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Effects of Saffron (*Crocus sativus* L.) and its Active Constituent, Crocin, on Recognition and Spatial Memory after Chronic Cerebral Hypoperfusion in Rats

Hossein Hosseinzadeh,[1] Hamid Reza Sadeghnia,[2] Fatemeh Abbasi Ghaeni,[1] Vahideh Sadat Motamedshariaty[1] and Seyed Ahmad Mohajeri[1]*

[1]Pharmaceutical Research Center, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran
[2]Department of Pharmacology, Department of New Sciences and Technology, Neuroscience Research Center, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

Cerebral ischemia produces brain damage and related behavioral deficits such as memory. In this study, a rat model of chronic cerebral hypoperfusion was used to determine whether saffron extract and crocin, which are potent antioxidants and free radical scavengers, can reduce vascular cognitive impairment. Male adult Wistar rats were administered different doses of an aqueous solution of crocin or hydroalcohol extract of saffron intraperitoneally (i.p.) 5 days after permanent occlusion of the common carotid arteries. Spatial learning and memory were assessed in training trials, 7–11 days after common carotid artery ligation using the Morris water maze. The results showed that the escape latency time was significantly reduced from 24.64 s in the control group to 8.77 and 10.47 s by crocin (25 mg/kg) and saffron extract (250 mg/kg). The traveled distance to find the platform was also changed from 772 cm in the control group to 251 and 294 cm in the crocin (25 mg/kg) and saffron extract (250 mg/kg) groups. The percentages of time spent in the target quadrant, in comparison with the control group (24.16%), increased to 34.25% in the crocin (25 mg/kg) and 34.85% in the saffron extract (250 mg/kg) group. This study suggests that saffron extract and crocin improve spatial cognitive abilities following chronic cerebral hypoperfusion and that these effects may be related to the antioxidant effects of these compounds.

Keywords: Crocus sativus; crocin; memory; chronic cerebral hypoperfusion; antioxidant.

INTRODUCTION

Vascular dementia is widely considered one of the most important and common forms of dementia after Alzheimer's disease (AD) and some studies indicate that the incidence and prevalence of the disease increases exponentially after the age of 65 (Murray *et al*., 2007). Vascular dementia is caused by a cerebrovascular disease that generally occurs in the elderly. People with vascular dementia commonly experience a decline in thought processes (cognitive impairment), caused by cerebrovascular diseases with pathological features of ubiquitous cerebral arteriosclerosis and infarction (He *et al*., 2008). Due to the increasing number of elderly people in the world, dementia, which is known by the progressive loss of memory and cortical functions, has given rise to enormous socioeconomic problems (Liao *et al*., 2004). It has been proved that vascular dementia is related to ischemia, hypoxia or hemorrhagic damage to specific corresponding regions involved in cognition and memory (Decarli, 2004).

Little is known about the role of antioxidants in the pathogenesis of vascular dementia and its associated neuronal damage. Increasing the free radical formation, together with reducing the antioxidant defense, may cause neuronal injury. A low concentration of antioxidants may influence the development of vascular dementia. In a study by Ryglewicz *et al*., low levels of plasma alpha-tocopherol were observed in patients with vascular dementia indicating a reduced antioxidant defense in these subjects (Ryglewicz *et al*., 2002).

Saffron is the dried stigmas of *Crocus sativus* and one of the most expensive spices. It could be used as a drug, textile dye and culinary adjunct (Mohajeri *et al*., 2010). Chemical analysis of its stigmas has indicated the presence of crocin (Fig. 1) as a water-soluble carotenoid, monoterpene aldehyde and its glucoside (safranal and picrocrocin) and flavonoids (quercetin and kaempferol) (Pitsikas *et al*., 2007). Saffron is commonly cultivated in Iran, Spain, India, Switzerland, Italy and other countries (Hadizadeh *et al*., 2010). The saffron and its colored carotenoid (crocin) have anticonvulsant (Hosseinzadeh and Talebzadeh, 2005; Hosseinzadeh and Sadeghnia, 2007), antioxidant (Hosseinzadeh and Sadeghnia, 2005; Hosseinzadeh *et al*., 2005b; Ochiai *et al*., 2007; Hosseinzadeh *et al*., 2009; Goyal *et al*., 2010; Mousavi *et al*., 2010), antitumor (Molnar *et al*., 2000) and antidepressant (Akhondzadeh *et al*., 2004; Hosseinzadeh *et al*., 2004; Moshiri *et al*., 2006) effects. It also has memory improving properties (Pitsikas *et al*., 2007). Zhang *et al*. indicated that saffron and its colored components reduced ethanol-induced

* Correspondence to: Assistant Professor Seyed Ahmad Mohajeri, Pharmaceutical Research Center, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran.
E-mail: mohajeria@mums.ac.ir

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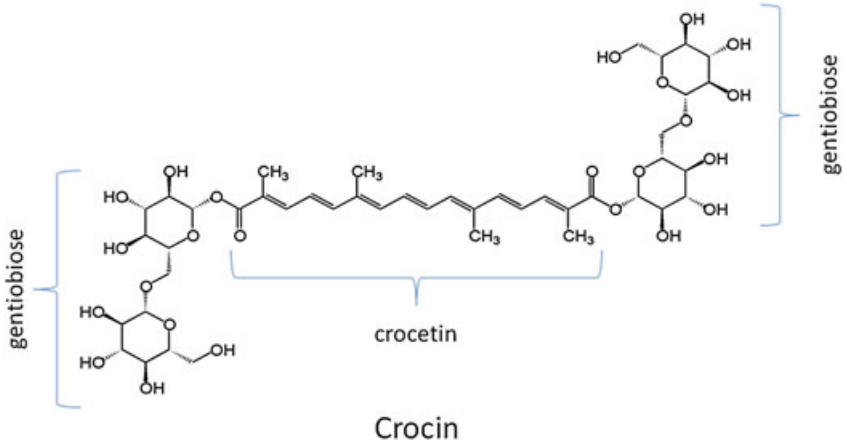

Figure 1. Chemical structure of crocin.

memory impairment in the passive avoidance test in mice (Zhang *et al*., 1994). Saffron extract counteracted recognition memory deficits and antagonized scopolamine-induced performance impairments in the passive avoidance task in the rat (Pitsikas and Sakellaridis, 2006). In a study by Pitsikas *et al*., the efficiency of crocin to counteract scopolamine-induced detrimental effects on spatial memory was reported (Pitsikas *et al*., 2007). In this study, the vascular dementia model was produced by permanent bilateral ligation of the common carotid arteries, which can induce hypoperfusion and spatial memory impairment (Guang and Du, 2006; He *et al*., 2008). Our aim was to determine whether saffron or its main constituents, crocin, as antioxidants have beneficial effects in the cerebral ischemia model in rats.

MATERIALS AND METHODS

Chemicals. The total saffron extract (Saharkhiz Saffron Co.) and crocin were isolated from the red dried stigmas (saffron) of *Crocus sativus*, as described previously (Hadizadeh *et al*., 2010). Briefly, saffron stigma powder (10 g) was suspended in 25 mL ethanol 80% at 0°C and shaken by vortex for 2 min. After centrifugation at 4000 rpm for 10 min, the supernatant was separated. Then 25 mL of 80% ethanol was added to the sediment and the extraction was repeated again. This step was repeated six more times. The total volume of solvent used for 10 g saffron stigmas in the extraction process was 200 mL (8 × 25 mL). For preparation of the total hydroalcohol extract of saffron, the resulting solution was dried in a rotary evaporator system in darkness at 35°C. For preparation of crocin (Hadizadeh *et al*., 2010), the resulting solution was kept in a thick walled glass container at −5°C for 24 days in darkness. The container was sealed during this period. The obtained crystals were separated from the solution and washed with acetone to remove the remaining water. The purity of the crocin crystals was tested with HPLC and was more than 97%. The total amount of crocin in the saffron extract was determined as 10–15%. The saffron extract and crocin were dissolved in normal saline (0.9%) before injection. Xylazine and ketamine were obtained from Loughrea, Co. (Galway, Ireland) and Rotexmedica GmbH (Germany), respectively.

Animals. Male Wistar rats weighing 200–230 g were obtained from the animal facilities of the Pharmaceutical Research Center, BuAli Research Institute of Mashhad University of Medical Sciences. The animals were housed five per cage with a 12/12 h light/dark cycle at 21 ± 2°C and had free access to food and water. About 70 rats were divided into eight groups: (1) sham-operated animals underwent the same surgical procedure without ligation of the common carotid arteries (n=14); (2) control group received 0.9% saline solution (n=14); (3), (4) and (5) saffron groups received saffron extract (50, 100 and 250 mg/kg/day, n=7); (6), (7) and (8) crocin groups received crocin (5, 10, 25 mg/kg/day, n=7). Handling and experimental procedures for all animals were in accordance with the Mashhad University of Medical Sciences Ethics Committee Acts.

Surgery and experimental procedure. The rats were anesthetized with a mixture of ketamine (60 mg/kg) and xylazine (6 mg/kg) (i.p.). The surgical technique for the induction of cerebral ischemia was adapted from the earlier published method of Xu *et al*. (2010), with some modifications. In the ischemic rats, the left common carotid artery was exposed through a midline neck incision and double ligated with 4–0 type surgical silk. After 3 days, the right common carotid artery was ligated in the same way. The sham-operated rats received the same two-step operation procedure except the ligatures. During the surgery, their body temperature was monitored and maintained at 37.5 ± 0.5°C by means of a heating lamp. The animals received drugs or vehicle intraperitoneally 1 h after the second step of operation and the same dosage every day for 5 days.

Behavioral assessment. The spatial memory performances were evaluated using a Morris water maze 7 days after induction of hypoperfusion. The water maze was a black circular tank 136 cm in diameter and 60 cm in height. The tank was filled with water (20 ± 1°C) to a depth of 35 cm. The maze was located in a room containing extra-maze cues. The walls of the pool and the platform were dyed black to conceal the platform. The maze was divided geographically into four quadrants (northeast (NE), northwest (NW), southeast (SE), southwest (SW)) and starting positions (north (N), south (S), east (E), west (W)) that were equally spaced around the perimeter of the pool. A hidden platform (diameter: 10 cm)

was located in the SE quadrant (target quadrant), 1cm below the surface of the water. A video camera was mounted directly above the water maze to record the rats' swim paths. A tracking system was used to measure the escape latency time, traveled distance and swimming speed of each rat, and also the percentage of time in the target quadrant.

The rats were given four training trials each day on 5 consecutive days. For each training trial, the rats were placed in the water facing the pool wall at one of the four starting positions (north, south, east or west pole) in a different order each day and allowed to swim until they reached the platform located in target quadrant of the maze in every trial. The latency to reach the platform was recorded for up to 60s. They remained on the platform for 20s before being removed. The experimenter guided any rat that had failed to reach the platform within 60s to it for 20s and the maximum latency was scored. One final test trial with the platform removed was conducted 24h after the last training trial to assess the memory of the correct platform location. After the trials, the rat was dried with a towel and placed in a holding cage under a heating lamp before it was returned to the home cage (Nakagawa and Takashima, 1997; Hosseinzadeh *et al*., 2005a).

Statistical analysis. Data are expressed as mean±SEM. The traveled distance, swimming speed of each rat and the percentage of the time in the target quadrant were assessed by one-way analysis of variance (ANOVA) followed by the Tukey's post hoc test. Data obtained from the latency time tests were analysed by two-way ANOVA. A value of $p<0.05$ was considered statistically significant.

RESULTS

The mean latency time in finding the hidden platform decreased during the training period in all groups. The control group took longer to find the platform than the sham-operated rats. This prolongation of latency was shortened by the total saffron extract and crocin. This effect was dose dependent in the saffron extract and crocin groups (Fig. 2a and b). In the final test trial, the swimming time in the target quadrant was used to evaluate the spatial memory performance. The sham-operated group and the saffron and crocin-treated groups swam longer in the target quadrant than the control group (Fig. 3a and b). In the final test trial, the percentage of time traveled in the target quadrant in the sham-operated group was 35.66%, in the control group was 24.16%, in the saffron extract (50, 100 and 250mg/kg) groups this was increased to 25.18%, 32.84% and 34.85% and the data for crocin (5, 10, 25mg/kg) groups were 30.20%, 33.26% and 34.25%. But only the saffron 250mg/kg and crocin 10 and 25mg/kg groups were statistically different from the control group ($p<0.05$). Figure 4a and b represent the traveled distance to reach the hidden platform on day 5. The data indicated that in comparison with the control group, the traveled distance to find the platform decreased in the saffron (100 and 250mg/kg) and crocin groups (at all doses) and these results were dose dependent in the treated groups. Figure 5 shows the speed of the animals in each group. The results indicated that no difference was observed between the groups in 5days.

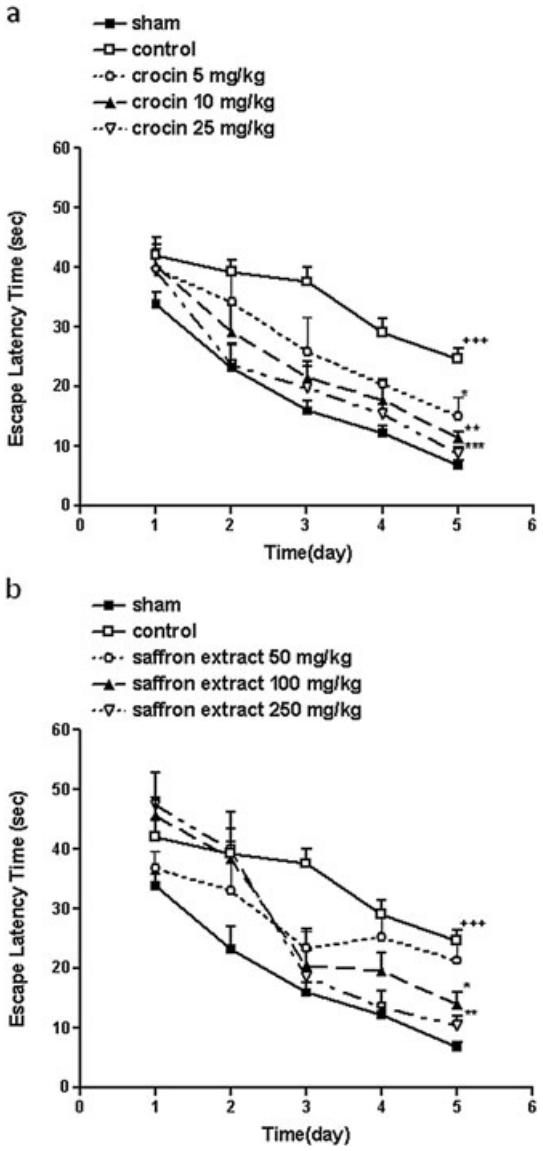

Figure 2. Escape latency time (mean±SEM) to reach hidden platform by crocin (a) and saffron extract (b) treated rats in the Morris water maze. $^{+++}$ $p<0.001$ compared with sham group, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared with control group ($n=14$, in sham and control group and $n=7$, in crocin and saffron extract groups).

DISCUSSION

Permanent bilateral occlusion of the common carotid arteries of rats can reproduce cerebral hypoperfusion and causes spatial memory dysfunction. Thus, this model is suitable for studying the memory deficits associated with cerebral circulation impairments (Farkas *et al*., 2004, 2005; Xu *et al*., 2010). In our experiment, the escape latency time during trials, the percentage of time spent in the target quadrant, the traveled distance to reach the hidden platform on day 5 and the speed of animals in each group were used to assess acquisition of the water maze task. The data showed that in comparison with the control group, crocin (at all doses) and saffron extract

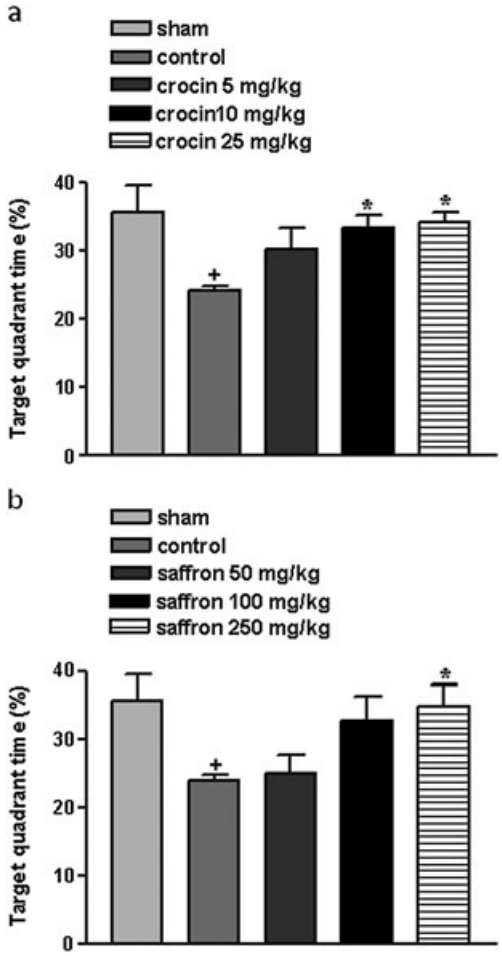

Figure 3. Percentage of time spent (mean + SEM) in the target quadrant of the pool in the final test trial by crocin (a) and saffron extract (b) treated rats in the Morris water maze. $^{+}p<0.05$ compared with sham group, $*p<0.01$, compared with control group ($n = 14$, in sham and control group and $n = 7$, in crocin and saffron extract groups).

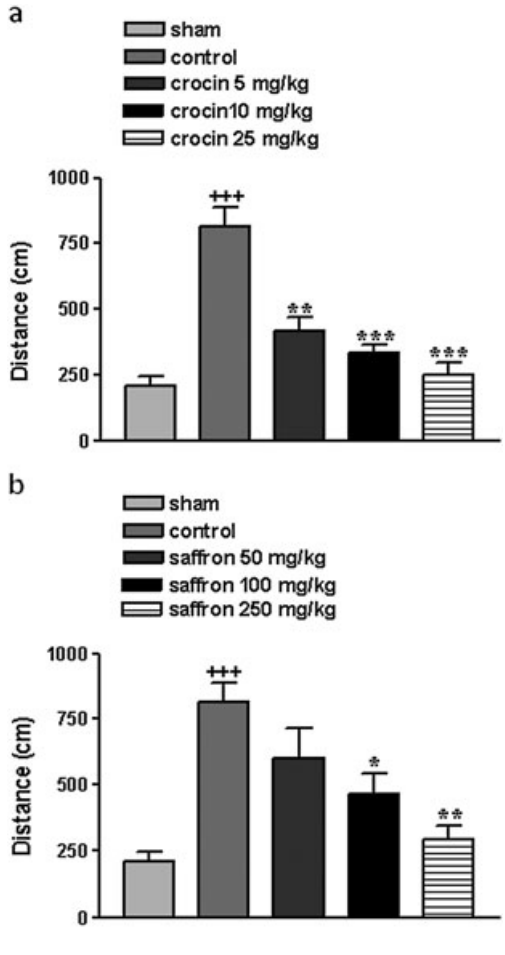

Figure 4. Distance swum (mean + SEM) to reach the platform on day 5 by crocin (a) and saffron extract (b) treated rats in Morris water maze. $^{+++}p<0.001$ compared with sham group, $*p<0.05$, $**p<0.01$, $***p<0.001$ compared with control group ($n = 14$, in sham and control group and $n = 7$, in crocin and saffron extract groups).

(at 100 and 250 mg/kg) could significantly reduce the escape latency time and the traveled distance in finding the hidden platform. Also, in the final test trial, the percentage of time traveled in the target quadrant, at higher doses of saffron extract (250 mg/kg) and crocin (10, 25 mg/kg) groups, was significantly increased in comparison with the control group. No significant difference was observed in the swim speed between groups (Fig. 5). It meant that decreasing the escape latency was not due to the effect of saffron extract or crocin on swimming speed. These data indicated that the saffron extract and crocin did not have any effect on motor ability. Therefore, the present study demonstrates that saffron extract and crocin improved cognitive deficits induced by chronic cerebral hypoperfusion in rats. This neuroprotective effect may be due to the antioxidative properties of saffron and crocin. Other workers have also reported that antioxidants could protect against cognitive deficits induced by chronic cerebral hypoperfusion. Xu *et al*. indicated that icariin has protective effects on learning ability and memory in a rat model of chronic cerebral hypoperfusion. Icariin is an antioxidant and a major constituent of flavonoids derived from the Chinese medicinal herb Epimedium brevicornum Maxim (Xu *et al*., 2010). In another study, edaravone as a potent antioxidant and free radical scavenger had protective effects against white matter lesions and endothelial injury in a rat chronic hypoperfusion model and suggested that edaravone is potentially useful for the treatment of cognitive impairment (Ueno *et al*., 2009). As shown in a study by Xu *et al*. (2010), green tea polyphenols, which are potent antioxidants and free radical scavengers, can attenuate vascular cognitive impairment (Xu *et al*., 2010). The ethanol root extract of *Pongamia pinnata* has also shown a protective effect in ischemia-reperfusion injury and long-term hypoperfusion in rats (Raghavendra *et al*., 2007). These studies indicate that the antioxidant effects of these compounds play an important role in improving the spatial cognitive abilities after chronic cerebral hypoperfusion.

It has been shown that saffron and crocin are potent antioxidants (Asdaq and Inamdar, 2010; Ochiai *et al*., 2004; Papandreou *et al*., 2006). They scavenge free radicals, especially superoxide anions and, thus, may protect cells from oxidative stress (Abe and Saito, 2000). In a study by Ochiai *et al*., crocin prevented the death of rat pheochromocytoma (PC-12) cells by its antioxidant effects being stronger than those of alpha-tocopherol (Ochiai *et al*., 2004). Crocin is likely to prevent ethanol-induced inhibition of hippocampal long term potentiation (LTP) by antagonizing the inhibitory effect of ethanol on NMDA receptors, though it is not

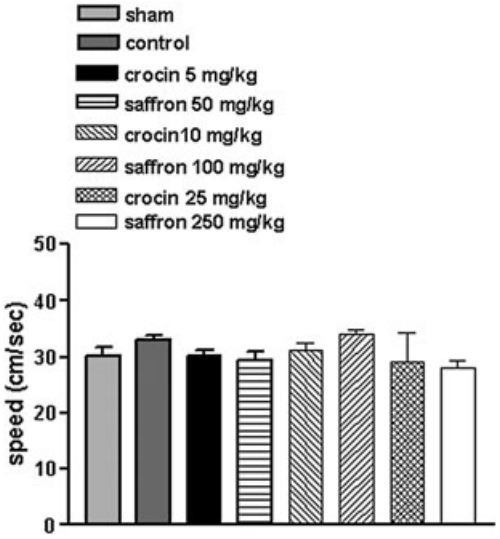

Figure 5. Swimming speed (mean + SEM) during the test trial by crocin and saffron extract treated rats in the Morris water maze, ($n = 14$, in sham and control group and $n = 7$, in crocin and saffron extract groups).

clear whether crocin acts directly on the NMDA receptor channel complex or indirectly modulates NMDA receptor functions (Abe *et al*., 1998; Abe and Saito, 2000). Other researchers indicated that saffron extracts significantly antagonized the scopolamine-induced memory performance deficits (Pitsikas and Sakellaridis, 2006). Thus, saffron extract and its constituents, especially crocin, improve the impairment of certain types of learning and memory through different mechanisms. Permanent cerebral hypoperfusion produces excess free radicals (Liao *et al*., 2004; Liu *et al*., 2007; Xu *et al*., 2010) and reactive oxygen species (ROS) that can damage the weakened antioxidant defense system of the brain, thereby inducing neuronal degeneration and death. After chronic cerebral hypoperfusion, antioxidant capabilities changed in the cortex and hippocampus (Xu *et al*., 2010). Liu *et al*. (2007) showed elevated superoxide dismutase (SOD) activity in the cortex and hippocampus of animals underwent permanent bilateral occlusion of the common carotid arteries and considered this a compensatory rise in antioxidant activity that indicated the brain's antioxidant machinery was activated when overwhelmed by oxidative stress. Huang *et al*. (2008) also found reduced antioxidant activity after chronic cerebral hypoperfusion. Saffron extract and crocin scavenge free radicals, especially superoxide anions, and thereby may protect cells from oxidative stress (Abe and Saito, 2000). Accumulating evidence suggests that cellular stress induced by free radicals is responsible for a variety of CNS neurodegenerative disorders, including Alzheimer's disease and Parkinson's disease (Abe and Saito, 2000). It is demonstrated that crocin significantly decreases the formation of peroxidized membrane lipids and restores SOD activity compared with α-tocopherol activity (Ochiai *et al*., 2004). The restoration of SOD activity suggests that crocin has an important role in modulating antioxidative effects. Crocin also suppressed the activation of caspase-8 caused by serum/glucose deprivation (Ochiai *et al*., 2004). Mousavi *et al*. found that saffron extract and crocin decreased the toxicity of glucose, in PC12 cells, by reducing the ROS production (Mousavi *et al*., 2010).

Crocin is the main active constituent and antioxidant in saffron. Therefore, it may be responsible for the memory enhancing effect of saffron extract. These results suggest that saffron and crocin as potent antioxidants may combat oxidative stress in neurons and could be useful in the therapy of brain neurodegenerative disorders such as vascular dementia.

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Conflict of Interest

The authors have declared no conflict of interest.

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