

## Inhibiting Post Traumatic Stress Disorder (PTSD) induced by electric shock using ethanol extract of saffron in rats

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The effects of ethanol extract of saffron and crocin on Post Traumatic Stress Disorder (PTSD) caused by electric foot shock were examined in male Wistar rats (250-300 g weight). One hundred and twenty eight rats were divided into two categories; the first one (8 groups, n = 7-9 rats/group) underwent bilateral cannulation in basolateral amygdala (BLA) and received intra-BLA saline (1  $\mu$ l per rat) extract or crocin (1, 5, 10  $\mu$ g per rat), whereas the second category (8 groups, n = 7-9 rats/group) received saline (1 ml  $\text{kg}^{-1}$ ) extract or crocin (1, 5, 10 mg  $\text{kg}^{-1}$ ) intraperitoneally. Both categories randomly received electric foot shock (0.1 mA) for 100 seconds over a period of 10 days. After returned to cages to repose for 21 days, the animals were put back into the stress box but received no stress. Plasma corticosterone levels, anorexic time and dopamine-dependent behaviors (rearing, sniffing, locomotion and freezing) were assessed. One-way ANOVA results showed that stress increased plasma corticosterone level and anorexic time but decreased dopamine-dependent behaviors. Intraperitoneal administration of the saffron extract and crocin reduced both plasma corticosterone level and anorexic time, but increased dopamine-dependent behaviors, whereas intra-BLA administration of saffron ethanolic extract and crocin had no significant effect. These findings indicate that saffron ethanolic extract and crocin can reduce hormonal, metabolic, and behavioral signs of PTSD. This effect, however, is exerted on some parts of the central nervous system outside of BLA.

**Key words:** saffron, crocin, Post Stress Traumatic Disorder, amygdala, rat.

### INTRODUCTION

The post traumatic stress disorder (PTSD) is among the most important mental disorders of our century which causes great stress and several complications for the afflicted person. Nowadays, the definition of PTSD comprises not only those affected by the accident, but also those who have witnessed it (APA, 1994). Previous studies indicate that the brain stress system, consisting of amygdala (particularly, basolat-

eral amygdala, BLA), hypothalamus, parts of the limbic cortex such as the prefrontal area and part of the reward system including the shell of the accumbens nucleus, is guilty for creation, sustention and recurrence of this disorder through the medial nucleus of amygdala. This disorder is accompanied by an increase in adrenergic activity, as well as an increase in noradrenaline levels in the above said regions of the brain (such as the prefrontal cortex). Since cerebral noradrenaline is mainly derived from locus coeruleus, it appears that parts of the brainstem are involved in this disorder besides those regions mentioned earlier (De Kloet *et al.*, 2006; Yehuda, 2009). In ad-

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dition, the main stress hormone (cortisol in human and corticosterone in rodents) is responsible for inducing the condition, although concrete data are lacking regarding increase or decrease of cortisol in serum levels of people with PTSD. On the other hand, it is unfortunate that no therapy has been developed for PTSD yet, and the current therapies mostly focus on symptom inhibition (De Kloet *et al.*, 2006; Yehuda, 2009).

Saffron has long been known as a healthy food additive and is still widely in use (Moshiri *et al.*, 2006). However, it is now being studied extensively for its medicinal features. Saffron has been in use for treating amenorrhea, pain, inflammation, throat diseases and depression (Karimi *et al.*, 2001; Akhondzadeh *et al.*, 2004). Older and recent studies indicate its anti-tumor, anti-convulsant, anti-depressive, and memory-aiding abilities (Abdullaev, 1993; Rios *et al.*, 1996; Akhondzadeh *et al.*, 2007; Schmidt *et al.*, 2007; Soeda *et al.*, 2007). Considering the lack of data about the effect of saffron extract or its main constituents (crocin) on PTSD and its complications, we have designed the present study to further assess the effects of saffron ethanolic extract and crocin on hormonal, behavioral and metabolic responses of male Wistar rats with PTSD induced by electric foot shock stress.

## MATERIALS AND METHODS

### *Animals and Plant Material*

One hundred and twenty eight male Wistar rats (W = 200-250 g) purchased from Pasteur Institute (Tehran, Iran), were used in this study. Four animals were kept per cage with 12/12 hrs dark and light cycle and free *ad-lib* access to food and water, except during the experiments.

Saffron was extracted from stigmas of *Crocus sativus*, provided by Talakaran-E-Mazraeh agricultural Corporation (Torbat Heydarieh, Khorasan, Iran). The extract was prepared as follows: 100 g of dried and milled stigmas were extracted with 1000 ml ethanol 100% by maceration procedure. The extract was dried by evaporation (30 to 35 °C). The yield of extraction was 21 mg of freeze-dried powder for 100 mg of the dry stigmas. The extract was dissolved in normal saline and was immediately administered intraperitoneally (i.p.) or intra-BLA to the animals and expressed as mg of extract per kg body weight or µg per rat, respectively. Crocin (Fluka Chemical Corporation, Germany) was also dissolved in normal saline and used immediately after preparation. The doses of the

extract and crocin for intraperitoneal was 1, 5 and 10 mg kg<sup>-1</sup> while their doses for intra-BLA administration was 1, 5, and 10 µg per rat.

### *Quantification of crocin in saffron extract*

Crocin in saffron extracts was analyzed according to Hosseinzadeh & Jahanian (2010) with some modifications (ISO 3632-2). All parameters were expressed as direct absorbance readings (bitterness at 257 nm, safranal at 330 nm and coloring strength at 440 nm, 93.3, 38.9 and 240 on dry basis, respectively). The crocin percentage of the extract was 17% and the doses used in conducted experiments were standardized according to their crocin content.

### *Experimental design and induction of stress*

Electric foot shock was applied for seven consecutive days. For this purpose, each animal was randomly assigned in stressed or control group. Animals (128 male rats, which were divided into 16 groups of 7 to 9 individuals each) were transferred to the experimental room 1 hr prior to the onset of the experiments for adaptation. Then, the animals were placed in the compartments individually and an electric foot shock was applied 30 min later.

To induce stress, a common apparatus (Rosales, 2002) was used with some modifications. Briefly, the communication box (Borj-e-Sanat Co., Tehran, Iran) consisted of 9 distinct compartments (16 × 16 cm), made by plexiglas, with 8 holes (2 mm in diameter) in their contact sides allowing reception of visual, olfactory and auditory cues. The floor of the compartment was equipped with stainless steel rods (4 mm in diameter) placed 1.3 cm apart. The rods were attached to the generator which was controlled by a PC and produced an electrical current of 0.1 mA to generate an electric shock foot for 100 sec.

After the termination of the electric foot shock, the animals were left in the compartment for additional 30 min and then they returned to their home cages. Controls were just placed in the compartment for 60 min without any shock foot. After stress completion, the animals were left in their cages for another 21 days without stress. On the 28<sup>th</sup> day, the animals were returned to the stress equipment and their behavior was recorded for 10 min. The anorexic time (time elapsed by the animals to start chow eating when they returned to their cages) was also recorded as an index of stress.

### Blood sampling

Blood samples were taken from retro-orbital sinus (0.5 ml of the blood in 0.5 ml sodium citrate 1%) between 12:00-14:00. The samples were centrifuged in 1400 g for 5 min in 4°C and the supernatant serum was collected for corticosterone detection. Corticosterone concentration was determined with ELISA kit (Rat Corticosterone ELISA kit, EIA-4164, DRG Instruments GmbH, Germany) in 450 nm.

### Dopamine-related behaviors

The behavior of each animal was digitally videotaped for 10 min. Dopamine-related behaviors, such as sniffing, rearing, coping, and locomotion, were recorded from these video-files by a person unfamiliar with the experiment and thus, objective (Fedele *et al.*, 1998).

### Surgery

Rats were anesthetized with Ketamine hydrochloride (70 mg kg<sup>-1</sup>, i.p.) and Xylazine (10 mg kg<sup>-1</sup>, i.p.) and one or two stainless steel cannulas (23-gauge) that were placed stereotaxically (Stolting instruments, USA) into the basolateral amygdala (BLA). The cannulas were implanted bilaterally 0.5 mm above the intended site of injection according to the atlas of Paxinos & Watson (1987). Stereotaxic coordinates for the BLA were: incisor bar (-3.3 mm), -2.56 mm anterior to the bregma, ± 4.1 mm lateral to the sagittal suture and 7 mm down from top of the skull. Cannulas were secured to jewelers' screws with dental acrylic. After completing the surgery, a dummy inner cannu-

la was inserted into the guide cannula and left in place until injections were made. The length of the dummy cannula matched with the guide cannula. The animals were allowed seven days to recover from surgery and anesthesia. For drug infusion, the animals were gently restrained by hand; the stylets were removed from the guide cannulas and replaced by 29-gauge injection needles (0.5 mm below the tip of the guide cannula). The solutions were slowly administered in a total volume of 1 µl per rat (0.5 µl in each side) over a period of 60 sec. Injection needles were left in place for an additional 60 sec to facilitate diffusion of the drugs.

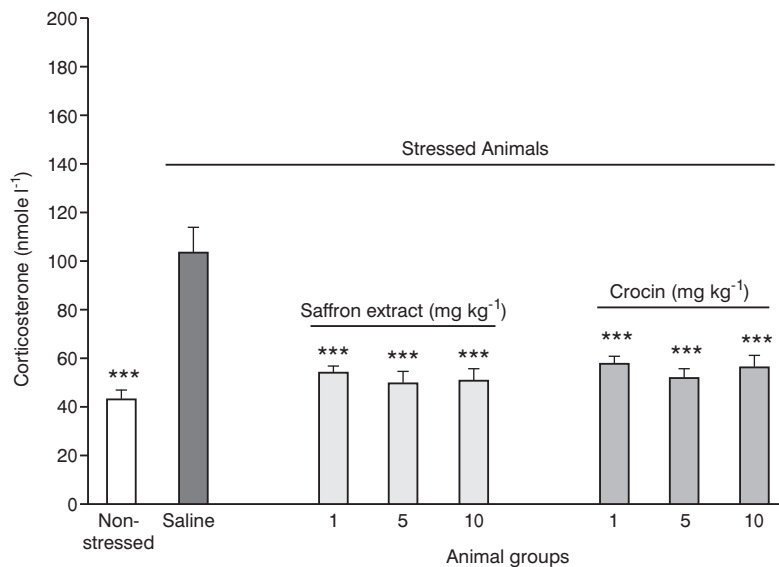
### Histology

After the completion of testing, all animals were anesthetized and received a transcardiac perfusion with 0.9% normal saline followed by 10% buffered formalin. The brains were removed, blocked and cut coronally in 40 µm sections through the cannula placements. The tissues were stained with cresyl violet and were examined by light microscopy by an unfamiliar observer to the behavioral data. Only the animals with correct cannula placements were included in the data analysis (Fig. 1).

### Statistical analysis

Data are presented as mean ± standard error (SEM). One-way analysis of variance (one-way ANOVA) and Tukey post hoc test (significance level at  $p < 0.05$ ) were used to assess the effects of saffron ethanolic ex-

FIG. 1. Plasma corticosterone level increment 21 days after electric foot shock stress termination in rats received intraperitoneal saffron extract or crocin. Data are shown as mean ± SEM for 8-9 rats. \*\*\* $p < 0.001$  different from saline stress group.



tract and crocin on (i) PTSD-induced plasma corticosterone level, (ii) PTSD-induced anorexia and (iii) PTSD-induced dopamine-dependent behaviors.

## RESULTS

### *Effects of saffron extract and crocin on the PTSD-induced plasma corticosterone and anorexia*

PTSD induced a significant increase at the plasma concentration of corticosterone, in animals injected either intraperitoneally or intra-BLA with saline only (see column marked with saline in Figs 1, 2). The PTSD-induced increase at corticosterone was abolished, when intraperitoneal administration of saffron

ethanolic extract or crocin took place [ $F_{(6, 48)} = 15.4, p < 0.0001$ ] (Fig. 1). On the contrary, intra-amygdala administration of saffron extract or crocin was accompanied by an increase at corticosterone concentration, which was not significantly different compared to control [ $F_{(6, 46)} = 0.7, p > 0.05$ ] (Fig. 2).

In addition, PTSD caused a significant increase at the anorexic time compared with non-stressed animals (Fig. 3, column marked with saline). Intraperitoneal administration of saffron ethanolic extract or crocin abolished the increase in anorexic time [ $F_{(6, 45)} = 10, p < 0.0001$ ] (see columns marked with saffron and crocin extract in Fig. 3). There was no significant difference in the mean value of anorexic time record-

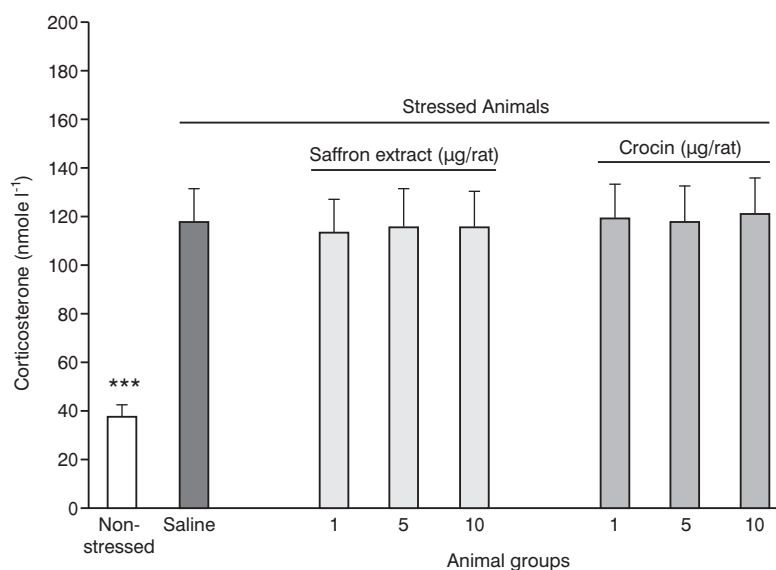


FIG. 2. Plasma corticosterone level increment 21 days after electric foot shock stress termination in rats received intra-amygdala saffron extract or crocin. Data are shown as mean  $\pm$  SEM for 7-9 rats. \*\*\*  $p < 0.001$  different from saline stress group.

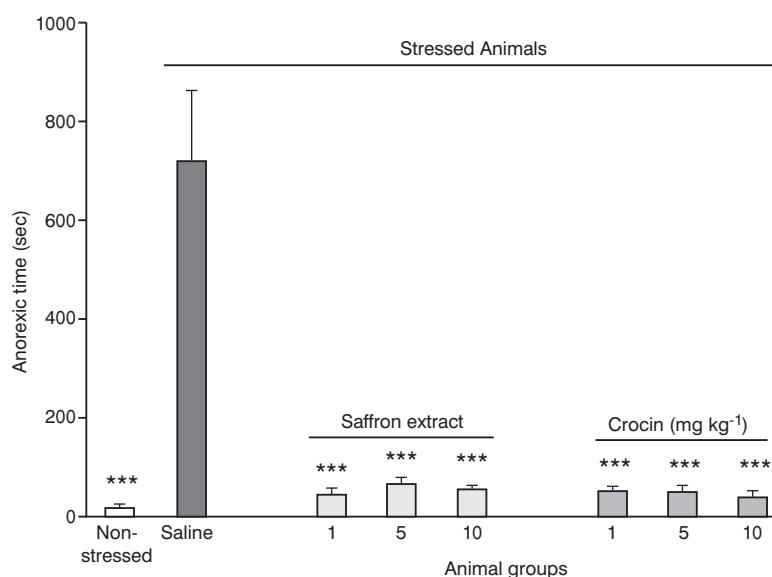


FIG. 3. The anorexic time (time elapsed for initiation of food consumption) 21 days after electric foot shock stress termination in rats received intraperitoneal saffron extract or crocin. Data are shown as mean  $\pm$  SEM for 8-9 rats. \*\*\*  $p < 0.001$  different from saline stress group.

FIG. 4. The anorexic time 21 days after electric foot shock stress termination in rats received intra-amygdala saffron extract or crocin. Data are shown as mean ± SEM for 7-9 rats. \*\*\**p* < 0.001 different from saline stress group.

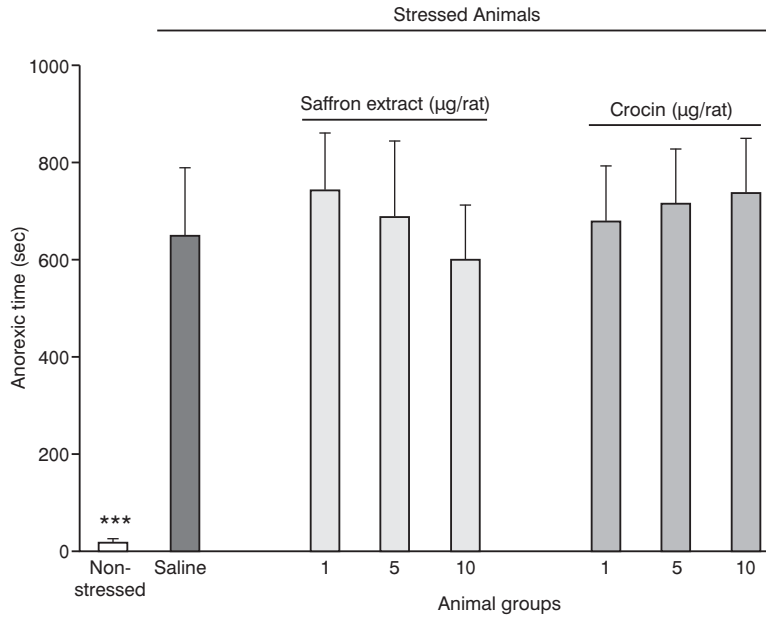


TABLE 1. Effects of intraperitoneal administration of saffron ethanolic extract and crocin on dopamine-dependent behaviors in rats 21 days after electric foot shock stress termination. Data are shown as mean ± SEM for 8-9 rats. \*\*\**p* < 0.001 different from saline stress group

Behavior / Groups	Freezing Time	Locomotion	Rearing	Sniffing
Non-Stressed	37.5 ± 5.88***	30 ± 4.42***	31.6 ± 3.54***	66.13 ± 10***
Saline	223.33 ± 20.76	1.83 ± 0.3	1.33 ± 0.33	7.5 ± 1.76
Saffron extract (1 mg kg <sup>-1</sup> )	41.66 ± 6***	4.66 ± 0.76***	35 ± 4.57***	70 ± 11.61***
Saffron extract (5 mg kg <sup>-1</sup> )	28.33 ± 3.57***	3.83 ± 0.7***	41.6 ± 11.01***	66 ± 11.45***
Saffron extract (10 mg kg <sup>-1</sup> )	45 ± 6.83***	4.33 ± 0.55***	41.6 ± 3.54***	61 ± 11.66***

TABLE 2. Effects of intra-amygdala administration of saffron ethanolic extract and crocin on dopamine-dependent behaviors 21 days after electric foot shock stress termination in rats. Data are shown as mean ± SEM for 7-9 rats. \*\*\**p* < 0.001 different from saline stress group

Behavior / Groups	Freezing Time	Locomotion	Rearing	Sniffing
Non-Stressed	37.5 ± 5.88***	35 ± 0.42***	31 ± 0.54***	66 ± 10.98***
Saline	223.33 ± 20.76	1.83 ± 0.3	1.33 ± 0.33	7.5 ± 0.76
Saffron extract (1 µg per rat)	210.83 ± 18.72	2.83 ± 0.6	1.66 ± 0.49	11.33 ± 1.05
Saffron extract (5 µg per rat)	176.66 ± 9.63	2.83 ± 0.3	2 ± 0.63	8.83 ± 0.7
Saffron extract (10 µg per rat)	198.33 ± 5.57	2.66 ± 0.42	1.16 ± 0.47	10.5 ± 1.11



ed from the animals that received intra-amygdala administration of saffron extract and crocin [ $F_{(6, 47)} = 1.1, p > 0.05$ ] (Fig. 4).

#### *Effects of saffron ethanolic extract and crocin on the PTSD-induced dopamine-dependent behaviors*

The effects of saffron and crocin on dopamine-dependent behaviors are summarized in Table 1. The time required for freezing was significantly increased ( $F_{(6, 48)} = 13.32, p < 0.0001$ ), whereas the time required for behaviours such as sniffing ( $F_{(6, 48)} = 12.43, p < 0.0001$ ), rearing ( $F_{(6, 48)} = 20, p < 0.0001$ ), and locomotion ( $F_{(6, 48)} = 11.2, p < 0.0001$ ) was significantly decreased in PTSD animals that received an intraperitoneal injection of saline only (Table 1). Intraperitoneal injections of either saffron extract or crocin eliminated the stimulatory effects of PTSD on freezing time and the inhibitory effects of PTSD on sniffing, rearing and locomotion time (Table 1).

Interestingly, intra-amygdala administration of the saffron extract only was accompanied by a significant increase in freezing time, and a significant decrease in locomotion, rearing and sniffing time. These effects in the case of saffron injection were similar and not significantly different compared to the mean values of time recorded in the case PTSD animals received an intraperitoneal injection of saline only (Table 2).

## DISCUSSION

Extensive studies have been conducted during the last decade concerning the long-term effects of stress as well as its impact on inducing long-term memory. Our study indicates that firstly, the stress of plantar electric shock may induce long-term memory over a period as long as 3 weeks, and secondly, peripheral administration of the ethanol extract of saffron and crocin can prevent the induction of this memory. It appears that the induction of long-term memory after this stress is somehow related to PTSD. Therefore, it may be predictable that the ethanol extract of saffron and crocin may be able to inhibit PTSD. Numerous studies have illustrated the fact that stress can induce memory through stimulation of many dopamine, glutamate, and cholinergic pathways in brain (Schultz, 1997; Kvetnansky et al., 2009). In this regard, numerous studies have been conducted concerning active and avoidance learning that use plantar electric shock in laboratory rats and mice to indicate memory-inducing effects of stress (McEwen, 2007; Yuena et al.,

2009). Other studies exist that indicate dendritic stimulation, synaptic degeneration and shrinking of hippocampus (Brunson et al., 2001; Avishai-Eliner et al., 2002) and prefrontal cortex as a result of stress (Van den Bergh et al., 2005). High blood levels of corticosterone in rodent following long-standing stresses have been considered as a causative agent for this phenomenon (McEwen, 2007). It must also be considered that it leads to disorders of metabolism for plasma cholesterol, glucose, and fatty acids and, through increasing stress oxidative agents, ultimately result in various metabolic diseases such as diabetes (Eizirik et al., 2008; Scheuner & Kaufman, 2008). Our study indicates that when the exposed animals, were returned to the stress environment after 21 days of termination of stimulus, showed reactions similar to the time of stress-induction; in other words, with an increase in their blood corticosterone level, they depicted severe dopamine-dependent behaviors, and their fecal and urine output increased. Thus, it seems that a strong learning system in the central nervous system controls the animal reaction to stress and creates long-term memory. Dopamine-dependent behaviors such as rearing, sniffing, locomotion and coping intensify on stress. The reason for this is the activation of the hypothalamic-pituitary-adrenal (HPA) axis on exposure to stress, which leads to an increase in the activity of the pathway, intensifying dopamine-dependent behaviors. Using antagonists of dopamine-receptors such behaviors may be decreased (Meaney et al., 2002).

The HPA axis activity is the first line of defense of the organism against stress; it includes an increase in production and secretion of CRH in hypothalamus followed by increased ACTH secretion from anterior pituitary and increased corticosterone secretion from adrenal cortex (Dunn & Swiergiel, 2008). Furthermore, researchers have illustrated that inducing stress activates the sympathetic and parasympathetic pathways and results in such reactions as increased heart rate, hypertension, hyperventilation, increased gut activity and increased fecal and urine output (Sapolsky et al., 2000; Carrasco & Van de Kar, 2002; McEwen, 2007). None of the animals in our case group showed any of these symptoms which consists an indication of improvement in their condition after receiving saffron extract or crocin. The change in plasma level of corticosterone in the case group indicates that the HPA axis either has not been activated or has stopped functioning midway. Experiments have observed that saffron extract can control pentylentetrazole-induced seizure in laboratory rats which is the result of

its interaction with nitric oxide and opioid pathways (Hosseinzadeh & Sadeghnia, 2007). Moreover, saffron extract may control morphine dependence (Hosseinzadeh & Jahanian, 2010), induce anesthesia and reduce inflammation (Akhondzadeh *et al.*, 2004). It has been illustrated that saffron extract attaches to glutamate NMDA receptor in the central nervous system and inhibits it (Lechtenberg *et al.*, 2008). In addition, the ethanol extract of saffron could induce the release of glutamate, serotonin, dopamine and noradrenalin in rat brain (Karimi *et al.*, 2001; Sarris, 2007). Thus, it appears difficult to explain how the main effect of stress (i.e. increased plasma level of corticosterone) did not occur in our case group. However, it must be noted that the saffron extract may have both central and peripheral effects. Peripherally, it seems that the saffron extract functions through inhibiting the peripheral receptors of the corticosterone-secreting cells of the adrenal cortex. These receptors are essential for releasing corticosterone and their inhibition may prevent the secretion of the substance from these cells. It must be noted that other impacts of stress, such as the behavioral and autonomic effects, were abolished in the case. Since these effects are summarized in the central nervous system, it seems that central mechanisms have also been involved in the effects of saffron extract and crocin. Considering the fact that saffron extract and crocin inhibit NMDA receptors (Lechtenberg *et al.*, 2008), it appears that part of the effects of the extract are exerted through inhibition of this type of receptors in the brain. As a corroboration of this statement, it is noteworthy that suppression of glutamate receptors in amygdala and hypothalamus may result in decreased CRF secretion, in addition to other hormones of the hypothalamus (Koob, 1999). On the other hand, suppression of glutamate NMDA receptors may lead to decreased activity of the mesocorticolimbic system, bringing about the decrease in cerebral dopamine and consequently the dopamine-dependent behaviors (Tzschentke, 2007). In any case, numerous experiments indicate that inhibition of glutamate receptors with MK-801 reduces the motor behaviors of these animals. Moreover, suppression of dopamine receptors by sulpiride suppresses dopamine-dependent behaviors. This indicates that the dopamine and glutamate pathways are interrelated (Tzschentke, 2007). In addition, our findings indicate that saffron extract and crocin inhibit these systems. The suppression of autonomic reactions in response to stress also demonstrates the central effect of the extract. There-

fore, we recommend for future studies to evaluate the permeability of saffron extract and its components through the blood-brain barrier. Our study indicated that injection of extract and crocin into amygdala has no effect on stress and the stress responses were observed after injection of saffron extract and crocin into amygdala. Therefore, although it appears that at least part of the effects of saffron extract and crocin are associated with their effect on the central nervous system, this effect is apparently not exerted through activation or suppression of stress pathways in amygdala as the most important area of the nervous system which responds to stress stimuli. It may be that other regions of the brain, such as hypothalamus, may be involved in manifestation of these effects.

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