Baden seems to have a volatiles profile very close to that of *S. italica*, both from a qualitative and a quantitative point of view (Dimaki et al. 2022).

Comparing the volatiles composition percentage between the two sampling areas for the raw material, it is possible to highlight some significant differences, which can be explained by many reasons, as for example phenological stage of collection, soil and environmental characteristics (Perrino et al. 2021). On the other hand, the comparison between the two extraction methods highlights the higher efficiency of the ethanol in terpenes extraction in respect to the water, but also the higher relative concentration of oxygenated terpenes in this later (linalool, α -terpineol, and α and β -bisabolol).

Conclusions

To the best of our knowledge, this is the first scientific report which deeply investigates the botanical, taxonomical, phytosociological, metabolomic characteristics of the Italian endemism *Stachys italica*. The collected samples were used for the production of ethanolic or watery extract, simulating the production of herbal liquor or tea. The obtained extracts, analyzed by advanced techniques based on mass spectrometry, revealed the presence of biological active substances in both type of extracts, highly beneficial for humans.

S. italica grows in rocky habitats, suggesting a good potential to grow organically in rural areas up to approximately 1.200 m a.s.l. Considering the biological effects associated with its metabolites this plant species, together with other similar species, should offer new source of income for a sustainable agricultural in marginal mountain areas. The valorization of *S. italica* and its potential use in foods and agronomics should play an important role in any future development program and strategy that aim at enhancing their territories and promote the resilience of communities and natural habitats, especially in rural areas, as evaluated in some environmental restoration projects at Mediterranean level [REACT4MED (<https://react4med.eu/>), EcoplantMed (<http://www.ecoplantmed.eu/project/>) projects].

Acknowledgements The authors wish to thank the curator of *Herbarium Horti Botanici Barenis* of Botanical Garden Museum of University of Bari (BI).

Author contributions EVP and AT conceived the original project, participated in the conception of research, executed experiments and analyzed results, supervised experiments, and finalized the article. EVP

wrote the first version of the article, conducted experiments in the field, and contributed to the writing of the article at every stage. AT conducted the laboratory experiments, and contributed to the writing of the article at every stage. GM analyzed data laboratory and completed the article at the last stage. RPW contributed data field and completed the article at the last stage.

Data availability All data generated or analysed during this study are included in this published article.

Declarations

Conflict of interest The authors declare that they have no conflict of interest to the content of this article.

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