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Chemical Composition, Antioxidant Activities and Protective Effects of *Sideritis italica* Extract on C2C12 Oxidative Stress

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Authors' contributions

This work was carried out in collaboration between all authors. Author LM provided plant material, defined experimental protocols and the interpretations of the results with statistical analysis, and prepared the draft of the manuscript. Author GP made the phytochemical analysis tests. Author BT performed the antiradical assays. Author LL managed the cell culture and biochemical analyses. All the authors have read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: *Sideritis italica* is a medicinal plant used for medical purposes mainly based on experiences rather than scientific evidence. Biological properties, composition of primary and secondary metabolites as well as the antioxidant capacity were investigated on samples from wild plant.
Methodology: The ultrastructure of aerial parts and quantitative distribution of pigments, including chlorophylls and amino acids, as well as the main class of secondary metabolites (phenols, flavonoids, flavonols and proanthocyanidins) were investigated. The extracts were tested by radical scavenging assays (DPPH, ABTS) and pharmacological assays (antiproliferative activity, effects on ROS production and protective effects against DNA damage induced by hydrogen peroxide) for their effects on C2C12 cell line.
Results: Scanning electron microphotography confirms the presence of pharmacognostic

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characteristics, such as glandular and non-glandular trichomes on aerial parts. The chemical analysis indicates that the leaves are the most important part of the plant, and ethanol/water 70/30 is the preferable extraction solvent. The highest concentration of all metabolites was found in 70% ethanol extract of leaves. The antiradical assays and the *in vitro* tests on mouse myoblast cells C2C12 confirm the biological activities of the extract. C2C12 culture medium supplemented with extract, at doses (5-200µg/ml) not interfering with cell viability, was seen to modulate the ROS production and balance the increased oxidative stress induced by hydrogen peroxide. The treatment of C2C12 cells with 200 µg/ml of extract results in a percentage reduction of ROS of -60% and -71%, compared to untreated and H_2O_2 treated groups, respectively, $P<.05$. The quantitative reduction of 8-hydroxy-2'-deoxyguanosine (8-OH-dG), which is a biomarker of free radical DNA damage, confirms the protective effect of *S. italica* extract on oxidative stress at basal condition as well as in presence of exogenous stimuli (-11 and -7%, at 20µg/ml, respectively versus untreated and H_2O_2 groups, $P<.05$).
Conclusion: The results obtained in the present study support the rational base for the medicinal use of plant and extracts in modulating the free radical metabolism and balancing the oxidative stress.

Keywords: Sideritis italica, C2C12 Mouse myoblast cell, reactive oxygen species, 8-hydroxy-2'-deoxyguanosine, plant extract.

1. INTRODUCTION

For the last decades, many researchers have targeted the genus *Sideritis* (Lamiaceae) as potential source of extract and phytochemicals with interesting biological and pharmacological activities.

The genus is present in Italy with the species *S. montana* L., *S. romana* L. and the endemic *S. italica* (Mill.) Greuter & Burdet [1].

The latter is locally used for medicinal purpose in the form of decoction of aerial parts as diuretic and digestive, while shepherds apply topically the leaves to treat wounds [2].

The chemical composition and pharmacological activities of the genus were investigated in recent revisions presented by Tsibranska and González-Burgos [3,4]. It is clear from these studies that the specie *S. italica* (syn*S. syriaca* L, *S. sicula* Ucria) was only marginally investigated.

Previous studies from our group pointed the attention to the chemical characterization of the essential oil and to the pharmacological activity of *Sideritis syriaca* samples collected from wild population of Italian origin. Our study highlighted some differences in the chemical composition compared to those from Greece confirming the rational use as anti-inflammatory agent [5,6].

Further investigation confirmed the presence of a biologically active fraction containing in particular phenolic compounds that are clearly related to the antiradical and antioxidant activities [7-9].

The present paper describes the results of chemical investigation on primary and secondary plant metabolites focused on pigments, amino acid, total proteins and main class of phenols

distribution in *Sideritis italica*. We also compared the composition of water, ethanol and hydroalcoholic extracts. The biological activity of extracts was also investigated as antimicrobial and antioxidant in chemical assays as well as in *in vitro* pharmacological assays on myoblast cell line as modulators of oxidative stress.

2. MATERIALS AND METHODS

2.1 Plant Material and Extraction

Fifty blooming plants of *Sideritis italica* (Mill.) Greuter & Burdet (syn *S. syriaca* L., *S. sicula* Ucria) were collected near Collarmele (AQ) (GPS coordinates: 42.073252, 13.651273, 800 meters above sea level), in Central Italy. The site is near the northern limit of the Italian area for this species, which is found in all of the southern regions, including Sicily [10].

The fresh plant material was manually divided into three major parts, flowers, leaves and roots and used for plant characterization, pigments and total protein determinations. For all other determination sample were dried in an oven (40°C for 48h) and stored in vacuum sealed bags protected from the light for no longer than 6 months. The dried material was powdered and extracted freshly before use. If not otherwise indicated, 2 g of powder were extracted twice in 50 ml of solvent in ultrasonic bath, for 30 min at room temperature. After each cycle the extract were separate by centrifugation and the liquid phases pooled and adjusted to a final volume of 100 ml. The solvent used were absolute ethanol (EtOH), 70% ethanol (EtOH 70%) and distilled water (H_2O).

2.2 Plant Characterization

Fresh aerial parts of *S. italica* were cut into segments of about 4 - 6 mm in length and fixed for 24 h in 6% glutaraldehyde in 0.05 M sodium cacodylate, rinsed in 0.05 M cacodylate buffer (pH 7.5) and dehydrated in a graded series of ethanol (20, 40, 60, 80 and 100% X 3) at 60 min per rinse. This was followed by critical point drying with liquid CO_2 (HCP-2 Critical Point Dryer, Hitachi Ltd., Japan). Samples were mounted on the aluminum specimen holder with double-sided carbon coated adhesive discs and sputter coated with gold-palladium (Eiko IB-3 Ion Coater). The surface of the sample was examined at varying magnifications using JEOL (Philips Cambridge 90B) SEM, operated at 10 – 15 kV acceleration voltage. All the representative features examined were captured and digitalized [11].

2.3 Metabolites Characterization

2.3.1 Pigments

Pigments were extracted from leaves and flowers by maceration with 80% acetone (2 g in 100 ml), in cold (4°C) and darkness. After 12 hours, the solution was centrifuged and the final volume was standardized. The extracts solutions were freshly prepared and directly used for quantitative determination. Chlorophyll determination was done on high performance liquid chromatography apparatus equipped with binary pump (Jasco PU-880 plus, Tokyo, Japan) and UV-Vis detector (Jasco MD-2010, Tokyo, Japan), according to the methodology described by Arar with some modifications [12,13]. This method uses C-18 reverse phase column (Spherisorb ODS-2, 250 mm × 4 mm i.d., 5 µm.) with guard column (Security Guard System, Cartridge C18, 4 x 30 mm, Phenomenex), flow in 1.0 ml/min and 20µl sample. The mobile phase was a gradient mixture of A (methanol: acetonitrile: water,

5:9:86, v:v:v) and B (methanol:ethyl acetate, 68:32, v:v). Peaks identification and quantification were done by direct comparison with pure standard solutions of neoxanthin, violaxanthin, lutein, β-carotene and zeaxanthin (Sigma-Aldrich).

2.3.2 Total protein determination

The total protein content was determined following the Lowry method with slight modifications [14,15]. Fresh plant material (2 g) was extracted in 15 ml of acetate buffer (pH 5). The solution was centrifuged (8000 g) and 5 ml of TCA (10%) was added to 5 ml of the supernatant in order to precipitate proteins. After centrifugation, the solid precipitation was dissolved in 2 ml NaOH (1N). 0.2 ml of the test solution or 0.02–0.1 ml of BSA protein calibrator (1 g/l) were diluted to 2 ml with phosphate buffer and 20 ml of Lowry reagent C. The latter solution was prepared by mixing 50 ml of solution A and 1 ml of solution B. Solution A was prepared by mixing Na_2CO_3 (2g), KNa tartaric (0.5g), NaOH (4g) in 100 ml of distilled water. Solution B was prepared by dissolving 0.5 g of $CuSO_4 \cdot 5H_2O$ in 100 ml distilled water. After 15 min a diluted (1:9) solution of Folin indicator was added and the test tubes were stored in the dark for 30 min. The absorbance of the assay mixture was measured at 600 nm by a spectrophotometer (Varian, Cary 50) using a reagent blank as zero. Quantification of total protein content was defined by comparison with calibration curve of BSA solutions at known concentrations.

2.3.3 Free amino acids

A slightly modified method of Hanczkò was used for the determination of free amino acids [16,17]. 5 g of fresh plant material was crushed in mortar with 30 ml of 70% ethanol (v/v). Sample was stirred at ambient temperature for 24 h. After centrifugation (5000 g for 10 min), 10 ml of water were added and a liquid-liquid extraction was performed with chloroform in order to remove pigments. The aqueous phase was then passed through Dowex resin (50WX8, 200-400 mesh) in order to remove sugars. Briefly, 3 g of resin were immersed in 400 ml of distilled water for 2 h, stirred and introduced into a glass column rapidly, continuing the water flow until the column height remained stable and the outgoing water became neutral. The aqueous phase was added to the top of the column and sugars were eluted with 10 ml of water three times. Then, amino acids were eluted with 15 ml of 7 N NH_4OH and 10 ml of ultrapure water. The collected solution was lyophilized and the residue dissolved in 10 ml of borate buffer (pH 10). For derivatization, 100µl were mixed with 500 µl of derivatizing reagent prepared as follows: 250 mg of *o*-phtaldialdehyde were diluted in 5 ml methanol and then made up to 50 ml with borate buffer (100 mM, pH 10). The chromatography apparatus is equipped with binary pump (Jasco PU-880 plus, Tokyo, Japan) and fluorescence detector (Jasco FP-920, Tokyo, Japan).

The chromatographic conditions were as follows: flow 0.7 ml/min; volume of injection 20 µl; solvent A, sodium phosphate buffer (10 mM, pH 7.3): methanol: tetrahydrofurane (80:19:1) and solvent B, sodium phosphate buffer (10 mM, pH 7.3): methanol (20:80). The gradient consisted of: 100% A during 5 min, from 0 to 15% of B in 6 min, 15% B is ocratically for 5 min, from 15 to 30% of B in 5 min, from 30 to 40% of B in 4 min, and from 40 to 80% of B in 12 min. Fluorimetric detection was carried out using excitation and emission wavelengths of 340 and 426 nm, respectively.

Quantification of each amino acid was done by direct comparison of Retention time (Rt) and area with those of known concentrations of pure commercial standards. Data were expressed as mean values±SD of triplicate analysis.

2.3.4 Total phenolics

A modified method of Folin-Ciocalteu, according to Singleton [18] was used: 7 ml distilled H_2O, 0.5 ml Folin-Ciocalteu reagent and 0.5 ml of extract (or standard solution of gallic acid) were mixed. After 3 min, 2 ml of 20% Na_2CO_3were added and incubated in darkness at room temperature for 90 min. The absorbance was measured at 685 nm and the results expressed in mg of gallic acid/100 g sample. All values were uniformly expressed referring to corresponding dry extract. All measures were repeated three times and averaged.

2.3.5 Total flavonoids

The flavonoids content was estimated by the $AlCl_3$ method: 1 ml of 70% ethanol extract solution was added to 1 ml of 2% methanolic $AlCl_3.6H_2O$. The absorbance was measured 10 min later at 430 nm comparatively to a rutin standard. The results were expressed as mg rutinin grams of dry extract. All measures were repeated three times and the results were averaged [19].

2.3.6 Total flavanols content

The flavanol content was calculated as mg of (-) epicatechin/100 g sample by comparison with standard (-) epicatechin treated in the same conditions. One ml of 70% ethanolextract was added to 5 ml of 0.1% (w:v) p-dimethylaminocinnamaldehyde in methanol : HCl (3:1; v:v). The absorbance was measured 10 min later at 640 nm. All values were uniformly expressed referring to dry extract. All measures were repeated three times and averaged [20].

2.3.7 Oligomericproanthocyanidin content

The proanthocyanidin content was measured as mg of cyanidin chloride/100 g sample after heating the extracts in *n*-butanol: HCl following the method of Porter [20]: 0.5 ml of 70% ethanol extract, 6 ml of *n*-butanol: HCl (95:5; v:v) and 0.2 ml of 2% (w:v) solution of $NH_4Fe(SO_4)_2{\cdot}12H_2O$ in 2 M HCl were mixed. The tightly capped tubes were heated at 92°C in a water bath for 40 min. After cooling, the colored solutions were measured at 550 nm. All values were uniformly expressed referring to corresponding dry extract. All measures were repeated three times and averaged.

2.3.8 Separation and Identification of Flavonoids

2 g of finely triturated sample (flowers, leaves, and roots) from *S. italica* were ultrasound-assisted extracted, at room temperature, with 100 ml 70% ethanol (v/v). Each extract was filtered, concentrated, washed with chloroform (3 x 20 ml), stirred with 15 ml of 4N HCl at 85°C for 45 min, and finally extracted with ethyl acetate (3 x 20 ml). The collected organic phases were dried on Na_2SO_4 and evaporated to dryness. The residue was dissolved in methanol (2 ml), filtered through 0.45 µm membrane filter (Millipore) and analyzed by HPLC. Analysis of flavonoids was carried out on all parts of the plant using an HPLC apparatus with binary pump (Jasco PU-2080, Tokyo, Japan) equipped with a 250 x 4.6 mm (particle size 5 µ) RP C18 column and coupled to a diode array detector (Jasco MD-2010, Tokyo, Japan). Eluents were 3% aqueous acetic acid (A) and methanol (B) using three ramps (A/B 84:16 from 0 to 20 min, A/B 70:30 from 20 to 22 min, A/B 84:16 from 22 to 30 min) along the elution time. The chromatogram was obtained using a reporting integrator and the composition recorded as a percent area. Identification of chemical constituents was based

on a comparison of retention times and UV spectra with those of commercially available pure compounds. Chromatogram at λ=340 was used for luteolin and apigenin quantification.

2.4 Antioxidant Capacity

2.4.1 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity

The antioxidant activity was determined also by the DPPH radical-scavenging method according to Peterson [22]. The degree of decoloration of stable DPPH radical solution indicates the scavenging efficiency of the added sample. 0.1 ml of test solution (H_2O, EtOH 70% and EtOH extracts from different plant organs) was added to 0.9 ml of pure methanol and 4 ml of DPPH solution (final concentration of DPPH: $2.0x10^{-4}$M). Thirty minutes later, the absorbance was measured at 517 nm. A blank solution was prepared with 100 ml of solvent. The scavenging activity on DPPH radical was expressed as Trolox equivalent units.

2.4.2 Scavenging activity of 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS)

The antioxidant activity was measured by the ABTS radical decolorization assay [23]. The $ABTS^{\circ+}$ radical was produced by the reaction between 7 mM ABTS in H_2O and 2.45 mM potassium persulfate. 0.8 ml of ABTS $radical^{\circ+}$ solution was added in different concentrations of the test solution in methanol. The mixture was incubated in the dark at 37°C. After 30 min of incubation, the absorbance was measured at 734 nm using a spectrophotometer. A standard curve was obtained by using trolox standard solution at known concentrations. The degree of ABTS radical-scavenging activity of test solution (H_2O, EtOH 70% and EtOH extracts from different plant organs) was calculated, based on the trolox standard curve, and expressed as trolox equivalent antioxidant capacity (TEAC, μmoltrolox equivalents referred to the test solution of 1 mg of dry extract/ml).

2.5 Antimicrobial Activity

The EtOH 70% extract of flowers, leaves and roots was preliminarily tested for their in vitro antimicrobial activity against fungi (*Candida albicans*), Gram negative (*Pseudomonas aeruginosa*, *Escherichia coli*) and Gram positive (*Staphylococcus aureus*) bacteria. In order to avoid interference of the solvent with the microbial growth, extracts were concentrated in rotary evaporator at 40°C and the residue suspended in distilled water using dimethylsulfoxide to the initial volume. Extract were tested at 500 μg/ml on liquid medium (inoculum $2x10^4$ cells) and the antimicrobial activity was evaluated as presence/absence of turbidity [24]. 100μl from clear test tubes were sub-cultured in fresh sterile medium and after 48 h of incubation the presence/absence of turbidity was intended as bacteriostatic/bactericidal activity, respectively. A blank with solvent was performed to confirm no interferences with microbial growth. Experiments were performed in triplicate.

2.6 Pharmacological Assays

2.6.1 Cell line

Mouse myoblast cells (C2C12) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin, in

humidified atmosphere of 5% CO_2 at 37°C, until confluent. The culture medium was changed twice every week, and the cells were subcultured at 1:4 ratio, once a week.

2.6.2 Cell viability

Cell viability was determined by the reduction of yellow 3-(4,5-dimethylthaizol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma Chemical Co.) into a purple formazane product by mitochondrial dehydrogenase of metabolically active cells, as already described [25]. Briefly, cells were seeded at a density of 2.5×10^5 cells/well into a 96-well plate. After overnight growth, the cells were treated with freshly prepared medium containing the extracts at concentration ranging from 50 to 200 µg/ml. After 48 h, 20µl of MTT were added, and incubation prolonged for 4h, then plates were centrifuged (900 g for 5 min) and supernatants were removed. The pellets were dissolved in 200 µl of dimethyl sulfoxide and the absorbance read at 540 nm on a scanning multi-well spectrophotometer (Cary 50 MPR, Varian). Each experimental condition was repeated in sixteen wells and the experiments and measurements were performed in triplicate.

2.6.3 Intracellular reactive oxygen species (ROS)

The DCF-DA fluorescence dye was used to detect the ROS scavenging activity [26,27] of *Sideritis* extracts in C2C12 cells in presence/absence of pro-oxidant H_2O_2 treatment.

Cellular ROS were quantified by the 2,7-dichlorofluorescin diacetate (DCFH-DA) assay using a microplate reader. For the assay, C2C12 were plated in black 96-well at a rate of 2×10^5 cells per well. 24 h later, the medium was changed and extracts diluted in culture medium at concentrations ranging from 50 to 200 µg/ml per well was added and incubated at 37°for 48h.

Thereafter, the cells were washed 3 times with DPBS, 100µl of 1X DCFH-DA media solution were added, and the plates were incubated at 37º C for 40 min. After washings two times with DPBS, the cells were treated with 100 µl of oxidant solution (H_2O_2 0.5 mM, in DMEM) for immediate fluorescence measurement. The fluorescence intensity is proportional to the ROS levels within the cell cytosol. Plates were read for kinetic analysis in increments from 1 to 5 minutes every 30 seconds. Fluorescence was measured by Microplate Fluorometer SPECTRAmax Gemini XS (Molecular Devices, Sunnyvale, CA, USA) using excitation and emission wavelengths of 480 nm and 530 nm, respectively, and analyzed by SOFTmax Prosoftware.

2.6.4 ELISA for 8-hydroxy-2'-deoxyguanosine (8-OHdG)

Isolation and digestion of DNA: Genomic DNA from our cells was obtained using a commercially available kit (DNeasy® Blood & Tissue Kit, Quiagen Sciences, Germantown, Maryland) for silica spin column capture accordingly to manufacturer instructions.

The DNA samples were then treated with 20 U of DNase I (in 0.15 M NaCl solution, pH 5.2) for 2 h at 37°C, followed by treatment with 10 U of alkaline phosphatase in reaction buffer [containing 50 mMNaCl, 10mm Tris–HCl (pH 7.9), 10mm $MgCl_2$ and 1mm dithiothreitol (DTT)] for 1h at 37°C.

C2C12 cells were pre-treated with extracts at different concentrations (5-100 µg/ml) for 24 h. Supernatants were finally collected for the detection of 8-OHdG, using an ELISA kit (Highly

Sensitive 8-OHdG Check, Cosmo Bio Company Ltd., Tokyo, Japan), according to the manufacturers' instructions.

2.7 Statistical Analysis

Analysis of variance (ANOVA), followed by Newman-Keuls multiple comparison test was performed using Graph Pad Prism version 5.01 for Windows (Graph Pad Software, San Diego, CA). Statistical significance was set at $P<0.05$.

3. RESULTS AND DISCUSSION

3.1 Plant Characterization

The foliar ultra-structures of *S. italic* are presented in Figs. 1-4. The SEM confirms the presence of two main types of trichomes present in the green part of the plant that were glandular and non-glandular trichomes. The linear pluricellular or single cell protective trichomes are densely distributed over the entire surface leading to the tomentose aspect of the aerial parts. As seen in Fig. 1, on the upper surface of the leaf a smooth epidermis covered by simple pluricellular (2-3 cells) hairs is evident. In Fig. 2 is evidenced the presence of smaller trichomes with short peduncle and glandular head constituted by secretory cells. These are present at a lower rate, and protective hairs of tenhidden their presence. In Fig. 3 the lower epidermis appears rugged and covered by similar type of trichomes, with higher density of the protective ones. In Fig. 4 is reported a peltate glandular trichome of flower epidermis with linear stalk and basal cells connecting the head constituted by secretory cells. The presence of glandular trichomes justifies the pleasant smell of *S. italica* and the small numbers can be related to low yield in essential oil (0.05 and 0.07%, respectively for leaves and inflorescences) [5].

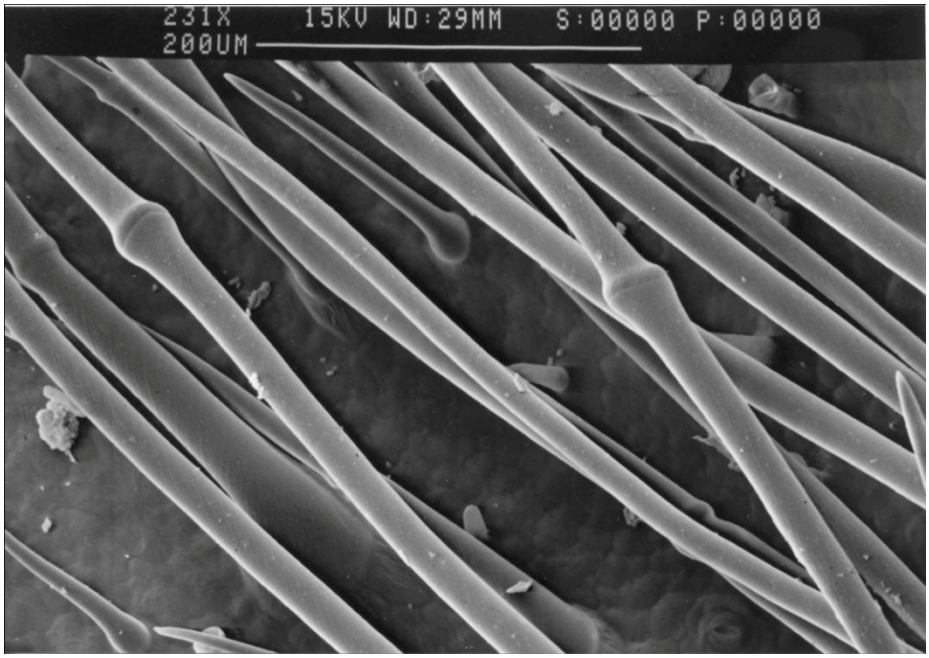

Fig. 1. SEM micrographs of upper foliar surface

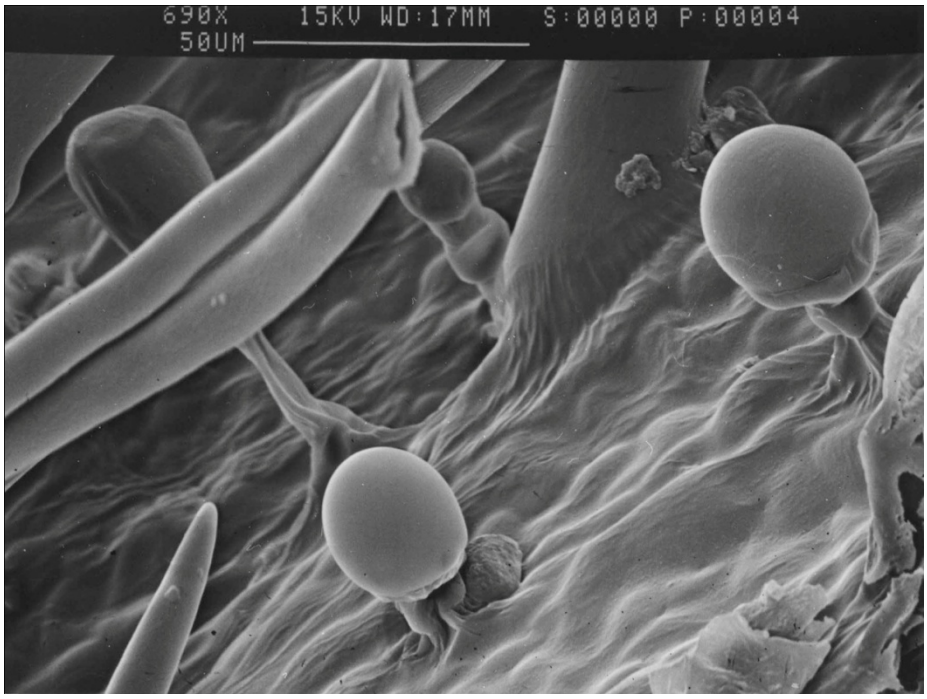

Fig. 2. SEM micrographs of upper foliar surface

Fig. 3. SEM micrographs of lower foliar surface

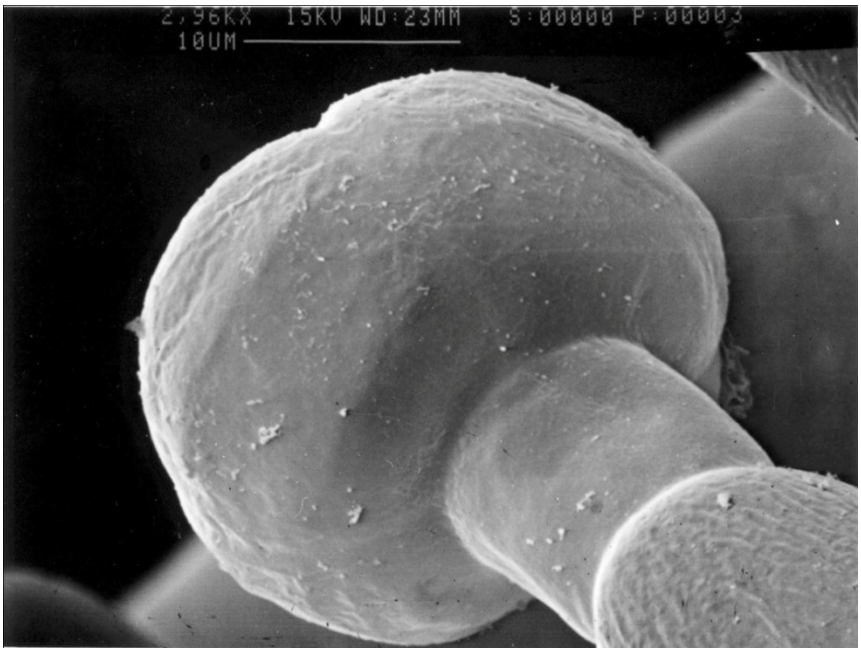

Fig. 4. SEM peltate glandular trichome on infloreresccence

3.2 Metabolites Characterization

Chlorophylls play a central role in plant metabolism considering their involvement in the photosynthetic process. Amino acids are considered precursors, in shikimate pathways, of different classes of flavonoids, so their quantification can be considered as an index of plant metabolic activity. Carotenoids and xanthophylls are present in chloroplasts as accessory pigments for photosynthesis and as protective agents against photo oxidative damage. They are also present in chromoplasts, particularly in flowers and fruits, for the vexillary function.

As reported in Table 1, distribution of all investigated pigments in aerial parts of *S. italica* are higher in leaves rather than in inflorescences. As expected, the chlorophylls are accumulated mainly in leaves. The presence of chlorophylls is related to multifactor agents, such as genetics, ecology, phenology and other biotic and abiotic interactions. In the tested materials the presence of chlorophylls a and b resulted in large variability. On the other hand, the chlorophyll a/b ratio results 2.4 and 2.9 and coherent with the physiologic range that is considered 2-3.5. The statistical analysis confirms that only the chlorophyll a content is statistically different in flower and leaf.

β-carotene is the main dietary precursor of vitamin A and is often the most abundant carotenoid in plants, together with xanthophylls with no provitamin A activity. In tested samples, despite a lack of statistical significance, the amount of β-carotene seems to be lower than lutein or at least similar to others xanthophylls, such as zeaxanthin and violaxanthin (in flowers).

The higher amount of pigments in leaves suggest that the primary function in this plant is related to photosynthesis and they are probably accumulated in chloroplasts rather than in other cell structures related to vexillary function, such as vacuole or chromoplasts.

The variation in pigment contents can be attributed to variation of lutein and β-carotene that are 4-5 fold lower in flower than in leaf, respectively. Neoxanthin showed the higher variation between leaf and flowers, but in term of absolute amount is the pigment present in lower quantity.

Table 1. Pigments composition of leaf and flower

Pigments	Leaf(µg/g of dry plant)	Flower(µg/g of dry plant)
chlorophyll a	2182±579	848±152*
chlorophyll b	737±237	354±40
Total	2920±807	1202±121*
a/b ratio	2.9	2.4
neoxanthin	7.46±4.0	1.36±1.6
violaxanthin	57.33±6.5	46.07±7.3
lutein	196.60±20.6	50.10±13.5
zeaxanthin	42.30±11.7	34.13±3.8
β-carotene	180.8±8.8	35.55±5.4

*ANOVA P<0.0001, *P<0.05, Data are mean value ±S.D of triplicate analysis.*

Amino acids and proteins are considered as primary metabolites involved in basal metabolic activity of the plant. The protein content can be related to nutritional values of different plant organs, while amino acids are protein precursor as well as metabolic intermediate in secondary metabolite synthesis, such as flavonoids.

The quantitative composition of amino acid and total proteins of leaf and flower extracts are reported in Table 2. Total amino acids result 3.262 and 2.895 µg/g of plant, respectively for leaves and inflorescences. The qualitative profile result similar, with slight difference in relative abundance that result Alanine+Tyrosine>Glutamic acid>Aspartic acid>Serine>Glutamine+Histidine for leaves and Alanine>Tyrosine>Aspartic acid>Glutamine+Histidine>Glutamic acid>Serine in flowers.

Table 2. Free amino acid and total protein composition in leaf and flower

Amino Acid	Leaf	Flower
ASP	0.493±0.25	0.363±0.25
GLU	0.498±0.15	0.273±0.18
ASN	0.104±0.08	0.135±0.03
SER	0.254±0.08	0.255±0.12
GLN+HIS	0.189±0.13	0.345±0.03
GLY+THR	0.217±0.07	0.215±0.07
ARG	0.021±0.01	0.019±0.01
ALA+TYR	0.857±0.32	0.729±0.36
CYS	0.017±0.01	0.045±0.02
TRP	0.043±0.01	0.094±0.03
MET	0.027±0.02	0.019±0.02
VAL	0.158±0.07	0.098±0.03
PHE	0.094±0.05	0.113±0.05
ILE	0.111±0.04	0.059±0.01
LEU	0.149±0.06	0.121±0.05
LYS	0.030±0.01	0.012±0.01
TOTAL PROTEIN µg/g calculated as BSA	606.7±141.9	1095.0±229.6

ANOVA evidenced no statistical difference between plant organs, Data are reported as mean values ±S.D.

Phenylalanine, that is directly related to the flavonoids biosynthesis [28], result in higher amount in flowers, in accordance with phytochemical data of total flavonoids. Notable also is the presence of tyrosine and tryptophan that are intermediate of metabolic pathways for large number of aromatic secondary metabolites.

3.3 Phytochemical Investigation

In Table 3 are reported results of phytochemical investigations. The solvent used for extractions strongly influences the quality of final product. The best solvent for polyphenols extraction results the hydro alcoholic solution that give the highest values, in accordance to bibliographic data [3]. In all extracts the flavonoid fraction is the most abundant and result due to apigenin-related aglycone. The quantitative distribution indicates leaves as the most useful part of the plants.

Table 3. Phytochemical investigation

	Extract	Flower	Leaf	Root
Total polyphenols	H_2O	9.23±1.02	6.76±0.81	5.47±0.96
mg/g d.e. as gallic acid	EtOH	13.33±1.63	9.15±1.24	11.20±1.93
	EtOH 70%	17.50±2.15	20.10±2.04	21.58±1.74
Total flavonoids	H_2O	0.99±0.03	0.90±0.07	0.18±0.06
mg/g d.e. as rutin	EtOH	2.44±0.09	3.22±0.31	0.39±0.07
	EtOH 70%	3.51±0.09	3.71±0.81	1.15±0.30
Total flavonols	H_2O	0.08±0.02	0.10±0.05	0.10±0.09
mg/g d.e. epicatechin	EtOH	0.07±0.01	n.d.	0.09±0.02
	EtOH 70%	0.14±0.08	0.43±0.11	0.08±0.02
Oligomer proanthocyanidins	H_2O	0.67±0.13	1.00±0.09	0.07±0.02
mg/g d.e. as cyanidinechloridrate	EtOH	1.05±0.11	0.18±0.07	0.09±0.03
	EtOH 70%	1.54±0.09	2.51±0.90	0.11±0.06
Luteolin (mg/gd.p.)	EtOH 70%	0.374±0.03	0.522±0.31	0.174±0.22
Apigenin (mg/g d.p.)	EtOH 70%	1.325±0.11	2.344±0.23	0.256±0.09

Data are mean value ±S.D. of triplicate analysis.

In Table 4 are reported data of the the antioxidant capacity of extracts obtained from different plant organs. The DPPH assay reveal that the activity is influenced by solvent used for extraction. For all plant organs, the EtOH 70% extract show the strongest activity. The root result the organ more active, with value that are always lower than those of other organs. Also in the values of TEAC the 70% ethanol is the better solvent for extraction, followed by H_2O and EtOH. Considering the plant organ, the flowers are the most active, followed by leaf and root.

Table 4. Antioxidant capacity

	Extract	Flower	Leaf	Root
DPPH				
mg of extract giving the activity	H_2O	28.5±4.1	40.4±6.5	13.5±2.8
of 1mg of Trolox	EtOH	15.0±2.2	12.3±2.1	3.4±0.5
	EtOH 70%	3.5±0.6	9.4±1.5	1.1±0.3
ABTS				
	H_2O	86.6±9.2	89.1±5.3	72.4±6.2
TEAC	EtOH	68.0±5.3	57.6±4.4	43.8±4.1
µmoltrolox eq.	EtOH 70%	139.8±8.4	114.6±6.1	95.3±6.2
(test solution 1 mg d.e./ml)				

Data are mean value ±S.D. of triplicate analysis.

3.4 Antimicrobial

The in vitro antimicrobial activity of the EtOH 70% extracts of *S. italic* was assayed in the broth dilution method [24] and is reported in Table 5, expressed as presence/absence of strain growth inoculated in medium with 500 µg/ml of tested extracts.

All microrganisms were sensitive to aerial part extracts, but only against *P. aeruginosa* and *C. albicans* the effect can be considered bactericide. At the tested doses extracts seem interfere with *S. aureus* and *E. coli*, with only a bacteriostatic activity. No antimicrobial activity was detected for root extract.

The experimental evidences are partially confirmed in bibliographic literature, where similar antimicrobial activity is reported only for other *Sideritis* species.

Table 5. Antimicrobial activity

		Turbidity			Sub-culture turbidity		
	Extracts (500 µg/ml)	**flower**	**leaf**	**root**	**flower**	**leaf**	**root**
Gram +	*Staphylococcus aureus*	-	-	+	+	+	+
Gram -	*Pseudomonas aeruginosa*	-	-	+	-	-	+
	Escherichia coli	-	-	+	+	+	+
Fungi	*Candida albicans*	-	-	+	-	-	+

Data are reported as presence/absence of turbidity in the culture medium (+/-, respectively).

3.5 Pharmacological Assays

3.5.1 Cell viability

Extracts were preliminary tested on MTT in order to evaluate cytotoxic effects before test the potential as modulator agent of cellular metabolism. Statistical analisys (One-way ANOVA) of the results obtained from the MTT assay (not shown) did not evidenced significant difference in cell viability between the extract-treated and the untreated groups. These results revealed that there was no toxicity of the extracts on C2C12 cell line at concentration up to 200 µg/ml.

3.5.2 Reactive oxygen species

DCFH-DA is a useful indicator of reactive oxygen species (ROS), in order to determine the antioxidant properties of molecules as well as phytocomplexes. DCFH-DA is a non-fluorescent ester dye that penetrates the cells and is hydrolyzed by intracellular esterases to the DCFH, which can be rapidly oxidized to the highly fluorescent 2,7-dichlorofluorescein (DCF) in presence of ROS.

The DCF-DA fluorescence dye was used to detect the ROS scavenging activity of *Sideritis* extracts in C2C12 cells. In Fig. 5 are reported the effects on ROS production in cells incubated in presence/absence (untreated cells) of different solvent extracts obtained from flower, leaf and root. At higher doses (200 µg/ml) only the aqueous extract of leaf, and the ethanol extracts from flower and leaf significantly reduce the ROS production compared to control untreated group. The hydro-alcoholic solvent strongly influences the quality of the extract, as revealed by a significant reduction of ROS for all plant organs. The ethanol 70% extract of leaf result the most active in modulation of ROS production, that results reduced at all tested doses.

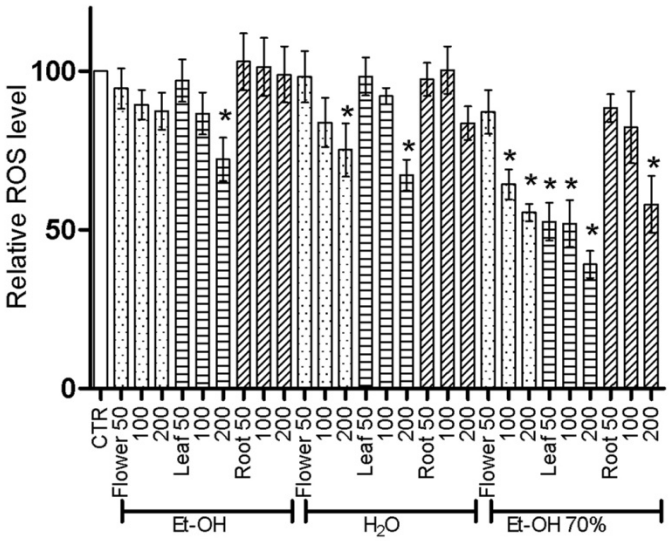

Fig. 5. Effect on ROS production in C2C12 cells after incubation in presence/absence of extracts

*ANOVA P< .001, *P< .05 vs untreated*

The extracts were also investigated for their protective effects against oxidative damage induced by exogenous stimulation with hydrogen peroxide. Apart for the ethanol extract from root at 50 µg/ml, the supplementation of all solvent extracts, from all organs, at all tested doses, reduce significantly the increased ROS production induced by hydrogen peroxide (at sub-toxic dose).

The more polar extracts (water and ethanol 70%) resulted more efficient in contrasting the pro-oxidant effects of the exogenous stimulus, as demonstrated by the quantitatively lower amount of induced ROS production. Also with a similar activity profile, data seems indicate the hydro alcoholic extract as the most active for intracellular ROS scavenging activity,

particularly those from the leaves that significantly reduce the basal ROS production at all tested doses (Fig. 6).

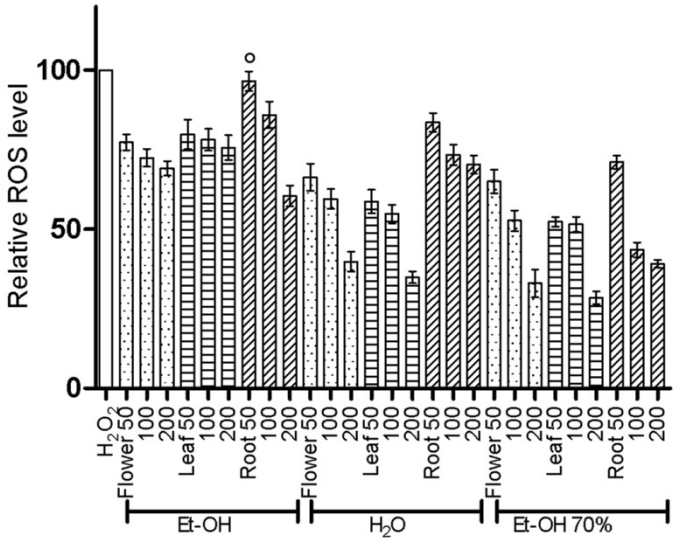

Fig. 6. Effect of hydrogen peroxide on C2C12 ROS production after incubation in presence/absence of extracts

ANOVA P< .001, Except°, all samples significantly differ for P< .05 vs untreated

3.5.3 Protective effect of extracts on 8-OHdG, a marker DNA damage

There is extensive experimental evidence that oxidative damage permanently occurs to lipids of cellular membranes, proteins, and DNA.

In nuclear and mitochondrial DNA, 8-hydroxy-2'-deoxyguanosine (8-OHdG) is one of the predominant forms of free radical-induced oxidative lesions, and has therefore been widely used as a biomarker for oxidative stress and carcinogenesis.

It can be used as biomarker to estimate the DNA damage induced by free radical metabolism. As biomarker has been used to estimate the DNA damage in humans after exposure to cancer-causing agents, such as tobacco smoke, asbestos fibers, heavy metals, and polycyclic aromatic hydrocarbons. In recent years, 8-OHdG has been used widely in many studies not only as a biomarker for the measurement of endogenous oxidative DNA damage but also as a risk factor for many diseases including cancer.

The aqueous extract was found to strongly reduce the profuse affliction to mtDNA by ROS generation. At all tested doses, the extract induces a significant reduction of DNA damage caused by hydrogen peroxide. Furthermore, the presence of extracts (20 µg/ml) in culture medium also determines a biomarker reduction compared to untreated groups, suggesting a potential role as preventive agent that could hinder the effects of exogenous oxidative stress on DNA. The relative mtDNA damage reduction (as observed by 8-OHdG analysis) induced by extract in basal and oxidative stress induced conditions are reported in Fig. 7.

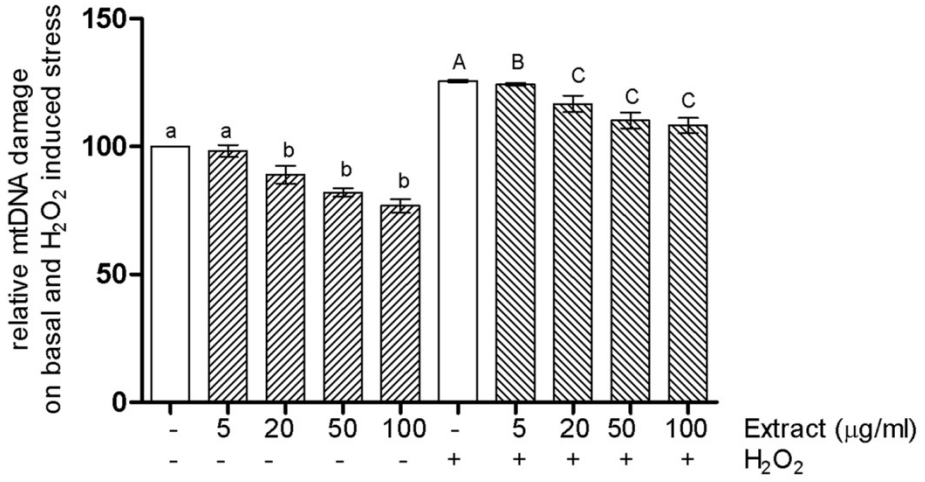

Fig. 7. Effect on 8-OHdG, a marker DNA damage, of extract on C2C12 cells in presence/absence of oxidative stimulus.

ANOVA P< .001, P< .05. Different letters indicate effects that statistically differ in basal or H_2O_2 group (lower case or upper case, respectively)

4. CONCLUSION

The genus *Sideritis* is a promising source of chemicals with interesting biological activities that can justify the use in traditional medicines as therapeutic agent.

In the present paper, we reported further chemical investigations on primary and secondary plant metabolites as well as in vitro pharmacological investigations of different plant part extracts, in order to evaluate the role as modulator of oxidative stress.

The extracts resulted not cytotoxic, as established by the MTT assay. The absence of any interferences in cell viability suggested us to further test two parameters related to the protection of cells against degenerative stress. In particular, we evaluated the production of ROS and 8-hydroxy-2'-deoxyguanosine that is a marker of DNA damage.

The hydroalcoholic extract resulted the most effective in protecting the cells from ROS. The protective effect is evident for basal conditions and after oxidative stress induced by H_2O_2.

Our data also suggest an important protective effect on DNA damage induced by free radicals, as indicated by the lower amount of 8-OHdG.

Sideritis extracts reduce the basal levels of ROS and hinder the increased production induced by the exogenous oxidant stimulus. We can speculate that the antiradical activity exert by the extracts could be the principal mechanism related to the prevention of the oxidative DNA damage.

Finally, the chemical investigation on secondary metabolites revealed that the highest amount are present in flower and leaf extracts. These data could justify, at least in part, the rationale of popular medicine that suggests collecting the blooming aerial parts to prepare traditional remedy.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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