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ORIGINAL CONTRIBUTION

Phytochemical composition of "mountain tea" from *Sideritis clandestina* subsp. *clandestina* and evaluation of its behavioral and oxidant/antioxidant effects on adult mice

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Abstract
Purpose The goals of this study were to monitor the effect of drinking of herbal tea from *Sideritis clandestina* subsp. *clandestina* for 6 weeks on behavioral and oxidant/antioxidant parameters of adult male mice and also to evaluate its phytochemical composition.
Methods The phytochemical profile of the *Sideritis* tea was determined by liquid chromatography-UV diode array coupled to ion-trap mass spectrometry with electrospray ionization interface. The effects of two doses of the herbal infusion (2 and 4% w/v, daily) intake on anxiety-like state in mice were studied by the assessment of their thigmotactic behavior. The oxidant/antioxidant status of brain (-Ce), liver and heart of adult male Balb-c mice following the consumption of *Sideritis* tea was also evaluated via the measurement of malondialdehyde (MDA) and reduced glutathione (GSH) levels using fluorometric assays. Our study was further extended to determine the antioxidant effects of the herbal tea on specific brain regions (cerebral cortex, cerebellum and midbrain).
Results The identified compounds were classified into several natural product classes: quinic acid derivatives, iridoids, phenylethanol glycosides and flavonoids. Our results showed that only the 4% *Sideritis* tea exhibited anxiolytic-like properties as evidenced by statistically significant ($p < 0.05$) decrease in the thigmotaxis time and increase in the number of entries to the central zone in comparison with the control group. Consumption of both tea doses (2 and 4% w/v) elevated GSH (12 and 28%, respectively, $p < 0.05$) and decreased MDA (16 and 29%, $p < 0.05$) levels in brain (-Ce), while liver and heart remained unaffected. In regard to the effect of herbal tea drinking (2 and 4% w/v) on specific brain regions, it caused a significant increase in GSH of cerebellum (13 and 36%, respectively, $p < 0.05$) and midbrain (17 and 36%, $p < 0.05$). Similarly, MDA levels were decreased in cerebellum (45 and 79%, respectively, $p < 0.05$) and midbrain (50 and 63%, respectively, $p < 0.05$), whereas cerebral cortex remained unaffected.
Conclusions Mountain tea drinking prevents anxiety-related behaviors and confers antioxidant protection to rodent's tissues in a region-specific, dose-dependent manner, and its phytochemical constituents are shown for the first time.

Keywords LC-MS/MS · Mountain tea · *Sideritis clandestina* subsp. *clandestina* · Oxidant/antioxidant parameters · Thigmotaxis test

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Abbreviations

MDA	Malondialdehyde
GSH	Reduced glutathione
TBA	Thiobarbituric acid
BHT	Butylated hydroxytoluene
TCA	Trichloroacetic acid
LC/DAD/ESI-MS^n	Liquid chromatography/diode array detection/electrospray ion-trap tandem mass spectrometry
NMR	Nuclear magnetic resonance

Introduction

The neurotoxic effects of oxidative stress and the consequent neurodegeneration in specific brain areas have been proposed as causal factors in Alzheimer's disease, Parkinsonism and aging process [1] The brain is the most susceptible organ to oxidative damage due to its high oxygen demand, high lipid content, especially polyunsaturated fatty acids, the abundance of redox-active transition metal ions, the low activity of antioxidant defense system and the reduced capacity for cellular regeneration [1]. Polyphenolic compounds supplied by nutritional sources [2] such as fruits, vegetables, wine and tea have displayed a wide range of beneficial biological actions [3], many of which have been attributed to their strong antioxidant properties [4].

Anxiety is a psychological and physiological state characterized by somatic, emotional, cognitive and behavioral components. Recent studies demonstrate a link between oxidative stress and anxiety-related behavior [5]. Some of these studies suggest that oxidative stress causes anxiety-related behaviors in humans with panic disorders and especially that oxidative metabolism can affect the regulation of anxiety [6], and others support a cause–effect relationship in humans and animal models but do not explain the underlying mechanisms [7].

The genus *Sideritis* (Lamiaceae) comprises about 140 species distributed in several countries of the Mediterranean region [8]. *Sideritis* species have been reported to present an array of biological activities, including anti-inflammatory and antinociceptive [9], antimicrobial [10] and antiulcer [11] activities. Interestingly, aqueous extracts derived from *Sideritis* species have been proposed to form the basis to design "functional foods" for the prevention of osteoporosis [12]. Furthermore, in vitro studies have shown moderate antioxidant activity for *S. javalambrensis*, *S. raeseri*, *S. euboea*, *S. perfoliata* subsps. *perfoliata* and *S. libanotica* ssp. *linearis* that was mainly attributed to flavonoids [13–17]. Rats drinking a tea from *S. caesarea* for 50 days were protected against chemical-induced oxidative injury [18]. We have previously found that consumption of a herbal tea from a different *Sideritis* taxon (*clandestina* subsp. *peloponnesiaca*) enhanced the antioxidant defense of the adult rodent brain in a region-specific manner; however, the phytochemical composition remained unknown [19].

The aims of the present study are (1) to characterize the composition of the aqueous extract of the herbal tea from *Sideritis clandestina* subsp. *clandestina* using LC/DAD/ESI-MS^n analysis, (2) to evaluate the effect of a 6-week consumption of 2 and 4% w/v of a herbal tea (commonly consumed tea doses) from the same taxon on the levels of anxiety and (3) to determine the oxidant/antioxidant activity of peripheral and brain tissues of adult mice by measuring malondialdehyde (MDA, index of lipid peroxidation) and reduced glutathione (GSH). This is the first study on the phytochemical composition of *S. clandestina* subs. *clandestina* ever, the first study on the composition of the aqueous and not organic solvent extract of the herbal tea from this taxon and one of the few studies to investigate its antioxidant effects in vivo after a period of drinking. With regard to the latter, we aimed to investigate whether the beneficiary effects, recorded after the consumption of another *Sideritis* taxon [19], would present a similar pattern or are taxon specific and whether these are dose dependent.

Materials and methods

Plant material, reagents and standards

The plant *Sideritis clandestina* subsp. *clandestina* used in the study was collected from Mainalo mountain (Central Peloponnese) between July and August (2008). Taxonomic identification of the plant material was carried out in the Division of Plant Biology, Department of Biology, University of Patras, by Prof. G. Iatrou, and a voucher specimen has been deposited in the local Herbarium.

MDA, GSH, TBA, BHT, TCA, *o*-phthalaldehyde and Chromasolv Plus acetonitrile and water for HPLC (99.9%) were obtained from Sigma–Aldrich. Analytical-grade acetic acid was provided by Merck (Darmstadt, Germany), and quinic acid (98%) was obtained from Aldrich (Steinhem, Germany).

Sample preparation and extraction

All parts of dry material (flowers, leaves and stems) in equal quantities were used for the extraction. For the preparation of the tea infusions, 2 or 4 g of leaves, flowers and stems of *Sideritis* taxa was extracted with 100 mL of boiling water for 5 min, in order to simulate actual brewing

conditions for tea consumed by human adults, and filtered after cooling. The filtrate was made to the volume of 100 mL. For the animal studies, the infusions were prepared daily. For the characterization of the phytochemical composition of the herbal tea, it was lyophilized and a residue (nice green powder) of 0.294 g (for the 2 g) was obtained.

LC-MS analysis

To evaluate the phytochemical composition of the aqueous *Sideritis* extract, we used liquid chromatography-UV diode array coupled to ion-trap mass spectrometry with electrospray ionization interface (LC/DAD/ESI-MS^n). All LC-MS^n experiments were performed on a quadrupole ion-trap mass analyzer (Agilent Technologies, model MSD trap SL) retrofitted to a 1,100 binary HPLC system equipped with a degasser, autosampler, diode array detector and electrospray ionization source (Agilent Technologies, Karlsruhe, Germany). All hardware components were controlled by Agilent Chemstation Software.

The *Sideritis* extract was dissolved in acetonitrile–water 50–50% to the desired concentration (4 mg mL^{-1} of extract). A 20-μL aliquot was filtered (0.45 μm) and injected into the LC-MS instrument. Separation was achieved on a 25 cm × 4.6 mm i.d., 5 μm Altima C18 analytical column (Alltech, Deerfield, USA), at a flow rate of 0.6 mL min^{-1}, using solvent A (water/acetic acid, 99.9: 0.1 v/v) and solvent B (acetonitrile). The gradient used in this experiment was as follows: 0–5 min, 95–92% A; 5–15 min 92% A; 15–20 min 92–75% A; 20–40 min 75–50% A; 40–50 min 50–20% A; 50–60 min 20–95% A. The UV/Vis spectra were recorded in the range of 200–400 nm, and chromatograms were acquired at 254, 280 and 330 nm.

Both precursor and product (MS^2 and MS^3) ions scanning of the phytochemicals were monitored between *m/z* 50 and *m/z* 1,000 in negative polarity. The ionization source conditions were as follows: capillary voltage, 3.5 kV; drying gas temperature, 350 °C; nitrogen flow and pressure, 12 L min^{-1} and 12 psi, respectively. Maximum accumulation time of ion trap and the number of MS repetitions to obtain the MS average spectra were set at 30 ms and 3, respectively.

NMR analysis

Nuclear magnetic resonance (NMR) experiments were performed at 298 K on a Bruker AV-500 spectrometer equipped with a TXI cryoprobe (Bruker BioSpin, Rheinstetten, Germany). Samples were dissolved in 0.5 mL DMSO-d_6 and transferred to 5-mm NMR tubes. The NMR system was controlled by the software TopSpin 2.1. Assignment of compounds present in the extract was determined on the basis of 2D ^1H–^1H COSY, ^1H–^{13}C HSQC and HMBC spectra. Relevant compound quantification was performed on the basis of the recorded integrals of resonance absorptions that are characteristic for specific compounds in the 1D ^1H-NMR spectrum of the extract.

Animals

Male, 3- to 4-month-old *Balb-c* mice (25–30 g BW) were kept in polyacrylic cages (38 cm × 23 cm × 10 cm) (8 per cage) under constant temperature (23–25 °C) and relative 50–60% humidity, with alternating 12-h light and dark cycles and ad libitum access to food. The mice were randomly divided into three groups consisting of sixteen animals each. Group I mice ($n = 16$) served as controls and received water ad libitum. Group II and III mice ($n = 16$/group) had ad libitum access to the tea from *S. clandestina* subsp. *clandestina* 2 and 4% (w/v), respectively, for a period of 6 weeks. Each of the above groups was divided into two subgroups ($n = 8$/subgroup), which were used for the isolation of a. brain, liver, heart and b. specific brain tissues (cerebral cortex, cerebellum and midbrain), respectively. The body weight, food and water or infusion intake were measured weekly in all the experimental groups throughout the treatment period. All procedures were in accordance with the Greek National Laws (Animal Act, PD 160/91).

Thigmotaxis test

At the end of the treatment period, animals were submitted to the open-field behavioral thigmotaxis test in order to evaluate the effects of herbal tea consumption on anxiety-related responses [20]. Thigmotaxis (walking close the walls of the apparatus), which was assessed by the time that mice spent close to the walls (<8 cm away from the walls), and the number of entries in the central zone of the open field are considered as an index of anxiety. All training and testing sessions were carried out during the light phase between 08:00 and 14:00 a.m.

Tissue preparation

One day after the behavioral test, all mice were killed by light ether anesthesia and brain (-Cerebellum) [referred as brain (-Ce)], liver, heart and specific brain regions (cerebral cortex, cerebellum and midbrain) were rapidly removed, weighted and stored at −80 °C until use. All tissues were homogenized (10% w/v) using a glass-Teflon homogenizer (Thomas, Philadelphia, USA, No B 13957) in ice-cold 30 mM Na_2HPO_4, pH 7.6. The homogenates were then centrifuged for 20 min at 15.000*g* at 4 °C in a Heal Force,

Neofuge 13R centrifuge. Supernatants were collected and used for the determination of GSH and MDA levels. Protein levels were evaluated using the Bradford assay.

Determination of MDA

MDA, one of the better-known secondary products of lipid peroxidation, was determined fluorometrically after reaction with thiobarbituric acid (TBA), as previously described [21]. The assay showed good linearity (0.05–10 μM, $y = 6.6034 \times -0.5959$, $R^2 = 0.9993$) and sensitivity. The level of MDA was expressed as μmol/g of tissue protein. All determinations were carried out at least three times and in triplicates.

Determination of GSH

GSH content of the brain and peripheral tissues was estimated fluorometrically after reaction with *o*-phthalaldehyde, as previously described [22]. A good linearity was obtained for GSH in the range of 0.5–100 μM ($y = 1.2353 \times -3.46$, $R^2 = 0.9969$). The levels of GSH were expressed as μmol/g of tissue protein. All determinations were carried out at least three times and in triplicates.

Statistical analysis

The results are expressed as mean ± SE. Statistical analysis was performed with the GraphPad Instat 3 software using the nonparametric Mann–Whitney test for evaluating statistically significant differences ($p < 0.05$) of each treated group from control and between the two treated groups.

Results

Phytochemical profile and identification of the main constituents of aqueous *Sideritis* extract

The total ion chromatogram of the investigated extract is illustrated in Fig. 1. The LC/DAD/ESI-MS^n analysis of the aqueous extract led to the separation and identification of the majority of the extract components, seventeen (17) in total, that belong to several classes of phytochemicals: quinic acid, melittoside, phenylpropanoids and flavonoid derivatives. Specifically, two quinic acid derivatives along with two melittoside derivatives, two phenylethanoid glycosides (*β*-hydroxyverbascoside or *β*-hydroxyisoverbascoside is described for the first time in *Sideritis* species) and one flavonoid 7-*O*-diglycoside were identified along with six acetylated flavonoid 7-*O*-diglycosides of apigenin and isoscutellarein and four isomers of apigenin 7-*O*-(coumaroyl)glucopyranoside (See Table 1 for a detailed list of compounds and Figures S1–S9 for representative MS spectra of selected compounds).

The identification of the compounds was based on their fragmentation pattern as has been documented in the literature [23–29]. On the basis of this methodology, we were not only able to determine the presence of well-known phenolic compounds in the extract, but also able to identify the two iridoid glycosides and quinic acid derivatives that could not be attributed to any known compound.

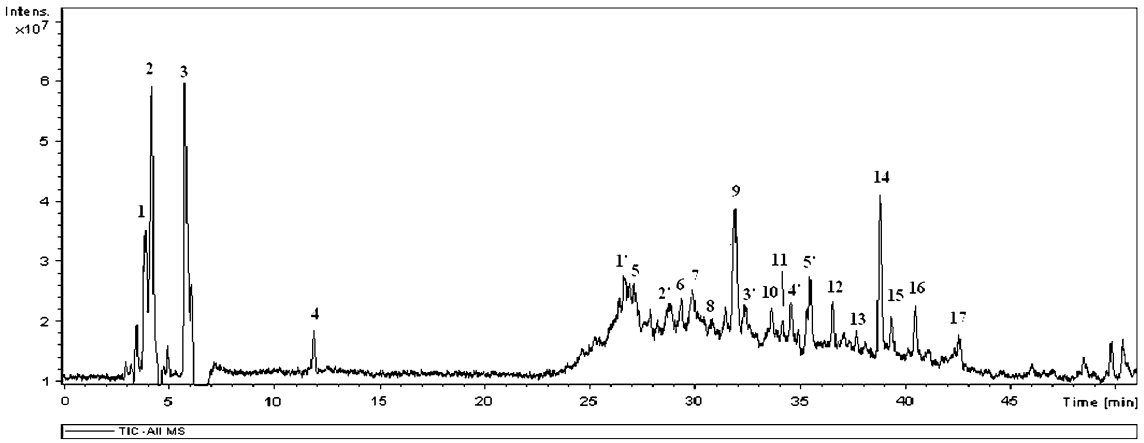

Fig. 1 Total ion chromatogram of *Sideritis clandestina* aqueous extract *1* Quinic acid derivative, *2* Quinic acid derivative, *3* Melittoside derivative, *4* Melittoside derivative, *5* *β*-Hydroxyverbascoside or *β*-Hydroxyisoverbascoside, *6* Apigenin 7-*O*-allosyl(1 → 2)glucoside, *7* Isoscutelarein 7-*O*-[6‴-*O*-acetyl]-allosyl(1 → 2)glucoside, *8* Apigenin 7-*O*-[6‴-*O*-acetyl]-allosyl(1 → 2)glucoside, *9* Apigenin 7-*O*-[6″-*O*-acetyl]-allosyl(1 → 2)glucoside, *10* Martynoside, *11* Isoscutellarein 7-*O*-[2‴,6‴-*O*-diacetyl]-allosyl(1 → 2)glucoside, *12* Apigenin 7-*O*-[2‴,6‴-*O*-diacetyl]-allosyl(1 → 2)glucoside, *13* Apigenin 7-*O*-acetyl-coumaroyl-allosyl(1 → 2)glucoside, *14–17* Apigenin 7-*O*- (coumaroyl)glucopyranoside isomers

Table 1 Compounds identified in *Sideritis clandestina* aqueous extract

Peak	R_t (min)	$[M–H]^-$ (*m/z*)	$–MS^2$ $[M–H]^-$ (*m/z*) (%)	$–MS^3$ [base peak]$^-$ (*m/z*) (%)	Compounds
1	4.2	421	191 (100)	389 (100), 127 (17), 173 (14)	Quinic acid derivative
2	4.2	533	191 (100)	173 (100), 127 (64), 150 (67)	Quinic acid derivative
3	5.9	654, 583, 523	523 (100), 179(6), 565 (8)	179 (100), 361 (30), 343 (24), 463 (11)	Melittoside derivative
4	11.9	583, 523, 665	523 (100), 565 (17), 179 (8)	179 (100), 343 (29), 361 (24), 463 (14)	Melittoside derivative
1′	26.7	449	389 (100), 167 (16), 329 (11)	329 (100), 161 (30), 179 (15), 251 (11)	Unknown
5	27.1	639	621 (100), 459 (8)	459 (100), 179 (13)	*β*-Hydroxyverbascoside or *β*-hydroxyisoverbascoside
2′	28.8	579	339 (100), 327 (44)	324 (100), 161 (30), 179 (15)	Unknown
6	29.3	593	269 (100), 431 (21)	269 (100)	Apigenin 7-*O*-allosyl(1 → 2) glucoside,
7	29.8	651	609 (100), 285 (4)	285 (100), 447 (28)	Isoscutelarein 7-*O*-[6‴-*O*-acetyl]-allosyl(1 → 2)glucoside
8	30.7	635	593 (100), 269 (7)	269 (100), 431 (24)	Apigenin 7-*O*-[6‴-*O*-acetyl]-allosyl(1 → 2)glucoside
9	31.9	431, 635	269 (100), 593 (51), 515 (8), 431 (7)	269 (100), 431 (26)	Apigenin 7-*O*-[6″-*O*-acetyl]-allosyl(1 → 2)glucoside
3′	32.1	783, 665	607 (100), 651 (53), 737 (41)	329 (100)	Unknown
10	33.6	651	475 (100), 505 (31), 457 (18)	161 (100), 329 (84)	Martynoside
11	34.1	693	651 (100), 633 (59)	285 (100), 489 (58), 609 (14)	Isoscutellarein 7-*O*-[2‴, 6‴-*O*-diacetyl]-allosyl(1 → 2) glucoside
4′	34.5	595	269 (100), 474 974), 513 (19)	269 (100)	Unknown
5′	35.4	733	698 (100), 714 (92), 634 (39)	714 (100), 673 (59)	Unknown
12	36.5	677	635 (100), 269 (63)	269 (100), 593 (31), 473 (67)	Apigenin 7-*O*-[2‴, 6‴-*O*-diacetyl]-allosyl(1 → 2) glucoside
13	37.6	781	739 (100), 721 (69), 269 (53), 635 (40), 575 (12)	269 (100), 593 (64), 577 (16), 431 (14)	Apigenin 7-*O*-acetyl-coumaroyl-allosyl(1 → 2)glucoside
14	38.7	577	269 (100), 431 (1), 413 (1)	225 (100), 269 (71), 149 (59), 117 (26)	Apigenin 7-*O*-(coumaroyl) glucopyranoside
15	39.2	577	269 (100)	225 (60), 269 (100), 149 (43)	Apigenin 7-*O*-(coumaroyl) glucopyranoside
16	40.4	577	269 (100)	225 (100), 269 (64), 149 (43)	Apigenin 7-*O*-(coumaroyl) glucopyranoside
17	42.5	577	269 (100), 431 (15), 413 (15)	225 (42), 269 (43), 149 (100)	Apigenin 7-*O*-(coumaroyl) glucopyranoside

Specifically, the unknown iridoid glycosides were compounds 3 and 4 (relevant peak fractions in Fig. 1). Compound 3 gave deprotonated molecular ions $[M–H]^-$ at *m/z* 654 (100%), 583 (90%), 613 (76%) and 523 (67%) (Figure S3). The fragmentation at *m/z* 583 showed the presence of one main fragment at *m/z* 523 (100%), 565 (8%) and 179 (6%). The MS^3 spectra of the ion at *m/z* 523 gave ions at *m/z* 179 (100%), 361 (30%), 343 (24%), 463 (11%) and 161 (11%). The ion at *m/z* 523 could belong to melittoside, and compound 3 could be attributed to a melittoside derivative. In the MS^3 spectra, the ion at *m/z* 361 corresponds to the loss of 162 amu (glucose) and the ion at *m/z* 179 corresponds to the loss of 182 amu, which are characteristic neutral losses for iridoid glycosides [25]. Melittoside has been isolated from different species of genus *Sideritis* [30], while recently a new melittoside derivative was isolated from an extract of the species *Sideritis lanata L.* [31]. Similarly, compound 4 eluting at 11.9 min with isocratic conditions gave deprotonated molecular ions $[M–H]^-$ at *m/z* 583 (100%) and 523 (64%) (Figure S4). The fragmentation at *m/z* 583 showed the presence of one main fragment at *m/z* 523 (100%), 565 (17%) and 179 (8%) again, while the MS^3 spectra of the ion at *m/z* 523 gave ions at *m/z* 179 (100%), 343 (29%), 361 (25%), 463 (14%), 439 (10%) and 161 (9%). Compounds 3 and 4 must have related structures, as they show similar product ions.

In order to ascertain the structure of compound 3, as it was the main constituent in the extract and since there is lack of available literature data regarding the fragmentation pattern of melittoside and its derivatives, we isolate it

through preparative liquid chromatography. NMR investigation (2D ^{1}H–^{1}H COSY, ^{1}H–^{13}C HSQC and ^{1}H–^{13}C HMBC) of the isolated compound confirmed that the base configuration was melittoside (Table S1), in accordance with literature data [32]. The full assignment is not completed due to extensive resonance overlapping problems.

Furthermore, the compound apigenin 7-*O*-acetyl-coumaroyl-allosyl(1 → 2)glucoside (compound 13) was identified and was characterized in the extract for the first time. The deprotonated molecular ion $[M–H]^-$ of compound 13 was detected at *m/z* 781 (Figure S9). After MS^2 of the compound, the base peak at *m/z* 739 (100%) was detected corresponding to the loss of an acetyl group, along with peaks at *m/z* 721 (69%), 269 (53%), 635 (40%) and 575 (12%). The MS^3 spectra of the ion at *m/z* 721 yielded the ion at *m/z* 269 (100%), 593 (64%), 577 (16%) and 431 (14%). The peak at *m/z* 593 was probably derived from the loss of a coumaroyl or rhamnosyl moiety (146 amu). The fragment at *m/z* 431 (14%) related to $[M–H-308]^-$ was derived from the loss of a glucose esterified with coumaric acid. The base peak $[(M–H)-324]^-$ that was detected indicated an *O*-glycosylation on a phenolic hydroxyl with a dihexoside ($[Y_0^7]^-$) [35]. The occurrence of loss of 162 amu (*m/z* 577, 16%) was indicative of 1 → 2 glycosylation between both sugars. This compound could be tentatively characterized as apigenin 7-*O*-acetyl-coumaroyl-allosyl(1 → 2)glucoside, and it is detected for the first time. Among the aforementioned compounds, the main constituents in the extract were found to be melittoside and quinic acid derivatives (Fig. 1). This was evident from the relevant integrals of characteristic resonance absorptions for the specific compounds in the ^{1}H-NMR spectrum of the crude herbal tea (Figure S10).

Evaluation of the anxiolytic-like effects of *Sideritis* infusion intake on adult mice

Due to the well-studied beneficial effects in human health of the basic structures of the main phytochemical constituents revealed in the studied extract, we could hypothesize that the *Sideritis clandestina* subsp. *clandestina* extract could also confer anxiolytic behavioral effects and also antioxidant defence in brain. Figure 2a and b illustrates the measurement of the thigmotaxis time and the number of central entries to the open field at 5-min time intervals during the 30-min testing session (except of the first 5 min). From this, it became evident that mice receiving 4% (w/v) herbal tea displayed statistically significant decrease (10–13%) in the thigmotaxis time and increase (70–180%) in the number of entries in the central zone compared with the control group. Similar but not statistically significant alterations in the above parameters were observed in *Sideritis* 2% (w/v)-treated group in comparison with the controls. The first 5 min of the testing session in both treated animal groups displayed no difference in the values of the above parameters when compared with the control group, as this time interval is a period of accommodation [20].

Evaluation of the antioxidant effects of *Sideritis* infusion intake on adult mice brain and peripheral tissues

Differences in the MDA and GSH levels among brain (-Ce) and peripheral tissues as well as among the three brain regions were observed in control mice. These differences are in accordance with previous studies [19, 33] and show that tissues exhibit a significant variation in the distribution of antioxidant defense [34] being closely related to the composition and the functional role of each tissue.

As illustrated in Table 2, the consumption of both doses (2 and 4%, w/v) of *Sideritis* herbal tea caused a significant decrease in MDA levels (16 and 29%, respectively) and an enhancement in the GSH levels (12 and 28%, respectively) in the brain (-Ce) in comparison with the control group, whereas liver and heart remained unaffected. Moreover, *Sideritis*-treated group that received the higher tea dose (4% w/v) exhibited significant further alterations in the

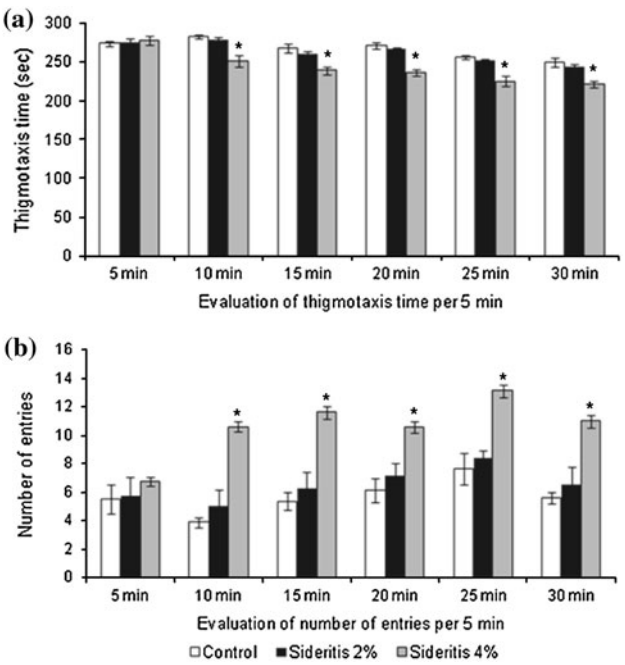

Fig. 2 Evaluation of **a** the thigmotaxis time and **b** number of entries in the central area during the first 30 min in the open field. Immediately after the introduction of naive mice into the open field of thigmotaxis test, the thigmotaxis time and number of central entries were measured during six consecutive periods of 5 min each. Data are means ± SE. of 8 mice per group. *Statistical difference between control and *Sideritis* 4%-treated mice

above oxidant/antioxidant indices of the brain (-Ce) in comparison with the treated mice that received the lower dose (2% w/v).

Taking into consideration that brain was the only tissue, which was influenced by the *Sideritis* tea intake, we further extended our study to determine the antioxidant effects of the herbal tea on specific brain regions, such as the cerebral cortex, cerebellum and midbrain. Our results showed that drinking of the herbal tea from *S. clandestina* subsp. *clandestina* for 6 weeks affected significantly the oxidant/antioxidant status of mice brain regions in a region- and dose-dependent manner. Particularly, as presented in Table 3, intake of both doses of the herbal tea decreased significantly the MDA levels of cerebellum (45 and 79%, respectively) and midbrain (50 and 63%, respectively), in comparison with the control mice, whereas cerebral cortex MDA levels remained unaffected. The consumption of the high dose (4%) of *Sideritis* infusion caused significant further reduction in midbrain and cerebellum lipid peroxidation levels, as compared to the low dose (2%). Furthermore, consumption of the low and high herbal tea dose increased significantly the GSH levels of cerebellum by 13 and 36% and of midbrain by 17 and 36%, respectively, in comparison with the control group. Neither of two tea doses affected the GSH content in the cerebral cortex.

Regular measurement of animal liquid and food intake exhibited no difference between the treated animal groups and the controls. Similarly, there was no difference in animal body weight between control and experimental group throughout the 6-week treatment period.

Discussion

The current study confirmed that drinking of the herbal tea from *S. clandestina* subsp. *clandestina* for 6 weeks affected significantly the oxidant/antioxidant status of mice brain regions in a region- and dose-dependent manner. Specifically, the significant alterations in MDA and GSH levels of the mice brain that accompanied the herbal tea intake exhibited a region specificity as cerebellum and midbrain were significantly affected in contrast to the cerebral cortex that showed remarkable stability. Significant stability in their oxidant/antioxidant status also displayed the liver and the heart. Accordingly, in our previous study, mice drinking a herbal tea (4% w/v) from another *Sideritis* taxon,

Table 2 Effect of herbal tea consumption (2 and 4% [w/v]) on MDA and GSH levels of mice tissues

Tissues	μmol MDA/g protein			μmol GSH/g protein		
	Control mice	*Sideritis* 2% w/v mice	*Sideritis* 4% w/v mice	Control mice	*Sideritis* 2% w/v mice	*Sideritis* 4% w/v mice
Brain (-Ce)	5.24 ± 0.17	4.42 ± 0.13* (↓16%)	3.74 ± 0.16*,† (↓29%) [↓15%]	11.80 ± 0.47	13.18 ± 0.41* (↑12%)	14.90 ± 0.38*,† (↑28%) [↑13%]
Liver	1.59 ± 0.16	1.52 ± 0.09	1.48 ± 0.09	7.06 ± 0.28	6.91 ± 0.17	6.93 ± 0.19
Heart	1.19 ± 0.16	0.95 ± 0.09	0.96 ± 0.11	5.05 ± 0.28	5.11 ± 0.15	4.77 ± 0.28

Data are mean ± SE ($n = 8$). Statistical analysis was performed by Mann–Whitney test. Percentage decrease (↓) of MDA values or increase (↑) of GSH values as to the control group. Percentage decrease [↓] of MDA values or increase [↑] of GSH values as to the *Sideritis* 2%-treated group

* Statistically significant difference between control and *Sideritis*-treated mice ($p < 0.05$)

† Statistically significant difference between *Sideritis* 2% and *Sideritis* 4%-treated mice ($p < 0.05$)

Table 3 Effect of herbal tea consumption (2 and 4% [w/v]) on MDA and GSH levels of mice brain regions

Tissues	μmol MDA/g protein			μmol GSH/g protein		
	Control mice	*Sideritis* 2% w/v mice	*Sideritis* 4% w/v mice	Control mice	*Sideritis* 2% w/v mice	*Sideritis* 4% w/v mice
Cerebral cortex	3.13 ± 0.22	2.95 ± 0.09	2.70 ± 0.10	12.47 ± 0.42	12.21 ± 0.41	12.28 ± 0.61
Cerebellum	2.10 ± 0.13	1.15 ± 0.04* (↓45%)	0.43 ± 0.008*,† (↓79%) [↓62%]	15.54 ± 0.70	17.52 ± 0.75* (↑13%)	21.07 ± 0.98*,† (↑36%) [↑20%]
Midbrain	1.45 ± 0.08	0.72 ± 0.035* (↓50%)	0.53 ± 0.04*,† (↓63%) [↓35%]	13.95 ± 0.44	16.27 ± 0.43* (↑17%)	19.01 ± 0.86*,† (↑36%) [↑17%]

Data are mean ± SE ($n = 8$). Statistical analysis was performed by Mann–Whitney test. Percentage decrease (↓) of MDA values or increase (↑) of GSH values as to the control group. Percentage decrease [↓] of MDA values or increase [↑] of GSH values as to the *Sideritis* 2%-treated group

* Statistically significant difference between control and *Sideritis*-treated mice ($p < 0.05$)

† Statistically significant difference between *Sideritis* 2% and *Sideritis* 4%-treated mice ($p < 0.05$)

S. clandestina subsp. *peloponnesiaca*, exhibited lower MDA and higher GSH levels in midbrain and cerebellum compared with their control littermates; cortex and liver remained unaffected [19]. The remarked stability of the cerebral cortex toward changes in its oxidant/antioxidant status has been also documented in the study of Haque et al. [35], where green tea catechins orally administered to rats exerted strong antioxidant effects in the hippocampus but not in the cerebral cortex. Conclusively, drinking of the herbal tea exerted a dose-dependent depletion of brain (cerebellum and midbrain) MDA levels with a simultaneous significant elevation of its GSH content. Since MDA, the end product of tissue lipid peroxidation, is highly reactive and responsible for cytotoxic effects, whereas glutathione is a tripeptide, which acts as a free radical scavenger preventing tissue damage, our findings support a neuroprotective action of the herbal tea through the inhibition of brain oxidative damage and the enhancement of its endogenous antioxidant defense.

In addition, thigmotaxis-testing results clearly show for the first time that *Sideritis* tea (4% w/v) consumption by adult mice exerts anxiolytic-like effects, although additional behavioral assessment is necessary. Therefore, the observed significant reduction of the anxious behavior that followed *Sideritis* tea consumption was accompanied by a significant antioxidant reinforcement of the mouse brain. There are also other reports that establish a strong link between oxidative stress and anxiety-like behaviors in rodents, but the underlying mechanisms are still unclear [5–7]. Despite the physiological role of cerebellum and midbrain in the control of motor functions, the anxiolytic-like effects of the herbal tea in the present study were combined with a significant elevation in the antioxidant capacity of the specific brain areas, while cerebral cortex remained unlikely stable. Nevertheless, the implication of cerebellum and midbrain in anxiety disorders has been recently demonstrated [36, 37]. Since behavior constitutes a complex brain process, future studies examining multiple biochemical indices of brain oxidant status and neurotransmitter systems may extend the current knowledge about anxiolytic-like and antioxidant properties of *Sideritis clandestina* tea.

In order to get a deeper understanding of the mechanism of action of the *Sideritis clandestina* tea, the network of interactions between individual constituents and cellular components needs to be characterized [38]. However, the identification of specific basic structures of phytochemicals, for which their activities have been investigated in the past, clearly supports the determined capacity of the studied extract to confer enhanced antioxidant activity, cognition-enhancing activities and neuroprotective actions. The biological and pharmacological properties of numerous derivatives bearing the same basic structure, as the main constituents identified in the current extract (quinic acid, melittoside (iridoid) and apigenin (flavonoid)), have been extensively studied in the literature. For instance, iridoids have shown to enhance the antioxidant capacity in primary hippocampal neurons by upregulating the antioxidative enzyme heme oxygenase-1 via the PI3 K/Nrf2-signaling [39]. Furthermore, another iridoid glycoside, loganin, was very recently found to improve learning and memory impairments induced by scopolamine in mice [40]. The iridoids *E*-harpagoside and 8-*O*-*E*-*p*-methoxycinnamoylharpagide significantly improved the impairment of reference memory in scopolamine-treated mice through both anti-acetylcholinesterase and antioxidant mechanisms [41]. The iridoid 8-*O*-*E*-*p*-methoxycinnamoylharpagide and its aglycone, harpagide, were very potent to protect primary cultured neurons against glutamate-induced oxidative stress primarily by acting on the antioxidative defense system and on glutamatergic receptors, respectively [42]. Concerning the activities of phytochemicals bearing the quinic acid basic structure, it has been shown to move through the blood–brain barrier and to present cognition-enhancing activities [43]. They have also demonstrated the neuroprotective effects through the upregulation of PGK1 expression and ATP production activation [44] as well as due to their metal chelation properties for metallotoxins (aluminum) [45]. Furthermore, quinic acid derivatives displayed neuroprotective action on amyloid Aβ-induced PC12 cell toxicity and neurotrophic activity by promoting neurite outgrowth in PC12 cells [46]. Moreover, they could effectively inhibit the formation of advanced glycation end products (AGEs) and rat lens aldose reductase [47]. In addition to these phytochemicals, the identified basic structure of apigenin in the extract has been shown to possess a variety of pharmacological actions on the central nervous system as well as antidepressant-like behavioral and neurochemical effects [48]. It also protects brain neurovascular coupling against amyloid-β_{25-35}-induced toxicity in mice [49] and inhibits the production of nitric oxide (NO) and prostaglandinE2 (PGE_2) in microglia and inhibits neuronal cell death in a middle cerebral artery occlusion–induced focal ischemia mice model [50]. Furthermore, apigenin was found to inhibit the oxidative stress–induced macromolecular damage in *N*-nitrosodiethylamine (NDEA)-induced hepatocellular carcinogenesis in rats [51], and also it can confer a protective role in the status of lipid peroxidation and antioxidant defense against hepatocarcinogenesis in rats [52].

In conclusion, the remarked anxiolytic-like effects and in vivo antioxidant capacity of *Sideritis clandestina* subsp. *clandestina* infusion accompanied by the phytochemical analysis of its aqueous extract support the beneficial role of regular drinking of herbal mountain tea from *Sideritis* taxa in the prevention of neurobehavioral diseases and the deleterious effects of aging.

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