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Inhibitory Activity on Amyloid-β Aggregation and Antioxidant Properties of *Crocus sativus* Stigmas Extract and Its Crocin Constituents

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Crocus sativus stigmas are one of the widely known spices (saffron) and consist of unusually polar carotenoids. Alzheimer's disease is characterized pathologically by deposition of amyloid β-peptide (Aβ) fibrils. Oxidation is thought to promote Aβ fibril formation and deposition. To identify agents inhibiting the pathogenesis of Alzheimer's disease, we examined in vitro the antioxidant properties of extract of *C. sativus* stigmas and its effect on Aβ_{1-40} fibrillogenesis. The antioxidant properties were determined by measuring the ferric-reducing antioxidant power and Trolox-equivalent antioxidant capacity, while its effects on Aβ-aggregation and fibrillogenesis were studied by thioflavine T-based fluorescence assay and by DNA binding shift assay. The water:methanol (50:50, v/v) extract of *C. sativus* stigmas possesses good antioxidant properties, higher than those of tomatoes and carrots, and inhibited Aβ fibrillogenesis in a concentration and time-dependent manner. The main carotenoid constituent, *trans*-crocin-4, the digentibiosyl ester of crocetin, inhibited Aβ fibrillogenesis at lower concentrations than dimethylcrocetin, revealing that the action of the carotenoid is enhanced by the presence of the sugars. Our findings suggest the possible use of *C. sativus* stigma constituents for inhibition of aggregation and deposition of Aβ in the human brain.

KEYWORDS: Alzheimer's disease; fibrillogenesis; antioxidant; *Crocus sativus*; crocin; stigmas

INTRODUCTION

Alzheimer's disease is the most common form of dementia among people over the age of 65 years old, which is characterized clinically by cognitive impairment and memory deterioration and pathologically by the presence of large numbers of neuritic amyloid plaques and neurofibrillary tangles in the neurons. The highly insoluble amyloid fibrils are comprised, primarily, of hydrophobic amyloid-β (Aβ) peptides of approximately 4 kDa, which are derived from the proteolytic cleavage of a longer precursor protein, termed amyloid-β protein precursor. The predominant forms of Aβ in amyloid deposits are those with 40 and 42 residues, Aβ_{1-40} and Aβ_{1-42} (*1*). Although extensive data support a central pathogenic role for amyloid-β peptide in Alzheimer's disease, the amyloid hypothesis remains controversial (*2*) and the neurotoxicity of Aβ is speculated to be linked to its state of aggregation (*3*). It has also been shown that oxidative stress itself is involved, at least in part, in the amyloid hypothesis (*4*). Nevertheless, accumulating experimental evidence suggest that Aβ can also increase oxidative damage (*5*).

Despite recent progress in the symptomatic therapy of Alzheimer's disease, an effective therapeutic approach that interferes with the accumulation/aggregation of Aβ in the brain is still eagerly awaited. Most of the antiamyloid strategies "applied" so far (e.g., immunotherapy agents, antiaggregants, secretase modulators, inhibitors of oxidative stress) have their own potential but unfortunately confer many side effects (e.g., acute allergic encephalitis, toxicity) in clinical trials. Recent studies are focusing on the development of drugs that will protect or delay the progression of the disease, giving opportunities for alternative methods of prevention/treatment. Supplementation with natural products from plants, such as *Hypericum perforatum* and *Ginkgo biloba* leaf extract EGb761, is popular for the delay of Alzheimer's disease (*6*). The cellular and molecular mechanisms of their therapeutic potential remain

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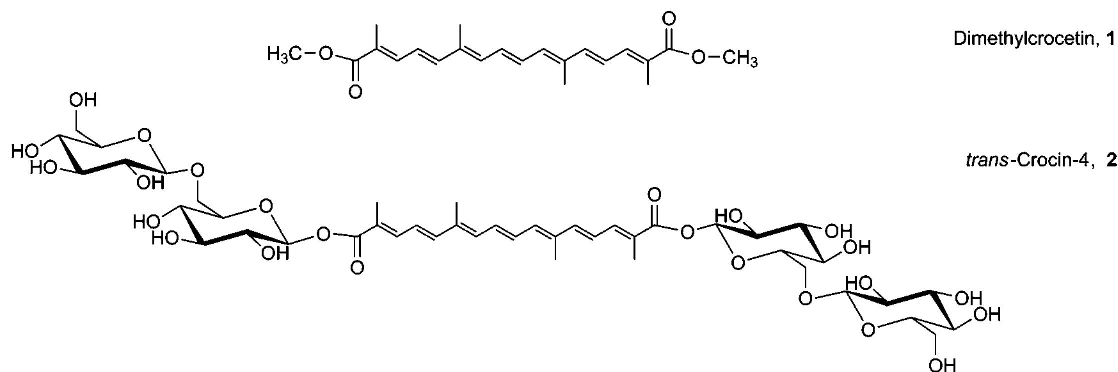

Figure 1. Chemical structures of dimethylcrocetin (1) and *trans*-crocin 4 (2).

largely unknown; however, their neuroprotective actions have been mainly attributed to the antioxidant and/or antiamyloidogenic properties.

Crocus sativus L., a stemless perennial herb of the Iridaceae family, is cultivated in many areas of the Mediterranean region and South Asia (mainly in Iran, India, Greece, Morocco, Italy, and Spain) for its red stigmas (style branches, commonly known as saffron) that are used for culinary purposes (spice, food colorant, and in alcoholic and nonalcoholic beverages) and are important ingredients in Mediterranean, Indian, and Chinese diet. Chemical analysis of *C. sativus* stigmas has shown the presence of unusually polar carotenoids (crocins) that are mono-, di- and triglucosyl esters of crocetin, a polyene dicarboxylic acid (8,8′-diapocarotene-8,8′-dioic acid) (**Figure 1**), small amounts of monoterpene aldehydes, like picrocrocin and safranal, and flavonoids (quercetin and kaempferol) (*7*, *8*). Saffron has been used as a drug in folk medicine, particularly in traditional Indian folk medicine, where it has been used for the treatment of various kinds of mental illnesses among other uses without any toxic side effects (*9*, *10*). Modern pharmacological studies have shown that oral administration of saffron capsules in humans (30 mg/day) was equally effective with imipramine and fluoxetine in the treatment of mild to moderate depression (*11*, *12*) and oral administration (125−250 mg/kg) or intracerebroventricular administration (51.2 nmol/brain) in mice reversed the loss of learning and memory caused by ethanol (*13*, *14*). Saffron also inhibited TNFα-induced apoptosis of PC12 cells that have been differentiated into neurons (*15*) and protected neurons from the neurotoxic activity of 6-hydroxydopamine hydrobromide (*16*). However, its direct or indirect effect on Aβ-fibrillogenesis has not yet been evaluated.

The aim of this study was to examine the antioxidant properties of *C. sativus* stigmas extract and its possible effect on Aβ fibrillogenesis, as well as those of the main crocin constituent (*trans*-crocin-4) and a seminatural crocin derivative (dimethylcrocetin).

MATERIALS AND METHODS

Chemicals. 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), ascorbic acid, ferrous chloride, sodium persulfate, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich (Athens, Greece). 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ) was from Fluka Chemica (Athens, Greece), and thioflavine T was from Merck (Darmstadt, Germany). Lamda FIX II vector from Stratagene was kindly provided by Anastasios Mintzas (Department of Biology, University of Patras) and $A\beta_{1-40}$ by Maria E. Figueiredo-Pereira (Department of Biological Sciences, Hunter College of City University of New York). All other chemicals used were of analytical reagent grade.

Plant Extraction. Stigmas (pure red Greek saffron) were kindly provided by the Cooperative Association of Krokos in Kozani, in West Macedonia, Greece. Harvesting, removal, and dehydration of stigmas was carried out by the growers by maintaining them at 20 °C during the first hours of the process and then at 30−35 °C until the moisture reaches the level of 10−12%. Dried stigmas of *C. sativus* were extracted in the dark, under magnetic stirring, with 125 mL/g of methanol:water (50:50, v/v) for 4 h. The extract was centrifuged, filtered through 0.2-μm filter, and evaporated to dryness in a Speed Vac system (Labconco Corp., Kansas City, MO). The dry residue was stored at −20 °C until further use.

Fresh vegetables were supplied from the local markets in Patras, Greece. Flesh samples (five fruits for each sample) were homogenized and freeze-dried. Extracts were prepared in methanol:water (50:50, v/v) (5 mL/0.2 g) for 2 h under magnetic stirring in the dark. The extracts were centrifuged, filtered through 0.2-μm filter, and evaporated to dryness, and the dry residues were stored at −20 °C.

HPLC of the Crude *C. sativus* Extract and Isolation of *trans*-Crocin-4. The crude *C. sativus* stigmas extract was analyzed by HPLC (Amersham Pharmacia), consisting of a P-900 AKTA purifier pump, UV-900 monitor, IV-907 and PV-908 valves, and M-925 mixer, on a 250 × 4.6 mm i.d., 5 μm, Supelcosil C18 column (Sigma-Aldrich, Athens, Greece) column, as previously described (*8*). In brief, the mobile phase was 10% methanol for 10 min followed by a linear gradient from 10 to 100% methanol in water for 60 min. Both solvents (10% and 100% methanol in water) contained 1% acetic acid. The solvent flow rate was 0.5 mL/min. Detection was performed simultaneously at 250 and 440 nm. Quantification was carried out by taking into account the molecular coefficient absorbance of *trans*-crocins (89 000 at 440 nm) and *cis*-crocins (63 350 at 440 nm) and expressed as the percentage of each crocin in relation to the total crocin content (*17*).

trans-Crocin-4 was isolated from the crude extract with semipreparative HPLC on a 250 × 8 mm i.d., 5 μm, Supelcosil C18 column (Sigma-Aldrich, Athens, Greece). The mobile phase was a linear gradient from 20 to 70% methanol in water solution in 70 min, while the solvent flow rate was 1.5 mL/min and the sample injection volume was 50−100 μL. The peak eluting at 39.3 ± 0.5 min, was collected and purified (>98%) after rechromatography on an analytical column. The identity of the pure crocin constituent was determined by analytical HPLC, ESI-MS on a Micromass-Platform LC instrument (Micromass, Manchester, UK) and UV/vis spectroscopy. HPLC analysis showed that one peak eluted at 40 min. The mass spectrum of *trans*-crocin-4 displayed an ion at *m/z* 999 [*trans*-crocin-4 + Na^+] and an additional signal at *m/z* 511.46 [*trans*-crocin-4 + $2Na^+$]/2. Furthermore, the UV/ vis spectra showed two absorption bands, one at 256 nm corresponding to glucosyl ester bonds of crocins and a double peak between 400 and 500 nm (maximum at 440 nm) characteristic of all *trans*-glycosidic carotenoids.

Preparation of Dimethylcrocetin. Five grams of *Crocus sativus* stigmas was extracted using 50 mL of petroleum spirit by ultrasound-assisted extraction. The ultrasound extraction was performed in a Sonorex, Super RK 255H type (300 × 150 × 150 mm internal dimensions) ultrasound water bath (indirect sonication), at the fixed-frequency of 35 kHz. The temperature of the sonicated water was 25 °C. The stigmas were sonicated five times for 10 min. The same procedure was repeated using diethyl ether as the solvent extractant. This procedure was done in order for the stigmas to be free from the essential oil. Then the stigmas were extracted in the dark, under

magnetic stirring, with 200 mL of methanol for 1 h. This procedure was repeated three times. The amount of the methanol extract (600 mL), containing the crocins, was hydrolyzed by 2 N KOH in methanol. In order to obtain the produced dimethylcrocetin, the hydrolyzed extract was centrifuged at 8000 rpm and 8 °C. Dimethylcrocetin was purified by repeated extractions (at least three times) using dichloromethane. The dichloromethane extracts were evaporated to dryness using a rotavapor. The purity and structure of the above compound was confirmed by FT-IR and the results were in total agreement with those reported in the literature (*18*).

Methods Determining the Antioxidant Capacity. The standard TEAC assay described by Re (*19*) was used with minor modifications. This assay assesses the capacity of a compound to scavenge the stable ABTS radical ($ABTS^{\bullet+}$), in comparison to the antioxidant activity of Trolox, a water-soluble form of vitamin E that is used as a standard. The blue-green $ABTS^{\bullet+}$ was produced through the reaction of 7 mM ABTS with 2.5 mM sodium persulfate ($Na_2S_2O_8$) (final concentrations) in the dark at room temperature for 12−16 h before use. The concentrated $ABTS^{\bullet+}$ solution was diluted with ethanol to a final absorbance of 0.8−0.7 at 734 nm. A 10-μL portion of sample (concentrations of 0.6, 0.3, and 0.1 mg/mL) was added to 990 μL of $ABTS^{\bullet+}$ solution, and the reduction in absorbance was measured 1 min after addition of Trolox (final concentration 1−20 μM) and up to 40 min after addition of the tested compounds. The stock solution of Trolox (2.5 mM) was prepared in ethanol. Absorbance was measured on a Biochrom460 UV/spectrophotometer (Pharmacia LKB).

TEAC showed good linearity for the standard substance used (Trolox) at concentrations of 1−20 μM ($R^2 = 0.9818$). The reduction in absorbance values of $ABTS^{\bullet+}$ was plotted versus the concentration of Trolox or the phytochemicals tested, respectively, at each time point. Calculation of the TEAC values was performed by dividing the regression coefficient of phytochemicals by the regression coefficient of Trolox, as earlier described (*19*).

The FRAP method measures the ability of antioxidants to reduce the $[Fe^{III}(TPTZ)_2]^{3+}$ complex to the blue-colored $[Fe^{II}(TPTZ)_2]^{2+}$ (*20*). Briefly, addition of ascorbic acid (between 1 and 120 μM) (used as a standard) or of samples into FRAP reagent (10 mM TPTZ and 20 mM $FeCl_3$ in 300 mM acetate buffer, pH 3.6) leads to an increase in absorbance readings at 490 nm. FRAP showed good linearity (5−250 μM, $y = 0.0028x + 0.0165$, $R^2 = 0.999$) and sensitivity with a detection limit of 5 μM ascorbic acid. The antioxidant capacity of the extract was expressed as the equivalent ascorbic acid concentration, which produces the same absorbance at 490 nm and was calculated by extrapolation of the absorbance values to the calibration curve. All determinations were carried out at least three times, and in triplicate, at each separate concentration of the standard and samples.

Thioflavine T Assay. $A\beta_{1-40}$ was dissolved in 100% dimethyl sulfoxide (DMSO) at a concentration of 10 mg/mL and was sonicated for 5−10 min. The stock solution of the peptide was stored at −70 °C. Just prior to use, the peptide 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 samples or without (control) (*21*). Aliquots (40 μL) were transferred into the measuring solution (960 μL of 10 μM thioflavine T in 25 mM phosphate buffer, pH 6.0) (*22*). Within 30 min after addition of thioflavine T, fluorescence was measured with a RF-1501 spectrofluorometer (Shimadzu), using an excitation filter of 435 nm and an emission filter of 482 nm, respectively. All fluorescence experiments were performed in triplicate and every measurement three times.

DNA Binding Shift Assay. Stock solution of monomeric 10 mg/mL $A\beta_{1-40}$ was diluted into phosphate-buffered saline (PBS) at a final concentration of 50 μM and incubated at room temperature for 5 days with samples at the final concentration of 0.1 mg/mL. For association reaction with nucleic acids, aliquots (38 μL) from the mixtures were incubated at 37 °C for 30 min with 60 ng of λDNA in a total volume of 40 μL (*23*). The content of DMSO did not exceed 2% (v/v). DNA samples were electrophoresed on a 1% agarose gel at 40 V for approximately 3−4 h and visualized by ethidium bromide staining. After electrophoresis, the gel was photographed and image analysis was performed using the program UNIDocMw version 99.03 for Windows (UVI Tech., Cambridge, UK).

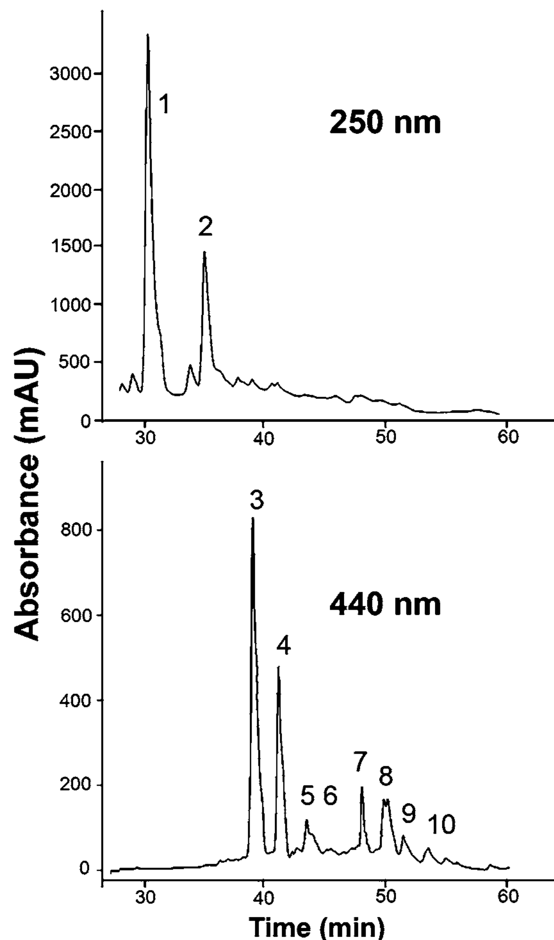

Figure 2. HPLC chromatograms of the *C. sativus* stigmas extract: (1) picrocrocin, (2) picrocrocin acid form, (3) *trans*-crocin-4, (4) *trans*-crocin-3, (5) *trans*-crocin-2′, (6) *cis*-crocin-5, (7) *cis*-crocin-4, (8) *trans*-crocin-2, (9) *cis*-crocin-3; (10) *cis*-crocin-2.

Statistical Analysis. Data are presented as means ± SE. Statistical analysis was performed with Microcal Origin 7.5. software, by applying one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Analysis of *C. sativus* Stigmas Extract. *C. sativus* stigmas extract was analyzed by HPLC according to the method of Tarantilis (*8*) and the fingerprint obtained is in complete agreement with previous reports (*8*, *17*). Ten peaks could be identified: two absorbing at 250 nm (picrocrocin and picrocrocin acid form) 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) (**Figure 2**). Quantitation confirmed that *trans*-crocin-4 is the main carotenoid constituent; *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%.

Antioxidant Properties of *C. sativus* Stigmas Extract. The antioxidant activity of *C. sativus* stigmas extract was determined by measuring in vitro the total ferric ion reducing power and the scavenging of free radicals by TEAC and compared to that of vegetables rich in carotenoids (tomatoes and carrots). Both FRAP and TEAC are end-point, single-electron-transfer based assays that do not differ greatly except that TEAC is carried out at neutral pH and FRAP under acidic conditions.

At all time points, *C. sativus* showed constantly higher ($p < 0.05$) TEAC values than tomato and carrot extracts (**Table 1**).

Table 1. TEAC Values (mmol/kg dry weight) at Different Time Points

samples	1 min	10 min	20 min	40 min
tomato	8.80 ± 0.16	13.25 ± 0.15	18.82 ± 0.21	16.18 ± 0.90
carrot	8.45 ± 0.77	13.75 ± 0.99	18.44 ± 0.80	24.88 ± 0.57
C. sativus stigmas	16.71 ± 1.84[a,b]	25.07 ± 1.67[a,b]	38.89 ± 3.03[a,b]	30.13 ± 3.03[a,b]

[a] Indicates statistically different ($p < 0.05$) values in comparison to tomatoes. [b] Indicates statistically different ($p < 0.05$) values in comparison to carrots.

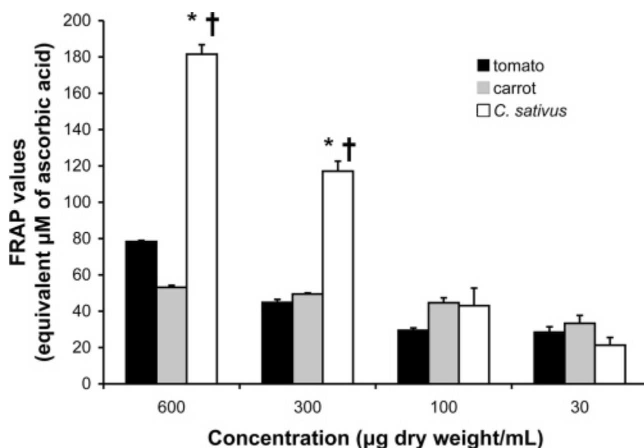

Figure 3. FRAP values of the methanol:water 50:50 (v/v) extracts of tomatoes, carrots and *C. sativus* stigmas. ∗ indicates statistically different ($p < 0.05$) values in comparison to tomatoes; † indicates statistically different ($p < 0.05$) values in comparison to carrots.

Our results show that the reaction of Trolox is essentially complete after 1 min, whereas the antioxidant activity of the vegetable extracts and saffron extract is constantly increasing within the time period of 20 min. Thus, these findings confirm earlier studies by Re (*19*), who showed that the time required for the completion of the reaction depends on the structure of the tested compounds, and therefore, the reported antioxidant activity is influenced by the selected time-point of measurement. TEAC values of *C. sativus* stigmas extract and tomato extract at 40 min are lower than those at 20 min, and this may be attributed to chemical oxidation or other transformation of their main constituents, i.e., open-chain carotenoids, which are acid- and light-labile and act as pro-oxidants when in high oxygen pressures (*24*).

The antioxidant properties of the plant extracts were also examined by FRAP, with ascorbic acid being used as a standard. The ferric ion-reducing activity of the extracts was concentration-dependent (**Figure 3**). At high concentrations (600 and 300 μg/mL) *C. sativus* stigmas extract had twice the antioxidant activity of tomato and carrot extracts, while at lower concentrations (100 and 30 μg/mL) all extracts had similar antioxidant activity. Therefore, both antioxidant assays used showed that the crude extract of *C. sativus* stigmas has good antioxidant activity, which is stronger than that of tomatoes and carrots. However, the antioxidant power of tomatoes and carrots should not be underestimated since the usual intake of these is much higher than that of saffron in humans.

Earlier study on the DPPH radical scavenging activity of the crude *C. sativus* extract, crocin-4, and safranal has shown that the antioxidant activity of the extract is attributed to the synergistic action of all phytochemicals; crocin exhibited higher antioxidant activity than safranal (*25*). Crocins can exert their effects by acting as high-efficiency free-radical scavengers, a property that appears to be closely related to their chemical structure. Treating the neuronally differentiated PC-12 cells deprived of serum/glucose with 10 μM crocin inhibited the formation of peroxidized lipids, partly restored the superoxide dismutase activity, and maintained the neuron morphology (*26*). Intraperitoneal administration of crocetin to rats for 7 days before the unilateral intrastriatal injection of 6-hydroxydopamine on day 8 protected the glutathione levels, increased the levels of antioxidant enzymes, and attenuated lipid peroxidation in substantia nigra (*16*). Therefore, the in vitro action seems to be transferred in vivo.

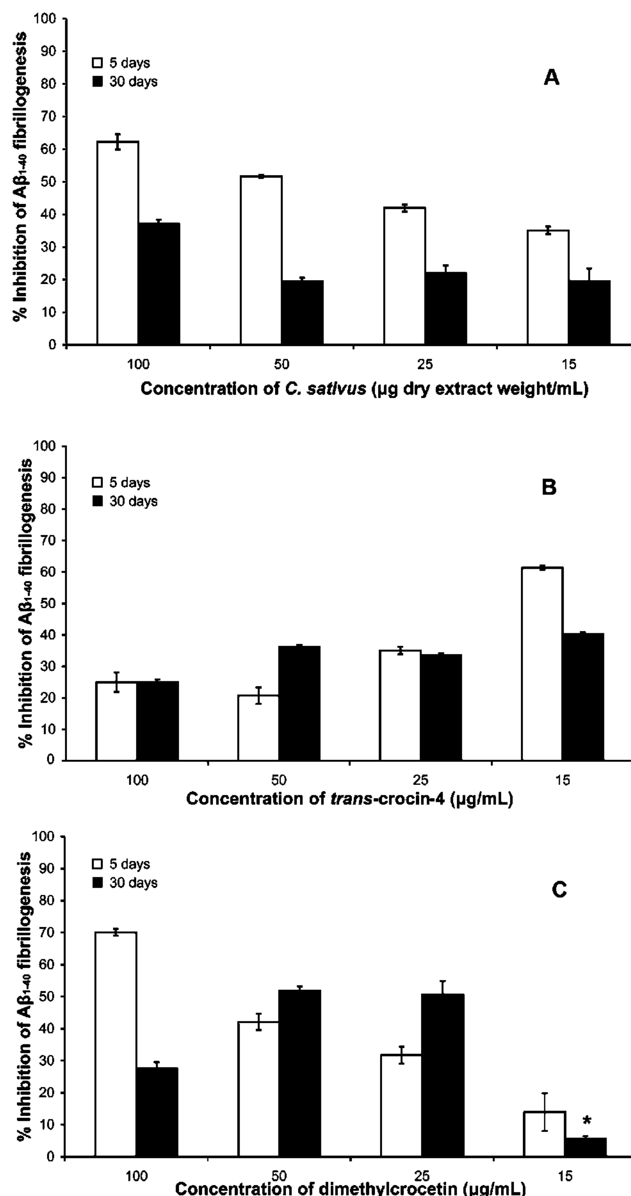

Figure 4. In vitro inhibition of Aβ_{1-40} fibrillogenesis in the presence of various concentrations of (**A**) *C. sativus* stigmas extract, (**B**) *trans*-crocin-4, and (**C**) dimethylcrocetin, as assayed with the thioflavine T fluorescence assay. The fluorescence absorbance of aged Aβ_{1-40} alone was considered as 100%. Mean ± SE, for $n = 12$. All tested phytochemicals exhibited statistically significant ($p \leq 0.05$) inhibition of Aβ_{1-40} fibrillogenesis, with the exception of dimethylcrocetin at 15 μg/mL, as indicated by *.

Effect of *C. sativus*, *trans*-Crocin-4, and Dimethylcrocetin on Fibrillogenesis of Aβ_{1-40}. β-Sheet formation and aggregation of Aβ are critical events in Alzheimer's pathology, and therefore, the possible effect of saffron extract, *trans*-crocin-4, and dimethylcrocetin on Aβ_{1-40} fibrillogenesis was examined by measuring the thioflavine T-based fluorescence of Aβ_{1-40}

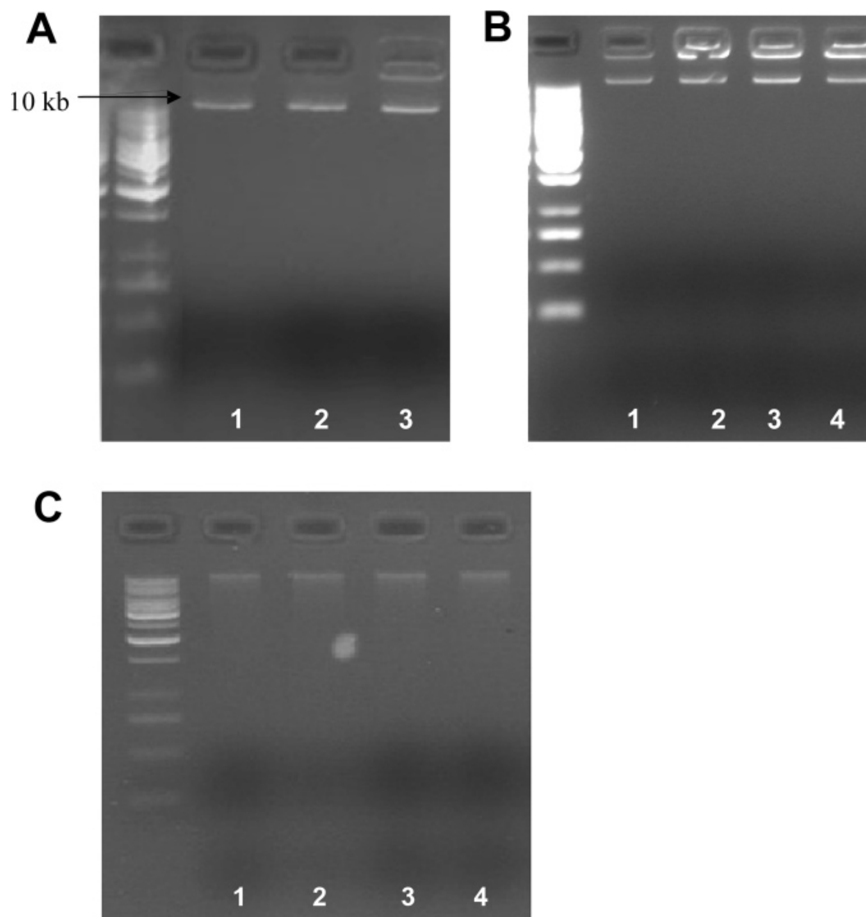

Figure 5. Effects of *C. sativus* stigmas extract, *trans*-crocin-4, and dimethylcrocetin (0.1 mg/mL each) incubated with and without Aβ_{1-40} for 5 days on the electrophoretic mobility of λDNA. (**A**) Lane 1, λDNA; lane 2, λDNA + Aβ_{1-40} freshly made; lane 3, λDNA + Aβ_{1-40} aged for 5 days. (**B**) Lane 1, λDNA + Aβ_{1-40} aged for 5 days; lane 2, λDNA + Aβ_{1-40} + saffron; lane 3, λDNA + Aβ_{1-40} + *trans*-crocin-4; lane 4, λDNA + Aβ_{1-40} + dimethylcrocetin. (**C**) Lane 1, λDNA; lane 2, λDNA + saffron; lane 3, λDNA + *trans*-crocin-4; lane 4, λDNA + dimethylcrocetin.

incubated for 5 and 30 days in the presence or absence of tested phytochemicals. The first time point was selected after 5 days, since it has been shown that the lag time until the first appearance of mature fibrils for Aβ_{1-40} is between 2 and 3 days and the plateau for ThT fluorescence is reached within 5 days (*27*).

Our results show, for the first time, that *C. sativus* stigmas extract significantly ($p < 0.05$) inhibited the formation of amyloid fibrils in a concentration- and time-dependent manner. The decrease of efficacy of saffron extract after 30 days of incubation with Aβ_1−40 (**Figure 4A**) might be attributed to oxidation or to other chemical transformation of the saffron constituents that render them less active. *trans*-Crocin-4, the major crocin constituent of stigmas, had inhibitory activity on Aβ-fibrillogenesis, which was higher (~60%) at low concentrations (15 μM) (**Figure 4B**). After 30 days of incubation of Aβ_1−40 with *trans*-crocin 4, the inhibitory activity of *trans*-crocin-4 at concentrations ranging from 15 to 50 μg/mL (15−50 μM) was constant (35−40%) and higher ($p < 0.001$) than that at 100 μg/mL. Therefore, by taking into account that *trans*-crocin-4 consists of about 10% of the crude extract (*28*) and the inhibitory values of the crude extract at 100 μg/mL (62.2 ± 2.3 and 37.1 ± 1.3%, at 5 and 30 days) and of *trans*-crocin-4 at 15 μg/mL (61.3 ± 0.7 and 40.3 ± 0.6%, at 5 and 30 days), it can be deduced that it is the main or one of the main active constituents.

In order, however, to investigate the impact of the saccharide constituents of *trans*-crocin-4 on its inhibitory activity, we also examined the inhibitory activity of dimethylcrocetin, which lacks the sugar constituents. Dimethylcrocetin had a different inhibition profile; the inhibitory activity positively correlated with its concentration after 5 days of incubation (**Figure 4C**). In contrast to *trans*-crocin-4, at the concentration of 15 μg/mL (44 μM), dimethylcrocetin had negligible effect on Aβ fibrillogenesis (~10% inhibition) after both 5 and 30 days of incubation. Similarly to *trans*-crocin-4, at the end of the long period of incubation the inhibitory activity at 100 μg/mL (291 μM) was lower ($p < 0.001$) than that at 50 and 25 μg/mL (145 and 73 μM, respectively).

The inhibitory action of *C. sativus* stigmas extract, *trans*-crocin-4, and dimethylcrocetin on β-amyloid aggregation was tested by the DNA mobility shift assay as suggested by Ahn (*23*). As shown in **Figure 5A**, incubation of λDNA with 50 μM monomeric Aβ_{1-40} did not affect the electophoretic pattern of the DNA. However, incubation with Aβ_{1-40} aged for 5 days resulted in the appearance of DNA bands that migrated slower (Aβ-associated DNA). In parallel, the intensity of the band of λDNA that was not associated with Aβ (nonassociated-DNA) was decreased by 48%. (**Figure 5A**). This confirms that DNA forms high molecular weight complexes with the aggregated Aβ_{1-40} (associated DNA). When Aβ_{1-40} was incubated for 5 days in the presence of the *C. sativus* stigmas extract, *trans*-crocin-4, and dimethylcrocetin, the intensity of the nonassociated λDNA band was significantly higher than that of aged Aβ alone (**Figure 5B**), suggesting that fewer Aβ aggregates were formed in the presence of these phytochemicals. In order to exclude any misrepresentation due to possible interactions of phy-

tochemicals with DNA, λDNA was incubated with the same concentrations of the extract and the carotenoids (**Figure 5C**), and the differences in intensity values from that of λDNA were subtracted from those of the samples. Furthermore, agarose gel electrophoresis of the extract and the carotenoids alone (without λDNA) showed no UV absorbance. Thus, in the presence of 0.1 mg/mL *C. sativus* stigmas extract, 0.1 mg/mL *trans*-crocin-4, and 0.1 mg/mL dimethylcrocetin, the intensity of nonassociated DNA was increased by 29%, 61%, and 27%, respectively, in comparison to the DNA that remained nonassociated in the presence of aged Aβ.

In agreement with Ahn (*23*), no conclusion could be reached about the associated DNA, since in contrast to the case of the nonassociated DNA, the intensity, the shape, and the number of the associated DNA bands were quite variable. Therefore, taking into account the changes in the intensity of the unassociated DNA, our results show that *C. sativus* stigmas extract and the crocins (both the di-gentibiosyl ester and the methyl ester of crocetin) inhibit Aβ aggregation; *trans*-crocin-4 produced the highest inhibition values.

Although the above results of thioflavine T-fluorescence and DNA mobility shift assay confirm the inhibitory action of *C. sativus* extract on amyloid-β aggregation and fibrillogenesis, they provide no information on the molecular basis of this phenomenon. The only assumption that can be made is that it is due to the chemical structure of crocins, i.e., the polyene backbone (seven double-bonds) and the gentibiose units attached to each end via ester bond formation. The inhibitory effect of dimethylcrocetin, which lacks the sugar units, demonstrates that the carotenoid backbone inhibits amyloid fibril formation. These results are in agreement to those of Ono (*29*) which also show that vitamin A and β-carotene dose-dependently inhibited formation and extension of Aβ fibrils, as well as destabilized preformed Aβ fibrils in the order of retinol = retinal > β-carotene > retinoic acid. However, the fact that *trans*-crocin-4 is much more effective at lower concentrations than dimethylcrocetin suggests that the sugar units contribute significantly to the antiamyloidogenic action. Earlier results (*30*) that small saccharides, like trehalose, have antiamyloidogenic activity add validity to this observation. Thus, it might be suggested that crocins have the capacity to interfere with amyloid-forming peptides either by binding to hydrophobic regions of Aβ via the hydrophobic carotene backbone, therefore inhibiting formation of fibrils (*29*, *31*); and/or by interacting with water molecules in the immediate environment of Aβ molecules via the sugar units, thus changing the entropy of the Aβ aggregation, making it less favorable; and/or by interacting with the polar ends of Aβ monomers via alcohol groups of sugars, making the polymerization more difficult. Generally, it is difficult to ascertain the mechanisms by which the secondary metabolites may afford any beneficial effect, but most of the scientists are focusing their attention either on the number of the hydroxyl groups present in each molecule (i.e., the more hydroxyl groups in a molecule, the higher the activity exerted) or their propensity to bind to specific sites of Aβ (*32*, *33*).

In conclusion, our study shows that *C. sativus* stigmas extract has antioxidant and antiamyloidogenic activity, thus reinforcing ethnopharmacological observations that *C. sativus* has a positive effect on cognitive function. The identification of *trans*-crocin-4 as one of the main active phytochemicals could be used for the development of new therapeuticals for Alzheimer's disease. Although the in vivo effectiveness of saffron and its carotenoid components remains to be investigated, our results indicate that saffron may be of value for prevention or delay of Alzheimer's disease.

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