HHS Public Access

Author manuscript

ACS Chem Neurosci. Author manuscript; available in PMC 2018 August 16.

Published in final edited form as:

ACS Chem Neurosci. 2017 August 16; 8(8): 1756–1766. doi:10.1021/acschemneuro.7b00101.

Crocus sativus Extract Tightens the Blood-Brain Barrier, Reduces Amyloid β Load and Related Toxicity in 5XFAD Mice

Yazan S. Batarseh[†], **Sonali S. Bharate**[‡], **Vikas Kumar**[‡], **Ajay Kumar**[‡], **Ram A. Vishwakarma**[‡], **Sandip B. Bharate**[‡], and **Amal Kaddoumi**[*,†]

[†]Department of Basic Pharmaceutical Sciences, School of Pharmacy, University of Louisiana at Monroe, Monroe, Louisiana 71201, United States

[‡]CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu-180001, India

Abstract

Crocus sativus , commonly known as saffron or Kesar, is used in Ayurveda and other folk medicines for various purposes as an aphrodisiac, antispasmodic, and expectorant. Previous evidence suggested that *Crocus sativus* is linked to improving cognitive function in Alzheimer's disease (AD) patients. The aim of this study was to in vitro and in vivo investigate the mechanism(s) by which *Crocus sativus* exerts its positive effect against AD. The effect of *Crocus sativus* extract on Aβ load and related toxicity was evaluated. In vitro results showed that *Crocus sativus* extract increases the tightness of a cell-based blood-brain barrier (BBB) model and enhances transport of Aβ. Further in vivo studies confirmed the effect of *Crocus sativus* extract (50 mg/kg/day, added to mice diet) on the BBB tightness and function that was associated with reduced Aβ load and related pathological changes in 5XFAD mice used as an AD model. Reduced Aβ load could be explained, at least in part, by *Crocus sativus* extract effect to enhance Aβ clearance pathways including BBB clearance, enzymatic degradation and ApoE clearance pathway. Furthermore, *Crocus sativus* extract upregulated synaptic proteins and reduced neuroinflammation associated with Aβ pathology in the brains of 5XFAD mice. Crocin, a major active constituent of *Crocus sativus* and known for its antioxidant and antiinflammatory effect, was also tested separately in vivo in 5XFAD mice. Crocin (10 mg/kg/day) was able to reduce Aβ load but to a lesser extent when compared to *Crocus sativus* extract. Collectively, findings from this

[*]Corresponding Author: kaddoumi@ulm.edu. Mailing address: Department of Basic Pharmaceutical Sciences, School of Pharmacy, University of Louisiana at Monroe, 1800 Bienville Dr., Monroe, LA 71201.

ORCID

Ram A. Vishwakarma: 0000-0002-0752-6238

Sandip B. Bharate: 0000-0001-6081-5787

Amal Kaddoumi: 0000-0001-9792-7766

Author Contributions

Extract preparation, characterization, and analysis experiments were executed by S. S. Bharate, V. Kumar, A. Kumar. R. A. Vishwakarma, and S. B. Bharate. In vitro and in vivo studies design and analyses for extract efficacy were performed by Y. S. Batarseh and A. Kaddoumi. Y. S. Batarseh, S. B. Bharate, and A. Kaddoumi wrote the manuscript.

Notes

The authors declare no competing financial interest.

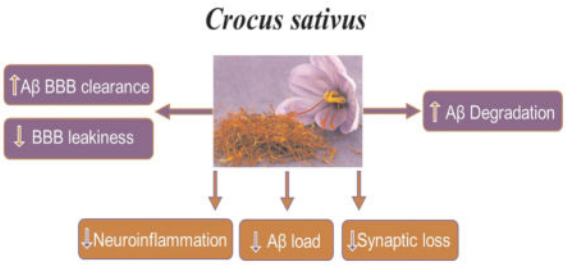
Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.7b00101.

HPLC chromatograms of hydroalcoholic extract of *Crocus sativus* stigma and crocin (obtained from stigma) (PDF)

study support the positive effect of *Crocus sativus* against AD by reducing Aβ pathological manifestations.

Graphical Abstract



Keywords

Crocus Sativus extract; crocin; amyloid-β clearance; BBB; Alzheimer's disease; neuroinflammation; 5xFAD

INTRODUCTION

Alzheimer's disease (AD) is one of the most common neurodegenerative disorders affecting elder patients.[1] AD is characterized by the presence of amyloid-β (Aβ) plaques in the brain tissue and blood vessels.[2] Aβ aggregates are assosiated with the symptomatic and pathological changes that accompany AD patients, with the majority of cases being related to insufficient Aβ clearance.[3] Aβ is a product of amyloid precursor protein (APP), which is a membrane-bound protein processed to produce Aβ_{40} and Aβ_{42}. Prior to Aβ plaques deposition, Aβ peptides form a structure called Aβ oligomers (Aβo), which significantly contribute to the pathology of AD.[3,4] Currently, only supportive treatments are available for AD which mainly include acetylcholinesterase inhibitors (donepezil, rivastigmine, and galantamine) and one glutamate receptor antagonist (memantine).[5] Although these approved AD treatments provide limited symptomatic improvement, none of them have a curative or disease modifying effect.[5] Therefore, there is a great need for research to develop AD modifying therapeutic approaches necessary to slow down AD pathology and enhance cognitive function.

In AD, blood-brain barier (BBB) dysfunction could range from minor disruption of tight junctions with increased BBB leakeness to chronic loss in integrity with altered transport of molecules across the BBB, brain hypoperfusion and inflammatory activation.[6] It has been suggested that BBB pathological changes in AD are related to the accumulation of Aβ peptides, affecting the BBB-endothelial cells integrity by reducing the expression and altering the relocalization of tight junction proteins.[7] In our laboratory, we established a high-throughput screening (HTS) assay to in vitro screen thousands of compounds for their ability to tighten an enothelial cells-based BBB model.[8] Findings from the HTS assay identified multiple hit compunds that were able to enhance the BBB model tightness. One of the identified hits was *Crocus sativus* extract.

Crocus sativus, commonly known as saffron or Kesar, is used in Ayurveda and other folk medicines for various purposes as an aphrodisiac, as an antispasmodic, and for expectorant effects.[9] Modern pharmacological studies have demonstrated that *Crocus sativus* extracts have antinociceptive, antiinflammatory,[9] antitumor,[10] radical scavenger,[11] anticonvulsant,[9] nephroprotective,[12] and neuroprotective effects.[13–15] Besides, *Crocus sativus* tested in AD patients showed to mildly enhance the patinets' cognitive function.[16,17] *Crocus sativus* contains more than 150 volatile and aroma-yielding compounds along with carotenoids including zeaxanthin, lycopene, and various α- and β-carotenes.[18] However, *Crocus sativus*'s golden yellow-orange color is primarily the result of crocins, which are considered as major constituents; whereas the picrocrocin and safranal are responsible for its bitterness and aroma, respectively. Crocetin is a aglycone portion of the crocins, which also occurs naturally in *Crocus sativus*. When crocetin is esterified with one or two water-soluble sugars, water-soluble pigments (crocins) are obtained. Crocins are esters formed from the conjugation of various sugars (glucose, gentiobiose, triglucose, and neapolitanoside) with dicarboxylic acid of crocetin. Furthermore, geometrically crocetin exists in all-*trans* and 13-*cis* forms. Thus, a total of 16 crocins are reported, all differing via a type of sugar moiety attached and all-*trans* or 13-*cis* geometry. The chemical structures of crocetin and crocetinesters (crocins) are shown in Figure 1.[10,18–20]

The aim of this study was to investigate the effect of *Crocus sativus* extract and its main active ingredient crocin on the BBB function and integrity, and on Aβ related pathology in vitro and in vivo in 5XFAD mice as a model for AD.

RESULTS AND DISCUSSION

Recent clinical studies reported the beneficial effect of *Crocus sativus* extract on the cognitive function of AD patients.[16,17] The preliminary evidence from these studies in patients with amnesic and multi domain MCI,[16] and in mild-to-moderate AD patients[17] indicates a potential therapeutic effect of *Crocus sativus* extract. The aim of the current study was to investigate the mechanism(s) by which *Crocus sativus* could provide its protective effect against AD. For this purpose, *Crocus sativus* (stigma) was extracted by EtOH: water (1:1, 3 times) to prepare the hydroalcoholic extract using a cold-maceration method. The extractive value was found to be 45%. The hydroalcoholic extract is primarily a mixture of crocins, which are crocetin glycosides. The HPLC and LC-MS analyses indicated that among the total 16 crocetin-esters (crocins) reported, *trans*-4-GG-crocin is the major crocetin-ester in the extract. The HPLC chromatogram (Figure S1, Supporting Information) showed the presence of primarily six crocins including *trans*-5-ng-crocin (t_R = 2.8 min), *trans*-4-GG-crocin (t_R = 3.8 min), *trans*-3-Gg-crocin (t_R = 5.0 min), *trans*-2-gg-crocin (t_R = 8.8 min), *cis*-4-GG-crocin (t_R = 9.7 min), and *cis*-3-Gg-crocin (t_R = 10.6 min). The *trans*-4-GG-crocin being the major crocin, it was previously isolated and characterized.[18] The % content of *trans*-4-GG-crocin in hydroalcoholic extract of *Crocus sativus* and crocin (purchased from Sigma) was determined using HPLC analysis. The hydroalcoholic extract and commercially procured crocin were found to contain 33.11 ± 3.25% (average of six different experiments) and 34.7 ± 1.41% of *trans*-4-GG-crocin. Thus, the % content of *trans*-4-GG-crocin in *Crocus sativus* (stigma) dried material was found to be 15.6%. This comparative HPLC analysis is indicative of the fact that Sigma crocin and hydroalcoholic

extract prepared by us are chemically similar. The % content of major crocin (*trans*-4-GG-crocin) is identical. Following isolation and characterization, *Crocus sativus* extract was evaluated for its biological activity in vitro and in vivo.

Cerebral levels of Aβ are regulated by the balance between its brain production and clearance.[21] Production of Aβ increases significantly in early onset familial AD where mutations in APP sequence, such as the Swedish and Florida mutations, increase Aβ production and propensity to aggregate with consequent reduction in its cerebral clearance.[22] Mounting evidence, however, suggests that Aβ accumulation in the brains of late-onset AD patients, which represent the majority of AD cases, is related to its impaired clearance from the brain.[23] Therefore, reduction in brain clearance of Aβ is considered an important component accounting for its accumulation and the development of AD. Moreover, experimental data show that soluble Aβ can cause increases in endothelial cell permeability,[24–26] that can further alter Aβ own clearance resulting in its accumulation and significant vascular and parenchymal Aβ deposition.[27,28] Available evidence, also, suggests that increased levels of cerebral Aβ impair the BBB integrity and function.[29] In AD, BBB impairment involves loss of integrity manifested by disruption of tight junction proteins, transport capacity, and brain perfusion.[6] Therefore, the development of therapeutics that enhance Aβ clearance, and BBB integrity and function could represent an important therapeutic approach to prevent or delay the onset of AD.

Results from the in vitro studies demonstrated *Crocus sativus* extract enhanced the tightness of bEnd-3 cells-based BBB model as observed by reduced permeability of LY in a concentration dependent manner in the range of 0.22–2.2 μg/mL (Figure 2A). Furthermore, at 2.2 μg/mL concentration *Crocus sativus* extract enhanced the transport of ^{125}I-Aβ_{40} from basolateral (B) to apical (A) compartment across the bEnd-3 cells by 15% (Figure 2B, $P < 0.01$). This increase in Aβ transport was associated with concentration-dependent increase in P-gp expression by 17% ($P < 0.05$) and 42% ($P < 0.01$) at 1.1 and 2.2 μg/mL, respectively (Figure 2C). However, LRP1 expression was not significantly altered by the examined concentrations.

The effect of *Crocus sativus* extract added to mice diet at 50 mg/kg/day for one month was then tested in vivo in wild-type mice. *Crocus sativus* extract enhanced the BBB clearance of microinjected ^{125}I-Aβ_{40}, measured as BEI%, by 16% (Figure 3A, $P < 0.01$), which was associated with a significant increase in Aβ major transport proteins LRP1 and P-gp[30] by 32% (Figure 3B, $P < 0.001$) and 19% (Figure 3B, $P < 0.05$), respectively, in mice brains' microvessles. When tested in vivo in an AD mouse model, findings from hippocampus immunostaining studies demonstrated a significant reduction in total Aβ and Aβo levels in the brains of 5XFAD mice fed with *Crocus sativus* extract enriched diet when compared to control mice (Figure 4); *Crocus sativus* extract consumption reduced total Aβ by 53% (Figure 4A, $P < 0.01$), and Aβo, a central player in causing neurotoxicity and initiating pathological and cognitive disturbances,[31] by 63% (Figure 4A, $P < 0.01$). While crocin consumption reduced total Aβ levels, this reduction did not reach a significant level. However, crocin significantly reduced Aβo levels by 31% (Figure 4A, $P < 0.05$). ELISA quantification also confirmed a significant reduction in the levels of monomeric Aβ_{40} and Aβ_{42} in brain homogenates from mice treated with *Crocus sativus* extract and crocin. *Crocus*

sativus extract consumption reduced Aβ_{40} by 73% (Figure 4B, $P < 0.001$) and Aβ_{42} levels by 63% (Figure 4B, $P < 0.001$). On the other hand, crocin consumption reduced Aβ_{40} by 25% (Figure 4B, $P < 0.05$) and Aβ_{42} levels by 29% (Figure 4B, $P < 0.05$).

To explain the observed reduction in brain levels of Aβ, multiple clearance mechanisms were evaluated, including clearance across the BBB, enzymatic degradation and clearance via the ApoE pathway. As shown in Figure 5A, *Crocus sativus* extract treatment significantly increased LRP1 and P-gp expressions by 19 ($P < 0.05$) and 35% ($P < 0.05$), respectively. Crocin treatment, however, did not show a significant effect on Aβ transport proteins expression (Figure 5A, $P < 0.05$). Besides increased clearance of Aβ across the BBB, both treatments significantly enhanced the expression of the Aβ degrading enzyme NEP in brain homogenates by 87% ($P < 0.001$) and 55% ($P < 0.01$) in *Crocus sativus* extract and crocin groups, respectively (Figure 5B). Neither treatment, however, had any effect on the expression of IDE levels (Figure 5B). NEP, a zinc metalloendopeptidase, has been identified as a critical Aβ-degrading enzyme.[32]

ApoE-dependent Aβ clearance is a well-established pathway known for its high efficiency in Aβ removal from the brain.[33] To investigate the effect of *Crocus sativus* extract consumption on ApoE pathway, ApoE, ABCA1 and their regulatory receptors were quantified and compared to the control group. ApoE and ABCA1 are transcriptionally regulated by the ligand-activated nuclear receptors, PPARγ and LXRs, which form obligate heterodimers with RXR.[34] The importance of ABCA1 expression in relation to Aβ clearance was confirmed in mice that do not express ABCA1 where its absence worsened Aβ deposition especially in ApoE4 expressing mice.[26] ABCA1 regulates both the level of ApoE as well as its state of lipidation via regulation of cholesterol efflux to ApoE. ApoE acts as a chaperone for Aβ by binding the peptide and altering its conformation, thereby influencing its clearance. Thus, increased levels of ABCA1 is expected to enhance ApoE lipidation, increase apoE/Aβ interaction and thus Aβ clearance.[35] Our findings here demonstrated that while ApoE brain levels were not altered (Figure 5C), *Crocus sativus* extract significantly increased the expressions of ABCA1 and PPARγ by 94% (Figure 5C, $P < 0.01$) and 26% (Figure 5C, $P < 0.05$), respectively, in 5XFAD brains, which could provide an additional mechanism that contributes to the decreased brain levels of Aβ. Crocin, on the other hand only increased ABCA1 expression by 61% (Figure 5C, $P < 0.05$). Collectively, these results suggest that *Crocus sativus* extract could exert its protective effect against Aβ pathology by reducing Aβ brain load, including toxic oligomers, in the brains of 5XFAD mice, which could explain the higher expression of Aβ transport proteins, degradation enzymes and ABCA1. Further studies are necessary to clarify whether *Crocus sativus* extract directly increased the proteins expression or indirectly reduced the deficiency of these proteins as a downstream effect to reduced Aβ load.

Besides its effect on Aβ clearance, the effect of *Crocus sativus* extract on the BBB tightness was also investigated. Integrity of the BBB is of utmost importance in maintaining the homeostasis of the brain. Available evidence suggests that in AD and aging brains have leaky BBB manifested by increased endogenous IgG extravasation to the brain.[36,37] The exact causes for BBB dysfunction in AD are not well-known, however impaired clearance of Aβ from the brain across the BBB as well as Aβ's role in mediating increases in endothelial

cell permeability,[25] have been proposed to enhance accumulation of cerebrovascular and parenchymal amyloid deposits.[38] Immunostaining results demonstrated that *Crocus sativus* extract significantly reduced IgG extravasation by 47% in 5XFAD mice brains (Figure 6A, $P < 0.001$), which was associated with increased expression of the tight junction protein cloudin-5 in brain microvessles by 38% (Figure 6B, $P < 0.05$). Crocin treatment, however, did not show a significant effect on BBB tightness except for inducing the expression of capillary cloudin-5 by 17% (Figure 6B, $P < 0.05$).

Treatment with *Crocus sativus* extract increased synaptic markers, and reduced inflammation. Aβ pathology is associated with loss in synaptic proteins such as GLT1 and the synaptic markers PSD-95 and SNAP-25. Both PSD-95, and SNAP-25 dysregulation may play important role in the pathological cascade of events caused by Aβ since their expression and function play a critical role in protein assembly, synaptic development and neural plasticity.[39] While *Crocus sativus* extract at the dose used did not alter the expression of GLT1 (Figure 7), it induced the expression of both PSD-95 and SNAP-25 by 36% ($P < 0.01$) and 26% ($P < 0.001$), respectively (Figure 7). Such effect could be observed as an indirect effect of the treatment on Aβ reduced levels, or by acting directly. Further studies are required to investigate this effect. On the other hand, crocin treatment only increased SNAP-25 by 15% ($P < 0.01$), but has no significant effect on GLT1 and PSD-95 expressions (Figure 7).

Prolonged astrocytes activation has damaging effects on neurons by promoting neuroinflammation characterized by secretion of cytokines and neurotoxic inflammatory mediators.[40,41] Reducing astrocytes reactivity has been identified previously as protective therapeutic approach.[42] Numerous studies demonstrated *Crocus sativus* antioxidant and anti-inflammatory effects.[43,44] Similarly, our results revealed that mice treatment with *Crocus sativus* extract significantly reduced brain levels of IL-1β by 22% as measured by ELISA (Figure 8A, $P < 0.01$), and astrocytes activation as determined by reduced GFAP optical intensity by 41% as measured by immunostaining (Figure 8B, $P < 0.01$), which provide an additional mechanism that could explain the beneficial effect observed. Crocin, however, has no effect on brain levels of IL-1β or GFAP optical density (Figure 8).

Available evidence showed crocin to improve induced-memory deficit in animal models,[9,45–47] and possesses antioxidant and anti-inflammatory properties,[47] which could partially contributed to the effect of *Crocus sativus* extract observed in this study, and therefore against AD pathology. Collective findings demonstrated that at the evaluated dose of 10 mg/kg/day crocin treatment was able to reduce Aβ load, however, to a much lesser extent compared to *Crocus sativus* extract. This lesser effect was also associated with insignificant or mild alteration on BBB tightness and other related Aβ pathology markers when compared to control. The reduced levels of Aβ load could be explained by the increased expression of ABCA1 and the degrading enzyme NAP. Further studies to evaluate different dosage regimens (dose and treatment time) are required to investigate its protective effect against AD. Yet, the effect observed with *Crocus sativus* extract could be the result of an additive or synergistic effect of crocin with other available components in the extract such as crocetin.

Aβ accumulation, especially Aβ oligomers, represents one of the upstream events in the pathology of AD.[48] Thus, reducing Aβ load may have contributed to the multiple protective mechanisms observed. Pathological mechanisms leading to synaptic loss and inflammation could directly be related to Aβ increased levels which results in aberrant neuronal signaling and cell death.[48] The activation of inflammatory pathways, such as NF-Kβ,[49] could augment inflammatory responses associated with Aβ pathology by increasing inflammatory markers such as ceramide and cytokines,[50] which activate apoptotic pathways and further enhance Aβ toxicity on neurons and BBB.[51] Whether *Crocus sativus* extract provided its positive effect against Aβ pathology by targeting such pathways, or others, are yet to be evaluated.

In conclusion, findings from this study showed that *Crocus sativus* extract consumption significantly reduced the total Aβ levels in 5XFAD brains hippocampi and decreased Aβ brain deposits. The reduction in Aβ levels could be explained, at least in part, by the enhanced Aβ clearance across the BBB via the up-regulation of P-gp and LRP1, upregulation of Aβ degrading enzyme NEP, and up-regulation of the ApoE-clearance pathway. In addition, *Crocus sativus* extract enhanced the tightness of the BBB limiting entry of unwanted large molecules, and demonstrated an anti-inflammatory effect where it reduced astrocytes activation and IL-1β brain levels. Collectively, these multiple mechanisms could explain, in part, the positive *Crocus sativus* reported in AD patients, and thus could provide a therapeutic option against AD.

METHODS

Preparation of Hydroalcoholic Extract of *Crocus sativus*

The authentic plant material of *Crocus sativus* (stigma) was purchased from local market of Srinagar (Jammu and Kashmir State, India). The plant material was taxonomically characterized, and a voucher specimen was deposited in the Janaki Ammal Herbarium at the CSIR-IIIM, Jammu. The crocin (CAS No. 42553-65-1) was procured from Sigma-Aldrich (St. Louis, MO).

Dried material (100 g) of *Crocus sativus* was extracted three times with water/ethanol (1:1) mixture. All three hydroalcoholic extracts were combined, and ethanol was evaporated from the mixture by vacuum assisted evaporation (temperature of 40 °C, vacuum of 250 mm/Hg) until the mass corresponded to approximately 50% of the mass of the mixture obtained. Concentrated mass was further dried on a rotary evaporator to get a dry powder extract (45.0 g). The extractive value on dry weight basis is found to be 45.0% (40–50%) w/w. The extract was standardized for the content of *trans*-4-GG-crocin (which is one of the major crocins) using HPLC. Similarly, crocin was analyzed for *trans*-4-GG-crocin content using same method. HPLC analysis was performed on the Shimadzu HPLC system connected to a PDA detector, and C8 (Intersil, 25 cm × 4.6 mm, 5 μ) column. Mobile phase consisted of acetonitrile (A) and 0.1% formic acid in water (B). The gradient system comprised 70% B (0.01 min), 70% B (2 min), 30% B (7 min), 30% B (15 min), 70% B (20 min), 70% B (25 min) at a flow rate of 1 mL/min.

Cell Culture

The immortalized mouse brain endothelial cell line, bEnd3, was obtained from ATCC (Manassas, VA) and used as a representative model for the BBB endothelium. bEnd3 cells, passage 25–35, were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin G (100 IU/mL, streptomycin (100 g/mL), 1% w/v nonessential amino acids, and glutamine 2 mM. Cultures were maintained in a humidified atmosphere (5% CO_2/95% air) at 37°C and media was changed every other day.

Lucifer Yellow Permeability Assay

On the day of the experiment, the barrier tightness of bEnd3 cells grown on inserts was evaluated by measuring Lucifer yellow (LY) permeation across the monolayer. LY is a small, water-soluble molecule that travels across bEnd3 cells, after achieving a monolayer only through passive paracellular diffusion.[52] Therefore, any changes in the tightness of bEnd3 monolayer will affect the permeation of LY. LY permeation study was performed as described previously.[8] At 24 h after treatment initiation, LY (100 μM) was loaded onto the apical side of 96-transwell filter. One hour later, the fluorescence intensity of LY was determined in apical and basolateral sides at excitation and emission wavelengths of 485 and 529 nm, respectively, using a Synergy 2 microplate reader (Biotek, Winooski, VT). Apparent permeation coefficient (Pc) was calculated as described previously.[8]

Amyloid-β Transport across bEnd3 Cell Based BBB Model

bEnd3 cellscultured on 24 well inserts were treated for 48 h with 2.2 μg/mL of *Crocus sativus* extract or vehicle. Aβ transport was performed as described previously.[53] Briefly, treatments were removed and replaced with fresh media for 4 h, as a washout period. The basolateral to apical transport (B → A) was initiated by the addition of media containing 0.1 nM ^{125}I-Aβ_{40} and ^{14}C-inulin (0.1 μCi/mL) to B side (lower chamber) and media alone to A side (upper chamber) for 30 min. Aliquots from both A and B sides were taken for radioactivity analysis using Wallac1470 Wizard Gamma and Wallac 1414 WinSpectral liquid scintillation counters (PerkinElmer Inc. Waltham, MA).

Animals

C57BL/6 (wild-type mice; Envigo, Houston, TX) and 5XFAD mice (Jackson laboratory, Bar Harbor, ME) were housed in plastic containers under the conditions of 12 h light/dark cycle, 22 °C, 35% relative humidity, and ad libitum access to water and food. 5XFAD mutations include: APP KM670/671NL (Swedish), APP I716V (Florida), APP V717I (London), PSEN1M146L, PSEN1 L286V, leading to early and aggressive Aβ accumulation associated with inflammatory astrocytes activation and cognitive decline.[54] All animal experiments and procedures were approved by the Institutional Animal Care and Use Committee of the University of Louisiana at Monroe and according to the National Institutes of Health guidelines.

Animals' Treatment

The effect of *Crocus sativus* extract on the clearance of Aβ across the BBB of C57BL/6 wild-type mice (male, 8 weeks old) was evaluated using the brain efflux index (BEI)

method.[53] The mice were divided into two groups, control group fed with regular diet (Teklad Laboratory diets, Harlan Laboratories, Madison, WI), and treatment group fed with *Crocus sativus* extract-enriched diet (50 mg/kg/day; $n = 6$/group) for one month. To evaluate the effect of *Crocus sativus* extract and its active compound crocin on the BBB and Aβ pathology, 5XFAD mice were divided into three groups ($n = 6$ mice/group, male mice); control group, group 2 was fed with *Crocus sativus* extract-enriched diet (50 mg/kg/day), and group 3 was fed with crocin-enriched diet (10 mg/kg/day). All treatments were started at the age of 4 months for 1 month, ending the experiment at the age of 5 months. Diet was changed every other day to maintain freshness. During the treatment period with all groups, animals body weights were measured every 2 weeks and health status and normal behavior were checked daily. Mice body weights were not significantly different between the treatment groups compared to the control group, ranging for from 27.5 ± 3.1 to 28.5 ± 2.3 g, respectively. At the end of treatment period, mice brains were extracted for immunochemical and biochemical analyses.

Brain Clearance of ^{125}I-Aβ_{40}

In vivo Aβ_{40} clearance was investigated using the BEI method in C57BL/6 wild-type mice as described previously.[53] Animals were anesthetized followed by the insertion of a stainless steel guide cannula into the right caudate nucleus of mice brains. A tracer fluid (0.5 μL) containing ^{125}I-Aβ^{40} (30 nM, PerkinElmer, MA) and ^{14}C-inulin (0.02 mCi, American Radiolabeled Chemicals, St. Louis, MO) prepared in extracellular fluid buffer (ECF) was microinjected in the mice brains. Thirty minutes later, brains were rapidly collected for ^{125}I-Aβ_{40} analysis and microvessels isolation as described below. Calculations of ^{125}I-Aβ_{40} clearance were performed as described previously.[53] ^{125}I-Aβ_{40} and ^{14}C-inulin radioactivity were determined in brain tissues using a Wallac beta and gamma counter. ^{125}I-Aβ_{40} BEI% was determined as described previously.[53]

Brain Microvessels Isolation

Brain microvessels were isolated from the brains of C57BL/6 and 5XFAD mice as described previously.[55] Brains were homogenized in ice-cold DPBS followed by the addition of one volume of 30% Ficoll 400 (Sigma-Aldrich). Homogenates were centrifuged at 8000g for 10 min and the resulting pellets were suspended in ice-cold DPBS containing 1% BSA and passed over a glass beads column to collect microvessels adhering to the glass beads. Isolated microvessels were used to determine P-glycoprotein (P-gp), lipoprotein receptor-related protein-1 (LRP1) and cloudin-5 expressions by Western blot.

Immunohistochemical Analyses

All cryostat brain slices (15 μm) were acetone-fixed then blocked for 1 h with 10% normal donkey serum in phosphate buffer saline (PBS). For total Aβ detection, brain tissue sections were immunostained with Alexa-fluor 488-labeled 6E10 human-specific anti-Aβ antibody at 1:200 dilution (BioLegend, San Diego, CA). For Aβo detection, brain tissue sections were immunostained with FITC-labeled antiamyloid oligomers antibody at 1:100 dilution (Abcam, Cambridge, MA).

To assess IgG extravasation from brain microvessels, hippocampal brain sections were fixed and blocked, as described above, then probed by dual immunohistochemical staining for collagen-IV and mouse IgG using rabbit anti-collagen-IV (Millipore, Temecula, CA) and fluorescein-conjugated donkey anti-IgG (Santa Cruz Biotechnology, Dallas, TX), respectively, both at 1:200 dilution. The secondary antibody used for collagen-IV was CFL594-conjugated donkey anti-rabbit IgG (Santa Cruz Biotechnology).

To evaluate the assosiation of reactive astrocytes with Aβ accumulation, hippocampal brain sections were fixed and prepared as above. Double immunostaining was performed using glial fibrillary acidic protein (GFAP; Santa Cruz Biotechnology) for astrocytes at 1:100 dilution and with Alexa-fluor 488 labeled 6E10 for total Aβ at 1:200 dilution. All images were captured using Nikon Eclipse Ti-S inverted fluorescence microscope (Melville, NY). Quantification of total Aβ load in the hippocampus was performed using ImageJ version 1.44 software after adjusting for threshold (National Institutes of Health, Bethesda, MD).

Western Blot Analysis

For the in vitro studies, bEnd3 cells were seeded in 10 mm cell culture dishes (Corning, NY) at a density of 1×10^6 cells per dish. When reached 50% confluency, cells were treated with *Crocus sativus* extract (1.1 and 2.2 μg/mL) or vehicle (0.1% DMSO) for 48 h in a humidified atmosphere (5% CO_2/95% air) at 37 °C. At the end of treatment period, cells were harvested and total protein was extracted for Western blot analysis. An amount of 25 μg of cellular protein was resolved on 7.5% SDS polyacrylamide gels and transferred onto nitrocellulose membrane. Membranes were blocked with 2% BSA and incubated overnight with antibodies for LRP1 (light chain, Abcam), P-gp (C-219, BioLegend), and actin (Santa Cruz Biotechnology). For proteins detection, the membranes were washed and incubated with HRP-labeled secondary IgG antibody for LRP1 (anti-rabbit), P-gp (anti-mouse), and β-actin (anti-goat) all from Santa Cruz Biotechnology.

For the in vivo studies, protein extracts were prepared from brain microvessels or brain tissues homogenate as reported previously.[55] Proteins analyzed were P-gp (C-219), LRP1, synaptic markers (PSD-95 and SNAP-25; GeneTex, CA), insulin degrading enzyme (IDE), neprilysin (NEP), ABCA1, apolipoprotein E (ApoE), liver-X receptor (LXR), retinoid-X receptor (RXR), peroxisome-proliferator-activated receptor-γ (PPARγ), GLT1, and GAPDH (Santa Cruz Biotechnology). For detection, the membranes were washed free of primary antibodies and incubated with HRP-labeled secondary IgG anti-mouse antibody for P-gp, ABCA1, PSD-95, and GAPDH; anti-rabbit antibody for LRP1, NEP, PPARγ, GLT1, and SNAP-25; and anti-goat antibody for ApoE and IDE. All secondary antibodies were from Santa Cruz Biotechnology.

The bands were visualized using a Pierce chemiluminescence detection kit (ThermoScientific). Quantitative analysis of the immunoreactive bands was performed using Li-Core luminescent image analyzer (LI-COR Biotechnology, Lincoln, NE), and band intensity was measured by densitometric analysis. Three independent Western blotting experiments were carried out for each treatment group.

Aβ ELISA

For detection of soluble $A\beta_{40}$ and $A\beta_{42}$, 5XFAD mice brain tissues were homogenized in RIPA buffer containing 1× protease inhibitors, and run on anti-human $A\beta_{40}$ and $A\beta_{42}$ ELISA kits according to the manufacturer instructions (Thermo Scientific). All samples were run at least in triplicates and corrected to the total protein amount in each sample using BCA assay.

Interleukin-1β (IL-1β) ELISA

For detection of IL1-β levels in brain homogenate, anti-mouse IL-1β Quantikine ELISA kit (R&D Systems, Minneapolis, MN) was used according to the manufacturer instructions. All samples were run at least in triplicate and corrected to the total protein amount in each sample.

Statistical Analysis

All data were expressed as mean ± SEM. Data was statistically analyzed using one way ANOVA followed with posthoc analysis using Dunnett's test, or by Student's *t* test. A *P*-value less than 0.05 was considered statistically significant. All statistical analyses were done using GraphPad Prism, version 5.03.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding

This work was supported by Louisiana Board of Reagent's Research Competitive Program under Grant Number LEQSF-(2013–16)-RD-A-16, and by National Institute of Neurological Disorders and Stroke under Grant Number R15NS091934 (A. Kaddoumi).

References

1. Citron M. Alzheimer's disease: strategies for disease modification. Nat Rev Drug Discovery. 2010; 9:387–398. [PubMed: 20431570]
2. Selkoe DJ. Alzheimer's disease: genes, proteins, and therapy. Physiol Rev. 2001; 81:741–766. [PubMed: 11274343]
3. Hardy J. The amyloid hypothesis for Alzheimer's disease: A Critical Reappraisal. J Neurochem. 2009; 110:1129–1134. [PubMed: 19457065]
4. Lehman EJ, Kulnane LS, Lamb BT. Alterations in beta-amyloid production and deposition in brain regions of two transgenic models. Neurobiol Aging. 2003; 24:645–653. [PubMed: 12885572]
5. Casey DA, Antimisiaris D, O'Brien J. Drugs for Alzheimer's disease: are they effective? Pharm Ther. 2010; 35:208–211.
6. Zlokovic BV. The blood-brain barrier in health and chronic neurodegenerative disorders. Neuron. 2008; 57:178–201. [PubMed: 18215617]
7. Gonzalez-Velasquez FJ, Kotarek JA, Moss MA. Soluble aggregates of the amyloid-beta protein selectively stimulate permeability in human brain microvascular endothelial monolayers. J Neurochem. 2008; 107:466–477. [PubMed: 18702666]
8. Qosa H, Mohamed LA, Al Rihani SB, Batarseh YS, Duong QV, Keller JN, Kaddoumi A. High-Throughput Screening for Identification of Blood-Brain Barrier Integrity Enhancers: A Drug

Repurposing Opportunity to Rectify Vascular Amyloid Toxicity. J Alzheimer's Dis. 2016; 53:1499–1516. [PubMed: 27392852]

9. Khazdair MR, Boskabady MH, Hosseini M, Rezaee R, AMT. The effects of Crocus sativus (saffron) and its constituents on nervous system: A Review. Avicenna J Phytomed. 2015; 5:376–391. [PubMed: 26468457]
10. Escribano J, Alonso GL, Coca-Prados M, Fernandez JA. Crocin, safranal and picrocrocin from saffron (Crocus sativus L.) inhibit the growth of human cancer cells in vitro. Cancer Lett. 1996; 100:23–30. [PubMed: 8620447]
11. Abdullaev FI. Biological effects of saffron. Bio Factors. 1993; 4:83–86.
12. Hosseinzadeh H, Sadeghnia HR, Ziaee T, Danaee A. Protective effect of aqueous saffron extract (Crocus sativus L.) and crocin, its active constituent, on renal ischemia-reperfusion-induced oxidative damage in rats. J Pharm Pharm Sci. 2005; 8:387–393. [PubMed: 16401388]
13. Abe K, Sugiura M, Yamaguchi S, Shoyama Y, Saito H. Saffron extract prevents acetaldehyde-induced inhibition of long-term potentiation in the rat dentate gyrus in vivo. Brain Res. 1999; 851:287–289. [PubMed: 10642859]
14. Zhang Y, Shoyama Y, Sugiura M, Saito H. Effects of Crocus sativus L. on the ethanol-induced impairment of passive avoidance performances in mice. Biol Pharm Bull. 1994; 17:217–221. [PubMed: 8205119]
15. Papandreou MA, Kanakis CD, Polissiou MG, Efthimiopoulos S, Cordopatis P, Margarity M, Lamari FN. Inhibitory activity on amyloid-beta aggregation and antioxidant properties of Crocus sativus stigmas extract and its crocin constituents. J Agric Food Chem. 2006; 54:8762–8768. [PubMed: 17090119]
16. Tsolaki M, Karathanasi E, Lazarou I, Dovas K, Verykouki E, Karakostas A, Georgiadis K, Tsolaki A, Adam K, Kompatsiaris I, Sinakos Z. Efficacy and Safety of Crocus sativus L. in Patients with Mild Cognitive Impairment: One Year Single-Blind Randomized, with Parallel Groups, Clinical Trial. J Alzheimer's Dis. 2016; 54:129–133. [PubMed: 27472878]
17. Akhondzadeh S, Shafiee Sabet M, Harirchian MH, Togha M, Cheraghmakani H, Razeghi S, Hejazi SS, Yousefi MH, Alimardani R, Jamshidi A, Rezazadeh SA, Yousefi A, Zare F, Moradi A, Vossoughi A. A 22-week, multicenter, randomized, double-blind controlled trial of Crocus sativus in the treatment of mild-to-moderate Alzheimer's disease. Psychopharmacology. 2010; 207:637–643. [PubMed: 19838862]
18. Srivastava R, Ahmed H, Dixit RK, Dharamveer, Saraf SA. Crocus sativus L.: A comprehensive review. Pharmacogn Rev. 2010; 4:200–208. [PubMed: 22228962]
19. Tarantilis PA, Tsoupras G, Polissiou M. Determination of saffron (Crocus sativus L.) components in crude plant extract using high-performance liquid chromatography-UV-visible photodiode-array detection-mass spectrometry. J Chromatogr A. 1995; 699:107–118. [PubMed: 7757208]
20. Carmona M, Zalacain A, Sanchez AM, Novella JL, Alonso GL. Crocetin esters, picrocrocin and its related compounds present in Crocus sativus stigmas and Gardenia jasminoides fruits. Tentative identification of seven new compounds by LC-ESI-MS. J Agric Food Chem. 2006; 54:973–979. [PubMed: 16448211]
21. Mawuenyega KG, Sigurdson W, Ovod V, Munsell L, Kasten T, Morris JC, Yarasheski KE, Bateman RJ. Decreased clearance of CNS beta-amyloid in Alzheimer's disease. Science. 2010; 330:1774. [PubMed: 21148344]
22. Mullan M, Crawford F, Axelman K, Houlden H, Lilius L, Winblad B, Lannfelt L. A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of beta-amyloid. Nat Genet. 1992; 1:345–347. [PubMed: 1302033]
23. Bell RD, Zlokovic BV. Neurovascular mechanisms and blood-brain barrier disorder in Alzheimer's disease. Acta Neuropathol. 2009; 118:103–113. [PubMed: 19319544]
24. Tai LM, Holloway KA, Male DK, Loughlin AJ, Romero IA. Amyloid-beta-induced occludin down-regulation and increased permeability in human brain endothelial cells is mediated by MAPK activation. J Cell Mol Med. 2010; 14:1101–1112. [PubMed: 19438816]
25. Pop V, Sorensen DW, Kamper JE, Ajao DO, Murphy MP, Head E, Hartman RE, Badaut J. Early brain injury alters the blood-brain barrier phenotype in parallel with beta-amyloid and cognitive changes in adulthood. J Cereb Blood Flow Metab. 2013; 33:205–214. [PubMed: 23149553]

26. Qosa H, Abuasal BS, Romero IA, Weksler B, Couraud PO, Keller JN, Kaddoumi A. Differences in amyloid-beta clearance across mouse and human blood-brain barrier models: kinetic analysis and mechanistic modeling. Neuropharmacology. 2014; 79:668–678. [PubMed: 24467845]
27. Weller RO, Boche D, Nicoll JA. Microvasculature changes and cerebral amyloid angiopathy in Alzheimer's disease and their potential impact on therapy. Acta Neuropathol. 2009; 118:87–102. [PubMed: 19234858]
28. Cirrito JR, Deane R, Fagan AM, Spinner ML, Parsadanian M, Finn MB, Jiang H, Prior JL, Sagare A, Bales KR, Paul SM, Zlokovic BV, Piwnica-Worms D, Holtzman DM. P-glycoprotein deficiency at the blood-brain barrier increases amyloid-beta deposition in an Alzheimer disease mouse model. J Clin Invest. 2005; 115:3285–3290. [PubMed: 16239972]
29. Attems J, Yamaguchi H, Saido TC, Thal DR. Capillary CAA and perivascular Abeta-deposition: two distinct features of Alzheimer's disease pathology. J Neurol Sci. 2010; 299:155–162. [PubMed: 20850138]
30. Qosa H, Abuznait AH, Hill RA, Kaddoumi A. Enhanced brain amyloid-beta clearance by rifampicin and caffeine as a possible protective mechanism against Alzheimer's disease. J Alzheimer's Dis. 2012; 31:151–165. [PubMed: 22504320]
31. Li S, Hong S, Shepardson NE, Walsh DM, Shankar GM, Selkoe D. Soluble oligomers of amyloid Beta protein facilitate hippocampal long-term depression by disrupting neuronal glutamate uptake. Neuron. 2009; 62:788–801. [PubMed: 19555648]
32. Iwata N, Tsubuki S, Takaki Y, Watanabe K, Sekiguchi M, Hosoki E, Kawashima-Morishima M, Lee HJ, Hama E, Sekine-Aizawa Y, Saido TC. Identification of the major Abeta1–42-degrading catabolic pathway in brain parenchyma: suppression leads to biochemical and pathological deposition. Nat Med. 2000; 6:143–150. [PubMed: 10655101]
33. Kline A. Apolipoprotein E, amyloid-ss clearance and therapeutic opportunities in Alzheimer's disease. Alzheimer's Res Ther. 2012; 4:32. [PubMed: 22929359]
34. Chawla A, Boisvert WA, Lee CH, Laffitte BA, Barak Y, Joseph SB, Liao D, Nagy L, Edwards PA, Curtiss LK, Evans RM, Tontonoz P. A PPAR gamma-LXR-ABCA1 pathway in macrophages is involved in cholesterol efflux and atherogenesis. Mol Cell. 2001; 7:161–171. [PubMed: 11172721]
35. Wahrle SE, Jiang H, Parsadanian M, Legleiter J, Han X, Fryer JD, Kowalewski T, Holtzman DM. ABCA1 is required for normal central nervous system ApoE levels and for lipidation of astrocyte-secreted apoE. J Biol Chem. 2004; 279:40987–40993. [PubMed: 15269217]
36. Lee P, Kim J, Williams R, Sandhir R, Gregory E, Brooks WM, Berman NE. Effects of aging on blood brain barrier and matrix metalloproteases following controlled cortical impact in mice. Exp Neurol. 2012; 234:50–61. [PubMed: 22201549]
37. Takechi R, Pallebage-Gamarallage MM, Lam V, Giles C, Mamo JC. Aging-related changes in blood-brain barrier integrity and the effect of dietary fat. Neurodegener Dis. 2013; 12:125–135. [PubMed: 23128303]
38. Cordonnier C, van der Flier WM. Brain microbleeds and Alzheimer's disease: innocent observation or key player? Brain. 2011; 134:335–344. [PubMed: 21257651]
39. Tu S, Okamoto S, Lipton SA, Xu H. Oligomeric Abeta-induced synaptic dysfunction in Alzheimer's disease. Mol Neurodegener. 2014; 9:48. [PubMed: 25394486]
40. Heppner FL, Ransohoff RM, Becher B. Immune attack: the role of inflammation in Alzheimer disease. Nat Rev Neurosci. 2015; 16:358–372. [PubMed: 25991443]
41. Tong L, Balazs R, Soiampornkul R, Thangnipon W, Cotman CW. Interleukin-1 beta impairs brain derived neurotrophic factor-induced signal transduction. Neurobiol Aging. 2008; 29:1380–1393. [PubMed: 17467122]
42. Fuller S, Steele M, Munch G. Activated astroglia during chronic inflammation in Alzheimer's disease–do they neglect their neurosupportive roles? Mutat Res, Fundam Mol Mech Mutagen. 2010; 690:40–49.
43. Cicerale S, Lucas LJ, Keast RS. Antimicrobial, antioxidant and anti-inflammatory phenolic activities in extra virgin olive oil. Curr Opin Biotechnol. 2012; 23:129–135. [PubMed: 22000808]
44. Bogani P, Galli C, Villa M, Visioli F. Postprandial anti-inflammatory and antioxidant effects of extra virgin olive oil. Atherosclerosis. 2007; 190:181–186. [PubMed: 16488419]

45. Asadi F, Jamshidi AH, Khodagholi F, Yans A, Azimi L, Faizi M, Vali L, Abdollahi M, Ghahremani MH, Sharifzadeh M. Reversal effects of crocin on amyloid beta-induced memory deficit: Modification of autophagy or apoptosis markers. Pharmacol, Biochem Behav. 2015; 139:47–58. [PubMed: 26484504]

46. Naghizadeh B, Mansouri MT, Ghorbanzadeh B, Farbood Y, Sarkaki A. Protective effects of oral crocin against intracerebroventricular streptozotocin-induced spatial memory deficit and oxidative stress in rats. Phytomedicine. 2013; 20:537–542. [PubMed: 23351962]

47. Finley JW, Gao S. A perspective on Crocus sativus L (Saffron). Constituent Crocin: a potent water soluble antioxidant and potential therapy for Alzheimer's Disease. J Agric Food Chem. 2017; 65:1005–1020. [PubMed: 28098452]

48. Kayed R, Lasagna-Reeves CA. Molecular mechanisms of amyloid oligomers toxicity. J Alzheimer's Dis. 2013; 33(Suppl 1):S67–78. [PubMed: 22531422]

49. Bonaiuto C, McDonald PP, Rossi F, Cassatella MA. Activation of nuclear factor-kappa B by beta-amyloid peptides and interferon-gamma in murine microglia. J Neuroimmunol. 1997; 77:51–56. [PubMed: 9209268]

50. Jazvinscak Jembrek M, Hof PR, Simic G. Ceramides in Alzheimer's Disease: Key Mediators of Neuronal Apoptosis Induced by Oxidative Stress and Abeta Accumulation. Oxid Med Cell Longevity. 2015; 2015:346783.

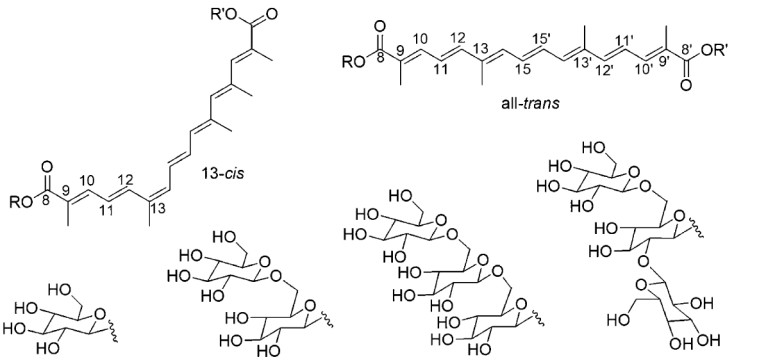
51. Erickson MA, Banks WA. Blood-brain barrier dysfunction as a cause and consequence of Alzheimer's disease. J Cereb Blood Flow Metab. 2013; 33:1500–1513. [PubMed: 23921899]

52. Omidi Y, Campbell L, Barar J, Connell D, Akhtar S, Gumbleton M. Evaluation of the immortalised mouse brain capillary endothelial cell line, b. End3, as an in vitro blood-brain barrier model for drug uptake and transport studies. Brain Res. 2003; 990:95–112. [PubMed: 14568334]

53. Qosa H, Batarseh YS, Mohyeldin MM, El Sayed KA, Keller JN, Kaddoumi A. Oleocanthal enhances amyloid-beta clearance from the brains of TgSwDI mice and in vitro across a human blood-brain barrier model. ACS Chem Neurosci. 2015; 6:1849–1859. [PubMed: 26348065]

54. Oakley H, Cole SL, Logan S, Maus E, Shao P, Craft J, Guillozet-Bongaarts A, Ohno M, Disterhoft J, Van Eldik L, Berry R, Vassar R. Intraneuronal beta-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: potential factors in amyloid plaque formation. J Neurosci. 2006; 26:10129–10140. [PubMed: 17021169]

55. Qosa H, Mohamed LA, Batarseh YS, Alqahtani S, Ibrahim B, LeVine H 3rd, Keller JN, Kaddoumi A. Extra-virgin olive oil attenuates amyloid-beta and tau pathologies in the brains of TgSwDI mice. J Nutr Biochem. 2015; 26:1479–1490. [PubMed: 26344778]



Crocin/ crocetin	isomer	sugar name (R/R')	no. of glucose	mass
trans-5-tG-crocin	*trans*	trig/gen	5	1138.4
cis-5-tG-crocin	*cis*			
trans-5-nG-crocin	*trans*	nea/gen		
cis-5-nG-crocin	*cis*			
trans-4-tG-crocin	*trans*	nea/glu	4	976.4
cis-4-nG-crocin	*cis*			
trans-4-GG-crocin	*trans*	gen/gen		
cis-4-GG-crocin	*cis*			
trans-3-Gg-crocin	*trans*	gen/glu	3	814.3
cis-3-Gg-crocin	*cis*			
trans-2-G-crocin	*trans*	gen/H	2	652.3
cis-2-G-crocin	*cis*			
trans-2-gg-crocin	*trans*	glu/glu		
cis-2-gg-crocin	*cis*			
trans-1-g-crocin	*trans*	glu/H	1	490.2
cis-1-g-crocin	*cis*			
trans-crocetin	*trans*	H/H	-	328.2
cis-crocetin	*cis*	H/H	-	328.2

Figure 1.

Chemical structures of crocins (in literature, varying nomenclature has been given to different crocins; therefore for better clarity we have shown the name of isomer followed by number of sugars it contain and name of sugars for each crocin. e.g. *trans*-5-tG-crocin is the *trans*crocetin ester comprising tri-β-D-glucoside (t) and β-D-gentiobioside (G) sugars).

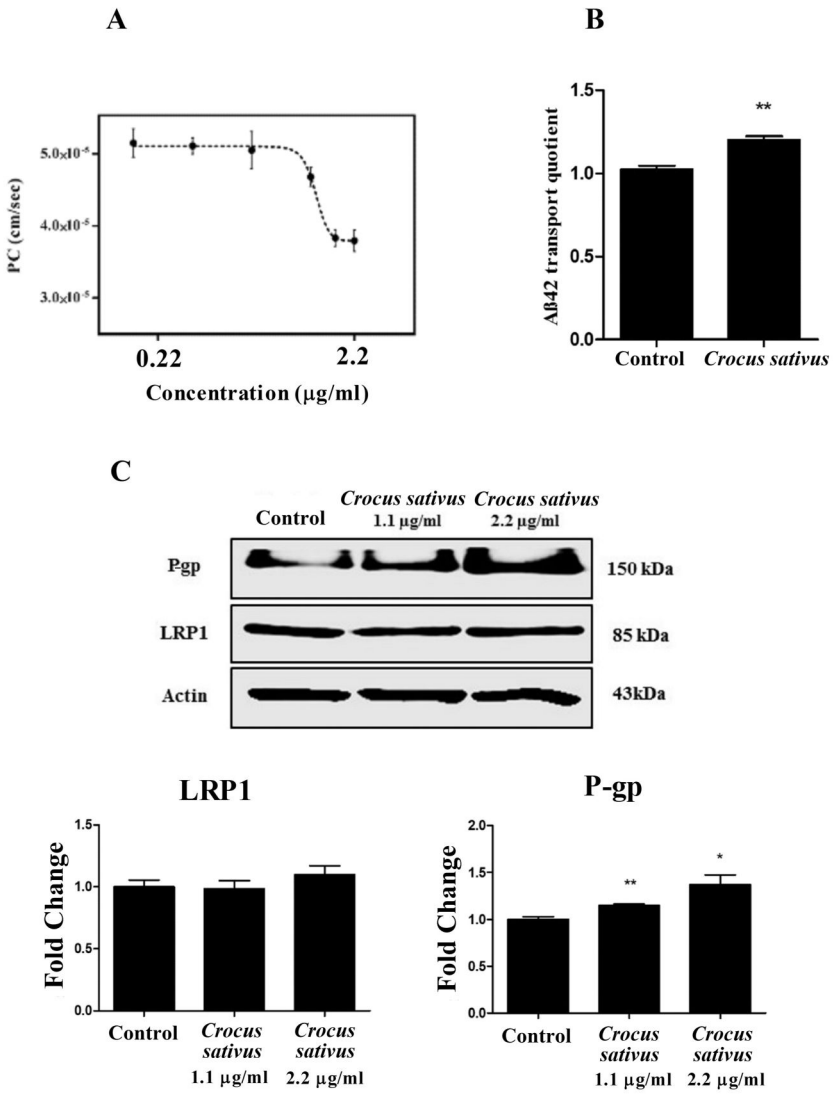


Figure 2.

Crocus sativus extract increased BBB tightness and induced Aβ transport in vitro. (A) Concentration-dependent decrease in LY permeability (PC) across the bEnd-3 cells-based BBB model. (B) *Crocus sativus* extract (2.2 μg/mL) increased Aβ transport quotient from basolateral to apical compartment of the in vitro BBB model. (C) Representative blots and densitometry analysis of LRP1 and P-gp in bEnd-3 cells treated with *Crocus sativus* extract. Data are presented as mean ± SD of three independent experiments (*$P < 0.05$, **$P < 0.01$ compared to control group).

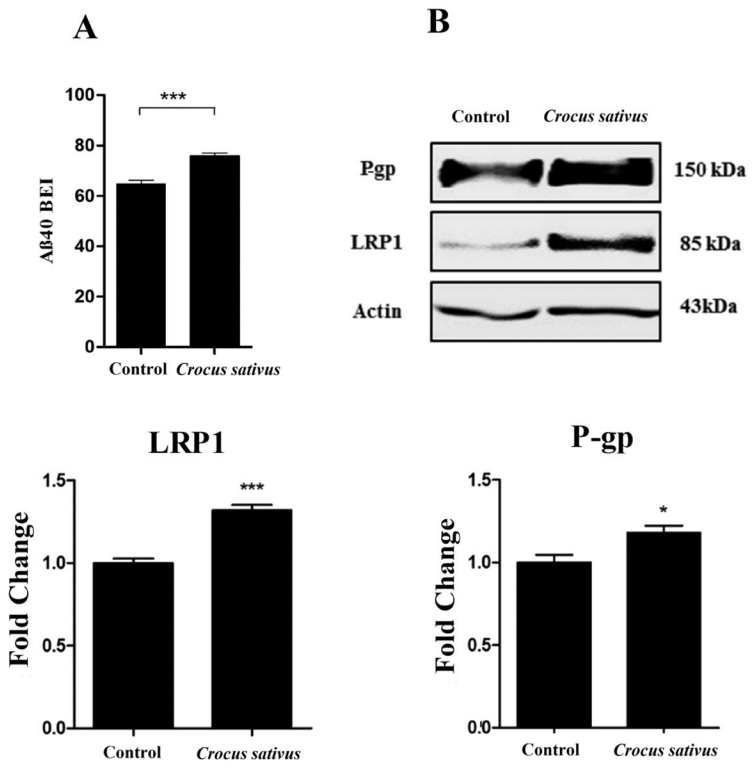


Figure 3.

Crocus sativus extract (50 mg/kg/day, one month treatment) enhanced ^{125}I-Aβ_{40} clearance across the BBB of the brains of wild-type mice. (A) Increased BEI% of exogenously adminstered ^{125}I-Aβ_{40} from the brains of wild-type mice. (B) Representative blots and densitometry analysis of LRP1 and P-gp in microvesicles isolated from of wild-type mice brains treated with *Crocus sativus* extract. Data are presented as mean ± SEM of six mice in each group (*$P < 0.05$, ***$P < 0.001$ compared to control group).

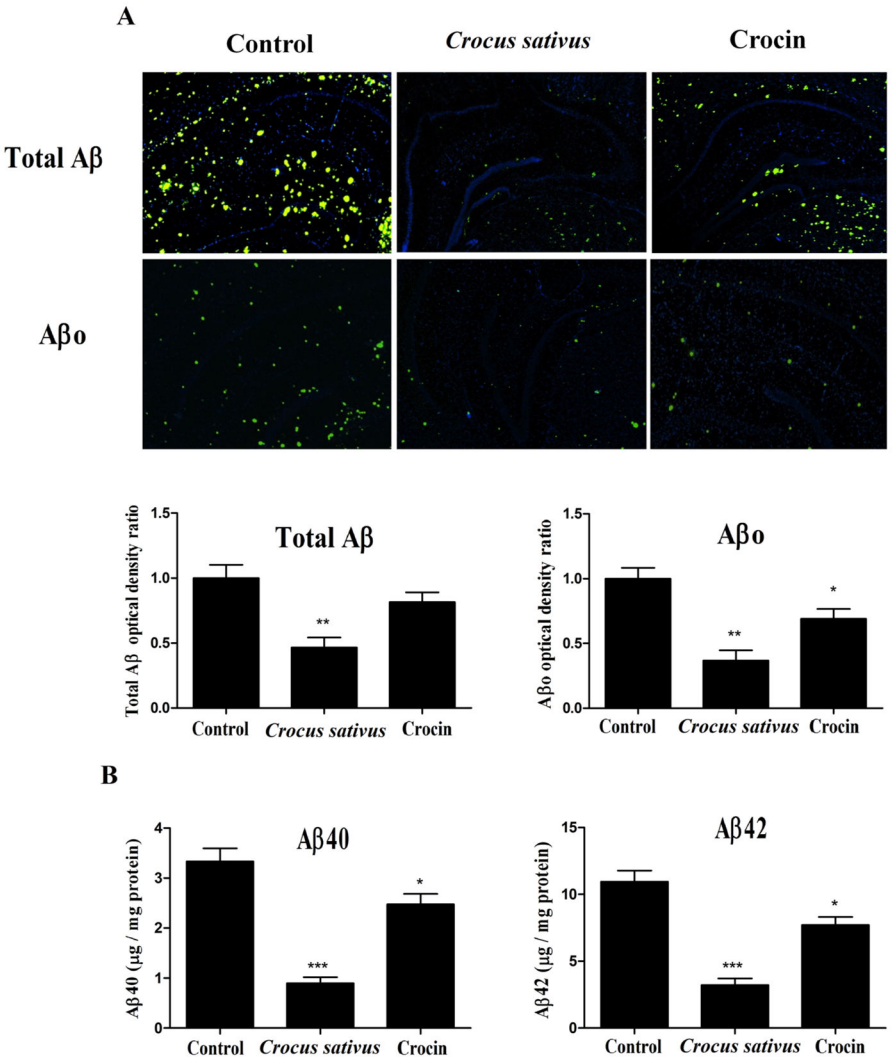


Figure 4.

Crocus sativus extract (50 mg/kg/day, one-month treatment) and to a lesser extent crocin (10 mg/kg/day, one month treatment) consumption significantly reduced Aβ burden in the hippocampus of 5XFAD mice. (A) Representative hippocampus sections and optical density quantification in 5XFAD mice from control, treated with *Crocus sativus* extract, and crocin; stained with 6E10 antibody against Aβ to detect total Aβ load (green) and Anti-Aβo antibody to detect Aβo load (green). DAPI (blue) was used to stain nuclei. (B) Soluble Aβ levels in the brains of 5XFAD mice from control, treated with *Crocus sativus* extract, and crocin. Data are presented as mean ± SEM of six mice in each group (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to control group).

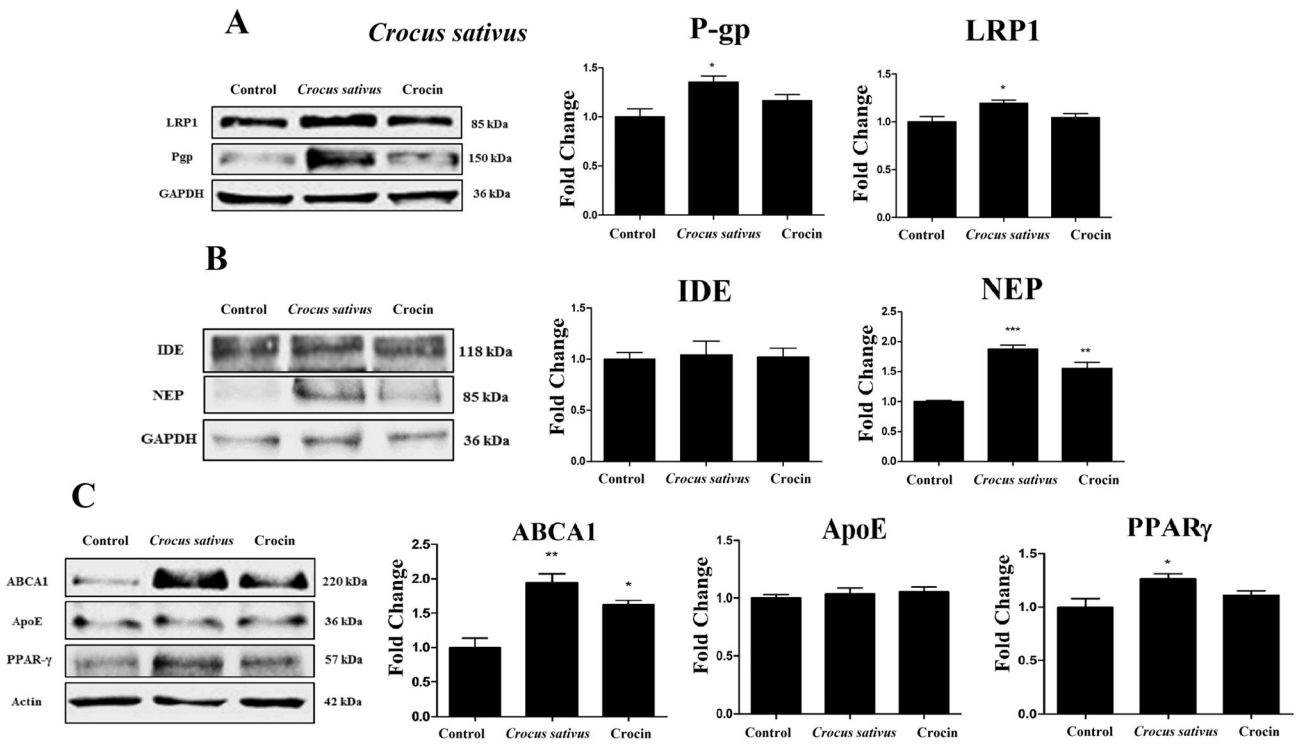


Figure 5.

Effect of *Crocus sativus* extract (50 mg/kg/day, one month treatment) and crocin (10 mg/kg/day, one month treatment) consumption on the expression of Aβ major transport proteins, Aβ degrading enzymes, and ApoE clearance pathway proteins in the brains of 5XFAD mice. (A) Representative blots and densitometry analysis of LRP1 and P-gp in microvessels isolated from 5XFAD mice brains. (B) Representative blots and densitometry analysis of IDE and NEP in brain homogenates. (C) Representative blots and densitometry analysis of ABCA1, ApoE and PPAR-γ in brain homogenates. Data are presented as mean ± SEM of six mice in each group (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to control group).

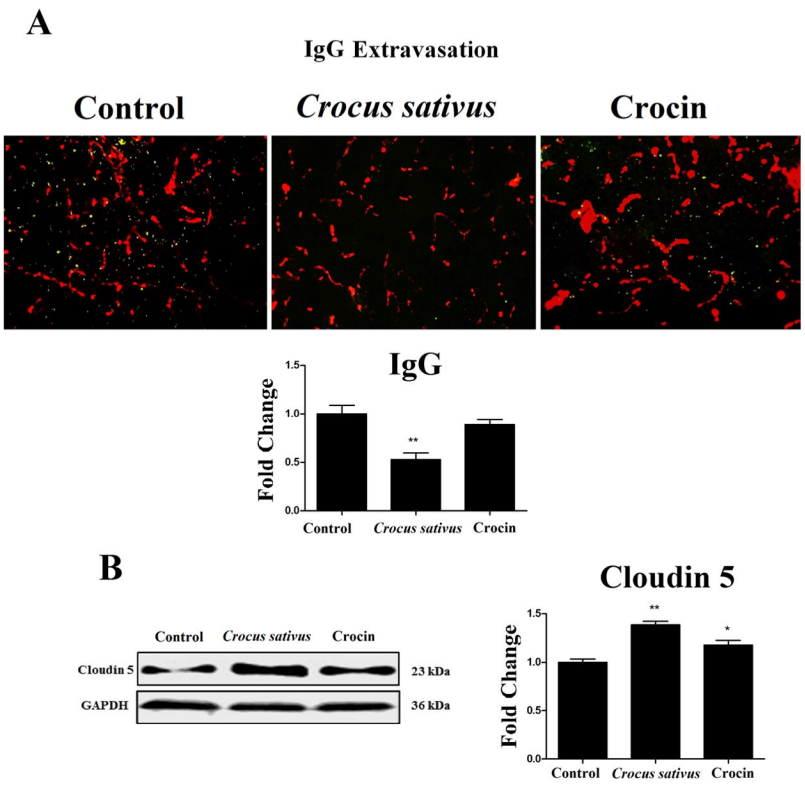


Figure 6.

Effect of *Crocus sativus* extract (50 mg/kg/day, one-month treatment) and crocin (10 mg/kg/day, one month-treatment) consumption on BBB tightness. (A) Representative brain sections stained with anti-IgG antibody to detect IgG extravasation (green) and anticollagen antibody (red) to detect microvessels, and their optical density quantitation. (B) Representative blots and densitometry analysis of cloudin-5 in microvessels isolated from 5XFAD mice brains. Data are presented as mean ± SEM of six mice in each group (*$P < 0.05$, **$P < 0.01$ compared to control group).

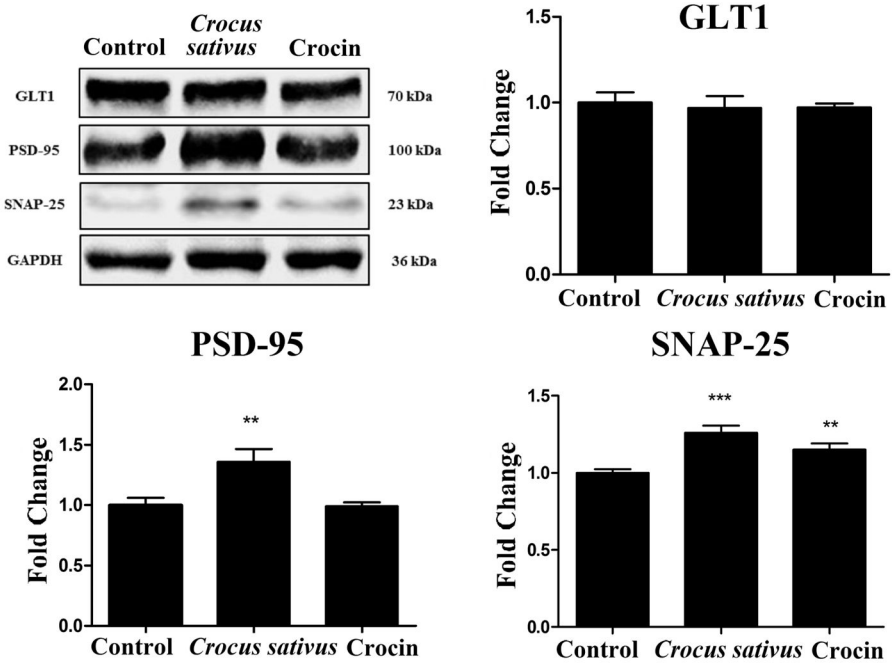


Figure 7.

Crocus sativus extract (50 mg/kg/day, one-month treatment) consumption significantly up-regulated neuro-synaptic proteins PSD-95 and SNAP-25 in the brains of 5XFAD mice. Representative blots and densitometry analysis of GLT1, PSD-95 and SNAP-25 in mice brains homogenates from control, mice treated with *Crocus sativus* extract and crocin (10 mg/kg/day, one month treatment). Data are presented as mean ± SEM of six mice in each group (** $P < 0.01$, *** $P < 0.001$ compared to control group).

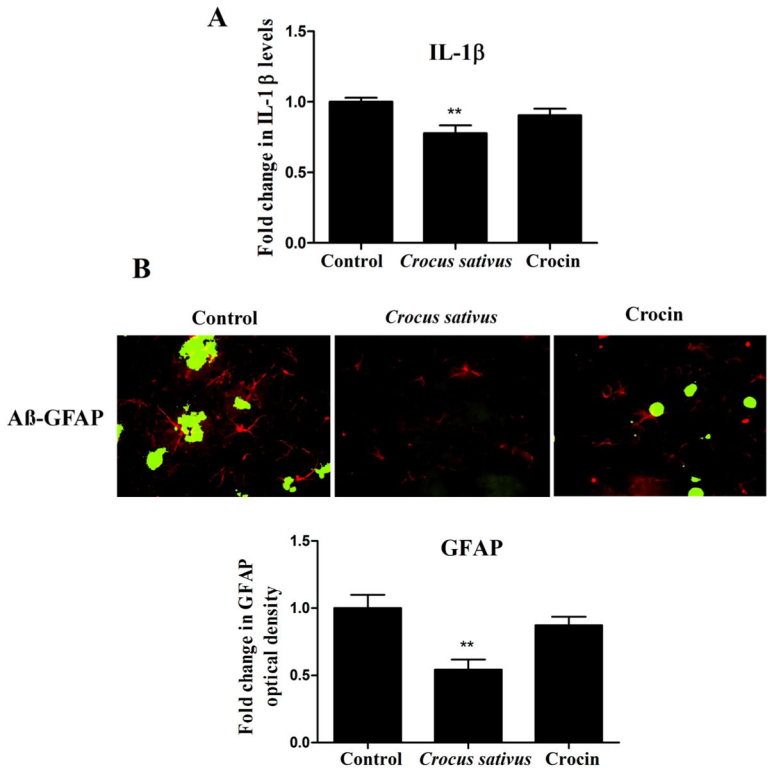


Figure 8.

Crocus sativus extract (50 mg/kg/day, one-month treatment) consumption significantly reduced neuroinflammation in 5XFAD mice. Neuroinflammation was assessed by measuring IL-1β levels, and astrocytes activation monitored by GFAP intensity and astrocytes morphology. (A) IL-1β levels in mice brains homogenates from control, mice treated with *Crocus sativus* extract and crocin (10 mg/kg/day, one-month treatment). (B) Representative brain sections stained with Anti-GFAP antibody(red) to detect activated astrocytes and 6E10 antibody (green) to detect Aβ in control, mice treated with *Crocus sativus* extract and crocin, with GFAP optical density quantification. Data are presented as mean ± SEM of six mice in each group (**$P < 0.01$ compared to control group).