Research report

Memory enhancing effects of saffron in aged mice are correlated with antioxidant protection

Magdalini A. Papandreoua, Maria Tsachaki b, Spiros Efthimiopoulos b, Paul Cordopatis c, Fotini N. Lamari c, Marigoula Margarity a,

a Laboratory of Human & Animal Physiology, Department of Biology, University of Patras, 26504 Patras, Greece
b Division of Animal & Human Physiology, Department of Biology, University of Athens, Greece
c Laboratory of Pharmacognosy & Chemistry of Natural Products, Department of Pharmacy, University of Patras, Greece

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A B S T R A C T

Brain aging is characterized by cognitive decline and memory deficits that could be the result of oxidative stress and impaired cholinergic function. In this study, the effects of a daily, 7-day, intraperitoneal administration of saffron on cognitive functions were examined in both healthy adult (4 months old) and aged (20 months old), male Balb/c mice (n = 8/group), by passive avoidance test. Whole brain homogenates (minus cerebellum) were collected for examination of brain oxidative markers, caspase-3 and acetylcholinesterase (AChE) activity. Results showed that saffron-treated mice exhibited significant improvement in learning and memory, accompanied by reduced lipid peroxidation products, higher total brain antioxidant activity and reduced caspase-3 activity in both age groups of mice. Furthermore, salt- and detergent-soluble AChE activity was significantly decreased only in adult mice. Thus, we showed, for the first time, that the significant cognitive enhancement conferred by saffron administration in mice, is more closely related to the antioxidant reinforcement. Next, we compared the effect of saffron (1–250 μg/mL), crocetin and safranal (1–125 μM) on H2O2-induced toxicity in human neuroblastoma SH-SY5Y cells. Both saffron and crocetin provided strong protection in rescuing cell viability (MTT assay), repressing ROS production (DCF assay) and decreasing caspase-3 activation. These data, together with earlier studies suggest that crocetin is a unique and potent antioxidant, capable of mediating the in vivo effects of saffron.

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1. Introduction

Normal aging is associated with a slow decline in brain functions such as sensory and motor performance, and at times, this decline is accompanied by progressive memory loss, dementia and cognitive dysfunctions, ultimately resulting in limited functionality. In both aged humans and rodents, cognitive impairment has been correlated to the accumulation of oxidative damage to lipids, proteins, nucleic acids [8,16,44] and the vulnerability of various neurotransmitters/neurotrophin systems activity to oxidative stress [21,40,60].

In addition, an age-related decline in cholinergic function is thought to be partially responsible for the memory disorders occurring during senescence. One of the major markers of cholinergic function is the activity of the enzyme acetylcholinesterase (AChE) which is known to be decreased with aging in various cerebral areas [54] and synaptic plasma membranes [20]. AChE activity is also known to be decreased by free radicals and increased oxidative stress [41]. This data has led to the suggestion that various antioxidant supplements and phytochemical components might be beneficial for preserving brain functions and forestalling the age-related deficits [64].

Crocus sativus L. is cultivated in many countries for the culinary uses of its styles (saffron). For more than 3000 years saffron is in constant use as a drug. Particularly in traditional Indian medicine, saffron has been used for the treatment of cogni-
tive dysfunctions. Chemical analysis has revealed the presence of unusual carotenoids glycosides (crocins), which are mono-, or di-glycosyl derivatives of crocetin, (2E,4E,6E,8E,10E,12E,14E)-2,6,11,15-
tetramethyl-2,4,6,8,10,12,14-hexadecahepta-enedioic acid, and small amounts of monoterpen aldehydes, like picrorcin and safranal [71]. Saffron is efficient in enhancing cognitive behavior in adult rodents which have previously been exposed to amnestic agents (e.g. scopolamine, ethanol or acetaldehyde) [57,58,69,70,79]. Saffron was effective in inhibiting TNF-a-induced apoptosis of PC12 cells [66] and protected neurons from the neurotoxic activity of 6-hydroxydopamine hydrobromide [2]. Crocin constituents also inhibited amyloid aggregation in vitro [54], while saffron was shown to be similarly effective with donepezil in patients with mild-to-moderate Alzheimer’s disease [3]. Previous in vitro studies [51,54] have shown that saffron has antioxidant properties, albeit moderate. However, despite the continuously increasing literature on safron neuroprotective effects in vitro and in various “disease-models”, none has so far examined its effect either on healthy adult and aged mice, or on cholinergic neurotransmission.

The aim of the present study was to investigate the effect of a 7-day intraperitoneal administration of saffron [60 mg/kg body weight (B.W.)] on learning and memory of healthy, adult and aged, male Balb-c mice, their brain ACHE activity, caspase-3 activity and oxidative stress. Results showed that the short-term supplementation of both healthy adult and aged mice significantly enhanced cognitive performance in the passive avoidance test and attenuated brain oxidative stress markers, whereas ACHE activity was attenuated significantly only in adult mice. In order to further investigate the antioxidant properties of saffron in stress conditions and identify the bioactive components, human neuroblastoma SH-SY5Y cells were incubated with saffron extract, crocin and safranal. Results showed that both saffron and crocetin provided strong protection and inhibited ROS production and caspase-3 activation.

2. Materials and methods

2.1. Plant material and extraction

Greek saffron (red threads) was kindly provided by the Cooperative Association of Krokos in Kozani, in West Macedonia, Greece. The preparation of the extract was performed as previously described [54]. Crocetin (CRE) (purity >98%) was prepared by saponification of saffron extract, as previously described [28], while safranal was purchased from Sigma–Aldrich Corporation, St. Louis, MO, USA.

2.2. Animals

The animals used in this study were adult (4-months-old) and aged (20-months-old) male Balb-c mice. Gross examination of the animals at sacrifice suggested aging related changes (i.e. hair loss) but not overt disease (e.g. tumors, dermatopathies etc). Mice were kept in polyacrylic cages (38 cm × 23 cm × 10 cm) with eight animals per cage and housed in a room under controlled temperature 24–26 °C, relative humidity 50–60% and with 12 h light–dark cycles. Food in form of dry pellets and water were available ad libitum. The inbred first generation male mice (n = 16, 4 months old; 35.8 ± 1.3 g BW) were randomly divided into 2 groups: a vehicle group (Control) and a saffron (60 mg/kg BW)-treated group (n = 8/group). Respectively, aged animals (n = 16, 20 months old; 39.0 ± 2.1 g BW) were accordingly divided to a Control and a saffron (60 mg/kg BW)-treated group (n = 8/group). All procedures were in accordance with Greek National Laws (Animal Act, PD 160/91).

Saffron extract was administered i.p. daily at a final volume 20 mL/kg, while control mice received 20 mL of saline. Saffron solution was prepared fresh daily, right before administration, by dissolving the dry extract in saline and by filtering through membrane filters of 0.2 μm internal diameter.

2.3. Behavioral testing: passive avoidance task

Mice were subjected to a passive avoidance test [27,29] on day 6, after a double training and an initial acquisition trial on day 5. The test is based on negative reinforcement to examine long-term memory [43,50]. It was performed according to previously described procedures [53] using a two-compartment passive avoidance apparatus (white/dark, separated by a black wall with a guillotine door in the middle part). Results were expressed as the mean initial (IL, max. time 120 s) and step-through (STL, max. time 300 s) latency times recorded once animals had crossed into the dark compartment, before, and 24 h, after the delivery of a mild foot shock (25 V, 3 mA, 5 s) to their paws. All training and testing sessions were carried out during the light phase (08:00 to 14:00 h).

2.4. Tissue preparation

Mice were killed on day 7 by transcardial perfusion with ice-cold 0.95% NaCl (10 mL/100 g BW) and the brain (minus the cerebellum) was weighed and homogenized (10%, w/v, 30 mM Na2HPO4, pH 7.6) with a glass homogenizer (Thomas, Philadelphia, USA, No. B 13957) at 9500 rpm, thrice. The homogenates were then centrifuged (23,113 × g, 4 °C, 2 h) to recover the salt-soluble fraction (SS) of ACHE. The pellets were re-extracted with an equal volume of 30 mM Na2HPO4, pH 7.6, containing 1% Triton X-100 and the suspensions were centrifuged at 23,113 × g at 4 °C for 2 h to recover the detergent-soluble fraction (DS) [11]. Protein concentrations were determined by the Bradford assay.

2.5. Acetylcholinesterase (ACHE) activity determination

ACHE activity was determined using the colorimetric assay of Ellman, as previously described [14,53], with acetylthiocholine iodide (ATC) as a substrate. The ACHE activity was expressed as mmol/min/g of tissue protein.

2.6. Measurement of caspase-3 activity in mice brain

Tissue samples (100 mg/mL) were assayed with the caspase-3 colorimetric assay (CASP-3-S, Sigma–Aldrich) kit, according to the manufacturer protocol, using acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) as substrate. Caspase-3 was expressed as nmol/h/mg of protein. The assay showed good linearity (1–500 μM, y = 0.0022x – 0.0104, R2 = 0.9971).

2.7. Assessment of antioxidant parameters

Ferric-reducing antioxidant power (FRAP assay) measures the ability of antioxidants to reduce the [Fe(TPTZ)]3+ complex to the blue-coloured [Fe(TPTZ)]2+ [6] and was assayed as previously described [53]. Ferrous sulphate (FeSO4) was used as a standard and the assay showed good linearity (10–1000 μM, y = 0.0016x + 0.0009, R2 = 0.9986) and sensitivity. The antioxidant capacity of the brain samples was expressed as μmol FeSO4/g of wet tissue.

In the above mentioned set-up for the FRAP assay, incubation with ascorbate oxidase from Corcula sps (Sigma–Aldrich) (4/1) μmol/L, prior to the addition of the FRAP reagent, enables the determination of ascorbic acid (FRASC assay) [47]. The experiment was performed as previously described [6]. FRASC showed good linearity (5–250 μM, y = 0.0018x + 0.0017, R2 = 0.9993) and sensitivity with a detection limit of 5 μmol ascorbic acid.

Malondialdehyde (MDA) was determined fluorometrically after reaction with thiobarbituric acid, as previously described [22,26,53]. The assay showed good linearity (0.05–5 μM, y = 6.7697x – 0.29, R2 = 0.9907) and sensitivity. The level of MDA was expressed as μmol/g tissue protein.

The level of reduced glutathione (GSH) was estimated fluorometrically after reaction with o-phthalaldehyde as previously described [40,53]. A good linearity was obtained for GSH in the range of 0.5–100 μM (y = 1.492x – 0.5683, R2 = 0.9972). The level of GSH was expressed as μmol/g of protein.

2.8. Cell culture and drugs treatment

Human neuroblastoma SH-SY5Y cell line was obtained from the American Type Culture Collection (ATCC). Cultures were grown in DMEM containing 10% heat-inactivated fetal bovine serum, 4.5 g/L glucose, 2 mM L-glutamine, 100 μg/mL penicillin and 100 μg/mL streptomycin sulphate. 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 × 104 cells/well. All experiments were carried out 24 h after the cells were seeded. Prior to all treatments, cells were incubated in serum-free media, for 24 h. Finally, cells were co-incubated with 250, 500 and 750 μM H2O2 and the tested phytochemicals (1–250 μg/mL saffron and 1–125 μM CRT and safranal), or vehicle control (medium containing max. 0.1%, v/v DMSO), for 18 h. For 2+3 experiments, cells were incubated with 250 μM H2O2 in the presence or absence of the tested phytochemicals (1–250 μg/mL saffron and 1–125 μM CRT and safranal), or vehicle as control (medium containing max. 0.1%, v/v DMSO), for 12 h.

2.9. Assessment of cell viability and reactive oxygen species (ROS) levels

Cell viability was estimated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-
tetrazolium bromide (MTT reagent) [59]. Briefly, on completion of the treatment, cells were washed with pre-warmed PBS and incubated with DMEM w/o containing MTT at the final concentration of 2.5 mg/mL for 150 min. Supernatants were carefully aspirated and the resultant formazan product was dissolved in DMSO and detected by a UV–vis spectrometer (Denley WellScan, West Sussex, UK) at 545 nm. The formation of ROS was evaluated by means of the probe 2,7′-
dichlorofluorescein diacetate (DCF-DA) (Sigma–Aldrich Corporation, St. Louis, MO, USA) modified by [76]. Cells were washed with pre-warmed PBS and incubated
was considered necessary for statistical significance.

The effect of saffron on initial latency (IL) and step-through latency (STL) in the double trial passive avoidance test, in (A) adult and (B) aged Balb-c mice. Individual values are presented as circles (closed for IL and open for STL) for control animals and triangles (closed for IL and open for STL) for saffron-treated ones, whereas lines represent the mean values. IL: max. time allowed 120 s and STL: max. time allowed 300 s. Statistical analysis was performed with GraphPad Instat 3 software, using the non-parametric Mann–Whitney test (n = 8 animals/group). (a) p < 0.01 statistical difference in comparison to control. (b) p < 0.01 statistical difference between the two age groups of mice receiving saffron extract.

with 50 μM DCF-DA in PBS at 37 °C for 30 min. Fluorescence was then quantified in a microplate-reader (Bio-Tek Instruments Inc., Ville St. Laurent, Que, Canada) at excitation wavelength 485 nm and emission wavelength 530 nm.

2.10. Measurement of caspase-3 activity in SH-SY5Y cells

Caspase-3 activity was measured with acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) as the substrate, as per the manufacturer's protocol (CASP-3-C, Sigma-Aldrich). Liberation of p-NA was measured at 405 nm with a UV–vis spectrometer (Denley WellScan). The changes in absorbance were standardized using graded concentrations of free p-NA solutions (1–200 μM). The assay showed good linearity (y = 0.0022x + 0.0077, R² = 0.998). Results were calculated as release of p-NA in pmol/min/mg protein.

2.11. Statistical analysis

Data are presented as means ± S.E. 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 necessary for statistical significance.

3. Results

3.1. Effect of saffron on rodent passive avoidance test learning ability

Passive avoidance behavior is based on negative reinforcement and is used to examine long-term memory. Step-through latency reflects the long-term memory of animals. Significant increase in step-through latency value shown improvement in memory. The effect of saffron injected i.p. to mice for 6 days on the step-through latency is shown in Fig. 1. No signs of writhing syndrome or changes in the emotional state of mice were observed after saffron administration. As shown in Fig. 1, the performance of each mouse was largely concise, although the motor activity of aged mice appeared slightly reduced. The latter observation is reflected by lower mean initial and step-through latencies of both groups of aged mice than those of adult mice. For instance, control aged mice showed a 69–77% decrease in their latency times, in comparison to their control adult littermates.

In the control and saffron-treated groups of adult mice, the mean initial latency was not significantly different (32.75 ± 9.02 and 23.63 ± 5.62, respectively) indicating that both groups behaved the same in the training trial. The same behavior was observed in the control and the saffron-treated aged mice (7.64 ± 1.56 and 10.99 ± 1.91, respectively), suggesting no major age-related differences in latency time prior to training. Saffron-treated adult mice exhibited significant increase (149%, p = 0.03) in step-through latency, as compared to the control group (246.25 ± 35.95 vs. 94.25 ± 34.74). Accordingly, saffron-treated aged mice exhibited significantly higher latencies than their control littermates (68.38 ± 12.60 vs. 29.62 ± 3.76). Overall, saffron administration facilitated learning in both adult and aged mice, as is evident by the delay of transfer in the dark chamber (shock) 1 day after the acquisition trial.

3.2. Effect of saffron on brain AChE activity

Given the implication of the cholinergic system in maintaining normal cognitive function, the activity levels of AChE were measured in mice brain, with the salt- and detergent-soluble fractions of brain homogenates as a marker of central cholinergic status. The molecular forms G4 and G1 of AChE are predominantly present in

### Table 1

The effect of saffron on AChE activity in adult and aged mice brain.

<table>
<thead>
<tr>
<th>Animal Groups</th>
<th>Salt soluble (SS)-AChE (nmol/min/g of tissue protein)</th>
<th>Detergent soluble (DS)-AChE (nmol/min/g of tissue protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult Control</td>
<td>0.211 ± 0.008</td>
<td>0.818 ± 0.049</td>
</tr>
<tr>
<td>Saffron-treated</td>
<td>0.102 ± 0.003**</td>
<td>0.509 ± 0.022**</td>
</tr>
<tr>
<td>Aged Control</td>
<td>0.111 ± 0.007**</td>
<td>0.667 ± 0.033*</td>
</tr>
<tr>
<td>Saffron-treated</td>
<td>0.103 ± 0.002</td>
<td>0.754 ± 0.053</td>
</tr>
</tbody>
</table>

* Animals were administered with an i.p. daily injection (20 μL) of either saline (control group) or 60 mg/kg B.W saffron extract (saffron group) for 7 days (n = 8 animals/group). The mice brain AChE levels are expressed as mean ± S.E. of 8 values each analyzed in triplicate. Statistical analysis was performed with GraphPad Instat 3 software, using the non-parametric Mann–Whitney test.

** p < 0.0001 statistical difference in comparison to control.

* p < 0.05 statistical difference between the two age groups of control mice.

** p < 0.01 statistical difference between the two age groups of control mice.
The control) and 25% in aged mice (39.5 ± 2.1 nmol pNA/h/mg protein). Caspase-3 levels are expressed as mean ± S.E. of 8 values each analyzed once, parametric Mann–Whitney test.

**3.3. Effect of saffron on brain oxidative status and caspase-3 activity**

Since oxidative stress can affect both the aging process and AChE activity [8], we examined the existence of a possible correlation between age-related alterations in AChE and brain oxidative status and caspase-3 activity. As shown in Table 2, the whole brain homogenates of control aged mice had statistically significant lower total antioxidant activity (26%) and ascorbic acid (23%) levels than control adult mice. Of statistical importance were also the increases observed in the whole brain lipid peroxidation products (∼22%) in aged mice, which was accompanied by a ∼23% decrease in GSH levels (Table 2).

Saffron administration significantly increased the total brain antioxidant activity, ascorbic acid and GSH levels in both age groups of mice. Saffron administration also caused a marked decrease in MDA levels (44–63%) (Table 2).

Comparison of the control littermates of both age groups showed a statistically significant increase (17%, p = 0.02) in the levels of caspase-3 in aged mice (52.9 ± 2.1 nmol pNA/h/mg vs. 45.4 ± 1.4 nmol pNA/h/mg protein), which is in agreement with the increased levels of MDA, previously mentioned. Administration of saffron attenuated caspase-3 activity by 13% in adult mice (39.9 ± 1.5 nmol pNA/h/mg vs. 45.4 ± 1.4 nmol pNA/h/mg protein of the control) and 25% in aged mice (39.5 ± 1.3 nmol pNA/h/mg vs. 52.9 ± 2.1 nmol pNA/h/mg protein of the control), respectively.

Thus, it appears that saffron administration conferred significant antioxidant protection and attenuated caspase-3 activity in both age groups of mice.

**3.4. H2O2-induced toxicity on human neuroblastoma SH-SY5Y cell line**

H2O2 can freely penetrate the cell membrane and generates the highly reactive hydroxyl radical, *OH, within the cell, ultimately inducing multiple pathways that will eventually result into an apoptotic cascade of events. To determine the effective concentrations of H2O2, SH-SY5Y cells were treated with various concentrations of H2O2 (50–1000 μM), for 18 h, and both the viability and free-radicals production of the cells were assessed by the MTT-reducing activity and DCF-assay. Treatment with 50 and 100 μM H2O2 for 18 h had virtually no great effect on the viability of the cell line (data not shown). Treatment with 250, 500 and 750 μM H2O2 decreased cell viability by 43 ± 1%, 52 ± 1% and 63 ± 1%, respectively, compared to untreated cells.

ROS accumulation was also significantly increased in the SH-SY5Y cell line after incubation with H2O2 (250 μM), as shown by the increase in relative fluorescence units (73,026 ± 4566 DCF/MTT values) compared to the untreated (control) cells (45,973 ± 2535 DCF/MTT values).

To determine whether the H2O2-induced cytotoxicity was due to apoptosis, activation of caspase-3 was determined, using Ac-DEVD-pNA as a substrate. As shown in Table 3, caspase-3 was significantly activated (up to 52%) after incubation of the neuroblastoma SH-SY5Y cells with 250 μM H2O2, for 12 h.

**3.5. Effects of saffron against H2O2-induced toxicity on SH-SY5Y cell line**

As illustrated in Fig. 2 treatment of SH-SY5Y cells with saffron and crocetin for 18 h, significantly prevented the cell death caused by the hydroxyl radical, H2O2.

### Table 2

Effect of saffron on biochemical parameters of mice brain oxidant status.

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>FRAP (μmol FeSO4/g wet tissue)†</th>
<th>Ascorbic acid (μg/g wet tissue)†</th>
<th>MDA (μmol/g protein)†</th>
<th>GSH (μmol/g protein)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.11 ± 0.13</td>
<td>115.90 ± 6.86</td>
<td>2.18 ± 0.16</td>
<td>25.83 ± 1.08</td>
</tr>
<tr>
<td>Saffron-treated</td>
<td>8.02 ± 0.24†</td>
<td>152.50 ± 6.05†</td>
<td>2.10 ± 0.11†</td>
<td>29.94 ± 1.21†</td>
</tr>
<tr>
<td>Aged</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.25 ± 0.30†</td>
<td>89.19 ± 11.75†</td>
<td>2.80 ± 0.18†</td>
<td>19.78 ± 1.35†</td>
</tr>
<tr>
<td>Saffron-treated</td>
<td>8.59 ± 0.32†</td>
<td>219.88 ± 11.51†</td>
<td>1.56 ± 0.10†</td>
<td>23.46 ± 0.74†</td>
</tr>
</tbody>
</table>

† Data are the mean ± S.E. of 8 values each analyzed once, parametric Mann–Whitney test.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>250 μM H2O2 (12 h)</th>
<th>+10 μg/mL saffron (12 h)</th>
<th>+10 μM CRT (12 h)</th>
<th>+10 μM safranal (12 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80 min</td>
<td>2.80 ± 0.10</td>
<td>4.16 ± 0.15†</td>
<td>2.99 ± 0.09†</td>
<td>2.84 ± 0.17†</td>
<td>3.31 ± 0.05†</td>
</tr>
<tr>
<td>120 min</td>
<td>1.84 ± 0.07</td>
<td>2.80 ± 0.10†</td>
<td>2.21 ± 0.31†</td>
<td>1.96 ± 0.10†</td>
<td>2.08 ± 0.07†</td>
</tr>
</tbody>
</table>

†† Absorbance of the hydrolysis of caspase-3 substrate was measured at 405 nm for 80 min and 120 min, respectively, after treatment of the SH-SY5Y cells with 250 μM H2O2, in the presence or absence of saffron (10 μg dry extract/mL) and its constituents (10 μM CRT or safranal), for 12 h. Results were calculated as release of p-NA in pmol/min/mg protein. Caspase-3 levels are expressed as mean ± S.E. of four independent experiments analyzed in duplicate. Statistical analysis was performed with GraphPad Instat 3 software, using the non-parametric Mann–Whitney test.

† † *p < 0.05 statistical difference in comparison to control.

† † † *p < 0.001 statistical difference in comparison to control.

### Table 3

Effect of saffron, crocetin and safranal against H2O2-induced activation of caspase-3 levels in SH-SY5Y cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>250 μM H2O2 (12 h)</th>
<th>+10 μg/mL saffron (12 h)</th>
<th>+10 μM CRT (12 h)</th>
<th>+10 μM safranal (12 h)</th>
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<td>120 min</td>
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<td>2.21 ± 0.31†</td>
<td>1.96 ± 0.10†</td>
<td>2.08 ± 0.07†</td>
</tr>
</tbody>
</table>

† † *p < 0.05 statistical difference in comparison to H2O2.
Fig. 2. The effects of the tested phytochemicals on H2O2-induced cytotoxicity on SH-SY5Y human neuroblastoma. Cells were co-treated with varying concentrations of H2O2 (250–750 μM) and the tested phytochemicals (1–250 μg dry extract/mL for saffron and 0.1–125 μM for CRT and safranal, respectively) for 18 h. Cell viability was determined by the MTT assay. Experimental values are the mean ± S.E. values of six determinations and each included quadruple sets. Statistical analysis was performed with GraphPad Instat 3 software, using the non-parametric Mann–Whitney test.* \( p < 0.0001 \) vs. H2O2.

Fig. 3. Effect of saffron and its constituents on H2O2-induced ROS accumulation on wild type (SH-SY5Y) human neuroblastoma cells. Quantitative analysis of DCF fluorescence intensity after treatment with 250 μM H2O2 in the presence/absence of test compounds at various concentrations for 18 h. Values are mean ± S.E. calculated from six different experiments performed in sextuple. Statistical analysis was performed with GraphPad Instat 3 software, using the non-parametric Mann–Whitney test. * \( p < 0.05 \) vs. H2O2 alone. ** \( p < 0.05 \) vs. control. Percentages indicate the percentages of decrease observed after incubation with the tested phytochemicals in the presence of H2O2.

by H2O2, in a non-concentration dependent manner, with the percentages of viability ranging from 69 to 98% in relation to the control group (100% viability). Although safranal co-incubation led to significantly higher viability (56–71%) compared to H2O2-treated, it failed to preserve viability levels equal to those in untreated cells.

Moreover, co-incubation of SH-SY5Y cells with the tested phytochemicals and H2O2 completely abolished H2O2-induced ROS accumulation, in a non-concentration-dependent manner (Fig. 3A–C). Saffron and safranal treatment preserved ROS levels equal to those in untreated cells, whereas crocetin reduced, further, ROS levels at all tested concentrations.

Furthermore, both saffron and its constituents attenuated H2O2-induced caspase-3 activation, in the order of CRT (30–38%) > safranal (21–31%) > saffron (21–38%) (Table 3), even up to 120 min of absorbance measurement, with the enzyme’s levels being near to those of the untreated cells. Thus, cell culture results showed that saffron and crocetin provide strong protection in
rescuing cell viability, repressing ROS production and decreasing H2O2-induced caspase-3 activation.

4. Discussion

Aging is characterized by a deterioration of cognitive function, including learning and memory. In the present investigation, in order to determine if Balb-c mice displayed age-related memory impairments, the retention of an aversive experience was measured. Passive avoidance paradigm is widely utilized for testing learning and memory in rats and mice [27,29]. In this procedure, the acquisition latency (initial latency) to enter the compartment on the first day is an indicator of visual ability and motor activity and the retention latency (step-through latency) to enter the compartment on the second day indicates memory of the aversive experience. In the present investigation, the mean initial latency and step-through latency values for the control aged mice were significantly lower (69–77%) than those of the control adult mice. Our findings are in agreement with numerous studies showing that aging is associated with decreases in fear conditioning in rats and mice [30,65].

A substantial part of age-related memory impairments have been attributed to alterations in various neurotransmitter systems [7,38], among which is the cholinergic: deteriorations in the integrity of basal forebrain cholinergic neurons have been shown to occur along with many other changes in limbic/cortical systems [13,15,17,35,45]. The cholinergic hypothesis of geriatric memory dysfunction suggests that the reduction of cholinergic markers, such as acetylcholine (ACh), choline acetyltransferase (ChAT) and AChE, is a critical component for the age- and dementia-associated memory deficits [5,56,72]. Additional lines of mostly indirect evidence establishing a relationship between age-related changes in mnemonic function and the cholinergic system come from studies in both humans and rodents, after either brain lesions and/or administration of anti-cholinergic drugs [9,10,17,18]. The alterations occurring in the cholinergic system of mouse brain as a function of aging were examined in the present study by recording AChE activity, the main enzyme responsible for the metabolism of ACh to choline and acetyl-CoA. AChE exists into different molecular forms, each of which presents different localization in the neuron. The G1 form, for instance, is cytosolic, while the G4 form is membrane bound and requires detergent for solubilization. Therefore, the SS- and DS-fractions of AChE contain predominantly the G1 and G4 forms, respectively [36].

AChE is an important therapeutic target. Reversible inhibitors of this enzyme have been used as cognitive enhancers in the treatment of Alzheimer’s disease and other dementia disorders [31,33,73]. Total AChE activity is found to be lower in AD than in normal brains [9,30,50], while decreases in AChE activity have also been reported in whole brain of C57BL/10 mice (16-months-old) [52], and in the hippocampus of C57BL/6J mice [75], but not in the cerebellum. In the light of these findings, it seems that there are regional [68] and dynamic age changes in AChE, since the largest declines occurred in specific brain areas and before 12–16-months of age, suggesting thus, that the reason behind AChE changes is far more complicated than initially thought of and might be more related to development than aging [63]. This assumption is interesting in view of the conflicting reports from both human and animals on the “validity” of the cholinergic hypothesis, with the studies showing age-related changes in cholinergic markers being about equal in number to those that failed to demonstrate this loss. Reasons mentioned to account for these discrepancies are differences in species and strains of experimental animals, age and sex, tissue sampling and methods of assay.

Although it is not possible, at present, to assign a definite factor to explain the pattern of deficit observed in the enzyme activity in old mice, previous studies in both rodents and humans have shown that there is a close correlation between age-related alterations in AChE and increased oxidative stress [41], as well as between age-related losses of cognitive function with oxidative protein damage in the brain [16,46]. Specifically the current study has demonstrated higher caspase-3 activity and MDA levels, along with decreased antioxidant activity (GSH, total antioxidant power and ascorbic acid levels) in control aged mice (Table 2), Similar age-dependent declines have also been reported in the literature in most tissues in aged rodents [23,25,67].

Saffron administration forestalled/reversed those age-related deficits. In particular, short-term administration of saffron resulted in enhanced cognitive function in both adult and aged mice, as evidenced by the significantly high STL values (131–149%) 1 day after the aversive experience. These results are in line with other reports on the long-term memory enhancing effects of saffron administration on chemically-induced amnesic rodents [1,48,57,69,70], with the protective effects been ascribed largely to the competition with the inhibitory actions of the amnestic agents (ethanol/or acetaldehyde) on binding to the NMDA receptors [1].

The exact mechanism(s) through which peripherally administered saffron exerts its cognitive enhancing effects remain yet to be elucidated, although our results showed to be only partially dependent on cholinergic markers functions, such as AChE (Table 1), since saffron extract failed to affect AChE activity in whole brain fractions of aged mice, in contrast to the marked decreases (~38–52%) observed in adult mice. The discrepancy observed between the enhanced cognitive behavior of saffron-treated aged mice with their “unaltered” brain AChE activity levels may be due to the implication of other neurotransmitter systems system in maintaining cognitive function.

Short-term administration of saffron also resulted in significant alterations in rodent brain oxidative stress parameters and antioxidant mechanisms, as evidenced by the decreased caspase-3 and MDA levels and increased total brain antioxidant activity, ascorbic acid and GSH levels in both adult and aged mice (Table 2). Similar protective effects of saffron by modulation of lipid peroxidation, antioxidants and detoxification systems have also been observed in genotoxins-induced oxidative stress in Swiss albino mice [61], in renal ischemia–reperfusion (IR)-induced oxidative injury in rats [24] and in reperfusion-induced oxidative/nitritary injury to cerebral microvessels after global cerebral ischemia in mice [80]. Thus, our in vivo results indicate that the significant cognitive enhancement conferred by saffron administration in aged mice, is more closely related to the antioxidant reinforcement, rather than to AChE inhibition, per se, not precluding other synergistic/pleiotrophic effects. Furthermore, it confirms the notion
that memory is a complex process requiring the coordination of many neurotransmitter system, apart from the cholinergic [34]. Only in healthy adult mice were the “memory-enhancing” properties of saffron further correlated with cholinergic function as evidenced by the decreased AChE levels, indicating the need for an optimal balance/preservation level to exist between neuronal cholinergic transmission and cognitive performance.

In order to further investigate the antioxidant properties of saffron in stress conditions and identify its bioactive components, the human neuroblastoma SH-SY5Y cell line was incubated with the tested phytochemicals in the presence of H2O2. Exposure of cultured cells to H2O2 resulted in a dose-dependent viability loss at high H2O2 concentrations (Figs. 2 and 3, Table 3). Similar protective effects afforded by the carotenoid constituents of saffron against cellular oxidative damage are also reported in the literature [47, 48, 55].

Overall, our results showed that crocetin, the aglycon crocin metabolite, is a unique and potent antioxidant capable of combating oxidative stress, even at the higher applied concentrations of H2O2. The latter is in agreement with other reports on PC12[49] and hepatic cells derived from rats [19, 74], showing protective effects of crocetin against ROS-induced hepatotoxicity and genotoxicity. In particular, in experiments performed in rats, crocetin showed protective effects in the striatum and substantia nigra of a hemi-Parkinsonian (6-hydroxydopamine, 6-OHDA) [2] model. Crocetin has been demonstrated to be one of the main metabolites of orally administered crocin in rodents, which when absorbed, is partially metabolized to mono- and di-glucuronide conjugates [4, 77] and could be mainly responsible for these pharmacological activities, although it still remains to be elucidated its ability to cross the blood-brain barrier. The latter is of particular importance since it will allow us to elucidate whether the central measures are a direct effect of saffron/or CTR, or whether this is secondary to a peripheral effect on other tissues.

In conclusion, our findings show, in vitro and in vivo, the antioxidant properties of saffron and its cognitive enhancing effect in both adult and aged mice. The latter seems to be more closely related to the higher brain antioxidant properties and only partially related to the inhibition of brain AChE activity. Protective effects were also afforded by saffron and crocetin against H2O2-induced toxicity in SH-SY5Y cells. Despite the fact that the mechanisms underlying these effects are still unknown and require more pharmacological and neurochemical research to establish any therapeutic advantage, saffron seems to be valuable in the prevention/treatment of age-related diseases and oxidative stress.

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