A new validated SPE-HPLC method for monitoring crocetin in human plasma—Application after saffron tea consumption

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A B S T R A C T
Saffron (stigmas of Crocus sativus L.) is a well-known spice with many attributed therapeutic uses throughout centuries. Although studies have demonstrated that crocetin and crocins from saffron have various biological functions, issues concerning the route and way of saffron administration, the absorption and metabolism of saffron carotenoids in humans have not been answered yet. In the present study, an isocratic reversed-phase liquid chromatographic method was developed and validated for the determination of crocetin in plasma. Samples were pre-treated by solid phase extraction (recoveries >72%) and were chromatographed on a Luna C-18 column (4.6 mm × 250 mm, 5 μm) with a mobile phase consisting of methanol–water–trifluoroacetic acid (75.0:24.5:0.5, v/v/v) at a flow rate of 1.0 mL min⁻¹. The HPLC method developed resulted in sharp peaks at 10.7 (trans-crocetin) and 18.6 min (cis-crocetin), whereas the calibration curve of total crocetin in plasma displayed a good linearity for concentrations of 0.020–20 μM (R² = 0.999). Specificity, precision, accuracy and stability were studied with spiked plasma samples and were acceptable. The developed method was applied to the determination of crocetin levels in plasma of four healthy human volunteers before and after consumption of one cup of saffron tea (200 mg of saffron in 80 °C water for 5 min). Results showed that the concentration of crocetin was high after 2 h (1.24–3.67 μM) and still determined after 24 h (0.10–0.24). Interestingly, the percentage of the cis-isomer ranges from 25 to 50%, suggesting in vivo isomerization.

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1. Introduction

Saffron, the dried stigmas (the term styles is also used by certain botanologists [1]) of Crocus sativus flowers, is a spice important in the Mediterranean, Indian and Chinese diet and has been continuously used in folk remedies for more than 90 diseases for over 3000 years [2]. Recent research on its biomedical properties supports its use for many of these indications, although the great majority of the studies are conducted in vitro and in experimental animals [3]. Its composition is also quite unique: the main constituents are hydrophilic carotenoids, the crocins, which are monodi- and diglycosyl-esters of crocetin and are responsible for the characteristic golden yellow color they confer.

Besides culinary uses, saffron is consumed as a herbal tea per se or is added in other herbal infusions for its unique flavor characteristics and its attributed medicinal uses. According to the WHO monograph issued in 2007, daily doses of up to 1.5 g of saffron are thought to be safe; at doses of 5.0 g or more, Stigma Croci may cause serious adverse reactions and overdose (12.0–20.0 g/day) may be fatal [3]. Intraperitoneal administration of saffron ethanolic extract to Wistar rats at the high doses of 0.35, 0.70 and 1.05 g kg⁻¹ body weight for two weeks caused mild to severe hepatic and renal tissue injuries, weight loss, significant reductions in the haemoglobin and haematocrite levels and total red blood cell counts; intraperitoneal median lethal dose (LD50) value of ethanolic extracts was found to be 3.5 g kg⁻¹ body weight [4]. Modaghegh et al. showed that consumption of 200 and 400 mg saffron capsules by healthy humans for 7 days is safe [5]. Administration of 200 mg saffron capsules to men with erectile dysfunction for 10 days was safe and had a positive effect on sexual function [6], whereas administration of 30 mg capsules to patients with mild–to-moderate Alzheimer’s disease [7] and to women with symptoms of premenstrual syndrome [8] had a favorable outcome. A reduction in lipoprotein oxidation in 20 human subjects was also reported after administration of 50 mg saffron in milk twice a day for 3–6 weeks [9].

However, issues concerning the route and way of saffron administration, the absorption and metabolism of saffron carotenoids in humans have not been answered. Experiments conducted in rodents, showed that orally administered crocins are hydrolysed to crocetin before being incorporated into blood circulation and
that crocetin is likely to be metabolized to glucuronide conjugates both in the intestinal mucosa and in the liver of mice[10,11]. The aim of this study was the development and validation of a chromatographic analytical method for the determination of crocetin in human plasma and application of that method to the determination of plasma crocetin levels after saffron tea consumption by healthy volunteers. In this context, we screened saffron tea composition to investigate if tea preparation induces changes in saffron components, i.e. carotenoid isomerization.

2. Materials and methods

2.1. Reagents and chemicals

Methanol (MeOH) and acetonitrile (AcCN) of HPLC grade were supplied from Merck KGaA (Darmstadt, Germany). Dimethylsulphoxide (DMSO), ammonia solution, hydrochloric acid and solid reagents (ammonium acetate, sodium chloride and sodium phosphate), of analytical grade were also purchased from Merck. Trifluoroacetic acid (TFA) and butylated hydroxytoluene phosphoric acid, of analytical grade were also purchased from Sigma–Aldrich (St. Louis, USA) whereas trans-crocetin [(2E,4E,6E,8E,10E,12E,14E)-2,6,11,15-tetramethyl-2,4,6,8,10,12,14-hexadecahexaenoic acid] (purity >98% for the sum of both isomers) was purchased from Extrasynthese (Genay Cedex, France). Saffron in the form of dried styles was supplied from Cooperative de Saffron (Krokos Kozanis, Greece). The dried plant material was identified by Professor Gregoris Iatrou, Department of Biology, University of Patras, Greece.

2.2. Preparation of crocetin standards

Stock solutions of crocetin were prepared in DMSO at an initial concentration of 10 mM and diluted to working concentrations (0.005–100 μM) in methanol. Methanolic solutions of the analyte were kept at −20 °C in a dark place, until further use. The solutions were stable for at least one month. Pooled plasma, obtained from young healthy volunteers (25–35 years old), was used for the preparation of spiked plasma standards. For spiked solutions of crocetin in plasma, 25 μL of crocetin was diluted in plasma to a final volume of 500 μL.

2.3. Plasma sample pretreatment

During the optimization of plasma sample pretreatment, samples were submitted to solid phase extraction (SPE) procedure for the removal of matrix interferences during the analysis. Reversed phase Strata-X cartridges (200 mg/3 mL) consisting of a surface modified with styrene–divinylbenzene polymer were obtained from Phenomenex (Torrance, CA, USA). The cartridges were conditioned with methanol and equilibrated in loading buffer. In the proposed method, 500 μL of plasma sample was diluted in 500 μL of MeOH:CH₃COOH (25 mM) containing 10% NaCl (w/v) (10:90, v/v), centrifuged and the supernatant was loaded onto the cartridges. Washing was performed with 1 mL of MeOH:CH₃COOH (25 mM) (5:95, v/v). The retained crocetin was eluted with 6 mL NH₃–MeOH–water (5:90:5, v/v/v). Finally, the eluate was evaporated to dryness in a speed Vac system (Labconco Corp., Kansas city, MO, USA). The residues were stored at −20 °C until the next day. Dry residues were redissolved in 100 μL of the HPLC mobile phase.

2.4. HPLC determination of crocetin in plasma

The chromatographic system consisted of an Ultimate 3000 Pump (Pump LPG-3400 A, Dionex Corporation, Sunnyvale, CA, USA) with a 20 μL Rheodyne 8125 injector (Rheodyne, Ronhert Park, CA, USA). The Column Compartment (TCC-3100) was stabilized at 40 °C and UV-detection was performed with a diode array detector (DAD), Ultimate DAD-3000. Data were collected, stored and integrated on a Chromeleon v 6.80 Systems software. Separation of analytes was performed on a Luna C-18 reversed–phase column (250 nm × 4.6 mm I.D., 5 μm particle size) from Phenomenex (Torrance, CA, USA). Elution was performed with methanol–water–TFA (75:0.2:4.8, v/v/v) for 30 min. The flow rate of the mobile phase was 1 mL min⁻¹. Spectra were monitored by the diode array detector.

2.5. Method validation

The proposed method was validated according to the official guidelines of FDA for bioanalytical methods[12]. Selectivity was investigated by analysis of blank samples (plasma) from at least 6 healthy individuals. The calibration curve was obtained by plotting the sum of peak areas (y) of trans- and cis–crocetin versus their concentration (x). Nine different concentrations (0.002, 0.02, 0.2, 0.5, 1, 2, 5, 10, 20 μM) were used for total crocetin. Good linearity was determined through the correlation coefficient (R²), which should be better than 0.99. The LOD was determined as the analyte concentration yielding signal with a signal-to-noise (S/N) ratio of 3:1, whereas the LOQ was defined as the analyte concentration yielding signal with S/N ratio 10:1. The noise was evaluated as the largest deviation of detector signal of baseline. Intra-day accuracy and precision were estimated with five analyses on the same day of plasma samples spiked with crocetin at three different concentration levels: 0.15, 3, 15 μM. The accuracy was calculated by comparison of each concentration measured to the nominal concentration of the standard solution and was expressed as % of the relative error values whereas precision was numerically expressed by relative standard deviation (RSD) of values. Inter-day accuracy and precision of the method were estimated with three analyses of the plasma samples spiked with crocetin (0.15, 3, and 15 μM) ten days later. Precision and accuracy should be less than 15% except for the LOD, where the values should not exceed 20%. The stability of crocetin is determined by analysis of the low (0.15 μM) and the high (15 μM) spiked plasma standards after three freeze (at −20 °C and −70 °C) and thaw cycles, and after 24-h storage at room temperature. SPE recovery of total crocetin was calculated by comparison of the determined concentrations obtained from analysis of spiked plasma samples after SPE to those calculated from the analysis of the respective concentrations of crocetin standards in methanol (5 times the concentration of spiked plasma samples).

2.6. HPLC analysis of various saffron infusions

Saffron infusion was prepared by adding 150 mL of hot water (80 °C) to 200 mg of saffron and leaving the stigmas steep for 5 min. The infusion was centrifuged and the supernatant was used directly for HPLC analysis. In order to compare the HPLC profile to that of the standard aqueous methanol extract, saffron was extracted with methanol:water (1:1, v/v) (3 mL/50 mg) for 4 h at 25 °C with continuous stirring. The extract was centrifuged, filtered through a 0.2-μm filter and evaporated to dryness. The residue was stored at −20 °C until further use and redissolved in methanol:water (1:1, v/v). All procedures were performed in the absence of light.

Chromatographic analysis was performed on a Supelcosil C18 (5 μm, 25 cm × 4.6 mm, Sigma–Aldrich) column on a Mod.10 AKTA instrument (Amersham Biosciences, Piscataway USA) as described previously by minor modifications[13,14]. Elution was performed with methanol:water (20:80, v/v) for 2 min, a gradient of methanol (20–70%) for 50 min, with a gradient of methanol (70–100%) for 5 min and 100% methanol for 1 min with a flow rate 0.7 mL/min. Both solvents (water and methanol) contained 1% (v/v) acetic
acid. Detection wavelength was 440 nm. Semi-quantification was carried out by multiplying the peak area of each crocin with the respective molecular coefficient absorbance value (89,000 for trans-crocetin and 63,350 for cis-crocetin) and expressed as the percentage of each crocin in relation to the total crocin content [15].

2.7. Method application: study design and biologic material

For the preparation of saffron infusions, hot water (80 °C, about 150 mL) was added to one cup containing 200 mg saffron stigmas which were allowed to steep for 5 min. Four young (25–35 years old, 3 female and 1 male, normal BMI) healthy volunteers participated in the study and their written informed consent was obtained. The collection of the biologic material and the procedure followed conformed to the ethical standards of the Helsinki Declaration of 1975 and its latest revisions. The study was approved by the local Bioethics Committee of the University of Patras and all procedures took place in the University Hospital of Patras under medical supervision. All volunteers drank the saffron tea at about 09:00–10:00 a.m. after overnight fasting. Blood was collected in EDTA coated tubes immediately before (0 h), 2 and 24 h after tea consumption. Plasma was prepared by centrifugation of blood at 1000 g for 15 min at 4 °C and then kept at −20 °C till analysis (next day).

3. Results and discussion

3.1. Method development

Initial experiments were performed with pure crocin standards in methanol. For the determination of the mobile phase, a number of elution systems were examined. The use of a gradient of AcCN:water (15–75% of the organic phase in 25 min) showed a very broad peak at 5 min. Addition of ammonium acetate at the final percentage of 0.1% (w/v) in both solvents resulted in two broad and tailing peaks (a main one at 13 and a minor at 20 min). Better HPLC profiles with good resolutions but still broad and tailing peaks (main peak at 9 and minor at 20 min) were observed with isocratic elution with MeOH–water–acetic acid (75:23.2, v/v/v), as earlier suggested [11]. Decrease in the percentage of acetic acid led to longer elution times. Replacing acetic acid with TFA to the optimum mobile phase composition of MeOH–water–TFA (75.0:24.5:0.5, v/v/v) resulted in sharp peaks at 10.7 and 18.6 min (Fig. 1). A higher amount (>0.5%) of TFA causes broadening of the peaks and increase in the peak area of the late-eluting substance.

Monitoring of spectra of the two peaks by the diode array detector showed that both of them share maximum double peaks at 420–460 nm, characteristic of the carotenoids; the earlier eluting peak at 10.7 min corresponds to trans-crocetin and the second one at 18.6 min to cis-crocetin.

This is the first report stressing that during HPLC analysis of trans-crocetin (high purity standard both commercially available and that prepared by alkaline hydrolysis of saffron extract as earlier described [10,16,17]), a small amount (about 10%) of the cis-isomer always appears irrespective of the matrix (i.e. in pure crocin standards or in plasma spiked with crocetin), and is well resolved from the trans-isomer, i.e. it elutes much later; in our optimum conditions it elutes 8 min later. The existence of the cis-isomer is probably a result of the susceptibility of carotenoids to various factors, e.g. light and acids. Earlier HPLC studies on determination of plasma crocetin by Xi et al. and Asai et al. do not report the existence of the cis-isomer [10,11].

Purification and condensation of plasma samples were accomplished via SPE. All experiments were conducted with plasma samples spiked with crocetin. Having in mind the acid–base balance of the carboxylic groups of crocetin at different pH values, reversed phase SPE columns were loaded with 500 μL plasma diluted in water (1:1, v/v), washed with MeOH–phosphate buffer (50 mM, pH 3.0) (20:80, v/v) (2 mL), and eluted with 6 mL NH₃–MeOH–water (5:90:5, v/v/v). About 50% of crocetin was recovered but nearly 30% of crocetin was lost in the washing procedure, probably because of strong interactions with plasma proteins eluted at that step. Indeed, repetition of the procedure without MeOH in the washing step had no difference on recovery values. Non-specific binding of crocetin to human serum albumin via H-bonding, the stability of those complexes and the changes on protein secondary structure have been earlier shown by Kanakis et al. [18]. In order to disrupt those complexes, a high concentration of salt, a small percentage of methanol and low pH values were used. In the proposed method, 500 μL of plasma sample was diluted in 500 mL of MeOH–CH₃COOH (25 mM) containing 10% NaCl (w/v) (10:90, v/v). Samples were loaded on cartridges after centrifugation, and washing was performed with 1 mL of MeOH–CH₃COOH (25 mM) (5:95, v/v). The retained crocetin was eluted with 6 mL NH₃–MeOH–water (5:90:5, v/v/v), as in previous tests. Those changes led to high recovery values; 94.8% at the concentration of 0.2 μM, 83.3% at 0.5 μM and 72.3% at 20 μM.

3.2. Method validation

Method’s quality parameters were studied under optimum separation parameter conditions using plasma spiked with crocetin at final concentrations ranging from 0.002 to 20 μM. The calibration curve of total crocetin (sum of areas of two peaks) displayed a good linearity for concentrations of 0.020–20 μM (R² = 0.999, y = 1.131x − 0.054). In detail, calibration curves were linear from...
Table 1
Accuracy and precision data (intra- and inter-day) for analysis of plasma samples spiked with crocetin expressed as relative error (% RE) and relative standard deviation (% RSD), respectively.

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>Intra-day</th>
<th>Inter-day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Accuracy (% RE)</td>
<td>Precision (% RSD)</td>
</tr>
<tr>
<td>0.15</td>
<td>5.55</td>
<td>6.34</td>
</tr>
<tr>
<td>3</td>
<td>-1.25</td>
<td>2.11</td>
</tr>
<tr>
<td>15</td>
<td>5.72</td>
<td>1.05</td>
</tr>
</tbody>
</table>

Table 2
Percentages (%) of crocins extracted by methanol–water (1:1, v/v) and by hot water (infusion).

<table>
<thead>
<tr>
<th>Crocins</th>
<th>50% aqueous MeOH for 4 h</th>
<th>Infusion in 80 °C water for 5 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trans-crocin-4</td>
<td>50.1 ± 1.4</td>
<td>59.5 ± 0.2</td>
</tr>
<tr>
<td>Trans-crocin-3</td>
<td>21.1 ± 0.5</td>
<td>21.0 ± 0.4</td>
</tr>
<tr>
<td>Cis-crocin-4</td>
<td>9.1 ± 2.9</td>
<td>5.0 ± 0.1</td>
</tr>
<tr>
<td>Trans-crocin-2</td>
<td>3.8 ± 1.3</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>Cis-crocin-3</td>
<td>7.8 ± 2.2</td>
<td>8.2 ± 0.2</td>
</tr>
<tr>
<td>Cis-crocin-1</td>
<td>1.8 ± 0.4</td>
<td>1.1 ± 0.3</td>
</tr>
</tbody>
</table>

Crocin-4, crocetin-di-β-D-gentiobiosyl ester; crocin-3, crocetin-(β-D-glycosyl)-(β-D-gentiobiosyl) ester; crocin-2, crocetin (β-D-gentiobiosyl) ester; crocin-1, crocetin (β-D-glycosyl) ester.

0.020 to 20 µM for trans-crocetin ($R^2 = 0.999$) and from 0.569 to 10 µM for cis-crocetin ($R^2 = 0.978$). LLOD values are 0.002 and 0.170 µM for trans- and cis-crocetin, and the respective LLOQ values are 0.020 and 0.569 µM.

The selectivity of the method was determined by analysis of blank plasma samples of six different healthy individuals. HPLC analysis showed no endogenous peaks at the retention times of the crocetin isomers (Fig. 2A).

The intra and inter-day accuracy and precision for the total amount of crocetin were less than 15% and at the LLOQ less than 20% (Table 1). Results are presented as sum of all isomers. These values show that the proposed methodology is reproducible and suitable for the quantitative determination of crocetin in human plasma samples. Peak asymmetry and theoretical plates for trans-crocetin are 1.249 ± 0.134 and 3966 ± 408, respectively, whereas for cis-crocetin the respective values are 1.126 ± 0.093 and 4327 ± 437.

The short-term (up to 24 h in room temperature) and freeze–thaw (after three cycles) stability was also studied by comparing the mean back-calculated concentrations of treated samples to those of freshly prepared ones. Low-concentration samples (0.15 µM) were not stable at room temperature for 24 h and after three freeze (at −20 °C)–thaw cycles since differences were around 30 and 48%, respectively. High concentration samples (15 µM) were stable in all conditions (differences < ±15%).

3.3. Analysis of saffron infusions

Saffron infusions were analyzed by HPLC and the fingerprint obtained is in complete agreement with previous reports [14,15,19]. Ten peaks could be identified: two absorbing at 250 nm (picrocrocin and HTCC) and eight absorbing at 440 nm (trans-crocin-4, trans-crocin-3, trans-crocin-2, cis-crocin-5, cis-crocin-4, trans-crocin-2, cis-crocin-3, cis-crocin-2). Semi-quantification showed that saffron tea preparation does not induce crocin isomerization and hydrolysis (Table 2). Indeed, in saffron infusions, the only noteworthy changes concern trans- and cis-crocin-4: content of trans-crocin-4 is higher and of cis-crocin-4 is lower. The lower percentage of trans-crocin-4 in aqueous methanolic extracts might be explained by the higher extractability of less polar crocins in the presence of methanol; the low-degree forma-

Fig. 2. Plasma analysis before and after saffron tea consumption. HPLC chromatograms of human plasma before saffron administration (A) after 2 h (B) and after 24 h (C). I: trans-crocetin, II: cis-crocetin.
Table 3  
Concentration (µM) of total crocetin determined by SPE-HPLC-DAD analysis in human plasma samples and % percentage of cis-isomers.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total crocetin (µM)</th>
<th>cis-crocetin/total crocetin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2h</td>
<td>24h</td>
</tr>
<tr>
<td></td>
<td>2h</td>
<td>24h</td>
</tr>
<tr>
<td>1</td>
<td>3.67</td>
<td>0.12</td>
</tr>
<tr>
<td>2</td>
<td>2.00</td>
<td>0.10</td>
</tr>
<tr>
<td>3</td>
<td>1.24</td>
<td>0.24</td>
</tr>
<tr>
<td>4</td>
<td>1.27</td>
<td>0.13</td>
</tr>
</tbody>
</table>

ND, cis-crocetin was not detected.

3.4. Application to determination of plasma crocetin after saffron tea consumption

The developed SPE-HPLC analytical methodology was applied for the quantification of crocetin in plasma of healthy humans. Plasma was taken before (0h) and after (2 and 24h) saffron tea consumption. The results show that, after consumption of a cup of tea from 200 mg saffron, crocetin is detected in blood stream and trace amounts are found even after 24 h (Fig. 2, Table 3).

This is the first report showing crocetin concentrations in human plasma after saffron consumption. These results confirm previous studies performed in rodents, which showed that orally administered crocetin is quickly absorbed into blood plasma whereas crocins are hydrolysed to crocetin in the gastrointestinal tract before entering blood circulation [10,11]. Asai et al. observed a peak in crocetin concentration at 30 min after administration of a mixed micelle solution (0.2 mL) containing crocin or crocins (40 nmol each) [10] whereas Xi et al. showed that plasma concentrations of crocetin in rats began to decline after 1 h [11]. In the study of Asai et al., crocetin glucuronides are also identified probably indicating the partial metabolism of crocetin to the glucuronide conjugates in the intestinal mucosa, in the liver, or in both [10]. However, in our experiments, spectra monitoring of early eluting peaks does not reveal the existence of glucuronides in the chromatograms. This does not contradict earlier findings since glucuronides might be lost during SPE. Treatment with glucuronidase before SPE as suggested by Asai et al. will aid future pharmacokinetic studies.

Both isomers were detected in blood plasma 2 h after saffron tea drinking, whereas the cis-isomer was not detected after 24 h because it was below LLOD. In three out of four individuals, the cis-isomer accounted for about 25% of total crocetin, whereas in the other one it accounts for nearly 50%. The higher determined percentages of the cis-isomer in blood samples after saffron tea consumption compared to the usual 10% we observed in pure trans-crocetin standards and spiked plasma samples may be the combined result of in vivo isomerization and hydrolysis of ingested cis-crocins (about 15%) present in saffron infusion in the gastrointestinal tract. Various isomers of lycopene (mainly cis- and trans-) are also found in blood samples, with the ratio of the cis-isomer reaching the 60–80% of total lycopene [21] even though lycopene in foods occurs mainly in the all-trans form. The mechanisms underlying the formation, interconversion or biological roles of various crocetin isomers remain enigmatic.

4. Concluding remarks

An analytical SPE-HPLC method was developed for total crocetin determination in human plasma, which displays good selectivity, linearity, sensitivity, accuracy and precision. The method was applied to the determination of crocetin in blood plasma of healthy human individuals before and after saffron tea consumption. Crocetin was not detectable before tea drinking, but its concentration was high after 2 h (1.24–3.67 µM) and still determined after 24 h (0.10–0.24). This first study in humans confirms findings of previous pharmacokinetic studies in rodents showing that orally ingested crocins are hydrolysed to crocetin before entering blood circulation. This is the first report showing the determination of the cis-isomer of crocetin in small percentages (10%) in pure crocetin or spiked plasma samples due to interconversion of the trans-isomer during analysis to a small degree. The significance of this observation is manifested in the determination of crocetin in healthy human individuals after saffron tea consumption; percentages of the cis-isomer range from 25 to 50% suggesting in vivo isomerization. At last, we showed that saffron tea preparation does not induce changes in saffron composition concerning the presence of individual crocins. This HPLC method enables the determination of crocetin concentrations in humans and the knowledge of actual concentrations and the percentages of the trans- and cis-isomers after saffron consumption will aid future nutritional or pharmacokinetic studies.

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References


Differential Antioxidant Effects of Consuming Tea from *Sideritis clandestina* subsp. *peloponnesiaca* on Cerebral Regions of Adult Mice

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1Laboratory of Human and Animal Physiology, Department of Biology; 2Division of Plant Biology, Department of Biology; 3Laboratory of Pharmacognosy & Chemistry of Natural Products, Department of Pharmacy; University of Patras, Patras, Greece.

ABSTRACT Oxidative stress is involved in the pathophysiology of neurodegenerative diseases and aging. Many species of the genus *Sideritis* (mountain tea) are widely consumed in the Mediterranean region as herbal tea. This study evaluated the effect of supplementation of mice with herbal tea from *Sideritis clandestina* subsp. *peloponnesiaca* on the antioxidant status of different brain regions. To select the most bioactive herbal tea, the polyphenolic content (Folin–Ciocalteu method) and the antioxidant properties (ferric reducing antioxidant power [FRAP] and 2,2-diphenyl-1-picrylhydrazyl assays) of several taxa and different populations of the *S. clandestina* infusions were measured in vitro. Male adult mice had ad libitum access to water (control) or the herbal tea (4% w/v) for 6 weeks. At the end of the treatment period we assessed the total antioxidant power (FRAP assay) and the levels of malondialdehyde (indicator of lipid peroxidation) and reduced glutathione in the cerebral cortex, cerebellum, and midbrain. These biochemical measures have also been determined in liver samples used as a comparative reference peripheral tissue. Consumption of 4% herbal tea increased the total antioxidant power of the midbrain by 72% (*P* < .05); a significant (*P* < .05) decrease in malondialdehyde levels and increase in reduced glutathione content of the cerebellum (78% and 27%, respectively) and midbrain (59% and 32%, respectively) were also observed. These findings indicate that mountain tea consumption enhances the antioxidant defense of the adult rodent brain in a region-specific manner.

KEY WORDS: • antioxidant capacity • brain • reduced glutathione • lipid peroxidation • mountain tea • Sideritis

INTRODUCTION

*Sideritis* L. (Lamiaceae), growing mainly in the Mediterranean region, comprises approximately 150 species worldwide. The dried aerial parts of *Sideritis* species are widely consumed in Greece as an herbal tea, popularly termed mountain tea.1 It has been used extensively in folk medicine as a calming agent and as a treatment for inflammation, cough, and gastrointestinal disorders. Recent research has demonstrated that *Sideritis* species have anti-inflammatory2 and antimicrobial3 activities, which have been attributed to their chemical composition. Indeed, many reports indicated that these plants contain essential oils,4 polyphenols5 (especially flavonoids6), and diterpenoids,7 which are of particular pharmacologic and nutritional interest.

A moderate *in vitro* antioxidant action of extracts of *Sideritis* species has been described elsewhere,6 but to our knowledge no *in vivo* studies have examined their effect. Mammalian brain is particularly vulnerable to oxidative stress,8 a factor that is considered to play a pivotal role in the pathogenesis of several age-related neurodegenerative disorders.9 However, the brain areas exhibit differential vulnerability to oxidative damage, probably because of differences in their endogenous antioxidant defense system.10 Therefore, we sought to investigate the antioxidant effect of consumption of tea composed of 4% *S. clandestina* on the cerebral cortex, cerebellum, and midbrain of adult normal mice. We also assessed the effects on the mice’s livers, a reference peripheral tissue. This plant was chosen after *in vitro* evaluation of the polyphenolic content and the antioxidant properties of infusions of several taxa and different populations of *S. clandestina*, grown in different regions of the Peloponnese.

MATERIALS AND METHODS

Plant material and preparation of herb extracts

*S. clandestina* is a species endemic in Greece (specifically, the Peloponnese). It is taxonomically divided into 2
subspecies: *S. clandestina* subsp. *clandestina* prospers in the mountains of the southern Peloponese, and *S. clandestina* subsp. *peloponnesiaca* prospers in the mountains of the northern Peloponese. *S. clandestina* subsp. *clandestina* was collected between July and August 2008 from the Taygetos (southern Peloponese), Parnon (eastern Peloponese), and Mainalo (Central Peloponese) mountains; *S. clandestina* subsp. *peloponnesiaca* was collected from the Kyllini Mountain (northern Peloponese). Taxonomic identification was performed at the Division of Plant Biology, Department of Biology, University of Patras, where herbarium vouchers are deposited. Green tea (blended and packed in Sri Lanka under the direction of St. Dalfour, Freres & Cie, Cour Cheverny, France), in 2-g packs, was used as standard.

Infusions were prepared with addition of 4 g of equal amounts of leaves, flowers, and stems of *Sideritis* taxa to 100 mL boiling water for 5 minutes. After 5 minutes, the solution was filtered and the filtrate volume was adjusted to 100 mL. Green tea was also prepared in the same way. For the animal studies, the infusions were prepared daily. For the *in vitro* studies, deionized distilled water was used. The weight of the dry extract was recorded after lyophilization in a Labconco FreezeZone 6 freeze-dry system.

**Animals**

Adult (3–4 months old) male Balb-c mice were kept in the same room under a constant temperature (23–25°C) with alternating 12-hour light/dark cycles. The mice were allowed free access to food. Mice were managed according to the Greek National Laws (Animal Act, PD 160/91).

The animals were divided into 2 groups consisting of 8 animals each. Group 1 mice served as controls and received water ad libitum. Group 2 mice had *ad libitum* access to 4% (w/v) tea infusion of *S. clandestina* subsp. *peloponnesiaca* for 6 weeks (40 days), based on the protocol of Choudhary and Verma with slight modifications. Body weight was measured weekly in both groups and did not significantly differ between them at the end of the treatment period.

On the completion of the 40-day treatment period, all animals were sacrificed by light ether anesthesia. Liver and brain were excised immediately, and cerebral cortex, cerebellum, and midbrain were separated from the whole brain. The tissue samples were kept at −75°C until use.

**Preparation of tissue homogenates**

Brain regions and liver samples were weighed and homogenized (10% w/v) with a glass-Teflon homogenizer in ice-cold 1.15% KCl. Tissue homogenates were centrifuged for 5 minutes at 15000 g, and the supernatants were used to determine the antioxidant activity (by ferric reducing antioxidant power [FRAP] assay) and malondialdehyde (MDA) and reduced glutathione levels.

**Results and Discussion**

To select the most bioactive population of all *S. clandestina* samples among those collected, their polyphenolic
content and antioxidant activity were determined in vitro. Table 1 presents the total polyphenol content of all *Sideritis* taxa infusions tested and their solid residue contents. All tea infusions of *S. clandestina* taxa and populations had significantly (*P* < .05) lower polyphenolic composition than *Camellia sinensis* leaves, which were used as the standard. The polyphenolic content of 4 of 5 populations of *S. clandestina* subsp. *clandestina* was significantly lower than that of *S. clandestina* subsp. *peloponnesiaca*. The *in vitro* antioxidant properties of the plant extracts were measured by DPPH and FRAP assays (Table 1). The infusion of *S. clandestina* subsp. *peloponnesiaca* displayed significantly (*P* < .05) stronger *in vitro* antioxidant properties than the other taxa of *S. clandestina*. However, the *in vitro* antioxidant properties of *S. clandestina* subsp. *peloponnesiaca* were lower than those of green tea. Similarly, Triantaphyllou *et al.* showed that the water extract of China black tea displayed higher total phenol content and antioxidant activity than that of *S. reaseri* collected from a mountainous region of northern Greece.

Another study showed that not only the species but also many environmental factors, such as soil type, sun exposure, and rainfall, have a strong effect on polyphenol composition. This finding explains the variation observed among the different populations of *S. clandestina* subsp. *clandestina*. All *S. clandestina* infusions had significantly lower antioxidant activity than *C. sinensis*, in accordance with their lower polyphenolic content. Tunalier *et al.* in their study of the antioxidant properties and phenolic composition of 27 *Sideritis* species grown in Turkey, indicated a linear relationship between these 2 measures. Thus, the infusion of *S. clandestina* subsp. *peloponnesiaca* displayed high polyphenolic content and strong antioxidant properties *in vitro*, rendering it suitable for the further *in vivo* study.

To this end, the biological effects of 4% (w/v) herbal tea from *S. clandestina* subsp. *peloponnesiaca* consumption for 6 weeks by adult control mice were further investigated. We assessed the total antioxidant activity, the extent of lipid peroxidation, and reduced glutathione content of 3 brain regions (cerebral cortex, cerebellum, and midbrain) and a peripheral tissue (liver) in control and treated mice. As shown in Table 2, there is a hierarchy in antioxidant/reducing capacity and MDA levels of the examined brain regions in control animals: cerebral cortex > cerebellum > midbrain; the differences reached significance (*P* < .05) between all brain areas. However, with respect to their reduced glutathione content, the cerebellum ranks higher than the cerebral cortex. In accordance with our observation, Feoli *et al.* also reported a similar hierarchy in total antioxidant reactivity levels, as estimated by total antioxidant reactivity assay. Other studies also reported greater lipid peroxidation in the cerebral cortex than in the cerebellum or midbrain of young control rats and higher reduced glutathione content in rat cerebellum than in the cortex and midbrain. These regional differences could be attributed to the differences in structure and physiology of the brain areas, leading to differences in vulnerability to oxidative stress and activity of their endogenous antioxidant defense system.

It is known that the brain regions vary with respect to their fatty-acid composition because white matter rich in myelin contains fewer polyunsaturated fatty acids (the major targets of free radicals) than the grey matter. In this context, the midbrain, a heavily myelinated region, is expected to be more resistant to lipid peroxidation than cerebral cortex and cerebellum and thus demanding the development of lower reduced glutathione content, and a moderate level of lipid peroxidation in liver, in accordance with findings from a previous study in normal rats.

Consumption of the tea significantly increased (*P* < .05) the antioxidant capacity of midbrain by 72% but did not significantly influence the antioxidant power of the cerebral cortex, cerebellum, and liver (Table 2). Furthermore, herbal

<table>
<thead>
<tr>
<th><em>Sideritis</em> samples</th>
<th>Solid residue content of extracts (mg/100 mL)a</th>
<th>Concentration of polyphenols (µg gallic acid/mg)b</th>
<th>Antioxidant Activity</th>
<th>DPPH assay—IC_{50} (mg/mL)c</th>
<th>FRAP assay (µmol Fe³⁺/mg)d</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. clandestina</em> subsp. <em>clandestina</em> (Taygetos I)</td>
<td>302</td>
<td>36.8 ± 0.9**</td>
<td>7.75 ± 0.55**</td>
<td>0.43 ± 0.07**</td>
<td></td>
</tr>
<tr>
<td><em>S. clandestina</em> subsp. <em>clandestina</em> (Taygetos II)</td>
<td>332</td>
<td>34.6 ± 1.8**</td>
<td>7.31 ± 0.39**</td>
<td>0.49 ± 0.12**</td>
<td></td>
</tr>
<tr>
<td><em>S. clandestina</em> subsp. <em>clandestina</em> (Parnon)</td>
<td>404</td>
<td>30.3 ± 2.2**</td>
<td>7.58 ± 1.12**</td>
<td>0.56 ± 0.09**</td>
<td></td>
</tr>
<tr>
<td><em>S. clandestina</em> subsp. <em>clandestina</em> (Taygetos III)</td>
<td>262</td>
<td>44.7 ± 0.8**</td>
<td>16.18 ± 1.69**</td>
<td>0.27 ± 0.07**</td>
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</tr>
<tr>
<td><em>S. clandestina</em> subsp. <em>clandestina</em> (Mainalo)</td>
<td>500</td>
<td>23.7 ± 0.5**</td>
<td>8.90 ± 1.55**</td>
<td>0.33 ± 0.03**</td>
<td></td>
</tr>
<tr>
<td>Green tea (<em>Camellia sinensis</em>)</td>
<td>744</td>
<td>165.0 ± 8.8</td>
<td>0.38 ± 0.12**</td>
<td>4.84 ± 0.33</td>
<td></td>
</tr>
<tr>
<td><em>S. clandestina</em> subsp. <em>peloponnesiaca</em> (Kylini)</td>
<td>330</td>
<td>45.6 ± 1.7</td>
<td>2.79 ± 0.21**</td>
<td>1.19 ± 0.09</td>
<td></td>
</tr>
</tbody>
</table>

Values are the mean of 4 or 5 independent tea preparations ± standard error.

aExpressed as mg dry extract per 100 mL of infusion.

bExpressed as µg gallic acid equivalent per mg of dry extract.

cExpressed as IC_{50} values corresponding to the aqueous extract concentration (mg/mL) causing 50% inhibition of DPPH radical.

dExpressed as µmol Fe³⁺ per mg dry extract.

**P < .05 compared with *S. clandestina* subsp. *peloponnesiaca*.

DPPH, 2,2-diphenyl-1-picrylhydrazyl; FRAP, ferric reducing antioxidant power; IC_{50}, 50% inhibitory concentration.
tea intake led to a significant reduction in MDA levels (Table 2) in the cerebellum (78%) and midbrain (59%), with simultaneous significant elevation in reduced glutathione content of these regions (Table 2) by 27% and 32%, respectively, compared with the control mice. Conversely, in accordance with their total antioxidant power, the MDA and reduced glutathione levels of the cerebral cortex and liver remained unchanged after tea consumption. These findings clearly show that consumption of the tea infusion reinforces the antioxidant defense system of the mouse brain in a region-specific manner.

The diverse pattern of antioxidant effects of tea consumption obtained in different brain areas may be attributed to differences in regional antioxidant defense. This region specificity of antioxidant reinforcement in the brain by polyphenol-rich plant extracts has been also observed in previous studies. Ultimately, the cerebral cortex seems to have a stronger antioxidant defense system, resulting in resistance to changes in its oxidant/antioxidant status. The comparison between the brain regions and liver reveals that the cerebral cortex and liver share the same antioxidant response pattern in response to the herbal tea consumption. As in the cortex, the high antioxidant power of liver, shown in the present and previous studies, might render this tissue insensitive to changes in its oxidant/antioxidant status. This finding is somewhat expected because liver executes several vital functions of the organism.

Our findings conclusively indicate that the consumption of the herbal tea from *Sideritis* species induces a significant region-specific antioxidant reinforcement in the brain, with the cerebellum and midbrain the most affected brain areas. Although further studies should address the specific bioactive ingredients of *Sideritis* infusions, their absorption and bioavailability, and their mechanism of action, the present data strongly suggest that this herbal tea could be a good source of natural antioxidants. The tea might help prevent the age-related deficits and neurodegenerative disorders related to oxidative damage of the specific brain regions.

**ACKNOWLEDGMENT**

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**AUTHOR DISCLOSURE STATEMENT**

No competing financial interests exist.

**REFERENCES**

Wild blueberry (V. angustifolium)-enriched diets alter aortic glycosaminoglycan profile in the spontaneously hypertensive rat

Aleksandra S. Kristo, Christina J. Malavaki, Fotini N. Lamari, Nikos K. Karamanos, Dorothy Klimis-Zacas

Abstract

Glycosaminoglycans (GAGs) are essential polysaccharide components of extracellular matrix and cell surface with key roles on numerous vascular wall functions. Previous studies have documented a role of wild blueberries on the GAG profile of the Sprague–Dawley rat with a functional endothelium as well as in the vascular tone of the spontaneously hypertensive rat (SHR) with endothelial dysfunction. In the present study, the effect of wild blueberries on the composition and structure of aortic GAGs was examined in 20-week-old SHRs after 8 weeks on a control (C) or a wild blueberry–enriched diet (WB). Aortic tissue GAGs were isolated following pronase digestion and anion-exchange chromatography. Treatment of the isolated populations with specific GAG-degrading lyases and subsequent electrophoretic profiling revealed the presence of three GAG species, i.e., hyaluronic acid (HA), heparan sulfate (HS) and galactosaminoglycans (GalAGs). A notable reduction of the total sulfated GAGs and a redistribution of the aortic GAG pattern were recorded in the WB as compared to the C group: a 25% and 10% increase in HA and HS, respectively, and an 11% decrease in GalAGs. Fine biochemical analysis of GalAGs at the level of constituent disaccharides (GalAGs). A notable reduction of the total sulfated GAGs and a redistribution of the aortic GAG pattern were recorded in the WB as compared to the C group: a 25% and 10% increase in HA and HS, respectively, and an 11% decrease in GalAGs. Fine biochemical analysis of GalAGs at the level of constituent disaccharides revealed a notable increase of nonsulfated (18.0% vs. 10.7%) and a decrease of disulfated disaccharides (2.2% vs. 5.3%) in the WB aorta. This is the first study to report the redistribution of GAGs at the level of composition and their fine structural characteristics with implications for the endothelial dysfunction of the SHR. © 2012 Elsevier Inc. All rights reserved.

Keywords: Wild blueberry; SHR; Aorta; GAGs; Heparan sulfate; Chondroitin sulfate

1. Introduction

Numerous studies suggest a protective role of dietary polyphenols against cardiovascular disease (CVD) [1–3], due to direct antioxidant activity [4,5] and/or signaling effects [6,7]. Wild blueberries, a rich source of anthocyanins and other phenolics [8–10], have demonstrated beneficial properties for various vascular functions [11–13]. Glycosaminoglycans (GAGs) are linear polysaccharides composed of repeating disaccharide units of hexosamine, d-galactosamine or d-glucosamine, and uronic acid (UA), d-glucuronic acid or l-iduronic acid. Four major classes of GAG molecules, determined by the hexosamine and UA type as well as sulfation pattern, are present in the vascular wall: (a) hyaluronic acid (HA); (b) galactosaminoglycans (GalAGs), comprised of chondroitin sulfate (CS) and dermatan sulfate (DS); (c) heparan sulfate (HS) and (d) keratan sulfate [14]. Hyaluronic acid is the only GAG molecule that is not sulfated and not linked to a protein core, whereas HS and GalAGs occur as part of proteoglycan (PG) molecules that may contain more than one GAG type, such as HS- and CS-containing syndecan, glypican or CS- and DS-containing versican, decorin and biglycan [15].

Glycosaminoglycans, as prevalent components of the extracellular matrix (ECM) and cell surface, interact with numerous proteins and ligands, modulating crucial biological processes such as cell growth and development [16]. In particular, the glycocalyx associated with the vascular endothelium is increasingly gaining appreciation as a determinant of signaling, mechanotransduction, vascular permeability and inflammatory response and thereby the development of endothelial dysfunction and CVD [17,18]. Additionally, the initiation of atherosclerotic process by lipid accumulation and retention in the ECM is largely mediated by CS/DS proteoglycans [19].

Abnormalities in GAG synthesis, such as CS elevation and increased sulfation, observed in the vasculature of the spontaneously hypertensive rat (SHR) are among the early events that contribute to the vascular pathology of this animal model of endothelial dysfunction of the SHR.

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dysfunction [20–24]. In comparison with its normotensive genetic control, the Wistar Kyoto (WK) rat, the SHR aorta is characterized by a thicker subendothelial matrix and a higher CS synthesis leading to a higher concentration of total GAGs and elevated peripheral resistance and blood pressure [22]. The increased sulfate incorporation in PG molecules in the resistance arteries of the SHR [23] is another hallmark of endothelial dysfunction in this animal model [24].

Although the role of bioactive dietary ingredients on ECM and GAG metabolism has not been thoroughly examined to date, various plant-derived micronutrients [25], vitamins E and C [26], flavonoids [27], tea polyphenols [28], sorghum phenolics [29] and genistein [30] have been reported to positively affect signaling pathways and enzymatic processes related to ECM and GAG metabolism. Wild blueberries consumed with diet have been documented to elicit reduced sulfation and population redistribution of aortic GAGs in the Sprague–Dawley (SD) rat with a normal endothelium [31]. Hence, in the present study, the effect of a wild blueberry diet on the aortic GAG profile and fine structural characteristics was examined in the SHR model of endothelial dysfunction after 8 weeks of diet consumption.

2. Methods and materials

2.1. Chemicals

Pure NaCl, KCl, CaCl₂, MgSO₄, KH₂PO₄, NaHCO₃ and dextrate for the physiologic salt solution (PSS) as well as heparinase I, II, III, chondroitinase AC and ABC, standards for HA, HS and CS/DS analysis and standard preparations of sulfated o-disaccharides from CS/DS [Δ-di-di(2,6)-GlcA, Δ-di-di(2,4)-GlcA, Δ-di-mono6S, Δ-di-mono4S and Δ-di-non5S] were purchased from Sigma (St. Louis, MO, USA), Chondroitinase AC I was purchased from Seikagaku America (Ijamsville, MD, USA), the Blyscan assay kit from Accurate Chemical (Westbury, NY, USA) and PRONASE Protease from Calbiochem (San Diego, CA, USA).

2.2. Animal model and diets

The animal welfare and the experimental protocols conformed to the Animal Care and Use Committee of the University of Maine standards (IACUC Protocol A2008-2006-2005). Twenty male SHRs (Charles River Laboratories, Wilmington, MA, USA) at the age of 12 weeks were randomly assigned to one of two diets: control diet (C) (modified AIN-76, n=10) and wild blueberry diet (WB) (control diet+c.8% wt/wt freeze-dried wild blueberry powder substituting for dextrate, n=10), for a period of 8 weeks. The animals were housed in the Small Animal Facility at the University of Maine in individual stainless-steel mesh-bottomed cages in a room controlled for temperature (22°C) and light conditions (12:12 h light:dark cycle). Tap water and food were provided ad libitum. Food consumption was measured daily; and body weights, weekly. The diets were prepared in our laboratory from purified diet ingredients, stored at 4°C and used within 5 to 7 days. The diet ingredients, dextrate, egg white solids, vitamin mix (A.O.A.C. Special Vitamin Mixture), ß-methionine, bixin, corn oil, were purchased from Harlan Teklad (Madison, WI, USA); custom-made mineral mix was purchased from MP Biomedicals (Solon, OH, USA). Wild blueberries, provided as a composite by Wyman’s (Cherryfield, ME, USA), were freeze-dried and powdered with standard procedures by FutureCeuticals (Momence, IL, USA). Twenty-one different anthocyanins were detected in the wild blueberry powder with the main anthocyanins, malvidin 3-galactoside and peonidin 3-glucoside, representing approximately 13% of the total anthocyanin content (1.6±0.2 mg/100 mg) [8].

2.3. Aortic sample collection and preparation

After 8 weeks of dietary treatment, rats were anesthetized with 95%CO₂/5%O₂; the thoracic aorta was excised and transferred in PSS, composed of NaCl 118, KCl 4.7, CaCl₂ 2.6, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 12.5 and dextrate 11.1 mM. Aortas were cleaned of the adherent connective and fat tissue, frozen in liquid N2 and stored at −80°C. After all samples were collected, frozen specimens were powdered under liquid N2, defatted twice with methanol/chloroform solution (1:1, vol/vol), incubated for 16 h at 4°C, rinsed with acetone and vacuum dried.

2.4. Tissue digestion and separation of GAGs

Dry defatted aortas were rehydrated overnight at 4°C with 2× distilled H₂O, 30 µ/kg aorta. The rehydrated samples were digested overnight at 37°C with PRONASE (1.6 units/mg aortic tissue) in 50 mM Tris-HCl, pH 8.0. The reaction was stopped with 0.6 M NaCl and transfer of vials in 100°C for 1 min. Aortic digests were centrifuged for 5 min at 12,000g, and the collected supernatant was diluted in 4.5 volumes of ethanol solution with 2.5% sodium acetate. After overnight incubation at 4°C, samples were centrifuged for 5 min at 12,000g, supernatant was decanted, and the precipitate was dissolved in 2× distilled H₂O, 5 µ/µg aortic tissue.

2.5. Uronic acid and total sulfated GAG determination

Carbazole reaction modified by Bitter and Muir was applied on aliquots from each sample to determine UA content [32]. Individual samples were also analyzed colorimetrically for total sulfated GAGs (sGAGs) with Blyscan.

2.6. GAG fractionation and identification

The intra-differences in UA and total sGAGs within each diet group were not significant. Therefore, all GAG samples obtained from each diet group (C, n=10 and WB, n=10) were pooled due to limited tissue availability from each rat and applied to ion-exchange chromatography on a DEAE- Sephacel column (7×1.6 cm i.d.), eluted with 0.1 M NaCl (3 volumes) and a NaCl linear gradient (0.1 to 0.9 M, 10 volumes), as previously described [31]. Fractions of 0.8 ml were collected and analyzed for UA. Uronic acid recovery from the DEAE column was higher than 90% in both C and WB pooled samples. The UA-positive DEAE fractions containing distinct GAG populations were pooled, precipitated overnight with 4.5 volumes of absolute ethanol at 4°C and centrifuged at 12,000g for 15 min. The precipitate was then dissolved in 2× distilled H₂O at the final concentration of 1 µg/µl UA. Glicosaminoglycan populations obtained by DEAE fractions were identified by cellulose acetate membrane electrophoresis using known standards of HA, HS and CS/DS and a running buffer of 0.1 M pyridine–0.1 M formic acid, pH 3.1, at a constant current of 0.5 mA/cm of membrane width for 50 min. Toluidine blue in 15% vol/vol aqueous methanol was applied for a 10-min membrane staining. Membranes were rinsed with water and scanned immediately [31].

2.7. GAG degradation with specific lyses

Digestions of pooled samples with a mixture of chondroitinases AC and ABC (0.01 units/µg UA) in 0.1 M sodium acetate–0.1 Tris-HCl buffer, pH 7.3, and a mixture of heparin lyases I, II and III (0.05 units/25 µg UA) in 20 mM acetate buffer, pH 7.0, with 1 µmol calcium acetate were performed overnight at 37°C and stopped by transfer of samples in 100°C for 1 min [31].

2.8. High-performance capillary electrophoresis (HPCE) analysis

Following enzyme digestions, GAG profile analysis was conducted as previously described [31,33] on an HP™P/ACE instrument (Agilent Technologies, Waldron, Germany) with a built-in diode array detector set at 232 nm, using uncoated fused-silica capillary tubes (50 µm i.d., 64.5 cm total and 56 cm effective length) at 25°C. Phosphate buffer, 50 mM, pH 3.0, was used as a running buffer. NaOH 0.1 M (1 min), 2× distilled H₂O (1 min) and operating buffer (5 min) were used before each run to wash the capillary tube. Migration of Δ-disaccharides from the cathode to the anode by electrophoretic mobility and against the electroosmotic buffer flow was achieved by applying samples at the cathodic end under 50 mbar and 30 kV for 5 s.

2.9. Statistical analysis

Student’s t test was used for comparisons of food consumption, body weights, and liver and aorta weight between the diet groups. Two-way analysis of variance was applied in ranked observations of UA and sGAG concentration of individual aortic specimens. Statistical analysis was performed with Sigmasat Statistical Program version 2.0 (SPSS Inc., Chicago, IL, USA). Values are given as mean±standard deviation (S.D.) or mean±standard error of mean (S.E.M.); differences are considered statistically significant at P≤0.05.

3. Results

3.1. Food intake and animal growth

The daily food intake was similar between the control and the wild blueberry diet-fed animals: 20±0.4 g in both diet groups. Growth rate, assessed by weekly measurement of body weight, did not differ significantly between the two diet groups (data not shown). As

<table>
<thead>
<tr>
<th>Diet group</th>
<th>BW (g)</th>
<th>Liver (g)</th>
<th>Aorta (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>343.0±2.7</td>
<td>13.2±1.0</td>
<td>10.4±2.4</td>
</tr>
<tr>
<td>WB</td>
<td>353.0±5.3</td>
<td>12.8±0.9</td>
<td>10.0±2.2</td>
</tr>
</tbody>
</table>

a Mean±S.D., n=10 rats per diet group.
b Aorta dry weight.
presented in Table 1, no significant difference between diet groups was detected in the final body weights or liver wet weight. Furthermore, thoracic aorta dry weight did not differ between the two diet groups.

3.2. Wild blueberry diet affects total content of aortic sulfated GAGs

Aortic samples were analyzed for UA and total sGAGs content after tissue defatting and proteolytic digestion. No significant differences were found in the UA concentration of the aortas isolated from either diet group: 5.2±0.3 vs. 4.6±0.3 μg/mg aorta in the WB and C group, respectively (Table 2). Notably, the concentration of total sGAGs was significantly lower in the WB group (12.6±0.2) as compared to the control (13.7±0.2 μg/mg aorta, P<.05, Table 2).

3.3. Distribution of GAG content is substantially affected in WB vs. control rats

Fractionation, isolation and identification of GAGs were performed as earlier described [31]. In brief, UA-positive populations were separated with ion-exchange chromatography of C and WB aortic samples. The identity of these populations was confirmed with cellulose acetate electrophoresis before and after treatment with specific GAG-degrading hydrolases (not shown). Glycosaminoglycan concentration (μg/mg aortic tissue) estimated by the amount of recovered UA is presented in Table 3. In both diet groups, HS was quantitatively the major GAG population, followed by GalAGs and HA. Although the total GAG content between the two groups tested was similar, the distribution of GAG populations was notably different between the diet groups. Hyaluronic acid concentration was significantly higher (25% increase) in the WB (154.2 vs. 123.6 μg/mg in control group). Heparan sulfate was also 10% higher in the WB (1370.3 vs. 1242.2 μg/mg), whereas GalAGs content was 11% lower in the WB group (960.8 vs. 1081.4 μg/mg, Table 3, Fig. 1).

3.4. Wild blueberry diet increases nonsulfated GalAGs disaccharide content

Analysis of the isolated aortic GalAG population with HPCE after combined digestion with chondroitinases AC and ABC showed that Δdi-mono6S was the main GalAG disaccharide in both diet groups (52.4% in C and 49.5% in WB). The other monosulfated disaccharide, Δdi-mono4S, was also slightly lower in the WB group (30.3% vs. 31.6%). Notably, as shown in Table 4, the content of GalAGs in nonsulfated disaccharides increased twofold in WB group (18.0% vs. 10.7%), and that in disulfated disaccharides (Δdi-di(2,6)S) decreased by 60% (2.2% vs. 5.3%).

4. Discussion

This is the first study to document that a wild blueberry-enriched diet elicited multiple alterations of aortic GAGs in the SHR model of endothelial dysfunction. A significant reduction of total sulfated GAGs was observed in the SHR thoracic aortas after 8 weeks of wild blueberry consumption (Table 2). Although the total aortic GAG content was similar with only a slight increase (2%) in the WB group, wild blueberry consumption resulted in a redistribution of GAG populations. The levels of HA and HS were 25% and 10% higher and the GalAGs 11% lower in the aortic tissue isolated from the WB group (Table 3, Fig. 1). The GalAG disaccharide analysis revealed that the disulfated and monosulfated GalAG disaccharides were reduced due to the wild blueberry diet, whereas a higher percentage of nonsulfated GalAG disaccharides was observed in the SHR aortas from the WB group (Table 4).

The elevated peripheral resistance and blood pressure of the SHR are related to an increase of vascular PGs [22]. Furthermore, in comparison with the parental WK strain, the SHR aorta is characterized by a thicker subendothelial matrix and a higher CS synthesis leading to a higher concentration of total GAGs [22]. With most CSPGs contained in the media layer of the vascular wall, hypertrophy of this layer in small and large SHR vessels agrees with the elevated CS in the SHR [34]. Hypertrophy of the aortic media observed in young SHRs before the development of hypertension is most likely responsible for the lower compliance and distensibility of the SHR aortic wall [35]. Additionally, GalAGs seem to promote atherogenesis, since CS and DS are highly involved in low-density lipoprotein (LDL) binding [36], and especially biglycan is a critical CS/DS-containing PC for LDL retention [37]. Subendothelial retention of apoB100 lipoprotein, the major apolipoprotein of human LDL, is an early step in atherogenesis, with the atherogenicity of the LDL particle linked to its binding affinity for arterial PGs [19,38]. Another indicator of the role of GalAG in atherosclerosis is the recently documented predictability for the postoperative CS4 levels in patients undergoing coronary artery bypass surgery based on preoperative apoB and apoE levels [39].

In the present study, we observed lower GalAGs concentration after 8 weeks of WB treatment, although it was not determined whether the lower GalAGs reflected a decrease in CS or DS or both types. The decrease of GalAGs due to wild blueberry consumption was accompanied by higher concentrations of both HA and HS in our adult

| Table 2  |
|———|———|———|
| Uronic acid and total sGAGs concentration in aortic tissue of the two diet groups | | |
| Diet group | UA (μg/mg)a | sGAGs (μg/mg)b |
| C | 4.6±0.3 | 11.7±0.2 |
| WB | 5.2±0.3 | 12.6±0.2 |

Individual aortas were analyzed for uronic acid concentration with carbazole reaction and sGAG content with Blyscan. Values are given in μg/mg of dry defatted aortic tissue.

| Table 3  |
|———|———|———|
| Glycosaminoglycan-derived UA concentration* in each population isolated from pooled aortic samples with DEAE-Sepharose anion-exchange chromatography in the two diet groups | | |
| HA | WB |
| C | 123.6 (5.1%) | 154.2 (6.2%) |
| HS | 1242.2 (50.8%) | 1370.3 (55.1%) |
| GalAGs | 1081.4 (44.2%) | 960.8 (38.7%) |
| Total GAGs | 2447.3 | 2485.4 |

* μg UA/mg of dry defatted aortic tissue. Percentages of each type of GAG are provided in parentheses.
Wild blueberries, other than being a rich source of bioactive polyphenols and anthocyanins, are a good source of manganese. Phenolics from sorghum bran were reported to inhibit hyaluronidase activity, with implications in the balance between HA synthesis and degradation in the ECM and hence connective tissue remodeling and homeostasis [29]. Flavonoid compounds can inhibit the activity of HA-splitting enzymes based on the number of their hydroxyl groups [27]. Plant-derived compounds (ascorbic acid and quercetin) and tea extract induced alterations of the ECM composition leading to reduction of monocyte adhesion in the endothelium [25]. High concentrations of genistein can inhibit GAG synthesis through an effect on epidermal growth factor-dependent pathway in patients with mucopolysaccharidosis, a condition of excessive GAG accumulation in lysosomes [30]. Signaling processes initiated by vitamins E and C [26] or tea catechins [28] that modulate ECM environment with effects on vascular remodeling and maintenance of vascular wall function also indicate a positive role of dietary and bioactive compounds on vascular integrity.

Current biomedical research has been targeting the endothelium in an attempt to uncover vascular-directed agents for the prevention of atherosclerosis and CVD [49]. GAGs are rendered as such molecules, with special attention given to their chain elongation and sulfation patterns [48]. In the present study, wild blueberries were involved in primary aspects of GAG metabolism such as population redistribution and sulfation in the SHR aorta. We are the first to document the role of a wild blueberry diet on arterial GAG remodeling in the SHR. Wild blueberries modulate structural characteristics that potentiate cardiovascular risk, but most importantly enhance the protective features of GAG molecules associated with endothelial dysfunction and vascular pathology in the SHR. Therefore, wild blueberries may be employed as a component of endothelium-targeted nutrition to prevent endothelial dysfunction and CVD development.

**Acknowledgments**

The authors would like to express their gratitude to Maria I. Krevvata for her significant contribution to this project, the Wild Blueberry Association of North America (WBANA) for contributing the wild blueberries and FutureCeuticals (Momence, IL) for processing them. This work was supported by a WBANA grant to D.K.-Z. and a fellowship by the Greek State Scholarship Foundation to A.S.K. This is Maine Agricultural and Forest Experiment Station Publication Number 3157.

**References**


**Table 4**

Galactosaminoglucosan disaccharide composition as determined by HPCE analysis of pooled aortic samples after combined digestion with chondroitinases AC and ABC in the two diet groups

<table>
<thead>
<tr>
<th>Disaccharide</th>
<th>C</th>
<th>WB</th>
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<tr>
<td>Δdi-di(2,6)S</td>
<td>5.3</td>
<td>2.2</td>
</tr>
<tr>
<td>Δdi-di(2,4)S</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Δdi-monosS</td>
<td>31.6</td>
<td>30.3</td>
</tr>
<tr>
<td>Δdi-monos4S</td>
<td>52.4</td>
<td>49.5</td>
</tr>
<tr>
<td>Δdi-nonS</td>
<td>10.7</td>
<td>18.0</td>
</tr>
</tbody>
</table>

Dashes indicate nondetectable disaccharides.

A longer LDL retention induced by statin treatment was attributed to the decreased sulfation incorporation into smooth muscle cell PGs [48].

The attenuation of sulfation in the SHR aorta after 8 weeks of wild blueberry consumption in the present study suggests that dietary wild blueberries play an important role in aortic GAG remodeling and may improve structural characteristics of the vascular wall related to the development and progress of CVD. This is an important finding, especially in the light of the emerging role of GAGs as vascular agents and sulfation patterns [48]. In the present study, wild blueberries were involved in primary aspects of GAG metabolism such as population redistribution and sulfation in the SHR aorta. We are the first to document the role of a wild blueberry diet on arterial GAG remodeling in the SHR. Wild blueberries modulate structural characteristics that potentiate cardiovascular risk, but most importantly enhance the protective features of GAG molecules associated with endothelial dysfunction and vascular pathology in the SHR. Therefore, wild blueberries may be employed as a component of endothelium-targeted nutrition to prevent endothelial dysfunction and CVD development.

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A percentage of total recovered Δ-disaccharides.

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**References**


Cell-Line Specific Protection by Berry Polyphenols Against Hydrogen Peroxide Challenge and Lack of Effect on Metabolism of Amyloid Precursor Protein

Magdalini A. Papandreou, Maria Tsachaki, Spiros Efthimiopoulos, Dorothy Klimis-Zacas, Marigoula Margaritis and Fotini N. Lamari

INTRODUCTION

Alzheimer’s disease, a form of senile dementia, is an irreversible, progressive neurodegenerative disorder, resulting from a complex pathological cascade that includes the accumulation of beta-amyloid (Aβ) peptide aggregates or aberrant amyloid precursor protein (APP) processing. The disease is manifested years after its initiation with serious cognitive and neuropsychiatric deficits but a clear understanding of its pathogenetic factors is lacking, thus limiting the development of adequate prevention and treatment strategies. Multiple cellular/biochemical events (inflammation, oxidative damage, aberrant signaling, high metal concentrations, aberrant tau/APP processing) are known to contribute to cognitive deficits by disruption of neuronal signal transduction pathways involved in memory and finally neuronal and synaptic loss. There is still no effective therapy for Alzheimer’s disease and pleiotropic drugs or cocktails have been proposed to have a better chance for halting disease progression (Cole and Frautschy, 2010). In that aspect, natural products either individually or in plant extracts are good candidates since pleiotropy is their inherent characteristic.

Blueberries (fruits of various Vaccinium species) contain a wide range of compounds, including the red and blue colored anthocyanins, the flavonols and ellagic acid derivatives, and have the highest antioxidant capacity among fruits and vegetables. A number of studies have documented that rodent diet supplementation with berries is effective in reducing not only oxidative stress but also neuronal, behavioral and cognitive defects associated with aging, inflammation, ischemia and Alzheimer’s disease (Shukitt-Hale et al., 2008; Galli et al., 2006; Joseph et al., 1998, 1999, 2003). In particular, blueberry-fed (4 months of age) APP + PS1 transgenic mice showed no deficits in Y-maze performance (at 12 months of age) with no alterations in Aβ burden (Joseph et al., 2003). Enhancement of cognitive functions and antioxidant protection were also observed by Joseph and his co-workers after dietary supplementation for 8 weeks of aged Fischer 344 rats with blueberry extracts (Joseph et al., 1998, 1999), or of rats subjected to ischemia/reperfusion, with the latter exhibiting a reduction in the volume of infarction in the cerebral cortex and an increase in post-stroke locomotor activity, while they also had significantly lower caspase-3 activity in the ischemic hemisphere (Wang et al., 2005). Additional experiments have shown that blueberry supplementation for 10 weeks of young and old rats subjected to an
in vitro inflammatory challenge (LPS) restored the ability of the aged hippocampal cells to respond to an inflammatory challenge with a large production of inducible heat shock protein 70 (HSP70) (Galli et al., 2006). It was shown previously that a 6-day intraperitoneal administration of a polyphenol-rich wild blueberry extract (PrB) enhanced cognitive performance of healthy mice, brain antioxidant markers and inhibited total brain acetylcholinesterase (Papandreou et al., 2009). Thus, blueberry extracts either through diet or administered via other routes seem to prevent characteristic cognitive deficits of senescence and Alzheimer’s disease and confer antioxidant protection in rodents.

Some mechanisms have been postulated for the efficacy of the blueberry extract and its constituents, but many issues concerning their protective effects remain unresolved. Correspondingly, polyphenol and anthocyanin treatment were also shown to be effective against diverse oxidative stressors, such as H2O2-induced reactive oxygen species (ROS) generation in red blood cells (Youdin et al., 2000a, 2000b) and Aβ-induced toxicity in COS-7 cells (Joseph et al., 2002). It has been recently documented that blueberry extracts significantly enhance microglial clearance of Aβ, inhibit aggregation of Aβ1-42, and suppresses microglial activation, all via suppression of the p44/42 MAPK module (Zhu et al., 2008). Additionally, the whole blueberry extract and many fractions of it (i.e. anthocyanin, pre-C18, post-C18, proanthocyanidins, chlorogenic acid, etc) have been studied for their protective ability against dopamine-, Aβ22- and LPS-induced decrements in calcium buffering in primary hippocampal cells and the degree of protection differed according to the degree of fractionation and the stressor applied (Joseph et al., 2010). Oxidative stress induces the expression and misprocessing of APP, leading to the generation of amyloidogenic fragments that readily form aggregates. This process can result in a potentially vicious cycle whereby oxidative stress leads to Aβ production, and this production in turn results in increased oxidative stress, neuronal dysfunction and ultimately neuronal death (Miranda et al., 2000). The effect of blueberry polyphenols on APP metabolism has not yet been studied.

The aim of the present study was to examine the effects of the extract rich in blueberry polyphenols (PrB) against H2O2-induced cell death in different neuronal and non-neuronal cell lines, which have not been previously studied, and furthermore to study the effect of PrB on APP metabolisms and Aβ-aggregation in vitro. For this reason, the study used (a) the human neuroblastoma SH-SY5Y cell line, a well known model cell system for studying neuronal cell death induced by oxidative stress (Ruffels et al., 2004); (b) Chinese hamster ovary (CHO) cells stably transfected to express mutant human APP770 carrying the Val → Phe mutation at residue 717, were a kind gift of Professor Leonidas Stefanis from the Biomedical Research Foundation of the Academy of Athens (BRFAA). All cultures were grown in DMEM containing 10% heat-inactivated fetal bovine serum, 4.5 g/L glucose, 2 mM-glutamine, 100 μg/mL penicillin and 100 μg/mL streptomycin sulfate. Confluent cultures were subcultured using 0.5 g/L trypsin/0.2 g/L EDTA into 96-well plates at a density of 1.5 × 10^5 cells/well for SH-SY5Y cells and 5 × 10^3 cells/well for HEK293 and CHOAPP770 cells. All experiments were carried out 24h after the cells were seeded. Prior to all treatments, the cells were incubated in serum-free media for 24 h. In a pilot investigation, the cells were treated with H2O2 at concentrations ranging from 50 to 1000 μM, for 18h, and then examined for cell viability. Finally, the cells were co-incubated with 250 μM H2O2 and the tested phytochemical (1–250 μg/mL PrB), or vehicle as control (medium containing max. 0.1% v/v DMSO), for 18h.

Cell extracts were prepared by treating cells with 50 mM Tris, 150 mM NaCl, 2 mM EDTA, pH 7.6 containing protease inhibitors Complete (Roche Applied Science) and 1% Triton X-100 for 30 min on ice. The lyses were then centrifuged at 10000 g for 30 min at 4 °C. The supernatants were collected and the amount of total protein was measured using the bicinchoninic assay (Pierce, Bonn, Germany).

**METHODS AND MATERIALS**

**Plant material and extraction.** Wild blueberries were purchased as a composite from Wyman’s (Cherryfield, ME), freeze-dried with standard procedures by American Lyophilizer Inc. (Bridgeport, PA, USA) and powdered. Polyphenols were extracted from the above as described previously (Lohachoompol et al., 2004; Papandreou et al., 2009). In brief, 2 g of blueberry powder was extracted in the dark, under magnetic stirring with 15 mL/g of methanol, acetic acid and distilled water at a ratio of 25:1:24, respectively, for 2 h. The extract was evaporated to dryness and the dry residue was stored at −20°C until further use.

The dry residue was re-dissolved with 1 mL of 3% formic acid in water (w/v), centrifuged and the supernatant was absorbed on a C18 Sep-Pak cartridge. The cartridge was washed with methanol, equilibrated with 5 mL of 3% formic acid in water (w/v) and eluted with 5 mL 3% formic acid in 50% methanol (w/v). The polyphenols eluted from the cartridge were evaporated under vacuum until dryness and kept at −20°C until use.

Total phenolics were measured with the Folin-Ciocalteu reagent method (Singleton and Rossi, 1965). The total polyphenolic content was expressed as gallic acid equivalents (GAE), using a standard curve with 50–600 mg/L gallic acid. Total anthocyanin content was estimated according to Giusti and Wrolstad (2001) by UV-visible spectroscopy at 538 nm after dissolution in a mixture of methanol and 0.1 M HCl at a ratio of 85:15. The total anthocyanin content was expressed as cyanidin 3-rutinoside equivalents.

**Cell culture and drug treatment.** Human neuroblastoma SH-SY5Y and human embryonic kidney HEK293 cell lines were obtained from the American Type Culture Collection (ATCC), while the Chinese Hamster Ovary (CHO) control cells (wild type, wt) or CHOAPP770 cell, stably transfected to express mutant human APP770 carrying the Val → Phe mutation at residue 717, were a kind gift of Professor Leonidas Stefanis from the Biomedical Research Foundation of the Academy of Athens (BRFAA). All cultures were grown in DMEM containing 10% heat-inactivated fetal bovine serum, 4.5 g/L glucose, 2 mM-glutamine, 100 μg/mL penicillin and 100 μg/mL streptomycin sulfate. Confluent cultures were subcultured using 0.5 g/L trypsin/0.2 g/L EDTA into 96-well plates at a density of 1.5 × 10^5 cells/well for SH-SY5Y cells and 5 × 10^3 cells/well for HEK293 and CHOAPP770 cells. All experiments were carried out 24h after the cells were seeded. Prior to all treatments, the cells were incubated in serum-free media for 24 h. In a pilot investigation, the cells were treated with H2O2 at concentrations ranging from 50 to 1000 μM, for 18h, and then examined for cell viability. Finally, the cells were co-incubated with 250 μM H2O2 and the tested phytochemical (1–250 μg/mL PrB), or vehicle as control (medium containing max. 0.1% v/v DMSO), for 18h.

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Assessment of cell viability and reactive oxygen species levels. Cell viability was estimated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT reagent) (Plumb et al., 1989). Briefly, on completion of the treatment, the cells were washed with pre-warmed phosphate-buffered saline (PBS) and incubated with serum-free media containing MTT at a final concentration of 2.5 mg/mL for 150 min. Supernatants were carefully aspirated and the resultant formazan product was dissolved in DMSO and the absorbance was measured using a UV-Vis spectrometer (Denley WellScan, West Sussex, UK) at 545 nm. Wells without cells were used as blanks and were subtracted as background from each sample. The results were expressed as a percentage of control untreated cells.

Intracellular formation of ROS was assessed using the probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich Corporation, St Louis, MO, USA), which was used as a substrate (Wang and Joseph, 1999). All cell lines (SH-SY5Y: 1.5 × 10^5 cells/well, HEK293/CHOAPP770: 5 × 10^5 cells/well), growing in microtiter 96-well plates, were washed twice with pre-warmed PBS, and then incubated with 50 μM DCF-DA in PBS for 30 min at 37°C. Fluorescence was then quantified in a microplate-reader (Bio-Tek Instruments Inc., Ville St Laurent, Que, Canada) at an excitation wavelength 485 nm and emission wavelength 530 nm.

Assessment of malondialdehyde levels. CHOAPP770 cells were incubated in serum-free media, for 24 h, and then treated with PrB extract (10–50 μg/mL) for 48 h. The cells were then lysed, centrifuged and the collected supernatants were subjected to malondialdehyde (MDA) analysis. The concentration of MDA, a compound that is produced during lipid peroxidation was determined fluorometrically by the thiobarbituric acid (TBA) method, as previously described (Jentzsch et al., 1996; Papandreou et al., 2009). The assay showed good linearity (0.05–5.00 μM, y = 11.488x + 0.7415, R² = 0.9976) and sensitivity. The level of MDA was expressed as nmol/mg protein in order to correct for any variability in cell number dictated by cell treatment.

Thiolutavine T assay. Thiolutavine T (ThT) measurement was performed according to the method described previously (Tjernberg et al., 1999; Papandreou et al., 2006). For Aβ aggregate formation assay, Aβ1-40 (10 mg/mL) was diluted in PBS (10 mM, 150 mM NaCl, pH 7.4) to 40 μM and incubated at room temperature for 5 and 30 days, with various concentrations of the PrB extract or without (control) (Papandreou et al., 2006). Within 30 min after addition of thiolutavine T (10 μM), the fluorescence intensity was measured with a RF-1501 spectrofluorometer (Shimadzu), using an excitation filter of 435 nm and an emission filter of 482 nm, respectively. The percentage inhibition was calculated by comparing these fluorescence values with those found in control solutions with no PrB.

Western blot analysis of amyloid-precursor protein (APP) metabolism. CHOAPP770 cells were treated with the plant extract (10–50 μg/mL for PrB) for 48 h. The cells were then lysed, centrifuged and the obtained supernatant, as well as the cell culture media, were used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were separated on gels (12% for media and 16% for cell lysates) and transferred to nitrocellulose membranes, which were incubated overnight at 4°C with the primary antibodies (1:1000) (6E10: residues Aβ1-16 monoclonal; R1(57): anti-APP C-terminal polyclonal; 71-192wt-1147: residues APP590-596 (Parisiadou and Efthimiopoulos, 2007; Parisiadou et al., 2008). Bound antibodies were visualized using peroxidase-conjugated secondary antibodies (anti-rabbit used in 1/2000 for R1 (57) and 1/1000 dilution for 71-192wt-1147; anti-mouse used in 1/1,000 dilution for 6E10), followed by ECL+ detection (ChemBio Ltd).

Statistical analysis. Data are presented as mean ± SE for at least ten replications. Statistical analysis was performed with GraphPad Instat 3 software (GraphPad Instat Software, Inc. USA) using the nonparametric Mann-Whitney test. In all tests, a criterion of p ≤ 0.05 (two-tailed) was considered necessary for statistical significance.

RESULTS

H2O2-induced toxicity on SH-SY5Y, HEK293 and CHOAPP770 cell lines

To determine the effective concentrations of H2O2, all cell lines were treated with various concentrations (50–750 μM) of H2O2 for 18 h and the viability of the cells was assessed by the MTT assay. Treatment with 50 μM and 100 μM H2O2 had virtually no effect on the viability of all cell lines under the conditions employed, but the viability of cells exposed to concentrations higher than 100 μM showed a dose-dependent decrease. In the SH-SY5Y cell line, the cell viability decreased by 43.0 ± 0.5%, 56.0 ± 0.5% and 60.0 ± 0.9%, compared with untreated cells after treatment with 250 μM, 500 μM and 750 μM H2O2, respectively. Similarly, in the HEK293 cells, in the presence of 250 μM and 500 μM H2O2, the percentage of cell viability decreased by 30.0 ± 2.0% and 48.0 ± 0.9%, respectively, while at 750 μM H2O2, by 69.0 ± 0.8%. Treatment of CHOAPP770 cells with 250 μM, 500 μM and 750 μM H2O2, resulted in 35.0 ± 1.2%, 40.0 ± 0.8% and 49.0 ± 1.2% cell death, respectively. There was a significant injury in all three cell lines tested, with the H2O2-treated cells becoming round and some of them detaching from the plate. Moreover, the H2O2-induced oxidative damage to cells was also measured as ROS formation, as assayed by the conversion of the probe DCFH-DA to DCF. ROS accumulation increased significantly in all cell lines tested after incubation with H2O2 (250 μM), as indicated by the increase in relative fluorescence units (SH-SY5Y, 73400 ± 4585; HEK293, 38529 ± 2220; CHOAPP770, 48999 ± 1086 DCF/MTT) compared with the untreated (control) cells (SH-SY5Y, 58114 ± 2901; HEK293, 18406 ± 8433; CHOAPP770, 40861 ± 1381 DCF/MTT values), respectively.

PrB content in polyphenols and anthocyanins

The PrB extract, obtained from solid-phase extraction on Sep-Pak C18, had a total polyphenolic content of
13.01 ± 0.06 mg GAE/g dry weight and a total anthocyanin content of 3.78 ± 0.05 mg/g dry weight, as reported previously (Papandreou et al., 2009). Comparison of this composition with that reported for freeze-dried blueberry powder by others (Mazza et al., 2002) shows that the solid-phase extraction concentrated the extract in polyphenols about four times.

**PrB rescues viability and decreases ROS levels in H2O2-treated SH-SY5Y cells**

As illustrated in Fig. 1A, treatment of SH-SY5Y cells with PrB extract (1–250 μg dry extract/mL) significantly reduced the cell death caused by H2O2 in a non-concentration-dependent fashion. No difference was seen in cell viability between cells treated with PrB alone and controls (data not shown). Moreover, co-incubation of SH-SY5Y cells with PrB extract and H2O2 not only completely abolished H2O2-induced ROS accumulation but further reduced ROS levels by 57–63% compared with the untreated cells.

**APP metabolism is not affected by PrB**

When CHO APP770 cells were treated with PrB for 48 h, no alterations were observed either on sAPPα/β levels secreted into their conditioned media (Fig. 5A, B), or on cellular APP expression (Fig. 5C), as evidenced by western blot, using β-actin as an internal control.

**DISCUSSION**

Hydrogen peroxide has been used extensively as an inducer of oxidative stress in in vitro models. Co-treatment of cells with H2O2 and PrB showed that the PrB effects varied among the types of cell lines tested (Figs 1–3). In particular, in the human neuroblastoma SH-SY5Y cell line, the PrB extract reduced H2O2-induced cell death and generated ROS, to levels lower than those of the untreated cells; whereas in the or untreated cells even though viability was not different than that of the H2O2-treated cells (Fig. 3). Furthermore, the effect of PrB on membrane lipid peroxidation in CHO APP770 cells was investigated. As shown in Fig. 4, CHO APP770 cells producing considerable amounts of Aβ species, showed an increase in lipid peroxidation (~88%), in comparison with the wild-type CHO cells, as evidenced by determination of MDA levels (0.160 ± 0.005 nmol/mg protein vs 0.091 ± 0.002 nmol/mg protein). However, PrB prevented peroxidation of lipids compared with the control, by 51%, 25% and 31%, at PrB concentrations of 1, 10 and 50 μg/mL, respectively.

**Inhibition of Aβ aggregation in vitro by PrB**

The effect of PrB on the Aβ1-40 propensity to form aggregates was studied by the standard ThT-based fluorescence assay. The PrB extract significantly inhibited Aβ aggregation in vitro in a time-dependent manner (Fig. 6). Notably, the inhibitory effect at the lowest concentration (15 μg dry extract/mL) was 67% and 62%, at 5 and 30 days, respectively.

**Antioxidant effects of PrB in CHO APP770 cells**

The PrB treatment significantly reduced the cell death of CHO APP770 cells treated with H2O2 only at the highest applied concentrations of PrB (125 and 250 μg/mL) with a concomitant low (7–9%) but statistically significant decrease in ROS levels in comparison with H2O2-treated cells (Fig. 3). However, at concentrations of 1–50 μg/mL of PrB in the presence of 250 μM H2O2, the ROS levels were significantly higher than those of the H2O2-treated cell line with H2O2 alone (Fig. 2B).
HEK293 cells it exacerbated H\textsubscript{2}O\textsubscript{2} cytotoxicity. Since CHO\textsuperscript{APP770} cells overproduce APP and A\textsubscript{\textbeta} in turn, they can be considered as more stressed in comparison with wild type cells; MDA measurements confirm this phenomenon. Treatment of those cells with PrB reduced the lipid peroxidation levels significantly. This is in agreement with reports whereby blueberry polyphenols protected primary hippocampal neuronal cells (HNCs) against A\textsubscript{\textbeta}-induced stress (Joseph \textit{et al}., 2010). In our experiment, when CHO\textsuperscript{APP770} cells were additionally stressed with H\textsubscript{2}O\textsubscript{2}, PrB further increased ROS levels (the higher the concentration, the lower the increase), while cell viability was enhanced in comparison with H\textsubscript{2}O\textsubscript{2}-treated cells, only at the highest applied concentrations. The current findings of PrB-induced increases in ROS levels in H\textsubscript{2}O\textsubscript{2}-stressed CHO\textsuperscript{APP770} and HEK293 cells are in line with the recently published data by Joseph and his co-workers (Joseph \textit{et al}., 2010), whereby treatment of HNC cells with whole blueberry extract or certain fractions of it (e.g. proanthocyanidins, POST-C18 and to some extent PRE-C18), resulted in increases in ROS levels, even in the absence of the stressors (i.e. dopamine, LPS or A\textsubscript{\textbeta12}).

This type of polyphenol-induced cytotoxicity is not uncommon in cell culture experiments and has been largely ascribed to the nature of the phenolic compounds, which can act either as pro-oxidants or as cytotoxic agents, in certain conditions (Sergedie\textit{ne et al}., 1999; Shamim \textit{et al}., 2008). Recent findings have also implicated certain phenolic compounds (e.g. delphinidin) in the induction of H\textsubscript{2}O\textsubscript{2}, within commonly used cell culture media, possibly due to their unstable nature and their ability to undergo chemical transformations, resulting thus in the formation of by-products capable

Figure 2. HEK293 viability and ROS levels. Cells were co-treated with 250 \textmu M H\textsubscript{2}O\textsubscript{2} and PrB (1–250 \textmu g dry extract/mL), for 18 h, and both cell viability (A) and ROS levels produced (B) were measured by MTT and DCF assay, respectively, *p < 0.05 vs H\textsubscript{2}O\textsubscript{2}-treated cells. Percentage indicates the percentage of cell death in the presence of the H\textsubscript{2}O\textsubscript{2}. There was a statistically significant difference (p < 0.05) in viability and ROS levels of all treated cells from control untreated cells.

Figure 3. CHO\textsuperscript{APP770} viability and ROS levels. Cells were co-treated with 250 \textmu M H\textsubscript{2}O\textsubscript{2} and PrB (1–250 \textmu g dry extract/mL), for 18 h, and both cell viability (A) and ROS levels produced (B) were measured by MTT and DCF assay, respectively. *p < 0.05 vs H\textsubscript{2}O\textsubscript{2}-treated cells. *\textgreater p < 0.05 vs untreated control.

Figure 4. Effect of PrB extract on lipid peroxidation (MDA) levels. CHO\textsuperscript{APP770} cells were incubated with PrB (1–250 \textmu g/mL) for 48 h. *p < 0.05 vs untreated control, #p < 0.05 vs wild CHO cells.

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of causing cell-specific toxicity (Long et al., 2010). In our experiments, however, treatment of cells only with PrB did not affect viability, as assessed by MTT, although it is more difficult to delineate on the changes of the phenolic compounds contained within the extract, due to the experimental procedure followed, i.e. co-administration with H2O2.

In addition, the lack of dose-dependency observed in either cell-viability or ROS production after cotreatment of all three cell lines in the particular concentration range is not uncommon in studies of natural products. For instance, treatment of normal epithelial cells and cells derived from the salivary gland with epigallocatechin gallate, resulted in a dose-independent effect in ROS production (Yamamoto et al., 2003).

The fact that PrB is an extract with many different polyphenol entities, which act by a variety of mechanisms, implicates the principles of synergy, antagonism and pleiotropy in explaining this phenomenon. Taking all these data into consideration, it is concluded that the PrB extract selectively protected SH-SY5Y cell viability from H2O2 stress and significantly decreased ROS levels, in contrast to HEK293 cells and partly CHOAPP770 cells. The observed differences in response could be ascribed to the existence of distinct cell surface receptors, cellular membrane composition and intracellular retention transport. For example, although quercetin-3-glucoside protects both SH-SY5Y and HEK293 cells against H2O2-induced viability loss, the underlying mechanisms are not the same: it induces sterol regulatory element-binding protein-2-mediated cholesterol biosynthesis only in SH-SY5Y cells (Soundararajan et al., 2008).

Oxidative stress and ROS accumulation, apart from their implication in cellular damage, are also involved, at least in part, in the toxicity of Aβ-peptide and its aggregation/accumulation (Miranda et al., 2000), and thus, with the pathogenesis of neurodegeneration in AD. The active form of Aβ-amyloid consists of soluble oligomeric fragments, composed of 39–43 amino acids; Aβ1-40 and Aβ1-42, are the predominant forms. The latter peptidic fragments are derived from the proteolytic processing of the Aβ-amyloid precursor protein (APP) via two pathways, each resulting in distinct cleavage products: (1) a non-amyloidogenic which involves cleavage of APP to soluble APP (sAPP) by the α-secretase; and (b) a formation of amyloidogenic β peptides by the β- and γ-secretases. As the proportion of APP processed by β-secretase vs α-secretase may affect the amount of Aβ produced, regulation of these two pathways may be critically important to the pathogenesis of AD.

The examination of the PrB extract on CHOAPP770 cells, stably transfected to express human APP, revealed that the tested phytochemical had no effect on APP metabolism, as evidenced by western blot analysis (Fig. 5). The former observation might provide an explanation for previous experiments performed by Joseph et al. (2003) showing no effect on Aβ peptide production, deposition or amyloid load on transgenic PS1-APP mice brains, after supplementation with blueberries extracts (2% of diet) from 4 to 12 months of age. Similar results were also observed after treatment of both transfected murine N2A (Swedish mutant form of APP, SweAPP N2a) and primary neuronal cells derived from transgenic APPsw mice with the catechins, (−)-epigallocatechin and (−)-epicatechin (Rezai-Zadeh et al., 2005).

However, the PrB extract significantly inhibited in vitro Aβ-fibrillogenesis (up to 78.79 ± 3.63%, at a concentration of 0.1 mg/mL, after 5 days of incubation)
(Fig. 6) as evidenced by the thioflavin T assay. Inhibition of Aβ aggregation by blueberry polyphenols was also reported by Zhu et al. (2008) and Fuentealba et al. (2011), whereas Guo et al. (2010) have reported a marginal inhibitory effect using two different in vitro tests. Silver staining after gel electrophoresis showed that a blueberry-enriched polyphenol extract significantly inhibited the formation of small aggregates (e.g. 10, 40 kDa), which are considered to be toxic (Fuentealba et al., 2011). Considering the in vitro results and the results in transgenic PS1 + APP rodents (Joseph et al., 2003), it seems that although in vitro tests document antiaggregant properties of blueberry polyphenols, these might not be enough to prevent amyloid aggregation in vivo or might be more potent against the formation of small soluble aggregates (not observed with the dye techniques usually used for visualization of amyloid load). Questions on the metabolism, bioavailability in humans and the ability to prevent amyloid deposits at the onset of the disease remain to be answered.

In conclusion, our results demonstrated that the PrB extract of wild V. angustifolium protect or further damage cells against H2O2-induced oxidative stress according to the cellular environment and characteristics of the particular cell line investigated. Measurements of cell viability and scavenging of ROS production after co-treatment with H2O2 and various concentrations of PrB showed that the PrB extract significantly protected SH-SY5Y at all concentrations tested and CHOAPP770 only at high concentrations. On the contrary, co-treatment of HEK293 with H2O2 and PrB exacerbated further the oxidative damage to cells. Beyond antioxidant effects in CHOAPP770 cells (it lowered MDA levels in untreated CHOAPP770 cells compared with wild CHO cells), PrB extract did not induce changes in APP processing. These findings contribute significantly to the ongoing search for compounds with pleiotropic actions against Alzheimer’s disease and in particular to study of the neuroprotective properties of blueberries.

Acknowledgement

The authors would like to kindly acknowledge Professor Leonidas Stefanis from Biomedical Research Foundation of the Academy of Athens (BRFAA) for providing the human neuroblastoma SH-SY5Y and stably transfected Chinese Hamster Ovary CHO APP cell lines and the Wild Blueberry Association of North America for providing the wild blueberry powder.

Conflict of Interest

The authors have declared that there is no conflict of interest.


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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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
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 antiallergic [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-MS² 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* subsp. *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 Peloponnesse) 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). All LC-MS 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-$\mu$L aliquot was filtered (0.45 $\mu$m) and injected into the LC-MS instrument. Separation was achieved on a 25 cm $\times$ 4.6 mm i.d., 5 $\mu$m 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; 20–30 min 75–50% A; 30–40 min 50–20% A; 40–60 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 $^1$H–$^1$H COSY, $^1$H–$^{13}$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 $^1$H-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 $\times$ 23 cm $\times$ 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$_2$HPO$_4$, pH 7.6. The homogenates were then centrifuged for 20 min at 15,000g 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 x – 0.5959, R² = 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 α-phthalaldehyde, as previously described [22]. A good linearity was obtained for GSH in the range of 0.5–100 μM (y = 1.2353 x – 3.46, R² = 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 isoscutelearein 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.
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 ms³ 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 ms² 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 clandestina [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 ms³ 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]$^2$ was derived from the loss of a glucose esterified with coumaric acid. The base peak [(M–H)-324]$^-$ 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.

Evaluation of the anxiolytic-like 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

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].

[Fig. 2 Evaluation of thigmotaxis time and 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

<table>
<thead>
<tr>
<th>Tissues</th>
<th>μmol MDA/g protein</th>
<th>μmol GSH/g protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control mice</td>
<td><em>Sideritis</em> 2% w/v mice</td>
</tr>
<tr>
<td>Brain (-Ce)</td>
<td>5.24 ± 0.17</td>
<td>4.42 ± 0.13*</td>
</tr>
<tr>
<td></td>
<td>(16%)</td>
<td>(129%) [15%]</td>
</tr>
<tr>
<td>Liver</td>
<td>1.59 ± 0.16</td>
<td>1.52 ± 0.09</td>
</tr>
<tr>
<td>Heart</td>
<td>1.19 ± 0.16</td>
<td>0.95 ± 0.09</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Tissues</th>
<th>μmol MDA/g protein</th>
<th>μmol GSH/g protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control mice</td>
<td><em>Sideritis</em> 2% w/v mice</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>3.13 ± 0.22</td>
<td>2.95 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>(145%)</td>
<td>(189%) [162%]</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>2.10 ± 0.13</td>
<td>1.15 ± 0.04*</td>
</tr>
<tr>
<td></td>
<td>(145%)</td>
<td>(179%) [162%]</td>
</tr>
<tr>
<td>Midbrain</td>
<td>1.45 ± 0.08</td>
<td>0.72 ± 0.035*</td>
</tr>
<tr>
<td></td>
<td>(150%)</td>
<td>(163%) [135%]</td>
</tr>
</tbody>
</table>

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 depletions 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 antioxidant 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 metallo-toxins (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-β25–35-induced toxicity in mice [49] and inhibits the production of nitric oxide (NO) and prostaglandinE2 (PGE2) 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.
Acknowledgments. We are grateful to Dr. Garbis S. for helpful discussions and comments. This work was partially supported by BIOFLORA, network of University of Patras and Esthiri-Gkani Foundation, Ioannina. Special thanks are given to the Mass Spectrometry Unit of University of Ioannina for providing access to LC-MS/MS facilities and Mrs. Theodora Lamari for collecting the plant material. NMR data were recorded in the NMR center of the University of Ioannina.

References

Saffron as a Source of Novel Acetylcholinesterase Inhibitors: Molecular Docking and in Vitro Enzymatic Studies

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* Supporting Information

ABSTRACT: Inhibitors of acetylcholine breakdown by acetylcholinesterase (AChE) constitute the main therapeutic modality for Alzheimer’s disease. In the search for natural products with inhibitory action on AChE, this study investigated the activity of saffron extract and its constituents by in vitro enzymatic and molecular docking studies. Saffron has been used in traditional medicine against Alzheimer’s disease. Saffron extract showed moderate AChE inhibitory activity (up to 30%), but IC50 values of crocetin, dimethylcrocetin, and safranal were 96.33, 107.1, and 21.09 μM, respectively. Kinetic analysis showed mixed-type inhibition, which was verified by in silico docking studies. Safranal interacts only with the binding site of the AChE, but crocetin and dimethylcrocetin bind simultaneously to the catalytic and peripheral anionic sites. These results reinforce previous findings about the beneficial action of saffron against Alzheimer’s disease and may be of value for the development of novel therapeutic agents based on carotenoid-based dual binding inhibitors.

KEYWORDS: acetylcholinesterase, dual inhibitors, in silico, saffron, mixed inhibition, crocetin, safranal

INTRODUCTION

Alzheimer’s disease (AD) is a multifactorial dementia characterized by cerebral accumulation of extracellular amyloid-β (Aβ) protein aggregates (senile plaques) and intraneuronal hyperphosphorylated twisted filaments of tau protein (neurofibrillary tangles) in brain areas associated with learning and memory (e.g., cortex, hippocampus, nucleus basalis of Meynert), resulting, thus, in profound memory disturbances and irreversible impairment of cognitive function. The latter was ascribed primarily to the loss of cholinergic neurons in those areas, due to Aβ and tau buildups, and led to the formation of “the cholinergic hypothesis” of AD.2,3

The abnormalities in cholinergic metabolism seen in AD are not viewed as the cause of the disorder, but cholinergic involvement is significant because it is universal, correlates with cognitive defects, and is one of the few pathophysiologic phenomena that can be addressed with currently approved treatment options, such as cholinesterase inhibitors (ChEIs) [e.g., tacrine (THA), rivastigmine, and galanthamine (GNT)]. ChEIs enhance cognitive function by inhibiting the catabolism of the neurotransmitter acetylcholine (ACh) by acetylcholinesterase (AChE) increasing the action of ACh in the synapse. Although various clinical studies document the beneficial effects of current AChE inhibitors on cognition and behavior, their benefits are modest, suffer from short half-lives, and show serious side effects caused by activation of peripheral cholinergic systems.4–6 Potent AChE inhibitors belong to the class of alkaloids, due to the requirement for positively charged quaternary ammonium in the structure, such as GNT and physostigmine.

AChE, apart from its involvement in cholinergic synaptic transmission, is also known to accelerate the aggregation of Aβ during the early stages of AD, primarily via interactions through its peripheral anionic binding site (PAS).7–9 In light of these data, the search for new AChE inhibitors for AD now includes the search of dual binding molecules, being able to interact simultaneously with both the PAS and the catalytic subsite of AChE.10–12

Advances in X-ray crystallography and site-directed mutagenesis have enabled the detailed mapping of AChE’s active center. Human AChE (EC 3.1.1.7) is a globular protein containing a 20 Å deep groove (gorge), which includes the following loci: (1) the acyl-binding pocket Phe295(288) and Phe297(290) (values in parentheses represent the amino acid numbering positions in Torpedo californica AChE) at the base

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of the gorge; (2) the esteratic locus, which consists of two subsites, the oxyanion hole [Gly120(118), Gly121(119), and Ala204(201)] and the active site catalytic serine hydrolase triad [His447(440), Glu334(327), and Ser203(200)]; (3) the quaternary ammonium binding locus [Trp86(84)]; and (4) the PAS site [Tyr72(70), Tyr124(121), and Trp286(279)], situated >10 Å above the active site triad and near the gorge entrance.13,14

Saffron, derived from the stigmas of *Crocus sativus*, is a well-known spice with many reputed therapeutic uses, including its use as a tonic, nerve sedative, and antidepressant and its use against dementias.15,16 Its composition is rare because it is a known spice with many reputed therapeutic uses, including its moderate AD.16,23,24 However, the effect of saffron constituents tested, that is, crocin (CRC), crocetin (CRT), dimethylcrocetin (DMCRT) and safranal (SFR), as well as of AChE’s substrate acetylthiocholine iodide (ATCI) and of AChE known inhibitors THA and GNT, are shown in Figure 1. CRC, a natural carotenoid, is the diester formed by the disaccharide gentiobiose and CRT. CRT is the main metabolite in humans, whereas the synthetic analogue of DMCRT was used to augment the study of carotenoid structure–activity relationships, that is, to investigate the role of CRT carboxyl groups. SFR is a monoterpene aldehyde and the main component of the essential oil of saffron.

#### MATERIALS AND METHODS

**Plant Material and Extraction.** Commercially available pure red Greek saffron (stigmas of *C. sativus*) was kindly provided by the Cooperative Association of Krokos in Kozani, in West Macedonia, Greece. Saffron was extracted with methanol/water 1:1 v/v, as previously described.17 CRT (purity > 98%) was prepared by saponification of saffron extract, whereas DMCRT was prepared by saponification of saffron extract in the presence of methanol, as previously described.18,19 and its final purity was >98%. SFR (purity > 88%) was purchased from Sigma-Aldrich Corp., St. Louis, MO, USA.

**In Vitro AChE Inhibition Assay.** The assay for AChE activity was performed with the colorimetric method of Ellman et al.,30 utilizing ATCI as a substrate and AChE from the electric eel (Sigma-Aldrich Corp.). Brie...
Table 1. Enzyme Kinetics of the Tested Compounds on AChE Activity

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inhibitor</th>
<th>IC_{50} (μM)</th>
<th>V_{max} (μmol/L/min)</th>
<th>K_{m} (μM)</th>
<th>Type of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCI</td>
<td>control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCI</td>
<td>control</td>
<td>23.01 ± 2.70</td>
<td>65 ± 3.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCI</td>
<td>gallocithione (GNT)</td>
<td>1.93 ± 0.05</td>
<td>26.3 ± 3.02</td>
<td>65 ± 3.3</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>ATCI</td>
<td>safanal (SFR)</td>
<td>21.09 ± 0.17</td>
<td>18.5 ± 23.9</td>
<td>27 ± 2.4</td>
<td>90.6 ± 2.0</td>
</tr>
<tr>
<td>ATCI</td>
<td>crocin (CRT)</td>
<td>96.33 ± 0.11</td>
<td>19.30 ± 2.6</td>
<td>39 ± 2.2</td>
<td>280.0 ± 2.3</td>
</tr>
<tr>
<td>ATCI</td>
<td>dimethylcrocin (DMCRT)</td>
<td>107.1 ± 0.11</td>
<td>16.5 ± 1.9</td>
<td>38 ± 1.8</td>
<td>16.5 ± 2.4</td>
</tr>
</tbody>
</table>

*a The values of V_{max}, K_{m}, and K_{i} were calculated by monitoring hydrolysis of substrate (ATCI) by AChE at different substrate concentrations (0.125–64 μM) in the presence or absence of samples (mean results of 4 experiments, run in duplicate) (0.15–34 μM).

measured again after 5 min of incubation at room temperature in the dark. GNT (1–32 μM, final concentrations) (Sigma-Aldrich) was used as a reference inhibitor. In all experiments the final organic solvent percentage was never above 6% v/v; control experiments with the same percentage of organic solvent without sample were also conducted, and the values were subtracted. All determinations were carried out at least four times, and in duplicate, at each concentration in 96-well microplates, using a UV–vis microplate reader (Molecular Devices). Results were reported as percentage of inhibition of AChE activity in the absence of inhibitor/tested compounds (positive control). The concentrations of the tested extracts that inhibited the hydrolysis of substrate (ATCI) by 50% (IC_{50}) were determined by GraphPad Prism v4.0 (GraphPad Software, Inc., USA) from sigmoidal dose–response curves obtained by plotting the percentage of inhibition (Y) versus the log concentration (x, μM) of the tested samples, using the equation

\[ Y = \frac{100}{1 + 10^{(\log IC_{50} - x)}} \]

**Kinetic Analysis of AChE Inhibition.** Microplate wells were filled with 50 μL of 50 mM Tris-HCl, pH 8.0, containing 0.1% BSA, 25 μL of the tested phytochemicals (0–256 μM CRT and DMCRT and 0–120 μM SFR) or GNT (0–120 μM), 25 μL of AChE (0.22 U/mL), and 75 μL of 3 mM DTNB (dissolved in 50 mM Tris-HCl, pH 8.0, containing 0.1 M NaCl, 0.02 M MgCl_{2}, 6H_{2}O). The mixture of enzyme and inhibitor/substrate samples was mixed, and absorbance was read at 405 nm after 5 min of incubation at room temperature, in the dark. Immediately after addition of the substrate (25 μL ATCI, 1–512 μM concentrations, dissolved in water), the change of absorbance at 405 nm was monitored for 5 min, and the reaction rate was calculated according to the method of Ellman et al.\(^{30}\) Inhibition studies were analyzed using GraphPad Prism v4.0 software. The analysis of the type of inhibition of AChE activity was determined by the Lineweaver–Burk (LB) plot, whereas the kinetic parameters K_{m} and V_{max} were obtained by curve fitting according to the classical Michaelis–Menten equation. The inhibition constants (K_{i}) of the tested samples were calculated according to GraphPad Prism v4.0, from linear regression analysis of LB plots (S, hyperbolic; I, hyperbolic mixed model).

**In Silico Computational Methods (Molecular Modeling and Docking Calculations).** Molecular models of all compounds described in this work were built in 3D coordinates and their best, most stable (lower energy) conformations were detected by geometrical optimization of their structure in the gas phase, as implemented in the Spartan '08 Molecular Modeling program suite (Spartan '08 v. 1.2.0, Wave Function Inc., Irvine, CA, USA). The molecules' structures were initially optimized by conformational search using the Monte Carlo method with the MMFF94 molecular mechanics model. Geometry optimization was accomplished via quantum-chemical calculations by utilizing the ab initio Hartree–Fock method with the 6-31G* basis set. Docking calculations were carried out via the BioMedCACHem program, which is part of the CACHem package (CACHem WorkSystem Pro version 7.5.0.85, Fujitsu). Docking experiments employed full ligand flexibility and partial protein flexibility focused at the ligand binding site. AChE complex with various inhibitors has provided valuable knowledge of the interactions that mediate inhibitor binding.\(^{31}\) To identify the molecular determinants responsible for the binding mode of the studied compounds with AChE protein, we used the determined X-ray crystallographic structure of T. californica AChE in complex with the AChE inhibitor, THA (PDB accession no. 1ACJ).\(^{32}\) X-ray structure was obtained from the Brookhaven Protein Data Bank (RCSB Protein Data Bank, operated by the Research Collaboratory for Structural Bioinformatics).\(^{33}\) The produced compound–protein complexes were ranked by the energy score, including their binding conformations. 3D models of the above protein crystal structures have been developed after the deletion of the cocrystallized bound inhibitor. The docking procedure provides the treatment of ligand flexibility within the protein binding site by means of a four-point chiral pharmacophoric comparison between the ligand and the site. The final output of the docking procedure is a set of solutions ranked according to the corresponding scoring function values, each defined by the 3D coordinates of its atoms and expressed as a PDB file. The accuracy of BioMedCACHem has been shown to successfully reproduce experimentally observed binding modes, in terms of root-mean squared deviation (rmsd). BioMedCACHem provided excellent result as was observed for the low value of rmsd (best docked solution of 0.43 Å) between experimental and docked structures (this is also shown by superimposition of the above structures). The ability to accurately predict the binding conformation of AChE inhibitors, THA and GNT, to AChE, gave confidence that the BioMedCACHem could also predict, with a similar accuracy, the binding conformations of saffron’s bioactive constituents utilized in the current study. The PyMol molecular graphics system was used to visualize the molecules and the results of the docking experiments.

**Statistical Analysis.** Data are presented as the mean ± SE. Statistical analysis was performed with GraphPad Instat 3 software (GraphPad Instat Software Inc., USA) using the nonparametric Mann–Whitney test (p < 0.05). In all tests, a criterion of p ≤ 0.05 (two-tailed) was considered to be necessary for statistical significance.

**RESULTS**

**In Vitro Effects on AChE Activity.** To study if the tested phytochemicals directly inhibit AChE, the effects of saffron extract and of CRT, DMCRT, SFR, and GNT (positive control) on electric eel AChE activity were tested in vitro, and...
the results are presented in Table 1 and in Figure 3 in the Supporting Information. Saffron showed moderate AChE inhibitory activity (up to 30%). Dose-dependent inhibition of AChE was observed for GNT (Figure 3a of the Supporting Information), a standard AChE inhibitor, with an IC$_{50}$ of 1.93 $\mu$M, as well as for the pure compounds, in the order SFR $>$ CRT $\geq$ DMCRT (Figure 3c–e in the Supporting Information). The determined IC$_{50}$ value of GNT is in agreement with previous results.$^{31,33}$

To elucidate the mechanism of AChE inhibition, kinetic studies of enzyme activity were performed. The relationship between substrate concentration and reaction velocity was in good agreement with Michaelis–Menten kinetics. In the absence of inhibitors, the average $K_m$ for the substrate (ATCI) was 23.01 ± 2.70 $\mu$M. When the slopes or the intercepts in the Lineweaver–Burk plots were drawn in GraphPad Prism 4.0 as a function of inhibitor concentration and the kinetic standards determined (Figure 2), CRT, DMCRT, and SFR showed mixed-type inhibition; the $\alpha$ values of CRT and DMCRT are indicative of a mixed-type, uncompetitive inhibition, whereas the $\alpha$ values of SFR show a mixed-type competitive inhibition (Table 1).

In Silico Protein–ligand Docking Study. In silico molecular docking studies were also undertaken to gain insight into the interaction of CRT, DMCRT, CRC, and SFR with the residues of the ligand binding site of the AChE protein and to investigate the underlying mechanism(s) of action(s). The docking simulation studies on the crystal structure of target protein AChE aimed to explore the ability of the studied compounds to act as potent inhibitors of the AChE enzyme. The topography of the calculated binding sites of the studied compounds provides a clearer understanding of their potency differentiation in the inhibition of AChE.

The global energy of interaction (in kcal/mol) for each docking experiments is given as follows: CRT, $-32.24$; DMCRT, $-36.07$; CRC, $-30.63$; SFR, $-18.43$; THA, $-18.26$; ATCI, $-19.65$; and GNT, $-32.89$. The ligand binding contacts of best docked poses of the known AChE inhibitors GNT and THA and the AChE substrate, ATCI, with key amino acid residues and water molecules in the active site of AChE, are shown in Tables 2–4 in the Supporting Information.

CRT docked very close to ATCI (at a nearby binding position). Its stabilization to the protein’s binding pocket was mainly attributed to its formed hydrophobic contacts (23 total contacts): (a) by the hydrophobic residues Phe284, Phe290 (acyl-binding pocket), Phe330, Phe331, Ile287, Leu358; (b) by the acidic and basic charged polar residues Asp285 and Arg289; and (c) by the polar noncharged residues Tyr121 and Ser286 (Table 5 in the Supporting Information). Additionally, CRT was localized inside the binding cavity, making two hydrogen bond contacts with Tyr121 and Asp285. Water molecule wat634 also contributes to the ligand binding by forming a hydrogen bond with O13 of CRT. The binding contacts of CRT with Phe330 and wat634 were found to be common with those of ATCI, THA, and GNT, whereas Tyr121, Phe331, and the acyl-binding residue Phe290 contributed also to the binding of THA and GNT inhibitors. As illustrated from the ligand binding site architecture (Figure 3), CRT was inserted into the narrow aromatic gorge of the crystal structure of AChE, oriented in a way that the molecule’s polyene backbone could

Figure 2. Lineweaver–Burk plots in the absence (a) and presence of standard GNT (b) and saffron’s constituents (c–e): $1/V_{max}$ versus $1/[ATCI]$. dx.doi.org/10.1021/jf300589c1J. Agric. Food Chem. 2012, 60, 6131−6138
be stabilized via hydrophobic contacts with the amino acid residues of the wall of this prolonged cavity. CRT penetrated into the central cavity of the protein, where the catalytic active site is located, with one of its carboxylic ends to be positioned near the catalytic site triad and near the position occupied by ATCI, THA, and GNT. The other end of the molecule (situated near the opening of the aromatic groove at a distance ∼20 Å from the catalytic active site) protrudes from the protein’s molecule and is stabilized in its position via two H-bond contacts with the amino acid residue Asp285 and the water molecule wat634. Hydrophobic contacts between CRT’s atoms of the hydrocarbon skeleton and amino acid residues of the peripheral anionic site contributed further to the attachment of the molecule.

DMCRT superimposed with THA inhibitor is shown in Figure 4 to be anchored inside the ligand binding pocket of the protein. H-bond and hydrophobic interactions between the DMCRT compound and the amino acid residues of the ligand binding cavity of the target protein are shown in Figure 4b. The polyene backbone moiety of the compound is localized inside the anionic binding groove, making contacts with the following amino acid residues: Phe284, Tyr121 (PAS), Trp279 (PAS), and Tyr334. In contrast, Phe330 and Asp72 residues mainly contribute to the attachment of the molecule into the catalytic binding site (Figure 4b). The DMCRT molecule is stabilized in the ligand binding cavity mostly through hydrophobic interactions with residues Asp72, Phe330, Tyr334, Trp279, Phe284, and Asp285 (Table 6 in the Supporting Information). Phe330 was a common binding residue with the ATCI substrate, whereas Phe330 and Tyr121 were common with THA and GNT. The total number of binding contacts found between DMCRT and AChE protein was 21, of which 5 were...
H-bonds, 1 was a bridged H-bond, and the rest were hydrophobic.

Docking studies of CRC on AChE demonstrated that the ligand was complexed with the protein with the incorporation of both H-bond and hydrophobic contacts. The docking studies revealed that the CRC molecule anchors inside the aromatic groove, but in a different binding locus from that of THA (binding is stabilized at the peripheral site located at the gorge rim, which encompasses binding sites for allosteric ligands). CRC has a unique orientation along the active-site gorge, extending from the anionic subsite of the active site at the bottom, to the peripheral anionic site at the top, via aromatic stacking interactions with various aromatic acid residues. The superimposed structures of CRC and THA on the target protein are illustrated in Figure 5a. The best possible docking binding mode of CRC was mediated through residues Asn230, His398, Pro232, Asp285, Ser235, and Trp524 and water molecules wat640 and wat650 (11 H-bond contacts) and Arg289, Pro361, Cys357, Asp285, and Phe284 (17 hydrophobic contacts) (Figure 5b; Table 7 in the Supporting Information). No common binding residues with ATCI, THA, or GNT were observed.

The docking procedure positioned SFR into the AChE binding site almost at the same place occupied by THA (Figure 6a,b). Through interaction analysis, it was shown that SFR makes one hydrogen bond contact with the hydroxyl group of wat634, whereas the stabilization of the compound into the pocket was additionally attributed to seven hydrophobic contacts formed by residues Phe330, Gly118, His440, and Trp84 (Figure 6c; Table 8 in the Supporting Information). All binding contacts were found to be common with the corresponding AChE–ATCI/THA complexes, whereas Gly118, His440, and Trp84 were common with the AChE–GNT complex. The former amino acid contacts of SFR are important anchoring residues for the inhibitor and are the main contributors to the inhibitor’s interaction. (Data concerning ATCI, GNT, and THA are not shown in the text or tables; they are provided as additional Supporting Information available online.)

**DISCUSSION**

Our results showed that the crude aqueous methanolic saffron extract exhibited low dose-independent inhibitory values. Analysis of the in vitro effect of CRT, DMCRT, and SFR on AChE inhibition revealed a dose-dependent inhibitory profile, but the IC$_{50}$ values were higher than that of GNT. Kinetic analysis confirmed that GNT exerts a competitive type of inhibition, with a $K_i$ value of 3.36 μM in electric eel enzyme, but the majority of the tested saffron phytochemicals exhibited higher $K_i$ values and a mixed type of inhibition.

Molecular docking studies revealed that CRT and DMCRT were not docked at the same place as ATCI, TCH, and GNT inhibitors, but bound simultaneously to the catalytic and peripheral anionic sites of AChE. CRC, due to its bulky glycosidically linked sugar moieties, was unable to be inserted in depth to the aromatic gorge (did not reach the active site that is found deep in the pocket) and attached to a different locus from that of ATCI, THA, and GNT. The fact that the presence of glycosidically linked sugars alters/impairs binding characteristics might provide an explanation for the low relative inhibitory activity of the saffron extract in vitro. The ability of CRT to span the two “anionic” sites, being ~20 Å apart, and bind to both PAS and the catalytic site of the AChE enzyme opens new possibilities for the design and synthesis of novel dual binding AChE inhibitors, which would beneficially affect cholinergic transmission and Aβ aggregation.

On the other hand, SFR seems to be a more potent inhibitor ($IC_{50} = 21.09 \pm 0.17$, $K_i = 90.6 \pm 2.0$) than the carotenoids, but both kinetic and in silico data show that it completely enters transmission and Aβ aggregation. The CRC molecule anchors inside the aromatic groove, but in a different binding locus from that of THA (binding is stabilized at the peripheral site located at the gorge rim, which encompasses binding sites for allosteric ligands). CRC has a unique orientation along the active-site gorge, extending from the anionic subsite of the active site at the bottom, to the peripheral anionic site at the top, via aromatic stacking interactions with various aromatic acid residues. The superimposed structures of CRC and THA on the target protein are illustrated in Figure 5a. The best possible docking binding mode of CRC was mediated through residues Asn230, His398, Pro232, Asp285, Ser235, and Trp524 and water molecules wat640 and wat650 (11 H-bond contacts) and Arg289, Pro361, Cys357, Asp285, and Phe284 (17 hydrophobic contacts) (Figure 5b; Table 7 in the Supporting Information). No common binding residues with ATCI, THA, or GNT were observed.

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**Figure 5.** Bound CRC ligand (illustrated in stick mode and colored according to atom type with light pink C atoms) docked in crystal structure of AChE protein (PDB accession no. 1ACJ) depicted as spectrum-colored cartoon (a) or solid surface colored by chain (in purple-blue) with highlighted interacting amino acid residues of the ligand binding cavity (b) in complex with THA (hot pink C). Highlighted residues interacting through H-bond: Asn230, light orange; His398, pale green; Ser235, teal; and Trp524, yellow. Hydrogen-bonded water molecule wat650 is rendered as a light blue sphere. Hydrogen bond and hydrophobic interactions are shown as yellow-dotted lines. Hydrogen atoms are omitted from all molecules for clarity (final structure was ray traced).
hydrocarbon skeletal terpenoids with the AChE hydrophobic active center.

The hypothesis derived from the in vitro experiments revealed that the majority of the tested phytochemicals exhibited a mixed type of inhibition, as supported by the adopted in silico studies, where all saffron constituents (apart from SFR) were found to be complexed with the enzyme at a different locus (allosteric binding site) from that of ATCI and the tested inhibitors (THA and GNT). These findings showed, for the first time, that CRT, the main metabolite of CRCs in the living organism, inhibits AChE by binding at two different loci, the catalytic center and the PAS site. This finding may partly explain the beneficial effects of saffron in clinical trials against AD and provides a basis for the design and development of novel pleiotropic AChE inhibitors. The presence of glycosidically linked sugars impairs this activity. Overall, our results showed that the in vitro kinetic analysis, in conjunction with molecular modeling and docking predictions, could be important initial steps toward the development of novel AChE inhibitors.

ASSOCIATED CONTENT

Supporting Information

The ligand binding contacts of best docked poses of the known AChE inhibitors GNT and THA, the AChE substrate ATCI, and saffron constituents CRT, DMCRT, CRC, and SFR, with key amino acid residues and water molecules in the active site of AChE, are shown in Tables 2−8. The effectiveness of GNT, saffron, and its constituents in inhibiting AChE activity is shown in Figure 3. IC50 or pIC50 (when converted to the −log IC50 scale) represents the concentration of the tested phytochemical(s) that is required for 50% inhibition in vitro and is calculated by converting the log values back to μM. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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ABBREVIATIONS USED

Aβ, amyloid-β peptide; ACh, acetylcholine; AChE, acetylcholinesterase; AD, Alzheimer’s disease; ATCI, acetylthiocholine iodide; BSA, bovine serum albumin; ChAT, choline acetyltransferase; ChEIs, cholinesterase inhibitors; CRC, crocin; CRT, crocetin; DMCRT, dimethylcrocetin; DTNB, 5,5′-dithiobis(2-nitrobenzoate); GNT, galanthamine; PAS, peripheral anionic binding site; PDB, Protein Data Bank; SAR, structure−activity relationship; SFR, safranal; THA, tacrine.
REFERENCES

Investigation of the neuroprotective action of saffron (Crocus sativus L.) in aluminum-exposed adult mice through behavioral and neurobiochemical assessment

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ABSTRACT

In the present study, the possible reversal effects of saffron against established aluminum (Al)-toxicity in adult mice, were investigated. Control, Al-treated (50 mg AlCl3/kg/day diluted in the drinking water for 5 weeks) and Al + saffron (Al-treatment as previously plus 60 mg saffron extract/kg/day intraperitoneally for the last 6 days), groups of male Balb-c mice were used. We assessed learning/memory, the activity of acetylcholinesterase (AChE, salt- (SS)/detergent-soluble (DS) isoforms), butyrylcholinesterase (BuChE, SS/DS isoforms), monoamine oxidase (MAO-A, MAO-B), the levels of lipid peroxidation (MDA) and reduced glutathione (GSH), in whole brain and cerebellum. Brain Al was determined by atomic absorption spectrometry, while, for the first time, crocetin, the main active metabolite of saffron, was determined in brain after intraperitoneal saffron administration by HPLC. Al intake caused memory impairment, significant decrease of AChE and BuChE activity, activation of brain MAO isoforms but inhibition of cerebellar MAO-B, significant elevation of brain MDA and significant reduction of GSH content. Although saffron extract co-administration had no effect on cognitive performance of mice, it reversed significantly the Al-induced changes in MAO activity and the levels of MDA and GSH. AChE activity was further significantly decreased in cerebral tissues of Al + saffron group. The biochemical changes support the neuroprotective potential of saffron under toxicity.

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1. Introduction

Aluminum (Al) is the third most abundant element in nature, making human exposure unavoidable (Verstraeten et al., 2008). The main entry sites of Al into the body are the gastrointestinal and respiratory tract and the skin (Verstraeten et al., 2008), enabling distribution and accumulation in several tissues, like spleen, lungs, liver, kidneys, heart, bone and brain (Kumar and Gill, 2009), through systemic circulation. It has been demonstrated that Al crosses the blood–brain barrier, implicating metal binding to transferrin in the blood and a subsequent transferrin receptor-mediated mechanism of brain Al influx (Yokel et al., 1999).

High brain levels of Al induce cognitive deficiency and dementia and, thus, Al is a widely accepted neurotoxin (Kawahara and Kato-Negishi, 2011). Its implication in the pathogenesis of neurodegenerative diseases has been suggested decades ago, but is seriously debated till now (Kawahara and Kato-Negishi, 2011; Tomljenovic, 2011). However, some researchers consider that the model of chronic Al-induced neurotoxicity best describes Alzheimer's disease, since it manifests many of the pathological hallmarks (Walton and Wang, 2009; Yokel, 2006; Zhang et al., 2003).

Al neurotoxicity is manifested through several behavioral and neurochemical alterations, which display diversity depending on the animal species in question, the administration route and the chemical form of Al administered (Erasmus et al., 1993; Kumar and Gill, 2009). It has been demonstrated that Al promotes oxidative stress in the cerebral cortex and hippocampus of young and aged rats, and damage of lipids, membrane-associated proteins

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(Na+-K') ATPase and protein kinase C) and endogenous antioxidant enzymes (Sethi et al., 2008; Sharma et al., 2009; Tripathi et al., 2009). Negative impacts of Al administration have been also observed in neurotransmitter systems of rodent brain regions, including serotonergic (Kumar, 2002) and cholinergic systems (Julka et al., 1995). The effects of Al on AChE activity remain controversial as both inhibition and activation have been reported (Julka et al., 1995; Sharma et al., 2009), whereas the respective data concerning BuChE activity are limited, though BuChE enzyme is considered to play a supportive functional role in acetylcholine hydrolysis (Lané et al., 2006). Finally, behavioral deficits assessed with different behavioral tasks, have been observed in rats following Al exposure (Julka et al., 1995; Sethi et al., 2008; Tripathi et al., 2009).

Crocus sativus L. (Iridaceae) is a cultivated plant species in many countries including Greece, since its styles (saffron) constitute an exquisite spice. Interestingly, saffron has been used as a medicinal agent for millennia (Schmidt et al., 2007). In recent years beneficial effects of saffron have been demonstrated in models of neuronal, cardiovascular, respiratory and other disorders (Bathaie and et al., 2006). Finally, behavioral deficits assessed with different behavioral tasks, have been observed in rats following Al exposure (Julka et al., 1995; Sethi et al., 2008; Tripathi et al., 2009).

2.1. Plant material and extraction

2.2. Animals

Male adult (4 month-old) Balb-c mice were used in this study. The animals were housed in groups in standard laboratory cages (5 mice per cage) in a temperature controlled room (20 ± 2 °C) with a 12 h light/dark cycle. Food in form of dry pellets (feed composition: grain and grain by-products, oil seed products, minerals, vitamins and trace elements from Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) was available ad libitum. Animals were randomly divided into three groups (n=10/group). Control group: mice had access to normal drinking water over the experimental period. Al treated group: mice received orally AlCl3 (aluminum chloride anhydrous, purity >99%, Sigma–Aldrich, St. Louis, USA) (50 mg/kg body weight/day) dissolved in normal drinking water for a period of 5 weeks. Body weight and liquid intake were measured regularly to adjust the dose and achieve a constant intake of Al. Al + saffron treated group: mice received orally AlCl3 (50 mg/kg body weight/day) dissolved in normal drinking water for 5 weeks and saffron extract (60 mg/kg body weight/day, 30 μL injection volume) intraperitoneally for the last 6 days of the 5-week Al treatment.

AlCl3 solution was freshly prepared every 4 days over the experimental period, while saffron solution was prepared fresh daily, just before administration, by dissolving the dry extract in saline. During the 6-day saffron treatment, control and Al-treated mice received intraperitoneally 30 μL of saline. Al and saffron dosage and schedule of exposure were based on the literature (Jyoti and Sharma, 2006) and on our previous study (Papandreou et al., 2011), respectively. All procedures were in accordance with Greek National Laws (Animal Act, PD 160/91).

2.3. Behavioral testing: step-through passive avoidance task

This test is based on negative reinforcement to examine long-term memory (Kaneto, 1997). A two-compartment passive avoidance apparatus (white/dark, separated by a guillotine door) was used. Mice were subjected to single trial passive avoidance task according to previously described procedures (Kaneto, 1997; Otano et al., 1999; Papandreou et al., 2009). Briefly, on day 5 of saffron treatment an acquisition trial was performed 1 h after a 100 s-long habituation trial, while a retention trial was performed 24 h after the training session. Results were expressed as the mean initial (IL, max. time 120 s) and step-through (STL, max. time 300 s) latency time values recorded once mice crossed into the dark compartment, before and 24 h after the delivery of a mild foot shock to their paws. All training and testing sessions were carried out during the light phase (08:00–14:00 h), one hour after intraperitoneal administration of saffron.

2.4. Preparation of tissue homogenates

On the completion of the 5-week treatment period, all animals were sacrificed by cervical dislocation. Brain was excised immediately, and cerebellum was separated from the remaining whole brain. Liver was also removed and used as a reference peripheral tissue. All tissues were rinsed with saline immediately. From the tissue samples isolated, only six tissues per group were used for the biochemical assessments. The others were assayed for Al content by atomic absorption spectrometry. The tissues for biochemical measurements and crocin determination were weighed and homogenized (103, w/v) with a glass-Teflon homogenizer in ice-cold 30 mM NaHPO4 pH 7.6. The homogenates were then centrifuged at 15,000g for 20 min at 4 °C. Supernatants constituted the salt-soluble (SS) fractions and were stored at −75 °C. The pellets were re-suspended in an equal volume of 1% (w/v) Triton X-100 (in 30 mM NaHPO4, pH 7.6) and then centrifuged at 15,000g for 20 min at 4 °C. Supernatants, the detergent-soluble (DS) fractions, were collected and stored at −75 °C. This double-extraction method was performed in order to cover the cytosolic (SS fraction) and membrane-bound (DS fraction) isoforms of cholinesterases (Das et al., 2005). Protein concentrations were determined by the Bradford assay (Bradford, 1976).

2.5. Aluminum determination by atomic absorption spectrometry

2.5.1. Digestion of tissues

The whole brain (−ce), cerebellum and liver samples from control, Al-treated and Al + saffron treated mice were analyzed for Al concentration. Glassware was not used during sample preparation in order to avoid the possible contamination. Eppendorf tubes were used after immersing in a solution of nitric acid (HNO3): ethanal (1:9, v/v) for 48 h and washing with ultra-pure water (Milli Q). A two times dilution of the samples was performed before analysis. The dry weight of the tissues was measured after heating at 100 °C for 20 h. Then, the samples were digested with 1 ml/100 mg wet tissue (for whole brain) and 1 ml/25 mg wet tissue (for cerebellum and liver) of nitric acid: sulphuric
acid (HNO₃:H₂SO₄, 1:1 v/v) at room temperature overnight. Mixtures were centrifuged at 10,000 g for 10 min to remove insoluble material. Al levels were determined in the supernatants.

2.5.2. Atomic absorption spectrometry

Al content in the supernatants was measured employing Atomic Absorption Spectrometry (Perkin Elmer, Analyst 300 equipped with a HGA 700 Graphite Furnace and an AS-70 autosampler). The temperature of the furnace was programmed as described elsewhere (Johnson and Treble, 1992). Absorbance was measured at 309.3 nm.

Preparation of samples for Al analysis: All samples were prepared by homogenization in eppendorf tubes and analyzed immediately after preparation. A 5% v/v HNO₃ and 0.1% v/v Mg solution prepared by proper dilutions from HNO₃ solution (65%, Carlo Erba) and Mg stock standard solution (1000 μg/mL Mg²⁺ ± 2 μg to 5% HNO₃, Chem-Lab), was used as a matrix modifier in order to stabilize Al in the furnace.

The standard addition method was employed for the analysis. Four samples were prepared for each aliquot, adding known amounts (spikes) of the analyte (Al) from a stock standard solution (1000 ppm in 1 M HNO₃, Fisher Scientific, UK). The spiked concentration ranged from 0 (unspiked sample) to 80 ppb. The volume of the supernatants was kept equal for all samples by means of addition of the matrix modifier. Preliminary tests assured that the overall concentration of Al was within the linear range of the technique.

Analysis: Absorbance for each sample was measured three times. A calibration curve for each supernatant aliquot was constructed (OriginPro 8). The results are expressed as μg Al/g wet tissue.

2.6. HPLC determination of crocetin in brain

Crocetin determination in whole brain (-ce) of Al + saffron treated mice was performed by an isocratic reversed-phase liquid chromatographic method developed by Chrysanthi et al. (2011) for plasma analysis, with slight modifications. In detail, a high volume (3.5 mL) of the whole brain homogenate (100 mg/mL, SS fraction) was submitted to solid phase extraction on reversed phase Strata-X cartridges (200 mg/3 mL) consisting of a surface modified with styrene–divinylbenzene polymer that were obtained from Phenomenex (Torrance, CA, USA). The eluate was evaporated to dryness in a Speed Vac system and the dry residue was redissolved in 30 μL of the HPLC mobile phase. The samples were chromatographed on a Luna C-18 column (4.6 × 250 mm, 5 μm) with a mobile phase consisting of methanol–water–trifluoroacetic acid (75.0:24.5:0.5, v/v/v) at a flow rate of 1.0 mL min⁻¹. Crocetin was quantified using spiked brain standards at the concentrations of 0.5, 1.0, 1.5, 2.0 and 3.0 μM of total crocetin (Chembiotin, purity >98%). Results are expressed as nmol of total crocetin/g wet tissue.

2.7. Biochemical assays

2.7.1. Determination of cholinesterase activity

The activity of SS and DS isoforms of AChE and BuChE in brain and liver samples, was determined spectrophotometrically based on Ellman’s assay and previously described protocols (Ellman et al., 1961; Lassiter et al., 1998; Papandreou et al., 2009). Total ChE activity was measured by acetylthiocholine iodide (ATCh, Sigma–Aldrich, UK) as substrate, whereas BuChE activity was determined by 5-butyrylthiocholine iodide (BuTCh, Sigma–Aldrich, Switzerland) as substrate, since BuTCh is hydrolyzed only by BuChE (Lassiter et al., 1998). AChE activity was then calculated by subtracting BuChE activity from total ChE activity. Enzyme activity is expressed as nmol of substrate hydrolyzed/min/g of tissue.

2.7.2. Determination of monoamine oxidase activity

The activity of both isoforms of MAO, MAO-A and MAO-B, was assessed in mouse whole brain (-ce) and cerebellum by a fluorimetric assay based on previously described protocols (Mahmood et al., 1994; Xu et al., 2010). Since MAO is a mitochondrial membrane-bound enzyme, the DS fractions of brain tissues homogenates were used. Briefly, 50 μL of brain sample was preincubated at 37 °C for 20 min with 100 μL of either 1 μM KCl, 1,10-phenanthroline hydrochloride (Deprenyl, Sigma–Aldrich, USA) or 1 μM N-Methyl-N-propargyl-1-(2,4-dichlorophenyl)propylamine hydrochloride (Clorgyline, MAO-A inhibitor, Sigma–Aldrich, USA) in 100 μL 30 mM NaH₂PO₄, pH 7.6. Then 10 μL of 3.07 mM kynurenine hydrochloride (Sigma–Aldrich, USA) was added to the reaction mixture as substrate. The mixture was then incubated at 37 °C for 15 min again. After incubation, the reaction was terminated by adding 100 μL of 0.6 M perchloric acid and centrifuging the mixture at 1500g for 10 min to remove precipitated proteins. An aliquot of 0.3 mL of the supernatant was added to 2 mL of 1 N NaOH. The fluorescence intensity of the produced 4-hydroxyquinoline was detected at an excitation wavelength of 315 nm and an emission wavelength of 380 nm, using a fluorescence spectrometer. The concentration of product was estimated from a corresponding standard fluorescence curve of 4-hydroxyquinoline (1–100 μM) (Sigma–Aldrich, USA). MAO activity is expressed as nmol of 4-hydroxyquinoline formed/g tissue/ min.

2.7.3. Estimation of lipid peroxidation and reduced glutathione levels

Malondialdehyde (MDA) levels, an index of tissue lipid peroxidation, were measured in cerebral and liver samples (SS fractions of tissue homogenates) following a previously described fluorimetric assay (Papandreou et al., 2009). Lipid peroxidation levels are expressed as μmol MDA/g of tissue protein.

Reduced glutathione (GSH) content of brain and liver samples (SS fractions of tissue homogenates) was determined fluorometrically according to the procedure of Mokrash and Teshchke (1984). GSH levels are expressed as μmol/g of tissue protein.

2.8. Statistical analysis

Data are presented as mean ± S.E.M. Statistical analysis was performed with GraphPad Instat 3 software using the nonparametric Mann–Whitney test for evaluating statistically significant differences (p < 0.05) between the experimental groups.

3. Results

3.1. General observations

HPLC analysis showed that saffron extract contains a variety of crocins: trans-crocin-4 constitutes 46% of total crocin content of the crude extract, trans-crocin-3, 26%; cis-crocin-4, 12%; and trans-crocin-2, 7%, as previously described (Papandreou et al., 2006; Tarantilis et al., 1995).

All animals developed normally during the experimental period and no mortality was recorded during Al treatment. Also, there were no differences on body weight gain and liquid intake, between the experimental groups.

3.2. Effect of saffron on learning and memory ability of Al-exposed mice

Passive avoidance task was performed to examine long-term memory. The initial latency (IL) time, measured at training trial, is an indicator of motor activity, and step-through latency (STL) time, measured at testing trial, indicates memory of the aversive experience. Al intake resulted in impaired long-term memory of mice, as evidenced by the significantly lower (59%) mean STL time of Al-treated animals compared to mean STL time of the controls (Fig. 1). The lack of difference of mean STL time between Al+ saffron- and Al-treated groups (Fig. 1), shows that saffron extract co-administered with Al during the last 6 days of the treatment period was ineffective in reverting Al-induced memory
impairment. The mean IL time values were not significantly different among all experimental groups (Fig. 1), implying no difference in motor activity of mice in training session.

3.3. AI and crocetin levels in cerebral tissues

The levels of AI in whole brain (-ce), cerebellum and liver of control, Al-treated and Al + saffron treated mice are presented in Table 1. Al was not detected in whole brain (-ce) of control mice, whereas it was determined in high amounts in cerebellum. In all animal groups AI levels in cerebellum were significantly (p < 0.05) higher than those of the remaining brain. Also, AI concentrations were significantly (p < 0.05) increased in both cerebral tissues and liver of Al-treated mice compared to the controls and did not differ significantly (p < 0.05) in the cerebral and liver tissue of Al + saffron treated mice, compared to Al-treated group.

Pooled whole brain (-ce) homogenates of Al + saffron treated and control mice were analyzed by HPLC. HPLC chromatogram (Supplementary data) of Al + saffron treated brain (chromatogram B) shows a peak at 12.4 min, which is not detected in control brain (chromatogram A), and corresponds to trans-crocetin. UV–visible absorption spectrum of the peak presents a maximum double peak at 420–450 nm, characteristic of the carotenoids. Crocetin concentration, was 2.0 ± 0.1 nmol total crocetin/g tissue (n = 2) in whole brain of mice treated with saffron extract and not detected in brain of control mice.

3.4. Effect on brain and liver ChE activity

The impact of Al or saffron treatment on the activities of both isoforms (SS, DS) of AChE and BuChE in whole brain, cerebellum and liver of adult mouse is presented in Table 2. AI intake decreased significantly (p < 0.05) the activity of both SS and DS isoforms of AChE and BuChE in mouse whole brain (-ce) (26%, 17% and 21%) and cerebellum (23%, 15% and 22%, 19%) compared to the controls, except of the activity of DS-BuChE of whole brain (-ce) that remained unchanged. Further significant (p < 0.05) reduction of the SS- and DS-AChE activity in whole brain (-ce) (15%, 28%) and cerebellum (24%, 27%) was observed in Al + saffron treated mice in comparison with Al treated group, whereas saffron treatment did not further affect the activity of cerebral BuChE.

Also, Al-treated mice displayed significantly (p < 0.05) decreased liver BuChE (11% SS, 21% DS) and SS-AChE (25%) activities compared to the controls, while Al + saffron treated mice displayed further inhibition of liver BuChE (27% SS, 19% DS) and SS-AChE (31%) compared to Al-treated mice.

3.5. Effect on brain MAO activity

The effect of administration of either AI alone or in the presence of saffron extract at the end of the treatment period, on MAO isoforms activity of mouse whole brain (-ce) and cerebellum, is presented in Fig. 2. Al-treated mice displayed significantly (p < 0.05) higher activities of MAO-A (19%) and MAO-B (10%) in their whole brain (-ce) and significantly lower MAO-B activity (14%) in cerebel-

3.6. Effect on brain and liver oxidant/antioxidant indices

MDA and GSH levels, as indicators of lipid peroxidation and cellular antioxidant defense, respectively, were measured in mouse brain tissues and liver of all experimental groups. As shown in Fig. 3a, Al intake increased (18%) significantly (p < 0.05) MDA levels only in whole brain (-ce) in comparison with controls, while the subsequent short-term co-administration of saffron restored (decrease by 22%) MDA levels to normal. Short-term co-administration of saffron also improved the antioxidant status of cerebellum and liver by significantly lowering the levels of MDA, even though these tissues remained unaffected by Al treatment (Fig. 3a). Additionally, Al-treated mice displayed remarkable reduction in GSH content of whole brain (-ce) (44%), cerebellum (16%) and liver (29%), compared to the control animals, whereas Al + saffron treated mice exhibited elevated levels of GSH (29%, 14% and 22%, respectively) compared to Al-exposed group (Fig. 3b).

4. Discussion

The present study investigates the neuroprotective potential of a 6-day i.p. administration of saffron extract to adult mice that...
were previously exposed to high Al intake through their drinking water for a 5-week period. To this end, cognitive behavior and brain cholinergic, monoaminergic and oxidative indices were assessed. Our findings showed that long-term Al intake induced learning/memory decline in adult mice. Impaired performance of rats on passive avoidance task has been shown by Bhalla et al. (2010) after long-term administration of a double dose of AlCl₃ in drinking water, while Sethi et al. (2008) showed declined spatial learning abilities of rats in Morris-water maze test after administration of the same dose of AlCl₃ (50 mg/kg/day) in drinking water for 6 months. Saffron co-administration at the end of the treatment period failed to reverse Al-induced cognitive deficits. In our previous study, short-term saffron administration enhanced learning/memory in healthy adult and aged mice in passive avoidance task (Papandreou et al., 2011). However, in the current report the administered dosage of saffron extract and/or the duration of exposure (which are the same as in the study of Papandreou et al., 2011) were insufficient to counteract Al-induced memory deficit.

Al levels were determined in whole brain (-ce), cerebellum and liver of all animal groups prior to biochemical evaluation. The values of Al found in cerebral tissues, are comparable with those of other studies that use Al as neurotoxic agent, although different Al forms, doses and routes of administration are investigated (Esparza et al., 2005; Kaizer et al., 2008; Sánchez-Iglesias et al., 2007). Our findings showed high concentrations of Al in cerebellum of control mice (higher than liver), but none or below detection limits in whole brain (-ce), in accordance with Bellès et al. (1998). In the current study, long-term AlCl₃ intake through drinking water resulted in significantly increased Al concentrations in whole brain, cerebellum and liver of adult mice. Our results are

![Fig. 2. Effect of saffron administration on monoamine oxidase activity (MAO-A and MAO-B) of Al-exposed adult mouse brain. Al-induced activation of both MAO isoforms in whole brain (-ce), inhibition of MAO-B in cerebellum and reversal effects of saffron are evident. Data are the mean ± SEM (n = 6 animals/group). *p < 0.05 vs. the control group, †p < 0.05 vs. Al treated group (non-parametric Mann–Whitney test).](image-url)

![Fig. 3. Effect of saffron administration on (a) lipid peroxidation (MDA) and (b) reduced glutathione (GSH) levels of Al-exposed adult mouse brain and liver. Al-induced increase in MDA levels of whole brain (-ce), decrease in GSH levels of whole brain (-ce), cerebellum and liver, and reversal effects of saffron are evident. Data are the mean ± SEM (n = 6 animals/group). *p < 0.05 vs. the control group, †p < 0.05 vs. Al treated group (non-parametric Mann–Whitney test).](image-url)
in accordance with previous studies where AlCl₃ is administered to rodents (Bhalla and Dhawan, 2009; El-Maraghy et al., 2001). The entry of Al into the brain through the blood–brain barrier has long been established (Yokel et al., 1999) and is also confirmed by our results. The accumulation of Al in brain is consistent with the observed impaired learning/memory ability of mice in passive avoidance test. However, cerebral Al levels did not differ significantly between Al- and Al + saffron treated mice, showing that short-term saffron administration in the end of the treatment period did not affect Al bioavailability.

Additionally, for the first time, we determined crocetin, the major metabolite (aglycon) of the bioactive crocin glycosides of saffron, in whole brain of Al + saffron treated mice, which was absent from the brain of control mice. The detection of crocetin in mouse brain demonstrates for the first time that this compound crosses the blood–brain barrier when saffron extract is administered for a short term through intraperitoneal route. In recent report (Chryssanthi et al., 2011), the presence of crocetin in human plasma was shown after 2 and 24 h of saffron tea consumption, while only Yoshino et al. (2011) have demonstrated the distribution of crocetin in rat brain after a single oral administration of pure crocetin, up to now.

The fact that saffron short-term co-administration failed to reverse the cognitive decline raises questions on the dosage, the period and the route of administration, before definite conclusions can be drawn about its effectiveness. However, our study presents some remarkable brain biochemical alterations that followed saffron co-treatment.

Determination of ChE activity showed that the DS-AChE was predominant in cerebral tissues, while approximately equal values of the activity of the two BuChE isoforms were recorded. These observations are consistent with previous findings (Das et al., 2005; Fernández-Gómez et al., 2008). In accordance with other studies (Lassiter et al., 1998; Li et al., 2000), AChE activity constitutes the major fraction of total ChE activity in whole brain (ce) and cerebellum, whereas BuChE activity predominates in liver. In the current work, Al intake resulted in significant decrease of the activity of AChE and BuChE isoforms in mouse whole brain and cerebellum. Region-specific inhibition of the activity of brain membrane-bound AChE and BuChE after Al intoxication, has been shown by others (Julkas et al., 1995). Previous studies have shown necrotic rat hippocampal ultrastructural changes following the same Al administration protocol (Jyoti and Sharma, 2006), providing an explanation for our cholinotoxic and memory impairment results. However, Al has been suggested to interfere with the action of metabolotropic glutamate receptors, enhancing excitotoxicity and neuronal injury (Blaylock, 2012). Also, Al impairs hippocampal long-term potentiation (LTP) in rats (Platt et al., 1995). Thus, besides the cholinotoxic effects of Al in the present study, the involvement of possible Al-induced glutamatergic neurotransmission disturbances in the observed memory decline, should also be considered. In liver, both BuChE isoforms’ activity (the main liver ChE fraction) was significantly decreased following Al administration, while only SS-AChE activity was decreased significantly. However, increased activity of soluble and membrane-bound forms of rat liver BuChE after Al intake through diet for 100–115 days (Dave et al., 2002), has been previously demonstrated, but the Al dose and period of exposure are different.

Saffron short-term co-administration caused further significant reduction of cerebral AChE and liver BuChE isoforms activity. Cerebral BuChE activity was not further affected and only SS-AChE activity of liver was further significantly decreased after saffron co-treatment. The inhibitory effect of saffron on the activity of brain SS- and DS-AChE in adult healthy mice has been demonstrated in our previous study (Papandreou et al., 2011). However, the impact of saffron administration on brain BuChE and liver ChE activities, has not been studied before. The considerable higher percentage of inhibition of DS-AChE in whole brain of Al + saffron treated mice compared to SS-AChE, suggests greater susceptibility of the enzyme’s DS isoform to saffron treatment. Geromichalos et al. (2012) have recently demonstrated, for the first time, that crocetin inhibits AChE by binding at two different loci, the catalytic center and the peripheral anionic sites, as assessed by in vitro enzymatic and molecular docking studies. Thus, the observed significant cerebral AChE inhibitory activity conferred by saffron short-term co-administration, may be attributed to a direct interaction of crocetin with the enzyme, since crocetin was detected in brain of Al + saffron treated mice and was absent in controls’ brain; however, it remains to be investigated whether the crocetin concentration is sufficient for such an effect. Furthermore, other indirect mechanisms cannot be excluded.

Monoamine oxidase (MAO, types A and B) is a mitochondrial membrane-bound enzyme which catalyzes the oxidative deamination of monoamines, thus regulating monoaminergic neurotransmission. Increased brain MAO activity has been recorded in neurodegenerative process (Hanish Singh et al., 2011; Mallajosyula et al., 2008). Our results showed significant increase of whole brain MAO isoforms activity after Al intoxication, and an opposite effect on cerebellum MAO-B activity. Bhalla et al. (2010) have also presented increased and decreased MAO activity in rat cerebrum and cerebellum, respectively, after long-term oral administration of AlCl₃. Activation of MAO isotypes in rat brain by prolonged intake of Al has been also previously reported (Huh et al., 2005), but the underlying mechanisms have not yet been clarified.

Saffron co-treatment completely reversed Al-induced activation of brain MAO isoforms and inhibition of cerebellar MAO-B. The effect of saffron administration on cerebral MAO activity has not been studied before. MAO inhibitors have long been well characterized for their antidepressant properties (Bortolato et al., 2008). The first small-scale clinical trials of saffron in the treatment of mild to moderate depression, have shown significant benefits in the mood of patients after a 6-week treatment (Akhondzadeh et al., 2005; Noorbala et al., 2005). The antidepressant efficacy of saffron could, at least in part, correlated with brain MAO inhibitory activity of saffron extract and the presence of crocetin in brain that are presented in our current study.

Oxidative stress has long been implicated in the initial and later stages of neuronal degeneration (Melo et al., 2011). In the present study, Al intake elevated significantly lipid peroxidation in whole brain and decreased GSH content in both cerebral tissues and liver. Other reports have also demonstrated increased lipid peroxidation levels and decreased GSH content in rat brain regions after long-term oral administration of AlCl₃ (Jyoti and Sharma, 2006; Nehru and Bhalla, 2006). The resistance of liver to Al-induced lipid peroxidation, indicated by us and other studies (Kaneko et al., 2004), is expected, as it constitutes the major site of detoxification in living organism. Although Al is not a redox-active metal, there is extensive experimental evidence on oxidative stress-mediated Al neurotoxicity (Kumar and Gill, 2009). It has been demonstrated that Al induces oxidative stress in cultured rat hippocampal neurons by potentiating iron-mediated oxidative injury (Xie et al., 1996). Also, in vitro studies (Oteiza, 1994) have shown that Al stimulates iron-induced lipid peroxidation in isolated membrane fractions through binding to the membrane and promotion of changes in the arrangement of membrane lipids. Considering the byproduct (hydrogen peroxide) of MAO-mediated reactions, Al-induced activation of brain MAO isoforms provided by our results could, at least in part, contribute to the observed increased brain oxidative stress.

Reversal effects of saffron short-term co-administration were recorded against Al-induced brain lipid peroxidation and GSH content reduction in cerebral tissues and liver. In our previous study
(Papandreou et al., 2011), saffron administration significantly decreased MDA levels and increased GSH content in the brain of healthy adult and aged mice. Shati et al. (2011) also showed that intraperitoneal co-administration of saffron aqueous extract with AlCl₃ ameliorated the disturbances in mouse brain lipid peroxidation and antioxidant enzymes’ activity induced by the metal when injected alone. Additionally, in our previous in vitro work (Papandreou et al., 2011) both saffron extract and its crocetin component provided strong protection against H₂O₂-induced toxicity in human neuroblastoma SH-SY5Y cells, by repressing reactive oxygen species production. It has been reported that crocin, the di-gentiobiosyl ester of crocetin, promotes mRNA expression of γ-glutamylcysteiny1 synthase, the rate-limiting enzyme of GSH synthesis, in PC12 cells under hypoxic conditions (Ochiai et al., 2007). Accordingly, crocetin may mediate the observed brain antioxidant protection of saffron extract under Al neurotoxicity.

5. Conclusions

Our findings show that long-term intake of a relative high dose of AlCl₃ through drinking water resulted in metal accumulation in adult mouse brain tissues and exerted neurotoxic effects, as evidenced by the declined learning and memory capacity, metal-induced inhibition of ChEs, changes of MAO and the production of oxidative damage. On the other hand, short-term co-administration of saffron extract at the end of the treatment period beneficially affected mouse brain oxidative stress and antioxidant status markers and MAO activity that were disturbed by Al. The bioavailability of crocetin in mouse brain after saffron extract administration through intraperitoneal route demonstrated in the present study, supports the implication of this bioactive component in the observed neurochemical alterations. However, the particular saffron treatment scheme was ineffective in reversing cognitive defects induced by the metal, although it had beneficial action on biochemical markers of brain function. The findings support further investigation of the potential of saffron and its crocin constituents as neuroprotective agents.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jfct.2012.11.016.

References


Structure—Activity Studies of lGnRH-III Through Rational Amino Acid Substitution and NMR Conformational Studies

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ABSTRACT:
Lamprey gonadotropin-releasing hormone type III (lGnRH-III) is an isoform of GnRH isolated from the sea lamprey (Petromyzon marinus) with negligible endocrine activity in mammalian systems. Data concerning the superior direct anticancer activity of lGnRH-III have been published, raising questions on the structure–activity relationship. We synthesized 21 lGnRH-III analogs with rational amino acid substitutions and studied their effect on PC3 and LNCaP prostate cancer cell proliferation. Our results question the importance of the acidic charge of Asp6 for the antiproliferative activity and indicate the significance of the stereochemistry of Trp in positions 3 and 7. Furthermore, conjugation of an acetyl-group to the side chain of Lys8 or side chain cyclization of amino acids 1–8 increased the antiproliferative activity of lGnRH-III demonstrating that the proposed salt bridge between Asp6 and Lys8 is not crucial. Conformational studies of lGnRH-III were performed through NMR spectroscopy, and the solution structure of GnRH-I was solved. In solution, lGnRH-III adopts an extended backbone conformation in contrast to the well-defined β-turn conformation of GnRH-I.


Keywords: Lamprey GnRH-III; lGnRH-III analogs; solution structure GnRH-I; solution structure lGnRH-III; antiproliferative activity

INTRODUCTION
The decapeptide gonadotropin-releasing hormone-I (GnRH-I; pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2) has a pivotal role in orchestrating and maintaining normal reproductive events by regulating the secretion of pituitary gonadotropin hormones. In addition, GnRH-I and many of its analogs exhibit an antiproliferative effect on various cancer cells, which can be exerted by indirect or direct ways. Chronic administration of GnRH analogs desensitizes the pituitary gonadotroph cells, results in arrest of gonadotropin secretion, and thereby suppresses ovarian and testicular function (chemical castration). Thus, chemical castration is a therapeutic approach of hormone-dependent tumors such as prostate and breast cancer. Thousands of synthetic peptide analogs of GnRH have been synthesized since its discovery, and several of them (agonists or antagonists) have been used in breast and/or prostate cancer therapy. In addition, GnRH analogs also exert anticancer activity by directly affecting the hormone-dependent and -independent cancer cells, an effect which

Additional Supporting Information may be found in the online version of this article.

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could be mediated through the GnRH receptors (GnRHRs) highly expressed in those cells.

The discovery of lGnRH-III (pGlu-His-Trp-Ser-His-Asp-Trp-Lys-Pro-Gly-NH₂), a native GnRH analog isolated from sea lamprey (*Petromyzon marinus*)⁵ that stimulates the release of estradiol and progesterone in the adult female sea lamprey,⁶,⁷ has stimulated further research on their potential medicinal uses. Functional activity of lGnRH-III has been found in the hypothalamus of several (mammalian) species, including rats, cows, and sheep, and it was originally suggested that lGnRH-III might act as a mammalian Follicle-Stimulating Hormone (FSH) releasing factor.⁸–¹¹ However, the data concerning the effect of lGnRH-III on the selective secretion of FSH are inconsistent.¹²–¹⁴ While questions concerning the endocrine activity of lGnRH-III are yet not answered, studies concerning the direct anticancer activity of lGnRH-III have been published in the last decade.¹⁵–¹⁸ lGnRH-III differs in the sequence 5-8 from the mammalian GnRH (GnRH-I)⁵ and is a weak agonist of the mammalian GnRHR. The ability of lGnRH-III to inhibit proliferation of cancer cells, combined with the weak GnRH-I agonistic activity, makes it an excellent starting compound for the development of peptide analogs with high and selective anticancer activity. Currently, new GnRH analogs have been synthesized to enhance the anticancer potency of native lGnRH-III.⁵,¹³,¹⁶,¹⁷ Those studies have shown that modifications in positions 5-8 decrease the anticancer activity of lGnRH-III. Systematic replacement of residues 5-8 of lGnRH-III by Ala¹⁹ showed that mutation of Asp⁶ or Trp⁷ resulted in the loss of antiproliferative effect probably due to an ionic interaction between these residues, which might stabilize the biologically active conformation of lGnRH-III. In addition, the importance of indole rings of Trp³,⁷ was also proposed.¹⁶ However, further information on the structure–activity relationships of lGnRH-III is still lacking.

To further investigate the structure–activity relationships of lGnRH-III, we synthesized 21 new lGnRH-III analogs and studied their direct antiproliferative effect on prostate cancer cells. The analogs were arranged into three groups: (A) analogs with single amino acid changes in position 6, (B) analogs with modifications in positions 3 and/or 7, (C) analogs with amino acid changes in positions 1, 5, 8 and cyclic analogs (Table I). In particular, we studied the importance of the Asp in position 6 by incorporation of several amino acids [Asn, Asp(OMe), Glu, Gln]. DTrp and L/D-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic) were inserted in positions 3 and/or 7 to further investigate the role of the

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a Yields were calculated on the basis of the amino acid content of the resin. All peptides were at least 98% pure.
b For elution conditions, see Materials and Methods section.
c Data obtained by ESI-MS, observed (obsd) and calculated (calcd) m/z values of MH⁺.
indole ring of Trp and its stereochemistry. In the last group, the importance of the amino acids in positions 1 and 8 are mainly studied. The utility of backbone cyclization has been well established in peptides, and it has been demonstrated to increase biological activity and selectivity20,21 since they are usually more stable in metabolism than the parent linear molecules.22 Therefore, cyclopeptides are of great importance and interest both in pharmaceutical and chemical respect. According to these considerations, we also synthesized 1-8 and 1-5 side chain cyclic peptides and studied their antiproliferative activity on prostate cancer cells.

Moreover, until today, some sporadic attempts have been done to study the solution conformation of lGnRH-III, but there is still no NMR 3D model available. According to the results of a previous molecular dynamics simulation study by Lovas and coworkers,23 lGnRH-III has an extended helical conformation from residues 2 through 7, which is stabilized by intramolecular hydrogen bonds and slightly polar interactions. However, that conformational model has not been confirmed by other studies.17 The determination of the conformational characteristics of lGnRH-III is a crucial step for in depth study of the structure–activity relationship. In that direction, we studied the solution structure of lGnRH-III through NMR spectroscopy, and a conformational model of this decapeptide is presented. Moreover, the solution structure of GnRH-I (PDB 1YY1) was studied under the same experimental conditions to elucidate the conformational differences between GnRH-I and lGnRH-III.

### MATERIALS AND METHODS

#### Peptide Synthesis

The linear and the cyclic peptides (Table I) were rationally designed and synthesized manually. 9-Fluorenylmethoxy carbonyl (Fmoc)-protected amino acids and peptide reagents were obtained from CBL (Patras, Greece), Bachem (Bubendorf, Switzerland), and Novabiochem (Läufelfingen, Switzerland). All solvents and reagents used for solid-phase synthesis were of analytical purity. Purification of crude peptides was achieved by semipreparative high-performance liquid chromatography (HPLC) (Mod.10 AKTA, Amersham Biosciences, Piscataway, NJ) coupled to a UV/Vis detector from Amersham Pharmacia Biotech on a Supelcosil C18 (5 μm particle size, 8 mm × 250 mm, Sigma-Aldrich) Analytical HPLC (Waters, Malva, Milford, CT) equipped with a Waters C18 column (symmetry, 3.5 μm, 4.6 mm × 75 mm) produced single peaks with at least 98% of the total peak integrals (integrated with Empower software). Electro-spray ionization-mass spectrometry (ESI-MS, Micromass-Platform LC instrument, Waters Micromass Technologies) was in agreement with the expected mass. The physicochemical properties of the new analogs are summarized in Table I.

#### Linear Peptides

Sieber amide resin (0.45 mmol/g capacity) was used as a solid support for the linear peptides. All peptides were synthesized manually following a Fmoc strategy. First, the Fmoc protecting group on the resin was removed by treatment with 20% piperidine/N,N-dimethylformamide (DMF) and then, the coupling reaction was performed by using threefold molar excesses of activated Fmoc-amino acids throughout the synthesis. The amino acids were activated essentially either in situ using diisopropylcarbodiimide/1-hydroxy-benzotriazol (HOBt) in DMF for 2.5 h or with O-benzotriazol-1-yl-1,1,3,3-tetramethylenuronium tetrafluoroborate, HOBt, and N,N-diisopropylthethylamine (DIEA) (2.45:3:6). The Fmoc deprotection step was accomplished by treatment with 20% piperidine in DMF or by 2% 1,8 diazabiacyclo[5.4.0]undec-7-ene (DBU) in DMF containing 2% piperidine for 2–3 min24 to prevent the potential migration of Dde and DMB groups to other functional groups after the incorporation of Fmoc-1lys(Dde)-OH or/and Fmoc-Glu(DMab)-OH. Regarding analogs XVI, XVII, and XVIII after the removal of the DMB and/or Dde protecting groups by 2% hydrazine in DMF (3 × 2 min), on-resin acetylation of the N′-amino group of Glu1 peptide (XVIII) and of N′-amino group of Lys5 (peptides XVI, XVII and XVIII) was performed by 0.5 M Ac2O in DMF for 1 h. The resin was washed manually with DMF, dichloromethane (DCM), and diethyl ether successively to remove the solvents excess. The peptide resin was treated with the splitting mixture trifluoroacetic acid (TFA)/DCM/anisole/1,2-ethanediol (EDT)/H2O (92:3:2:1:2, v/v/v/v/v) for 3 h to remove the protecting groups and the peptide from the resin. The resin was filtered off, and the solution was concentrated. Peptides were isolated by precipitation with cold diethyl ether, centrifuged, dissolved in water with a few drops of acetic acid, and lyophilized. Purification of crude peptides was achieved by semipreparative HPLC (Mod.10 AKTA, Amersham Biosciences) on Supelcosil C18 (5 μm particle size, 8 mm × 250 mm, Sigma-Aldrich) Analytical HPLC (Waters, Malva) equipped with a Waters C18 column (symmetry, 3.5 μm, 4.6 mm × 75 mm) produced single peaks with at least 98% of the total peak integrals (integrated with Empower software). ESI-MS (Micromass-Platform LC instrument, Waters Micromass Technologies) was in agreement with the expected mass. The physicochemical properties of the new analogs are summarized in Table I.

#### Cyclic Peptides

The precyclic peptides (analog XIV, XV, XX, XXI) were synthesized on a Sieber amide resin (0.45 mmol/g capacity) utilizing a standard Fmoc solid-phase synthesis protocol described above. Fmoc-protected peptidyl resins were treated with 2% DBU in DMF containing 2% piperidine for 2–3 min. Boc-protecting group was inserted on the free terminal amino group of Glu1 peptide (XVIII) and of N′-amino group of Lys5 (peptides XVI, XVII and XVIII) was performed by 0.5 M Ac2O in DMF for 1 h. After that step, Dde and DMB protecting groups were removed by using 2% hydrazine in DMF (3 × 2 min). Then, the amide bond between γ-carboxyl group of Glu1 and the ε-amino group of Lys in position 8 (analog XIV, XV) or in position 5 (analog XX, XXI) was catalyzed by treating the mixture of PyBop/HOBt/DIEA (1.5 equiv./1.5 equiv./2.0 equiv, 3 × 4 h, RT-60 °C), monitoring the formation of the lactam bridge by Kaiser test. Cleavage from the resin and deprotection of the amino acid side chains of the cyclopeptides were performed as reported above. The crude product was precipitated with cold diethyl ether, centrifuged, and lyophilized. Cyclopeptides were analyzed by analytical RP-HPLC and characterized using the procedure described above. The
physicochemical properties of the new analogs are summarized in Table I.

All peptides were purified by semipreparative HPLC on a RP C18 support using a linear gradient from 20% to 35% of solvent A (0.1% TFA/H2O) and solvent B (0.1% TFA/acetonitrile) for 15 min at a 2 mL/min flow rate and UV detection at 215 and 280 nm. Eluted peptides were lyophilized immediately. The purity of each peptide was verified by an analytical reversed-phase C18 column at a 1 mL/min flow rate with the same solvent system as in the preparative HPLC. The molecular weight of the peptide was confirmed by using ESI-MS. The HPLC chromatogram showed that the purity of the peptides was >98%, while ESI-MS showed the correct molecular ion for the peptide.

Cell Culture and Proliferation Assay
The human prostate cancer epithelial cell lines PC3 and LNCaP (ATCC, American Type Culture Collection) were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cultures were maintained in 5% CO2 and 100% humidity at 37°C. Cell culture reagents were from BiochromKG (Seromed, Germany).

Slow-growing LNCaP and fast-growing PC3 cells were seeded into 48-well culture plates at 1 x 104 cells/well. After a 24-h incubation period, adherent cells were treated with the peptides in 2.5% (v/v) FBS-RPMI-1640. At the end of the incubation period, cells were fixed with methanol and stained with 0.5% crystal violet in 20% methanol for 20 min. After gentle rinsing with water, the retained dye was extracted with 30% acetic acid, and the absorbance was measured at 590 nm.

Comparison of mean values among groups was done using ANOVA and the unpaired Student t-test. Homogeneity of variance was tested by Levene’s test. Each experiment included at least triplicate measurements for each condition tested. All results are expressed as the mean ± SD of at least three independent experiments. Values of P less than 0.05 were taken to be significant (*P < 0.05, **P < 0.01, ***P < 0.001).

NMR Spectroscopy
Samples of synthetic GnRH-I and 1GnRH-III in deuterated dimethyl sulfoxide (DMSO-d6) were used for NMR studies. Data were acquired in a wide range of temperatures from 298 K up to 343 K on a Bruker Avance 400 MHz spectrometer. 1H 1D NMR spectra were recorded using spectral width of 12–17 ppm with or without presaturation of the H2O signal. 1H-1H 2D Total Correlation Spectroscopy (TOCSY)26,27 were recorded using the MLEV-17 spin lock sequence using τm = 80–100 ms, and 1H-13C HSQC spectra28,29 with 200,791 ppm spectral width in F1. 1H-13C TIPPI Nuclear Overhauser Effect Spectroscopy (NOEY)30,31 spectra were acquired using mixing time τm = 400 ms applying water suppression during the relaxation delay and mixing time. All 2D spectra were acquired with 10,014 ppm spectral width, consisting of 2K data points in the F2 dimension, 16–32 transients, and 512–1024 complex increments in the F1 dimension. Raw data were multiplied in both dimensions by a pure cosine- transients, and 512–1024 complex increments in the F1 dimension. Raw data were multiplied in both dimensions by a pure cosine- 

Effect on Prostate Cancer Cell Proliferation
To study the anticancer activity of lGnRH-III and 1GnRH-III analogs, we tested their effect on the anchorage-dependent proliferation of the well-established prostate carcinoma cell lines, LNCaP and PC3, that mimic different stages of the progression of prostate cancer. LNCaP cells are androgen-sensitive and express a large number of GnRHs, and PC3 cells are highly malignant, androgen-insensitive, and express lower number of GnRHs.6,38,39 lGnRH-III inhibited the proliferation of LNCaP cells in a concentration-dependent manner having a maximal effect (21.5% reduction) at the
of lGnRH-III and significant ($P < 0.01$) effect on PC3 proliferation, incorporation of DTic in positions 3 and 7 (analog X) led to increased antiproliferative effect on both cancer cell lines (Figure 2). The antiproliferative effect of [DTrp$^3$, DTic$^7$]-lGnRH-III (analog XI) and [DTic$^3$, DTrp$^7$]-lGnRH-III (analog XII) was higher than that of lGnRH-III. In detail, analog XI inhibited the proliferation of LNCaP and PC3 cells by 36.5 ± 0.9% ($P < 0.001$) and 32.4 ± 4.0% ($P < 0.001$), respectively, and peptide XII, by 36.7 ± 0.7% ($P < 0.001$) and 31.6 ± 2.7% ($P < 0.001$), respectively.

### Analogs with Amino Acid Changes in Positions 1, 5, 8 and Cyclic Compounds (Group C)

The antiproliferative effect of [NMeGlu$^1$]-lGnRH-III (analog XIII) on LNCaP cells was 30% lower than that of lGnRH-III whereas it had no effect on PC3 cell proliferation. Cyclic analogs [cyclo(Glu$^1$, Lys$^8$)]-lGnRH-III (XIV) and [cyclo(AcGlu$^1$, Lys$^8$)]-lGnRH-III (XV) had increased inhibitory effect on cell proliferation compared to lGnRH-III; their effect on LNCaP cells was 36.0 ± 2.0 ($P < 0.001$) and 35.8 ± 1.0 ($P < 0.001$), respectively, while on PC3 cells the observed effect was 33.8 ± 2.0 ($P < 0.001$) and 30.6 ± 3.0 ($P < 0.001$), respectively (Figure 3). Acetylation of $N^\alpha$ amino group of Lys$^8$ (analog XVI) led to significant increase in antiproliferative effect on both cell lines compared to lGnRH-III. Interestingly, further modifications on that analog in position 1 (analog XVII and XVIII) decreased the effect on LNCaP cell proliferation compared to [e-N-Ac-Lys$^8$]-lGnRH-III (Figure 3). On PC3 cell line, analog XVII had similar and XVIII had higher effect than analog with simple acetylation of $N^\alpha$ amino group of Lys$^8$. Analog XIX ([Lys$^5$, His$^8$]-lGnRH-III) resulting

#### FIGURE 1

Effect of lGnRH-III and analogs I–IV on the proliferation of LNCaP and PC3 cells at a final concentration of 20 μM, after 144 h incubation for LNCaP cells and 96 h for PC3 cells. The cell number was estimated as described under Materials and Methods section. Results are presented as % inhibition relative to the control and are the mean ± SEM of three independent experiments. ***$P < 0.001$.

#### FIGURE 2

Effect of lGnRH-III and analogs V–XII on the proliferation of LNCaP and PC3 cells at a final concentration of 20 μM, after 144 h incubation for LNCaP cells and 96 h for PC3 cells. The cell number was estimated as described under Materials and Methods section. Results are presented as % inhibition relative to the control and are the mean ± SEM of three independent experiments. **$P < 0.01$; ***$P < 0.001$. 

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after an exchange of positions between the two basic amino acids, His and Lys, had similar effect to lGnRH-III on LNCaP and PC3 proliferation. The antiproliferative effect on LNCaP cells of 1-5 cyclic analogs XX and XXI was lower than that of lGnRH-III and evidently decreased compared to counterparts 1-8 cyclic analogs (XIV and XV, Figure 3).

NMR Studies

Resonance Assignment. TOCSY maps of GnRH-I and lGnRH-III peptides were first analyzed to assign the individual spin patterns of amino acids through scalar connectivities (Figure 4). Sequential, medium-, and long-range connectivities were identified from NOESY maps acquired with $\tau_m = 400$ ms. Proton chemical shift peptides are reported in Supporting Information Tables S1 and S2.

As far as the GnRH-I peptide is concerned, NH—NH connectivities of the type $(i, i+1)$ are detected for the region spanning the residues His$_2$-Leu$_7$ while NH—NH $(i, i+2)$ NOE cross-peaks are detected for the residue pairs His$_2$-Ser$_4$, Trp$_3$-Tyr$_5$, Tyr$_5$-Leu$_7$, and Arg$_8$-Gly$_{10}$. On the other hand, H$_\beta$—NH connectivities of the type $(i, i+1)$ are observed for the fragments pGlu$_1$-Tyr$_5$ and between the residues Leu$_7$-Arg$_8$, while H$_\beta$—NH type $(i, i+1)$ connectivities have been observed for the N-terminal tetra-peptide segment (pGlu$_1$-Ser$_4$) and for the Tyr$_5$-Gly$_6$ pair. Moreover, H$_\beta$—NH $(i, i+2)$ NOEs are identified among amino acids almost all over the peptide sequence. More than 30 medium-range NOEs were observed in the NOESY maps acquired with $\tau_m = 400$ ms. The most characteristics among them are H$_\beta$—NH type connectivities between amino acids Ser$_4$-Gly$_6$ and protons of the aromatic ring of His$_2$ with all backbone protons of Ser$_4$ as well as NOEs between Leu$_7$-Gly$_{10}$ and Tyr$_5$-Arg$_8$. Long-range NOE signals involve the amide proton of N-terminal Serine at position 4 of the peptide sequence and the C-terminal Proline at the position 9 (four NOEs) as well as the amine protons of the CO-NH$_2$ terminal group (one NOE). These data provide a solid experimental evidence for the spatial proximity of the peptide’s termini.

**FIGURE 3** Effect of lGnRH-III and analogs XIII–XXI on the proliferation of LNCaP and PC3 cells at a final concentration of 20 $\mu$M, after 144 h incubation for LNCaP cells and 96 h for PC3 cells. The cell number was estimated as described under Materials and Methods section. Results are presented as % inhibition relative to the control and are the mean $\pm$ SEM of three independent experiments. ***$P < 0.001$.

**FIGURE 4** Characteristics $^1$H-$^1$H TOCSY fingerprint regions of GnRH-I and lGnRH-III peptides recorded at 400 MHz NMR and in DMSO-$d_6$ at 298 K.
Structure--Activity Studies of lGnRH-III

Although the 1GnRH-III exhibits a lower number of NOE cross-peaks compared to the GnRH-I, the number of identified NOEs is satisfactory, and 1GnRH-III can be defined by a single well-determined structure. The NOE pattern involving NH—NH connectivities, spanning the residues His5-Lys8, for this peptide is rather similar to these of the GnRH-I analog, while H2—NH and Hβ—NH (i,i + 1) type connectivities have been identified all over the peptide sequence except for Pro9. Both long- and medium-range NOEs involving backbone protons present in GnRH-I were totally absent in the 1GnRH-III analog. Only two Hβ—Hγ (i,i + 2) type connectivities among Trp3 and His5 as well as one NOE cross-peak between the εNH of Lys8 and the Hβ of Asp6 have been assigned. Moreover, the interaction among the aromatic rings of His5 and Trp7 is manifested by the existence of two medium-range NOEs between the Hα proton of His5 and the Hδ proton of Trp7.

Structure Calculation and Conformational Analysis

The NOE-derived structural information extracted from the analysis of NOESY spectra acquired in DMSO under identical experimental conditions (τm = 400 ms) was introduced to DYANA for structure calculations. The average target function for the DYANA family of 20 calculated models was found 9.2 × 10−2 ± 1.7 × 10−2 Å2 for GnRH-I and 0.36 ± 1.64 × 10−3 Å2 for 1GnRH-III, respectively. No consistent violations existed at the final DYANA run, and no constraint violation was found larger than 0.20 Å. The RMSD values for the GnRH-I 20 models’ ensembles were calculated as 0.52 ± 0.24 Å (BB) and 0.96 ± 0.19 Å (HA). Similarly, the 1GnRH-III DYANA 20 models’ ensembles exhibit pairwise RMSD values for all residues 0.70 ± 0.18 Å (BB) and 1.52 ± 0.60 Å (HA).

3D Solution Models

Despite the fact that a typical Hε—NH (i,i + 3) connectivity has not been observed for the fragment Ser5–Arg8, the presence of the Hε—NH and NH—NH (i,i + 2) connectivities—-together with the absence of Hε—NH (i,i + 1) type NOEs for Gly6—suggest the existence of a β-turn structure in this region. In this conformation, the N-terminal region is close to the C-terminal and the spatial proximity of the two termini is manifested by long-range NOEs between: (i) the NH proton of Ser5 and various Pro9 protons, and (ii) the NH proton of the C-terminal amide. Since these long-range constraints might be fundamental for the β-turn formation and the convergence of the two peptides’ termini, structure calculations were also performed without the above-mentioned long-range NOEs. The resulted ensemble of 20 DYANA models (PDB 1YY1) is also characterized by the formation of the β-turn and the highly flexible peptide termini. This conformation led us to conclude that the peptide preserves the hairpin-like conformation, and the NOE contacts that determine this conformation are all among the residues of the Ser4–Arg8 loop.

In the GnRH-I solution structure presented here, Gly6 is found in the tip of the loop formed by residues Ser4–Arg8 (Figure 5). NOEs of (i,i + 2) between Hε Ser4 and HN Gly6 as well as Gly6 H2—HN Arg8 indicate that achiral Glycine residue in position 6 allows the turn formation and the U-shape fold of the peptide. Additionally, the side chains of Tyr5 and Leu5 seem to be in a short distance, while the observed NOEs between the Tyr5 aryl protons and the Leu7 methyl protons support the parallel orientation of their side chains. Moreover, the side chains of His5 and Trp3 present different orientation, while Trp3 orients its indole ring to the same direction with the Tyr5 aryl ring.

The substitution of the GnRH-I tetra-peptide segments Tyr5–Gly6–Leu7–Arg8, which is located in the middle of the GnRH-I sequence with the His5–Asp6–Trp7–Lys8 segment in 1GnRH-III, seems to impose great structural changes in the stereocchemistry of the latter peptide. The spatial arrangement of both N- and C-terminal fragments is totally different with respect to the peptide representing the native hormone, since no long-range NOEs involving the terminal residues have been identified (Figure 6). Furthermore, there is no experimental evidence for a β-turn formation in the peptide segment 4–8, and consequently 1GnRH-III does not maintain the hairpin structure obtained in many GnRH analogs.40,41 Despite the lower number of NOE cross-peaks identified in the NOESY spectra of 1GnRH-III, the peptide seems to adopt a rather defined backbone conformation. Our NMR data suggest that the molecule adopts extended backbone geometry with three irregular turns/bends around amino acids Trp3, Ser5, and Pro9. The side chains of His5 and Trp3 still occupy different region in space, with the indole ring of Trp3 found on the opposite site of the peptide backbone level in
respect to the His$^2$ side chain and pointing towards the indole ring of Trp$^7$. Moreover, the aromatic ring of His$^5$ is found within NOE distance with Trp$^7$ indole ring supporting the parallel orientation of their side chains.

**DISCUSSION**

The ability of lGnRH-III to inhibit proliferation of cancer cells, combined with the weak GnRH-I agonistic activity, makes it an excellent starting compound for the development of peptide analogs with enhanced selectivity and anticancer activity. In this study, we report the synthesis of 21 new lGnRH-III analogs and their impact on the proliferation of two different prostate cancer cell lines. Moreover, to investigate the conformational resemblance or diversity in GnRH family, we studied the conformational properties of lGnRH-III and GnRH-I through NMR spectroscopy and their solution models are presented herein.

In our previous studies,$^{41}$ we have shown that GnRH-I does not exhibit significant antiproliferative activity on LNCaP and PC3 cells even at the concentration of 100 $\mu$M. Results of this study provide experimental evidence that the inhibitory effect of lGnRH-III, at the concentration of 20 $\mu$M, on LNCaP cell proliferation was 21.5 ± 0.9% while, on PC3 cell it was negligible. The absence of high affinity binding sites on PC3 cells$^{42}$ and the lower number of GnRHRs expressed on them$^{39}$ compared to LNCaP cells, may be related to the lack of responsiveness of PC3 cells following lGnRH-III treatment. However, many of the new analogs of lGnRH-III presented on this study had significant antiproliferative effect on both cancer cell lines. Our observations confirm the results of other studies that additional mechanisms may mediate the biological effects of GnRH analogs on cancer cells which have not yet been clarified.$^{42}$

In lGnRH-III, the position 6 is occupied by Asp, whereas mammalian GnRH (GnRH-I) bears Gly at this position. Although Gly$^6$ is often replaced by $\alpha$-amino acids to enhance the biological activity of GnRH-I analogs, previous studies report that removal of the Asp$^6$ in lGnRH-III resulted in complete loss of biological activity.$^{19,43}$ To further investigate the significance of this position, we substituted Asp$^6$ by Asn, Asp(OMe), Glu, and Gln. Incorporation of Asp(OMe) instead of Asp$^6$ significantly increased the antiproliferative activity of lGnRH-III on LNCaP cell line. Furthermore, Asp(OMe)-analog was the only analog of that group, with significant effect on PC3 proliferation. Insertion of Gln was well tolerated, while no antiproliferative activity on LNCaP cells was observed when Glu or Asn substitutes Asp$^6$. These findings suggest that neither the acidic function of the side chain of Asp$^6$ nor the putative salt bridge (Asp$^6$-Lys$^8$) are crucial for the antiproliferative activity of lGnRH-III, and we presume that different intramolecular interactions, that deserve further investigation, may occur and preserve the bioactive conformation.

According to Heređi-Szabó et al.,$^{19}$ indole rings of Trp$^3,7$ are crucial for the anticancer activity, probably due to interactions of these residues with the N-terminal which stabilizes the biological active conformation of lGnRH-III. In our study, the antiproliferative effect of the analog with DTrp in position 3 was significantly higher than lGnRH-III whereas DTrp$^7$ analog was ineffective. Introduction of DTrp instead of Trp$^3,7$ decreased the anticancer activity while, combined modification on the C-terminal resulted in a significant increase of the anticancer potency in both cancer cell lines. Tic is a conformationally restrained imino acid that has been incorporated to GnRH antagonists as local structure determinant.$^{44}$ In addition, we have previously shown that substitution of Trp$^3$ in GnRH-I by $\alpha$- or $\beta$-Tic increased the antiproliferative effect of the analogs.$^{45}$ In this study, results show that substitution of Trp$^3,7$ by Tic decreases the antiproliferative effect of lGnRH-III on LNCaP cells and slightly improves the effect on PC3 cancer cells. In contrast, the inhibitory activity of the analogs [DTic$^3,7$,lGnRH-III (X), [DTic$^3$, DTrp$^7$,lGnRH-III (XI), and [DTic$^3$, DTrp$^7$,lGnRH-III (XII) was significantly higher. Based on these findings, we presume that introduction of DTic, although lacks the indole ring, preserved the biologically active conformation of lGnRH-III. Furthermore, the importance of the stereochemistry of the residues in positions 3 and 7 is also indicated.

Conjugation of an acetyl-group via the side chain of Lys$^8$ significantly increases the antiproliferative activity of lGnRH-III suggesting that the putative salt bridge between Asp$^6$ and Lys$^8$ in lGnRH-III, proposed in an earlier structure activity study$^{16}$ and demonstrated by our findings, is not important for the anticancer activity. Similar elevation of the antiproliferative effect was observed by analogs with side chain

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cyclization of amino acids 1-8 (peptides XIV, XV) probably due to a more constrained conformation. On the other hand, although exchanging places between the weakly basic His5 and the more basic Lys8 did not alter the anticancer activity of the parent hormone, simultaneous 1-5 side chain cyclization in analogs XX and XXI resulted in lower antiproliferative effect.

In our attempt to elucidate conformational characteristics of lGnRH-III, the solution model was determined through NMR spectroscopy in DMSO, a solvent widely used for solubility reasons and is an acceptable medium of mimicking a prototypical hydrophobic environment. Furthermore, the 3D solution model of GnRH-I was also determined to shed light on the conformational differences between the two peptides. The obtained results show substantially different conformational features between the lGnRH-III and the GnRH-I (Figure 7). According to our NMR study, the network of sequential NOE connectivities present in GnRH-I spectra suggest the existence of β-turn structure for the Ser4-Arg8 segment. Long-range NOE signals between the N- and C-termini provide solid experimental evidence for the spatial proximity of the two termini. On the other hand, lGnRH-III does not exhibit the GnRH-I conformation (U-shape). In spite of earlier publications suggesting the existence of some helical properties,23 such backbone conformation cannot be supported by our results. In contrast, the 3D models of lGnRH-III can be characterized by the extended backbone conformation forming a kink around Asp6 and two more turns/bends around Trp3 and Pro9. The present lGnRH-III models are in agreement with Mezo et al.,17 where a relatively ordered extended like backbone conformation is proposed for the central region of the lGnRH-III peptide.

The substitution of the small and flexible residue Gly in position 6 of the GnRH-I sequence with Asp6 in lGnRH-III does not favor the formation of the β-type conformation in the 4-8 segment of the peptide sequence. Moreover, the substitution of Leu7 with the bulky Trp7 differentiates remarkably the conformation of the peptide. This also becomes evident by the observed NOE interactions between His5 and Trp7, which do not favor the spatial proximity of the two termini. As a consequence, the hydrophobic core present in GnRH-I, is lost in lGnRH-III.

Furthermore, the proposed ionic interaction between Asp6-Lys8 residues16 is in agreement with our findings. Trp7 changes its orientation pointing away from Lys8 and consequently no NOE interaction is found between their side chains (as in the Leu7-Arg8 case in GnRH-I), and Asp6 is found to be within NOE distance with Lys8 giving rise to one interaction between εNH of Lys8 and Hβ of Asp6.

In conclusion, we synthesized 21 new lGnRH-III analogs and the structure–activity studies show that several of them had similar or higher activity than that of the parent hormone. Analogs II, V, VIII, X, XI, XII, XIV, XV, and XVI had higher antiproliferative activity than lGnRH-III on LNCaP cells and significant activity on PC3 cell. The modifications of those vary significantly but their inhibitory effect is almost the same, hampering thus the identification of one particular “lead compound.” However, we have studied “key” positions of lGnRH-III, providing new information regarding the structure–activity relationship of lGnRH-III. Our observations are illustrating that lGnRH-III is a promising molecule for the development of peptide analogs with increased and potentially selective anticancer activity.

REFERENCES

Saffron administration prevents selenite-induced cataractogenesis

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Abstract

Purpose: The present study sought to investigate whether *Crocus sativus* stigmas (saffron) extract prevents selenium-induced cataractogenesis in vivo, and to study the possible protective mechanism.

Methods: Wistar rat pups were randomized into three groups. Group I (control) received subcutaneous injection of normal saline on postnatal day 10. Groups II (selenite-treated) and III (selenite+saffron-treated) received subcutaneous injection of sodium selenite (20 µmol/kg bodyweight) on postnatal day 10. Group III also received intraperitoneal injections of saffron extract (60 mg/kg bodyweight) on postnatal days 9 and 12. On postpartum day 21, rats were sacrificed and the lenses were isolated and examined for cataract formation. Activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) and glutathione levels, as markers of antioxidant defense, were measured in the isolated lenses. Levels of the indicator of lipid peroxidation, malondialdehyde (MDA), and protein oxidation (sulfhydryl content) in the lens were also determined. The effect of the different treatments on lens protein profile was evaluated with the estimation of the soluble to insoluble protein ratio and SDS–PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of the water-soluble fraction (WSF) of lens proteins.

Results: Saffron demonstrated significant protection against selenite-induced cataractogenesis in vivo. The mean activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and glutathione levels were significantly increased in group III compared to the selenite-treated group. Saffron significantly prevented selenite-induced lipid peroxidation, protein oxidation, as well as proteolysis and insolubilization of the lens WSF.

Conclusions: Saffron extract prevented selenite-induced cataract formation in Wistar rats, possibly by reinforcement of antioxidant status, reduction of the intensity of lipid peroxidation, protection of the sulfhydryl groups, and inhibition of proteolysis of the lens WSF. These findings highlight the anti-cataractogenic potential of saffron by virtue of its antioxidant property.

Introduction

Cataract is a degenerative condition and is the consequence of the loss of the crystalline lens transparency. According to data provided by the World Health Organization, cataract is one of the major causes of visual impairment globally and the first cause of...
blindness, accounting for 51% of cases worldwide [1]. It is estimated that about 40 million
people will eventually have severely reduced vision due to cataract by the year 2020 [2],
especially in the developing countries of Asia and Africa [3]. The only available treatment
for cataract is the surgical removal of the opaque lens and its replacement with an artificial
one to restore visual acuity. The aforementioned procedure has been optimized over the last
decades so as to become an everyday surgery in the-developed countries. Nevertheless,
cataract-induced blindness remains a severe health problem in the-developing
countries, due to the low socioeconomic status of the patients and limitations in the
acceptability, accessibility, and affordability of cataract surgical services [4].

Since surgical treatment is not widely available, many researchers seek for have investigated
pharmaceutical agents to prevent this disease. It has been estimated that a delay of 10 years
in the onset of cataract, by any means, would halve the number of patients needing surgery
[2]. Actually, over the last few years, great emphasis has been laid placed on exploring the
possible protective effects of several natural products against cataract formation. Natural
products are privileged candidates for drug discovery, since they are often endowed with
multiple functions. Cataractogenesis is considered to be a multi-factorial disease, correlated
with various pathogenetic mechanisms, that have not been completely clarified. There is a
large body of evidence demonstrating that oxidative stress (i.e., measurably increased levels
of reactive oxygen species ROS and oxidized substrate molecules—lipids,
sulphydryls sulfhydryls, nucleic acids) is compulsory to cataract development [5-9].
Consequently, enhancement of the antioxidant defenses of the lens could prevent or delay the
onset of cataract. Based on that premise, numerous natural products, with known antioxidant
properties, have been evaluated during in the last decades.

Saffron consists of the dried stigmas of Crocus sativus L., which is a bulbous perennial plant
of the family Iridaceae. Saffron is has been used and consumed since antiquity, and is one of
the most expensive spices; it is used for flavoring and coloring food preparations, as a
perfume, and also as a dye. It is cultivated in many Mediterranean countries, like including
Spain, Greece, and Turkey, as well as in India and Iran (its major producer) [10]. Saffron’s
The value of saffron is attributed to its characteristic phytochemical components—
specifically the unique hydrophilic carotenoids, i.e., crocins (glycosidic esters of crocetin),
picrocrocin (terpenoid glycoside responsible for the exquisite taste), and safranal (the main
essential oil component) [11-13]. Hippocrates and Dioscurides mention the use of saffron for
the treatment of ophthalmic disorders, among other uses. Since then, saffron has been
constantly used in traditional medicine, as therapy of several conditions such as insomnia, depression, bronchospasm, cardiovascular diseases, gastrointestinal disorders, menstrual pain, menopausal problems, as analgesic, and even against cancer [10,14]. Some of the medicinal-biologic properties of saffron, or its components, are attributed to its antioxidant features, which have been highlighted in several studies [15-21]. The objective of the present study is to investigate, for the first time in the literature, the effect of saffron extract against selenite-induced experimental cataract in vivo.

**Methods**

**Chemicals**

Commercially available saffron was kindly provided from the Cooperative de Safran (Krokos Kozanis), West Macedonia, Greece. Sodium selenite (44%–46%), catalase (CAT) from bovine liver (4966 U/mg), Purpald, N-ethylmaleimide, L-glutathione reduced, glutathione oxidized, glutathione peroxidase (GPx), and N-acetyl-cysteine were purchased from Sigma-Aldrich (St. Louis, MO 63,103, USA). A Superoxide Dismutase Assay Kit from Cayman Chemical Company (Ann Arbor, USA) was used for the determination of superoxide dismutase (SOD) activity.

**Preparation of saffron extract**

Saffron stigmas powder was extracted with methanol:water (50%v/v; 18 mL/250 mg) for 4 h at room temperature, in the absence of light and with continuous stirring. The extract was centrifuged, filtered, and evaporated to dryness using a Speed Vac System (Labconco Corp., Kansas City, MO). The composition was screened with HPLC on a Supelcosil C-18 column as previously described [13,20]. The residues were stored at −20 °C until further use. Samples were redissolved in normal saline and sterilized through membrane filtration (0.2 μm i.d.) for the injections.

**Animals**

New-born suckling Wistar rat pups were used in our experiment. The pups were housed along with their mothers in stainless-steel cages in well-ventilated rooms with controlled temperature (23 °C) and humidity conditions and 12 h:12 h light-dark cycles. The mothers were maintained on a standard laboratory animal diet in the form of dry pellets and provided tap water ad libitum throughout the experimental period. Animals were randomly assigned to three groups:
• Group I (control, n=9 animals), which received only subcutaneous injection of normal saline;

• Group II (Selenite-selenite-treated, n=8 animals), which received a subcutaneous injection of sodium selenite (20 µmol/kg bodyweight) on postnatal day 10; and

• Group III (Selenite+selenite+Saffron-saffron-treated, n=9 animals), which received a subcutaneous injection of sodium selenite (20 µmol/kg bodyweight) on postnatal day 10 and intraperitoneal injections of saffron extract (60 mg/kg bodyweight) on postnatal days 9 and 12.

Cataract formation could be evaluated from the 16th day, when the pups opened their eyes, with the help of an ophthalmoscope and later on with the naked eye. Bilateral cataracts were observed in the animals used for this study. On postpartum day 21, rats were anaesthetized with diethyl ether and then sacrificed by cervical dislocation. The eyes were enucleated and lenses were at once excised intracapsularly through an incision 2 mm posterior to the limbus under surgical microscope magnification. Both lenses were obtained from each rat and morphological examination for cataract formation was performed by gross examination of lenses under the magnification of the dissecting microscope against a background of black grid-lines.

Staging of the cataract formation was conducted by an examiner, blinded regarding the studied study groups, based on a scale 0 to 3 according to Geraldine et al. [22]. The degree of opacification was graded as follows:

• Grade 0: absence of opacification (gridlines clearly visible; Figure 1A);

• Grade 1: a slight degree of opacification (minimal clouding of gridlines, with gridlines still visible; Figure 1B);

• Grade 2: presence of diffuse opacification involving almost the entire lens (moderate clouding of gridlines, with gridlines faintly visible; Figure 1C);

• Grade 3: presence of extensive thick opacification involving the entire lens (total clouding of gridlines, with gridlines not seen at all; Figure 1D)

The experiments were conducted in strict compliance with the ARVO Association for Research in Vision and Ophthalmology Statement for Use of Animals in Ophthalmic and Vision Research and Guiding Principles in the Care and Use of Animals [23]. The study has been approved by the University Hospital Bioethics Committee.
Tissue treatment

Prior to biochemical analysis, the lenses were washed in ice-cold saline to remove blood and then weighed carefully. The lenses were homogenized in 30 mM phosphate buffer, pH 7.6, (10% w/v), and centrifuged at 15,000 rpm for 20 min at 4 °C. The supernatant obtained was stored at −30 °C, pending further analysis.

Analytical methods

Assay of superoxide dismutase SOD activity

Superoxide dismutase SOD activity was determined according to the Superoxide Dismutase Assay Kit, Cayman Chemical Company, which is based on the formation of a tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. The absorbance was read at 450 nm. The results are expressed as units/mL, where one unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide anion.

Assay of catalase activity

The peroxidative activity of catalase CAT was determined according to the modified method of Sinha [24]. The method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H2O2. The formaldehyde produced is measured colorimetrically at 540 nm with reaction with Purpald (4-amino-3-hydrozino-5-mercapto-1,2,4-triazole) and oxidation with KIO4. Standard concentrations of formaldehyde (0–75 μM) were used for the construction of a calibration curve. All determinations were performed in triplicate. The results are expressed as nmol/min/mL.

Glutathione Peroxidase peroxidase (GPx) activity

GPx activity was determined colorimetrically, according to following Rotruck et al. [25], to estimate the rate of the glutathione oxidation by H2O2. Reduced glutathione was used as substrate and DTNB [5,5′-dithio-bis(2-nitrobenzoic acid)] as a chromogen. Suitable aliquots of standard concentrations of GPx (0.025–1 U/mL) were used for the construction of the calibration curve. The color that was developed was read against a reagent blank at 412 nm. Results were expressed as units/mg tissue (one unit was the amount of enzyme that converted 1 μmol of reduced to the oxidized form of glutathione in the presence of H2O2/min).
**Estimation of reduced and total glutathione content**

A modified modification method of Hissin and Hilf’s method was used for the determination of reduced (GSH) and oxidized glutathione (GSSG) content in the lens [26]. The two forms of glutathione were estimated fluorometrically after reaction with \( o \)-phthalaldehyde (OPT). The samples were treated with 50% TCA and centrifuged to eliminate the protein content. The supernatant was extracted three times with diethyl ether. The aqueous fraction was used to determine the GSH and GSSG content; GSH selectively reacts with OPT (10 mg/ml in pure methanol) at pH 8.0 (500 mM Na\(_2\)HPO\(_4\) buffer), whereas after the addition of 5 mM NEM in the samples, only GSSG reacted with OPT at pH 12.0 (0.2 N NaOH). Standard concentrations of GSH and GSSG (0.75–15 \( \mu \)M) were used. The excitation wavelength was at 340 nm and the fluorescence intensity was determined at 420 nm. The respective concentrations in nmol/mg wet tissue were calculated from the respective calibration curves. The concentration of total glutathione was calculated by adding GSH and GSSG.

**Estimation of protein sulfhydryl content**

The protein sulfhydryl content was estimated using the Ellman’s procedure as slightly modified by Sedlak and Lindsay [27]. Briefly, the pellets from the samples treated with 50% TCA for the determination of glutathione were redissolved with a Tris-EDTA-guanidine HCl (3 mM: 3 mM: 3M) buffer, pH 8.9. In a 96-well microplate, 250 \( \mu \)l of the sample was mixed with 20 \( \mu \)l DTNB [5,5′-dithio-bis(2-nitrobenzoic acid)]. After incubation for 15 min at room temperature, the absorbance was read at 412 nm. The content of protein sulfhydryls was calculated using a calibration curve prepared with N-acetyl-cysteine (0.06 mM–1 mM). The protein content of each sample was evaluated using the Bradford assay [28]. Since NAC contains only one sulfhydryl group, the results were expressed as nmol –SH/mg protein.

**Determination of Lipid Peroxidation**

Lipid peroxidation was determined by the evaluation of malondialdehyde (MDA) levels in the rat lens. Due to the small volume of tissue sample, the fluorimetric method of Grotto et al. [29] and Jentzsch et al. [30] was used with slight modifications to measure MDA levels using a 96-well microplate. MDA was determined after the reaction with thiobarbituric acid and extraction with \( n \)-butanol. The excitation wavelength was at 515 nm and the fluorescence intensity was determined at 553 nm. The results were expressed as nmol MDA/g tissue against a standard curve (0.05–10 \( \mu \)M MDA).

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**Comment [Au12]:** Molecular Vision style requires that any abbreviated term be used at least three times. For two or fewer times, the term should be spelled out in full.

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**Comment [Au14]:** Molecular Vision style requires that any abbreviated term be used at least three times. For two or fewer times, the term should be spelled out in full.

**Comment [Au15]:** Should this be defined?
Sodium-dodecyl-sulfate polyacrylamide gel (SDS–PAGE) electrophoresis of water soluble proteins

Samples (30 μg protein) were loaded on a 12% polyacrylamide gel (1 mm thickness) according to a modified modification of method of Laemmli’s method [31]. Before loading, lens homogenate was mixed with sample buffer (35% v/v glycerol, 18% v/v 2-mercaptoethanol, 230 mM Tris/HCl pH=6.8, 10% w/v sodium dodecyl sulfate [SDS], 0.3% w/v bromophenol blue). Each mixture was heated at 100 °C for 5 min and then centrifuged for 3 min. The protein mix was left to cool down at room temperature.

Electrophoresis was performed in using a Cleaver Scientific Ltd (Warwickshire, UK) apparatus at 100V for approximately 2 h. For the visualization of protein lanes, gels were stained with 0.5% w/v Coomassie brilliant blue in methanol: acetic acid: water (3:1:6 v/v/v) and destained. Image J Launcher was used for the densitometric analysis of gels (at least four different experiments).

Determination of lens proteins

The determination of insoluble and soluble protein content in the rat lenses of each group was estimated by using the Bradford assay, using with BSA bovine serum albumin as a standard [28]; and the results were expressed as mg protein/ mg wet tissue. The ratio of soluble to insoluble proteins per sample was calculated.

Statistical analysis

All variables were tested for normality with the Kolmogorov–Smirnov test. Differences in clinical score between the groups were estimated with the Mann–Whitney U test. One-way ANOVA analysis of variance was used to detect differences in continuous variables among the three groups. The Bonferroni post hoc test was used for pairwise comparisons. A p value less than 0.05 was considered statistically significant. The Analysis analysis was performed using SPSS 15.0 software (SPSS, Inc., Chicago, IL).

Results

Morphological assessment of cataract formation

In group I, all the eyes were clear. In group II, 100% of the eyes developed moderate to severe cataract, indicating success in establishing the selenite-induced cataract model in our experiment. Stage 2 was the common endpoint of the cataractogenic process, accounting for 62.5% of the eyes (median 2, range 2–3). On the contrary, saffron coadministration (group III) significantly retarded selenite-induced cataractogenesis in vivo.
In this group, 38.9% of the lenses exhibited stage 1 cataract, 44.4% of the eyes developed stage 2 cataract, and only 16.7% developed dense cataract; (median 2, range 1–3; Table 1, Figure 1). The difference in exhibited cataract exhibited between in the control and selenite-treated group was significant (p<0.0001, Mann–Whitney U test). The difference of cataract frequency and severity between groups II and III was statistically significant (p<0.05, Mann–Whitney U test).

**Antioxidant status in the lens**

**Superoxide dismutase (SOD) activity**

The activity of SOD (mean±standard deviation [SD]) was significantly lower in the lenses of the group II compared to that of the normal lenses (p<0.001; Table 2). However, saffron co-administration (group III) resulted in significantly higher SOD activity levels compared to the group II, that which received only sodium selenite (p<0.05), although they were lower than those of the group I (control; p<0.05).

**Catalase activity**

The mean activity of CAT (mean±SD) in the lenses of group II rats was significantly lower than that of the lenses in control group (p<0.01; Table 2). In the group III, CAT activity was significantly higher than in group II (p<0.05). In fact, saffron coadministration resulted in the prevention of the loss of CAT activity, which was measured at levels similar to those of the control group (p>0.05).

**Glutathione Peroxidase (GPx) activity**

The mean activity of GPx (mean±SD) in lenses of group II was significantly lower than that of the lenses in the control group (p<0.001; Table 2). In the selenite+saffron treated group (III), GPx activity was significantly higher than in the selenite-treated group (p<0.001).

**Reduced and total glutathione**

For the further evaluation of the antioxidant status of the lenses, the reduced and total glutathione levels (mean±SD) were evaluated (Table 3). Selenite administration resulted in a significant reduction of reduced glutathione in comparison with normal lenses (p<0.05). Cotreatment with saffron (group III) restored the reduced glutathione concentration, which reached a level that was not statistically significantly different from the control group (p>0.05). The Lens lens total glutathione pool was depleted after selenite administration, while saffron showed a sparing effect on total glutathione content.
Levels of lens protein sulfhydryls

The level of protein sulfhydryl groups is an important indicator of tissue protein oxidation. In group II, protein sulfhydryl content (mean±SD) was significantly lower compared to control (p<0.001; Table 4). However, in group III, lens protein sulfhydryl content was significantly higher than the concentration observed in the selenite-treated group (p<0.05), demonstrating its protective effect against protein oxidative damage. Sulfhydryl content in the group III reached a level that was not statistically significant lower from than group I (p>0.05).

Levels of the indicator of lipid peroxidation in lens (malondialdehyde)

Malondialdehyde The MDA level (MDA; mean±SD) reflects the overall tissue lipid peroxidation [32]. MDA in group II, this was 91.6% higher than in group I (p<0.0001; Table 4). In group III, MDA it was 54.8% lower compared to the selenite-treated group (p<0.0001) and similar to those that of the control group (p>0.05).

Effect of Saffron on Lens Proteins

Calculation of the soluble to insoluble ratio (mean±SD) revealed a significantly lower ratio in the selenite-treated group when compared to the control group (p<0.0001; Table 4). In group III, saffron resulted in a significantly higher ratio in comparison to the group II (p<0.05). The ratio in group III was still significantly lower from than the that of normal lenses (p<0.001).

The relative changes in the water-soluble fraction (WSF) of lens proteins were detected by SDS–polyacrylamide gel electrophoresis (PAGE) analysis. Figure 2 shows a significant decrease of low molecular weight proteins in the selenite-treated lens; proteins between 10 and 30 kDa in group II were lower (~35%) than those in group I. Saffron coadministration seems to diminish the observed proteolysis of these proteins, since the densitometric analysis revealed a significant increase of protein (25–30 kDa) bands intensity (13%–27%) compared to group II. Particularly, the intensity of the 19–21 kDa protein band in group III was similar to that of the control (group I).

Discussion

Over the last In recent years, studies have been conducted to evaluate the possible beneficial effects of saffron or its components in ophthalmology. All the published data are with make reference to the pathology of the retina and its possible beneficial effects in conditions such as ischemic retinopathy, age-related macular degeneration, or other neurodegenerative conditions [33-38]. However, despite the continuously increasing literature on the
neuroprotective effects of saffron or its components in the retina, to date, no study has been
carried out to evaluate saffron's possible effects against cataract formation.
The purpose of our study was to examine in vivo the potential anti-cataract effects of saffron
extract in selenite-induced cataract. Our clinical observations of the morphological
examination of the lenses showed that cataract formation caused by selenite administration
was attenuated by the administration of saffron, suggesting its anti-cataract potential.
Furthermore, biochemical analyses were conducted to support the clinical observation.
The selenite cataract model was selected because of its rapid, effective, and
reproducible cataract formation [39,40]. Although the rate of lens opacification is much more
rapid than in human cataract, the selenite model shows an amount of general similarities to
human senile nuclear cataract, such as the formation of vesicles, increased levels of calcium,
proteolysis, a decrease of the in water-soluble proteins, the presence of insoluble proteins,
and diminished amounts of GSH. The mode of action of sodium selenite in cataractogenesis
has not been completely defined, but it is indicated that the main biochemical events are
induced by selenite overdose — are triggered by the selenite-induced oxidative stress
and the generation of reactive oxygen species (ROS) in the aqueous humor in combination
with a decrease in the activity of antioxidant enzymes such as SOD, CAT, GPx, glutathione
transferase, and glutathione reductase, and of as well as GSH content in the lens [40,41].
More precisely, it is assumed that oxidative damage occurs to critical sulphydryl groups of
proteins of the lens epithelium membranes. The aforementioned, in combination with
oxidative stress-induced lipid peroxidative damage of lenticular membranes, leads to the
inactivation of membrane proteins, such as Ca2+-ATPase. Inhibition of the function of Ca2+-
ATPase results in loss of calcium homeostasis and calcium accumulation in the lens. As a
consequence of calcium influx in rodent lenses, calpains, a family of well-characterized
calcium dependent proteases, are activated. Calpain activation, mainly m-Calpain and Lp82
in rodent lenses, induces rapid proteolysis of the water soluble proteins, that is, lens
crystallins, and cytoskeletal proteins. Proteolysis exposes the hydrophobic regions of lens
proteins, which then interact to form insoluble aggregates [39]. Insolubilization results in
precipitation and aggregation of lens fragmented proteins, and finally, in loss of lens
transparency [40].
SOD and CAT make up a primary line of defense against superoxide anion (O2−) and
hydrogen peroxide (H2O2), respectively. More precisely, SOD is a specific scavenger of
superoxide anion. It converts harmful superoxide radicals to H2O2 which is detoxified by
CAT to harmless by-products. GPx, which belongs to the family of selenoproteins, is present in relatively large amounts in the epithelium of the lens [42]. It catalyzes the reduction of a variety of hydroperoxides with the help of its reducing substrate, GSH. The enzyme GPx maintains the integrity of the phospholipid bilayer of membranes by putting a brake on the lipid peroxidation initiated by superoxide. In our experiment, administration of sodium selenite resulted in significant reduction of the activities of the antioxidant enzymes SOD, CAT, and GPx, whereas such a decline was prevented in the lenses of animals co-treated with saffron. It could be postulated that saffron improves the antioxidant defense mechanisms of the lens, leading to restoration of the levels of SOD, CAT, and GPx activities, in spite of exposure to sodium-selenite.

GSH, a tripeptide of glycine, glutamic acid, and cysteine, is found in unusually high levels in the crystalline lens and is believed to play a significant role in the maintenance of the reduced state in the lens. GSH (via its side sulphydryl group) is involved in the detoxification of potentially damaging oxidants such as H₂O₂ and dehydroascorbic acid, and in the protection against oxidation of membrane –SH groups, which are important for cation transport regulation. Finally, it protects cytoskeletal proteins and prevents the cross-linking of soluble crystallins, and, therefore, maintenance of its levels is vital for the preservation of lenticular transparency [43-45]. GSH levels decrease with age and are found reduced in most types of cataract [45,46]. Furthermore, selenite overdose has been proved to cause a significant depletion of lens GSH levels [47,48]. Restoration of GSH and total glutathione levels could be attributed to the beneficial effects of saffron treatment on the enzymes involved in glutathione synthesis [49,50].

The crystallins are subject to oxidative changes, which include the formation of disulfide and other inter- and intramolecular cross-links, resulting in their aggregation [51]. Furthermore, one of the primary events in selenite-induced cataractogenesis theory is the oxidative damage that occurs to proteins of the lens epithelium membranes [39]. Free sulphydryl (–SH) groups of proteins are mainly responsible for their antioxidant response, and they can serve as a sensitive indicator of oxidative stress [52]. Consequently, the reduced level of protein sulphydryl content is an indication of tissue protein oxidation. In the present study, in contrast to the lower level of lens protein sulphydryl content of the selenite-treated group, saffron coadministration kept the protein sulphydryl content near to control levels, indicating its protective effect against oxidative damage.
Free radical-induced lipid peroxidation is a highly destructive process and has been implicated as one of the main components of the pathogenetic mechanism of selenite-induced cataractogenesis [47]. The accumulated peroxidation products damage vital membrane structures [53]. MDA is a product of the breakdown of mainly unsaturated fatty acids through the oxidation mechanism [54]. In the present study, such a disturbance of membrane lipids from selenite probably resulted in the observed increase in MDA lens levels, when compared to normal lenses. The observed almost twofold reduction in the MDA level in the selenite+saffron-treated group suggests that saffron possibly preserved the structural integrity of the lens, thereby preventing its opacification.

Selenite cataract is characterized by a marked decline in water-soluble proteins through protein insolubilization either by excessive proteolysis by calpains or through structural alterations resulting from sulfhydryl oxidation [40]. The selenite-induced alteration in the lens protein profile has been depicted through the calculation of the soluble/insoluble proteins ratio in the selenite group in our experiment. Saffron supplementation prevented protein degradation and aggregation, resulting in less cataract formation. Previous studies showed that many of the soluble proteins in the approximate molecular weight ranges of 23–31 kDa are from β-crystallin, 19–20 kDa are from α-crystallin, and the smear of polypeptides from 21 to 23 kDa are γ-crystallins [55]. Saffron coadministration resulted in considerable preservation of the pattern of lens proteins with molecular size 19–21 kDa and 25–30 kDa, which probably denotes inhibition of crystalline proteolysis.

To conclude, in the present in vivo study, we showed, for the first time in the literature, that intraperitoneal administration of saffron extract could significantly protect against nuclear opacity formation in selenite-treated pups. This fact indicates that saffron components/metabolites can penetrate the blood-aqueous barrier and reach the aqueous humor. It has been recently shown that crocetin, the main crocin metabolite, is determined in cerebral tissue after intraperitoneal administration of saffron extract [56]. The extent of tissue damage caused by selenite and the protective effect of saffron were evaluated clinically and biochemically. Saffron’s modulatory effect of saffron seems to be associated with a reduction of the intensity of lipid peroxidation and protein oxidation, maintenance of antioxidant status, and inhibition of proteolysis of the lens WSF. This study has focused on the scientific validation of saffron as an anticataract agent, suggesting that saffron (or its components) exhibits a promising anti-cataractogenic effect. However, it is important to emphasize that certain differences exist between human and selenite experimental cataract.
This preliminary study is encouraging, but further research is required to determine whether a similar anti-cataractogenic potential can be demonstrated in humans.

Acknowledgments

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Figure 1. Transillumination pictures of lenses representative of each grade. Lens opacification of experimental animals corresponding to each grade. 

**A**: Grade 0: absence of opacification (gridlines clearly visible);  
**B**: Grade 1: a slight degree of opacification, with minimal clouding of gridlines;  
**C**: Grade 2: diffuse opacification involving almost the entire lens, with gridlines faintly visible;  
**D**: Grade 3: extensive dense opacification involving the entire lens (gridlines not visible).

*Comment [Au21]*: Molecular Vision style requires that all figure legends be written in complete sentences. Please rewrite all sentence fragments in this legend and review this point for all figures.

*Comment [Au22]*: Can these sentences be combined into one title?
Figure 2. Sodium-dodecyl-sulfate PAGE-polyacrylamide gel electrophoresis of the lens water soluble protein fraction. An equal amount of protein (30 μg) was loaded on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The first right lane indicates the molecular weight marker; Group I lane represents the protein profile of the normal untreated lens; Group II, the selenite-treated group; Group III, the selenite+saffron-treated group. In the normal untreated lens water-soluble fraction (WSF), the prevalence of
proteins with a molecular mass of 19–30 kDa (characteristic of crystallins) is shown. Selenite treatment led to a significant reduction (~35%) of these proteins, which was prevented by saffron coadministration.

**Table 1. Morphological assessment of cataract formation of each group’s isolated lenses**

<table>
<thead>
<tr>
<th>Group</th>
<th>Grade 0</th>
<th>Grade 1</th>
<th>Grade 2</th>
<th>Grade 3</th>
<th>Median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (n=18)</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0 (0)</td>
</tr>
<tr>
<td>II (n=16)</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>6</td>
<td>2 (2–3) *</td>
</tr>
<tr>
<td>III (n=18)</td>
<td>0</td>
<td>7</td>
<td>8</td>
<td>3</td>
<td>2 (1–3) *</td>
</tr>
</tbody>
</table>

Incidences and staging of cataract formation of the isolated lenses in each experimental group. Group I: Control, Group II: Selenite-treated, Group III: Selenite+Saffron-treated. The Mann–Whitney U test was used for pairwise comparisons. Different symbols indicate statistically significant differences between groups. * p<0.0001 compared with group I. ‡ p<0.05 compared with group II.

**Table 2. Activities of antioxidant enzymes of the lenses of each group.**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide dismutase (unit/ml)</td>
<td>0.37±0.07</td>
<td>0.18±0.05**</td>
<td>0.26±0.03‡</td>
</tr>
<tr>
<td>Catalase (nmol/min/ml)</td>
<td>0.07±0.01</td>
<td>0.04±0.005*</td>
<td>0.067±0.02‡</td>
</tr>
<tr>
<td>Glutathione peroxidase (μmol glutathione oxidized/min/mg tissue)</td>
<td>14.71±1.39</td>
<td>7.39±1.30**</td>
<td>12.34±1.93‡</td>
</tr>
</tbody>
</table>

Activities of the enzymes superoxide dismutase, catalase and glutathione peroxidase of the lenses of each experimental group. Group I: Control, Group II: Selenite-treated, Group III: Selenite+Saffron-treated. Each value represents the mean ± SD of six determinations. Statistical analysis was performed by one-way ANOVA with Bonferroni’s adjustment. Different symbols indicate statistically significant differences between groups of each enzyme activity. *p<0.01; **p<0.001; ‡p<0.05 compared with group I. †p<0.05; ††p<0.001 compared with group II.

**Table 3. Total and reduced glutathione levels.**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced glutathione (nmol/mg wet tissue)</td>
<td>0.33±0.1</td>
<td>0.17±0.02*</td>
<td>0.31±0.13</td>
</tr>
</tbody>
</table>
Reduced and total glutathione levels in the lens of Wistar rat pups of each group. Group I: Control, Group II: Selenite-treated, Group III: Selenite+Saffron-treated. Values represent the mean ± SD of six determinations. Statistical analysis was performed by one-way ANOVA with Bonferroni’s adjustment. Symbol indicates statistically significant difference between groups II and I (p<0.05).

Table 4. Levels of the indicators of protein (sulfhydryl content) and lipid (malondialdehyde) oxidation and ratio of soluble/insoluble proteins in lens of each group.

<table>
<thead>
<tr>
<th>Component analyzed</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein sulfhydryl (nmol -SH/mg protein)</td>
<td>0.93±0.1</td>
<td>0.67±0.08**</td>
<td>0.82±0.1‡</td>
</tr>
<tr>
<td>Malondialdehyde (nmol/g tissue)</td>
<td>56.64±12.38</td>
<td>108.51±13.63*</td>
<td>49.04±18.22†</td>
</tr>
<tr>
<td>Soluble/insoluble proteins</td>
<td>2.91±0.09</td>
<td>1.88±0.27*</td>
<td>2.28±0.27**</td>
</tr>
</tbody>
</table>

Quantitative analysis of protein sulfhydryl groups and malondialdehyde levels in the lenses of each experimental group. Calculated soluble to insoluble proteins ratio in each group lenses. Group I: Control, Group II: Selenite-treated, Group III: Selenite+Saffron-treated. Data represent the mean ± SD of six determinations. Statistical analysis was performed by one-way ANOVA with Bonferroni’s adjustment. Different symbols indicate statistically significant difference between groups. *p<0.0001; **p<0.001 compared with group I. †p<0.0001; ‡p<0.05 compared with group II.
Nikolitsa Koutroumani, Ioanna Partsalaki, Fotini Lamari, Athina Dettoraki, Andrea Paola Rojas Gil, Alexia Karvela, Eirini Kostopoulou and Bessie E. Spiliotis*

Protective mechanisms against oxidative stress and angiopathy in young patients with diabetes type 1 (DM1)

Abstract

Objective: Advanced glycation end-products (AGEs) via their receptor, RAGE, are involved in diabetic angiopathy. Soluble RAGE, an inhibitor of this axis, is formed by enzymatic catalysis (sRAGE) or alternative splicing (esRAGE). Malondialdehyde (MDA) is an oxidative stress marker, and ferric reducing ability of plasma (FRAP) is an anti-oxidant capacity marker.

Methods: In isolated mononuclear blood cells from 110 DM1-patients (P) and 124 controls (C) (4 – 29 years) RAGE mRNA (g) and protein expression (pe) were measured by RT-PCR and Western immunoblotting, respectively. Plasma levels of CML (AGEs) and sRAGE were measured by ELISA, MDA by flurometry and FRAP according to ‘Benzie and Strain’.

Results: P showed: (i) higher g of RAGE, especially in P>13 years of age and >5 years DM1, (ii) increased pe of esRAGE in DM1>5 years and (iii) increased FRAP and MDA.

Conclusions: The increased esRAGE and FRAP with increased levels of CML and MDA possibly reflects a protective response against the formation of diabetic complications in these young diabetic patients.

Keywords: advanced glycation end-products (AGEs); diabetes mellitus type 1; oxidative stress; RAGE; soluble RAGE.

Introduction

Diabetes mellitus type 1 (DM1) affects up to 35 per 100,000 children, adolescents and young adults every year, with a rising prevalence within the last decade (1). In adults it is commonly associated with microvascular complications (diabetic nephropathy, neuropathy and retinopathy) although these may rarely also develop in adolescents (2). The development of adult diabetic complications is strongly correlated to hyperglycemia and the duration of diabetes and several pathological mechanisms have been proposed that contribute to these complications.

One of these mechanisms is the production of advanced glycation endproducts (AGE), which are products derived from the non-enzymatic glycation and oxidation of plural substrates, such as proteins, lipids and nucleotides. Intermediate products of these processes are the amadori products, the best known of which is glycated hemoglobin A1C (HbA1c) (3). The accumulation of AGEs can directly modify the extracellular matrix (ECM) resulting in increased stiffness of the vasculature. They can also modify hormones, cytokines and free radicals.
that elicit oxidative stress in several tissues and subsequently evoke thrombotic and inflammatory processes intracellularly, via their receptor, RAGE. A common AGE that accumulates in diabetes is N-(carboxymethyl)-lysine (CML), which is believed to be lipid and protein adducts that have been found to impair nitric oxide (NO) production and activate the ‘nuclear factor kappa-light-chain-enhancer of activated B cells’ (NF-κB), thereby increasing atherogenesis, oxidative stress and inflammation (4).

Lipid peroxidation in diabetes has been shown to impair LDL uptake by the endothelial cells and it is one of the proposed mechanisms involved in diabetic microangiopathy and retinopathy (5). Malondialdehyde (MDA) has been found to be an important marker of lipid peroxidation responsible for the milieu of oxidative stress and its levels have been found increased in adult DM1 patients (6, 7). Furthermore, in DM1 adult patients with retinopathy the increased levels of MDA coexist with a reduction of their anti-oxidant power, as indicated by a reduction of the ferric reducing ability of plasma (FRAP) (8).

The most widely studied receptor for AGEs is RAGE. RAGE is a cell surface receptor that belongs to the immunoglobulin superfAMILY of receptors. Full-length RAGE receptor is comprised of one cytoplasmic domain, responsible for intracellular signaling, one transmembrane domain and two extracellular C-type domains preceded by one V-type immunoglobulin type domain that plays a critical role in ligand binding. Other forms of RAGE that appear to lack the cytosolic and transmembrane domains are known as soluble receptors for AGEs and can be detected in the circulating blood. These can be produced by alternative splicing of RAGE pre-mRNA transcripts forming the endogenous secretory RAGE (esRAGE) and by enzymatic cleavage of the full length RAGE, forming circulating soluble RAGE (sRAGE). Both esRAGE and sRAGE can bind AGEs in the circulation, thus preventing the adverse effects of the activated AGEs-RAGE axis intracellularly (9).

Limited reports have investigated the physiological mechanisms of AGEs accumulation in relation to oxidative stress in a milieu of type 1 diabetic children, adolescents and young adults. Therefore, the aim of our study was to investigate oxidative stress (MDA) and the anti-oxidant capacity of the body (FRAP) in children, adolescents and young adults with DM1 in relation to the activation of the AGEs (CML)-RAGE axis and its soluble isoforms, sRAGE and esRAGE, in order to investigate their possible role in the predisposition for, or the protection from, diabetic complications.

Patients and methods

Patient population

The study population consisted of 110 patients with DM1 (P) [90 children and adolescents (4–18 years) and 20 young adults (18–29 years)] and 124 age- and sex-matched (92 children and adolescents and 32 young adults) normal non-diabetic control subjects (C) (Table 1). In all the subjects, BMI (Body Mass Index), BMI percentile (BMI%), Tanner stage and waist circumference (WC) were obtained, together with measurements of glycosylated hemoglobin (HbA1C), total cholesterol, HDL and LDL cholesterol, triglyceride, liver enzymes (SGOT, SGPT), and urea and creatinine plasma levels (Table 1). In all the P, the duration of diabetes was also noted.

DM1 patients were followed-up on a regular basis in the Outpatient Clinic of the Division of Pediatric Endocrinology and Diabetes of the University Hospital of Patras, Greece. The inclusion criteria for the controls were: (a) absence of diabetes and any other related autoimmune disease and (b) absence of obesity. Informed parental consent and children’s assent were obtained in all cases. The study was approved by the Ethical Committee of the University Hospital of Patras (Patras, Greece).

Isolation of human peripheral blood mononuclear cells

The isolation of the human peripheral blood mononuclear cells (PBMCs) was performed as previously reported by our research group (10). Specifically, 20 mL of venous blood were obtained from all the subjects and this was layered over a Ficoll-Paque (Histopaque 1077; Sigma Aldrich, Dorset, UK) layer at a 2:1 ratio and centrifuged at 805 g for 35 min at 20°C. PBMCs were collected from the interface and washed with RPMI (Roswell Park Memorial Institute) media 1640 ( Gibco Invitrogen Corporation, Carlsbad, CA, USA) in order to remove the plasma and ficoll, according to the manufacturer’s instructions. The isolated pellets of PBMCs were stored at –80°C in order to isolate total RNA and proteins subsequently.

Total RNA extraction and reverse transcription PCR

Total RNA extraction from the PBMCs was performed according to the manufacturer’s instructions with the mammalian total RNA, miniprep kit (Sigma, Aldrich). DnaseI (Thermoscript RT, Invitrogen Corporation, Carlsbad, CA, USA) treatment of all the samples was included. Preparation of cDNA from total RNA was performed using a reverse transcription system (Thermoscript RT, Invitrogen Corporation). From the cDNA samples RAGE and β-actin mRNA were amplified with: RAGE primers: forward = 5’AAC-TGC-AGG-CTC-TGT-GGG-AGG-AT 3’, reverse = 5’CCT-TGC-CAA-CTG-TTC-TTC-TAC-AAC-C 3’ and β-actin primers: forward = 5’AAG-GCC-AAC-CGT-GAA-AAG-ATG-ACC 3’, reverse = 5’ACC-GCT-CTG-CTG-CAG-TGA-TGA 3’ under the following conditions: denaturation at 95°C for 1 min, annealing at 55°C for 30 s and extension at 72°C for 1 min, for 35 cycles. PCR bands were quantified by densitometry with scion image (version 4.0.3.2, Scion Corporation, Frederick, MD, USA).
Western immunoblotting

Protein samples of isolated PBMCs were lysed with laemnli sample buffer (2 × SDS sample buffer) prepared in the laboratory. Protein content in the samples was approximately estimated by SDS-PAGE electrophoresis and staining with Coomassie Blue. Whole cell lysates were separated by SDS-PAGE and proteins were transferred to nitrocellulose membranes (Amersham Biosciences plc, Buckinghamshire, UK). The membranes were blocked for 1 h at room temperature (RT) and then incubated with IgG: anti-RAGE (Biotechnology, Santa Cruz, CA, USA) diluted 1:150, overnight at RT. Furthermore, the protein measurements were corrected with the expression of β-tubulin, which was detected with an anti-β-tubulin antibody (Cell signaling Technology, Inc., Danvers, MA, USA) diluted 1:500, overnight at RT. Band detection was performed with chemiluminescence reagent ECL (GE Healthcare Bio-Sciences AB, Sweden). The Western blot signals (complexed protein bands) were quantified by densitometry with scion image (version 4.0.3.2, Scion Corporation).

Blood samples

Venous blood samples were obtained with heparinised syringes. The plasma was collected during the procedure of the PBMCs isolation and then stored at –80°C. The plasma samples were used to measure the levels of sRAGE, CML, FRAP and MDA.

Plasma assays

The plasma levels of the total soluble RAGE (esRAGE and sRAGE) (11) were measured by a sandwich ELISA kit (Human Immunoassay RAGE, Quantikine, R & D Systems, Minneapolis, MN, USA). Measurements were performed according to the manufacturer’s instructions and the intra-assay coefficient of variation for repeated measurements was 7.5%.

The plasma levels of CML were measured by a sandwich ELISA kit (Circulex™ Anti-CML Human autoantibody, Qzzuantikine, Cyclex Co, Ohara, Japan). Measurements were performed according to the manufacturer’s instructions and the intra-assay precision was 5.6%, whereas the inter-assay precision was 8.7%.

Malondialdehyde (MDA) was determined fluorometrically according to the protocol by Papandreou et al. (12). In brief, 25 μL of 90 mM butylated hydroxytoluene (BHT) was added to 250 μL of plasma samples or MDA standards (0.05 – 5 μM), to prevent further lipid peroxidation. Samples were hydrolysed in mild alkaline conditions at 60°C for 30 min in order to release bound MDA and the proteins were precipitated with 10% trichloroacetic acid (TCA). After being kept on ice for 10 min, mixtures were centrifuged and the supernatants obtained were incubated with 0.8% thiobarbituric acid (TBA) at 95°C for 90 min. The supernatants were then extracted with n-butanol and fluorescence was measured at 535 nm (emission wavelength) after excitation at 515 nm on a Perkin-Elmer LS55 fluorescence spectrometer. All determinations were carried out at least two times and in duplicates.

Table 1 Clinical and biochemical characteristics of the DM1 patients and the controls. Data are means ± SD. Statistical significance, p ≤ 0.05. NS, not significant.

<table>
<thead>
<tr>
<th></th>
<th>Diabetic patients</th>
<th>Controls</th>
<th>p-Value</th>
</tr>
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<tr>
<td>Subjects</td>
<td>110</td>
<td>124</td>
<td>NS</td>
</tr>
<tr>
<td>Sex, male/female</td>
<td>59/51</td>
<td>61/63</td>
<td>NS</td>
</tr>
<tr>
<td>Age, years</td>
<td>14.23±5.19</td>
<td>14.05±4.97</td>
<td>NS</td>
</tr>
<tr>
<td>Height, cm</td>
<td>154.39±19.02</td>
<td>150.57±16.50</td>
<td>NS</td>
</tr>
<tr>
<td>Height SDS</td>
<td>0.11±1</td>
<td>−0.38±1.18</td>
<td>0.002</td>
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<tr>
<td>Tanner stage</td>
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<td></td>
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<tr>
<td>I: 19</td>
<td></td>
<td>I: 11</td>
<td>NS</td>
</tr>
<tr>
<td>II: 17</td>
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<td>II: 11</td>
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<td>III: 4</td>
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<td>III: 7</td>
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<tr>
<td>IV: 18</td>
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<td>IV: 22</td>
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</tr>
<tr>
<td>V: 46</td>
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<td>V: 32</td>
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<tr>
<td>Weight, kg</td>
<td>51.15±17.8</td>
<td>44.44±14.33</td>
<td>0.003</td>
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<tr>
<td>Body mass index, cm/kg²</td>
<td>20.71±3.90</td>
<td>18.99±3</td>
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<td>Body mass index, cm/kg²%</td>
<td>65.60±22.88%</td>
<td>47.51±28.10%</td>
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<tr>
<td>Body mass index SDS</td>
<td>0.29±1.08</td>
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<td>Waist circumference, cm</td>
<td>70.78±9.6</td>
<td>66.93±8.9</td>
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<td>DM1 duration, years</td>
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<tr>
<td>HbA1c, %</td>
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<td>0.001</td>
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<td>Cholesterol, mg/dL</td>
<td>164.81±27.13</td>
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</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>94.05±29.1</td>
<td>95.18±25.76</td>
<td>NS</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>56.69±12.1</td>
<td>55.83±12.35</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>66.98±30.71</td>
<td>66.83±22.10</td>
<td>NS</td>
</tr>
<tr>
<td>SCOT, U/L</td>
<td>18.92±5.69</td>
<td>17.57±3.82</td>
<td>NS</td>
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<td>SGPT, U/L</td>
<td>17.8±9.3</td>
<td>16.5±5.5</td>
<td>NS</td>
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<td>Urea, mg/dL</td>
<td>31.3±6.83</td>
<td>23.57±6.02</td>
<td>NS</td>
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<tr>
<td>Creatinine, mg/dL</td>
<td>0.72±0.18</td>
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<td>NS</td>
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<tr>
<td>Micro-angiopathy</td>
<td>–</td>
<td>–</td>
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The ferric reducing ability of plasma (FRAP) was determined by a modified method of the Benzie and Strain protocol (13, 14). In particular, the working FRAP reagent was produced by mixing 300 mM acetate buffer (pH=3.6), 10 mM 2,4,6-tripyridil-s-triazine (TPTZ) solution and 20 mM FeCl\textsubscript{6}H\textsubscript{2}O in a 10:1:1 ratio just before use. This reagent was then loaded (150 \( \mu \)L) to each well of a 96-well micro titer plate together with a total of 5 \( \mu \)L of sample and 15 \( \mu \)L of ddH\textsubscript{2}O. A reading at 595 nm was taken and also a standard linear curve of Ferrous sulfate (FeSO\textsubscript{4} 7H\textsubscript{2}O) was produced by known concentrations (10 \( ^{-3} \) – 10 \( ^{-5} \) M). FRAP values were determined according to the standard curve.

### Statistical analysis

Our subjects were grouped according to age, DM1 duration and HbA1c levels (for DM1 patients), LDL cholesterol levels, pubertal stage, waist circumference, body mass index (BMI) and BMI % tiles. Expression levels of mean values with \( \pm \) standard deviation (SD) and correlations were performed using the SPSS computer program (17.0 version for Windows, Mann-Whitney or Kolmogorov 2-tailed t-test, Pearson’s or Spearman’s correlation coefficients). The threshold of statistical significance was defined as \( p \leq 0.05 \).

### Results

#### Clinical and biochemical characteristics of the study subjects

All the clinical and biochemical characteristics of the subjects are presented in Table 1. The diabetic patients exhibited significantly increased height SDS, weight, body mass index (BMI), BMI %, BMI SDS and waist circumference when compared to their controls, but these values were within the normal range, therefore they were not considered as risk factors for diabetic complications. Furthermore, the diabetic patients had not developed any micro- or macro-angiopathy and their glycemic profile showed that their diabetes was moderately controlled (HbA1c=8.0\%\pm1.8\%).

#### RAGE mRNA and protein expression in PBMCs

The gene expression (mRNA) of RAGE was increased in the overall sample of DM1 patients in comparison to the controls, \( (p=0.007) \) (Figure 1A). More specifically, RAGE mRNA expression was increased in the DM1 patients \( >13 \) years of age in comparison to their respective controls \( (p=0.039) \) (Figure 1B), and in the DM1 patients with \( >5 \) years duration of diabetes in comparison to the controls \( (p=0.002) \) (Figure 1C).

The protein expression of the non-glycosylated esRAGE isoform is detected at 46 kDa (15). The esRAGE protein expression tended to increase in the DM1 patients in comparison to their controls in the overall sample (Figure 2A) and this tendency remained even after the samples were grouped according to their age (\( \leq \) or \( >13 \) years of age) (Figure 2B). esRAGE, however, was significantly increased...
in the DM1 patients with >5 years duration of diabetes when these were compared with the DM1 patients with ≤5 years of diabetes (p=0.008) and also with the controls (p=0.041) (Figure 2C).

### sRAGE plasma levels

sRAGE plasma levels showed no significant differences between the DM1 patients and the controls (data not shown), but they had a tendency to increase in DM1 patients with the increasing levels of their HbA1c and especially with HbA1c >9% (Figure 3).

### CML plasma levels

CML plasma levels tended to increase in the DM1 patients when compared to the controls in the overall sample (Figure 4A) and this increase was significant in the DM1 patients that were ≤13 years of age (Figure 4B) and the DM1 patients with ≤5 years duration of diabetes (Figure 4C).

### MDA plasma levels

MDA plasma levels were increased in DM1 patients in comparison to the controls in the overall sample (p=0.001)
A B C
Controls Diabetic patients Controls ≤13 years Diabetic patients ≤13 years ≤5 years DM1 >5 years DM1 Controls
Diabetic patients >13 years Controls >13 years

Figure 4

CML plasma levels in DM1 patients and their controls in (A) the overall sample, (B) grouped according to age (≤ or >13 years) and (C) grouped according to DM1 duration (≤ or >5 years).

(A) The DM1 patients had a tendency to increase CML in comparison to their controls. (B) The DM1 patients ≤13 years of age had increased CML plasma levels in comparison to their age-matched controls (p=0.034). (C) The DM1 patients with ≤5 years of diabetes had increased CML plasma levels in comparison to the controls (p=0.027). (Data are plotted as mean±SE.)

Figure 5

MDA plasma levels in DM1 patients and their controls in (A) the overall sample, (B) grouped according to age (≤ or >13 years) and (C) grouped according to DM1 duration (≤ or >5 years).

(A) The DM1 patients had increased MDA plasma levels in comparison to controls (p=0.001) in the overall group. (B) The DM1 patients ≤13 and >13 years of age had increased MDA plasma levels in comparison to their controls (p=0.012). (C) The DM1 patients with ≤5 years of diabetes had increased MDA plasma levels in comparison to the controls (p=0.01). (Data are plotted as mean±SE.)

FRAP plasma levels

The FRAP plasma levels were significantly increased in the DM1 patients in comparison to the controls in the overall sample (p=0.005) (Figure 6A). More specifically, the FRAP plasma levels tended to increase in the DM1 patients regardless of age in comparison to their age-matched controls (Figure 6B) but they were significantly increased in the DM1 patients regardless of the duration of diabetes (≤5 years (p=0.012) and >5 years (p=0.012)) in comparison to the controls (Figure 6C).

Correlations

In the overall sample of the diabetic patients: (a) the sRAGE and MDA plasma levels were negatively correlated with the duration of the diabetes (r=-0.247, p=0.027 and...
r = –0.349, p = 0.020, respectively), (b) the protein expression of the 46 kDa isoform of RAGE (esRAGE) was positively correlated with the duration of the diabetes (r = 0.530, p = 0.005) and negatively correlated with the MDA plasma levels (r = –0.661, p = 0.038). (c) The gene expression of RAGE was negatively correlated with the FRAP plasma levels (r = –0.474, p = 0.015) and (d) the CML plasma levels were positively correlated with the FRAP plasma levels (r = 0.306, p = 0.021) (data not shown).

**Discussion**

Activation of the AGEs-RAGE axis plays a central role in the development of diabetic complications. This activated axis leads to diabetic angiopathy in adults (16). It has always been of clinical interest that the younger DM1 patients do not develop microangiopathy frequently, even with a duration of DM1 >5 years which is a crucial time period after which adults with DM1 present with these complications.

In our DM1 patients, we found a tendency of the plasma levels of CML (AGE) to increase, a tendency that was still evident when the subjects were grouped according to age and duration of diabetes. In particular though, the younger DM1 patients (≤ 13 years of age) and those with less DM1 duration (≤ 5 years) increased CML significantly. Previous reports have also shown significantly increased CML levels in DM1 children and adolescents that were not associated, though, with age and duration of diabetes, but were mostly associated with DM1 complications (17, 18). However, this significant increase was not associated with an increase in the RAGE mRNA expression in these young patients, possibly indicating that these increased circulating CML levels had not fully activated the AGEs-RAGE axis. Furthermore, RAGE mRNA expression was significantly increased in the older DM1 patients (> 13 years of age) and in those with a longer duration of diabetes (> 5 years). Previous studies have also reported increased mRNA expression of RAGE with increased duration of diabetes (19) but our study has shown, for the first time, that the age cut-off of 13 years is of great significance in childhood DM1.

Conversely, the protein expression of the endogenous inhibitor of AGEs, esRAGE (RAGE 46 kDa), increased with the duration of diabetes and the levels of total soluble RAGE (sRAGE and esRGE) tended to increase with the increased levels of HbA1C (> 9%) and negatively correlated with the duration of the patients’ diabetes. The increase in intracellular esRAGE in the patients with increased duration of diabetes (> 5 years) could be part of a protective mechanism that is activated in these young DM1 patients in an attempt to keep the circulating AGEs within ‘normal’ levels, as the CML levels did not significantly differ in this group from their respective controls. It is, however, of great interest that the sRAGE levels did not significantly change, but do show a negative correlation with the duration of diabetes. This negative correlation may possibly indicate that some of the AGEs’ homeostatic mechanisms are negatively affected by the duration of the DM1, even in these young patients. A clinical report (EURODIAB) in
adults with DM1 has shown that sRAGE increases in DM1 patients that already show macro- and micro-angiopathy when these were compared with diabetic patients with no angiopathy (20). Therefore, the tendency of sRAGE to increase especially with increased HbA1c may also reflect a counteractive mechanism in these children attempting to decrease their risk for diabetic complications.

In addition, we found increased MDA and FRAP plasma levels in the DM1 subjects. Previous reports have shown increased MDA plasma levels in children and adults with DM1 (6, 7, 21, 22). It has also been shown that MDA plasma levels progressively increase in the older patients with DM1 (23) in comparison to the younger ones. This increase in MDA plasma levels has not been shown by other studies to be accompanied by an increase in FRAP. Nowak et al. (8) showed in 2010 that DM1 patients who developed nephropathy have decreased antioxidant capacity as evaluated by their decreased FRAP plasma levels. Also, Firoozrai et al. in 2007 (7) correlated the decreased antioxidant defense (decreased FRAP) of their adult DM1 patients with their poor glycemic control. Therefore, it is possible that the increased FRAP in our young DM1 patients is in response to the increased MDA, showing that our young DM1 patients seem to still possess the ability to combat the oxidative stress mediators, which could put them in danger for vascular complications. This hypothesis is also supported by Astaneie et al. in 2005 (24), who showed the existence of increased anti-oxidant capacity in adult DM1 patients, through markers like FRAP and EGF, which also did not exhibit changes in oxidative stress markers like the lipid peroxidation marker of the thiobarburitic reactive substances (TBARS).

In conclusion, the increased protein expression of the endogenous inhibitor of AGEs, esRAGE, in our young patients with a duration of diabetes >5 years together with the tendency of sRAGE to increase with HbA1c >9%, seem to indicate that these protective factors are possibly recruited in the face of increased AGEs (CML), in these young DM1 individuals, in order to decrease the risk of AGE-induced complications. The countering effect of the increased FRAP levels in the face of the increased MDA levels, may also indicate that these young diabetic patients have possibly preserved their body’s ability to activate the physiological mechanisms recruited normally to reduce oxidative stress. It therefore seems that the younger patients with DM1 may recruit protective mechanisms against glycation and oxidative stress that may deem them capable of reducing their risk for diabetic complications.

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Medicinal Plants are plants which have a recognized medical use. They range from plants which are used in the production of mainstream pharmaceutical products to plants used in herbal medicine preparations. The Medicinal & Aromatic Plants for health are used as herbal treatments and therapies that can be new habits for culture. Plant, drug, or medicine yielding a fragrant aroma, as sage or certain spices and oils are known as aromatic plants.

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In cataract, opacification of the lens reduces visual acuity, finally leading to blindness if untreated. Cataract is the leading cause of blindness worldwide – there are about 50 million blind people in the world and cataract formation is the cause in half of these cases [1].

Today, cataract is treated by surgery. The cost of the surgery places an urgent need for less expensive, non surgical approaches to cataract treatment [2]. It was estimated that extra-capsular surgery used with 95% coverage of the population would aver more than 3.5 millions disability-adjusted life years (DAILY) per year globally [3]. In 2008, a study showed that the mean total cost of the operation per patient was €714 (SD = €57) and ranged from €437 in Denmark to €1087 in Italy [4]. The financial encumbrance is enormous considering that 28,000 new cases are reported daily [5]. Thus, pharmaceutical interventions that will maintain the transparency of the lens are intensively sought after.

Clearly, to develop such treatments, a precise understanding of the underlying pathophysiological mechanism of cataract formation is required. There are three discrete categories of cataract: the "age-related", that is associated with aging, "congenital cataract" that is present at birth indicating pathological changes during embryonic development of the lens, and "sugar cataract" which is associated either with diabetes mellitus [6-8] or galactosemia [9].

Sun light and oxygen, that the lens is exposed to, are associated with extensive damage to proteins and other constituents of the tissue. Other risk factors include environmental stress factors such as smoking, excessive UV-light exposure and electromagnetic radiation, life threatening diseases like coronary heart diseases [8], renal failure and many drugs [7]. These factors contribute to the depletion of antioxidants [e.g. vitamins C and E, carotenoids and glutathione (GSH)] and reduction of antioxidant enzymes [e.g. superoxide dismutase (SOD), catalase (CAT) and glutathione reductase/peroxidase (GSR/GPx)]. Furthermore, they may diminish protease (i.e. caspase, nuclease) activity, whose role is the second level of defense (e.g. removal of obsolete proteins).

Diabetes mellitus is another important risk factor. Hyperglycemia induces oxidative stress through a complicated pathway. Evidently, during hyperglycemia the accelerated flow of sorbitol through the polyol pathway and enhanced oxidative stress are implicated in the pathogenesis of secondary diabetic complications, such as cataractogenesis [10]. Aldose reductase (AKR1B1) catalyzes the first and rate-limiting step of the polyol pathway of glucose metabolism.

Several biochemical mechanisms are involved in the opacification of the lens and they have been thoroughly described: i) non enzymatic-glycation, ii) oxidative stress, iii) polyol pathway and iv) activation of calpain proteases. The established feature of cataract is the major structural modifications of the water soluble crystallins [11]. Ageing process, diabetes and oxidants lead to impaired membrane function, which results to pathologically elevated levels of intracellular Ca2+ [12,13]. Under these conditions, calpains (calpain 1 or μ-calpain, Lp85, calpain 2 or m-calpain, calpain 10 and Lp82) are over activated and the resulting deregulated proteolysis of soluble crystallins leads to their insolubilization and aggregation. Over activated calpains also degrade the cytoskeleton proteins, which can further elevate Ca2+ [14]. The above mechanisms compromise lens transparency and induce cataractogenesis. There are numerous models for in vitro and in vivo study of cataractogenesis. At in vitro experiments, cataractogenic agents such as galactose, glucose, naphthalene, selenite, transforming growth factor-β, methylglyoxal are added to the lens culture. The in vivo models include induction of cataractogenesis especially in rodents through streptozotocin-induced diabetes, galactose feeding, ionizing radiation, inhibition of cholesterol synthesis, steroid treatment, and administration of selenite overdose inducing oxidative stress [5].

Over the last decade, an increasing number of studies indicates the possible role of some plant extracts or isolated agents against cataract formation. Plant extracts, like extract of Ocimum sanctum [15], aqueous garlic extract [16], onion juice [17], as well as fraction of flavonoids extracted from Emilia sonchifolia [18], polyphenolic compounds of Camelia sinensis [19] and natural products such as curcumin [20], ellagic acid [21], luteolin [22], acetyl-L-carnitine [23,24], lycopene [25], resveratrol [26] have been proved to ameliorate selenite-induced cataract formation by enhancing antioxidant enzyme activity and inhibiting radical formation and lipid peroxidation. Additionally to their antioxidant potential, some plants or their compounds exhibit anticotactar action via maintaining Ca2+-ATPase, prevention of Ca2+ accumulation, thus inhibiting calpain activation. Such compounds are drevogenin D, an aglycone triterpene [27] and the flavonoid fraction extracted from Vitex negundo [28]. Furthermore, it has been found that the carotenoid, astaxanthin, delays lens crystallin precipitation [29], whereas ellagic acid prevents the alteration of lenticular, non-crystalin proteins [30].

Diabetic cataract and the possible preventing properties of some plants extracts or compounds have, also, been studied, but not as thoroughly as the selenite model. Zingiber officinalis (ginger) extract [31], Allium sativum methanolic extract [32], turmeric and curcumin [33] inhibited the polyol pathway and the advanced glycation of proteins, and protected lens antioxidant enzymes in streptozotocin–induced cataract model. Hydroxytryrosol and oleuropein from olive leaves, as well as caffeine protected lenses from oxidative stress in an alloxan- and a galactose-induced cataractogenesis model, respectively [34].
An increasing number of herbal extracts is documented to have anticataractogenic potential; whether the tested doses and dosage schemes can be translated in effective agents that will prevent cataract formation in humans is widely discussed. Most studies focus on the antioxidant properties of the natural products. Thus, members of the polyphenol family, i.e. flavonoids, galloyl glycosides, caffeinequimic acids, known for their antioxidant properties are privileged candidates for development of efficacious preventive agents. Beyond this approach, Babizhayef et al. [35-37] have shown that a natural histidine dipeptide can prevent and reverse cataract. N-acetylcarnosine has been used in several clinical studies as eye drops, in order to assess its ability to treat senile cataract. First results were encouraging, so Innovative Vision Products Inc. (INV) scientists developed the lubricant eye drops Can-C, designed as 1% N-acetylcarnosine (NAC) prodrug of L-carnosine and tested in 75 symptomatic patients 54-78 years old for 9 months. That small-scale study showed that NAC is safe and efficacious for prevention and treatment of senile cataract for long-term use.

The use of “omics” technologies might reveal new molecular targets and, thus, specificity in pharmacological targeting might reveal more effective therapeutics. Inhibition of aldose reductase is the commonest target in order to combat diabetic cataract. Previous studies using normal rats, dogs and mice have identified that AKR1B1 inhibitors are potential drugs to prevent high glucose- and galactose-induced cataracts. Nonetheless, the clinical utility of AKR1B1 inhibitors remains uncertain [38]. Another emerging molecular target is the lens calpains because calpain over activation is the convergence point of all implicated factors, i.e. ageing, diabetes, and oxidation. Among natural products, chalcones were indicated as calpain inhibitors in a recent in vitro study [39]. Besides that, the topical application as eye drops of those agents remains an attractive drug delivery means [35,40].

The screening of natural compounds for the identification of potent antacataract agents is a difficult and long-term goal. However, as far as cataract remains a significant public health problem and the only way of treatment is, the unprofitable for the society and for individuals, surgery operation, researchers have to keep seeking an agent that could prevent cataract formation. Nature has certainly arranged it for us.

References


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