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Research Article

Protective Effects of *Crocus Sativus L.* Extract and Crocin against Chronic-Stress Induced Oxidative Damage of Brain, Liver and Kidneys in Rats

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Abstract

Purpose: Chronic stress has been reported to induce oxidative damage of the brain. A few studies have shown that *Crocus Sativus L.*, commonly known as saffron and its active constituent crocin may have a protective effect against oxidative stress. The present work was designed to study the protective effects of saffron extract and crocin on chronic – stress induced oxidative stress damage of the brain, liver and kidneys.

Methods: Rats were injected with a daily dose of saffron extract (30 mg/kg, IP) or crocin (30 mg/kg, IP) during a period of 21 days following chronic restraint stress (6 h/day). In order to determine the changes of the oxidative stress parameters following chronic stress, the levels of the lipid peroxidation product, malondialdehyde (MDA), the total antioxidant reactivity (TAR), as well as antioxidant enzyme activities glutathione peroxidase (GPx), glutathione reductase (GR) and superoxide dismutase (SOD) were measured in the brain, liver and kidneys tissues after the end of chronic stress.

Results: In the stressed animals that receiving of saline, levels of MDA, and the activities of GPx, GR, and SOD were significantly higher ($P<0.0001$) and the TAR capacity were significantly lower than those of the non-stressed animals ($P<0.0001$). Both saffron extract and crocin were able to reverse these changes in the stressed animals as compared with the control groups ($P<0.05$).

Conclusion: These observations indicate that saffron and its active constituent crocin can prevent chronic stress–induced oxidative stress damage of the brain, liver and kidneys and suggest that these substances may be useful against oxidative stress.

Introduction

Stress is a state of physiological or psychological responses caused by adverse stimuli that tend to disturb the functioning of an organism.[1] During chronic stress, the balance between oxidant and antioxidant systems is lost.[2,3] Chronic stress increases the oxygen free radicals levels and influences on the function of antioxidant defense system enzymes.[4] One specific and classic method to induce psychological and physiological stresses simultaneously is immobilization/restraint stress[5] which alters either the activities of antioxidant enzymes or their capacities in some organs including brain, liver, and kidneys.[3] Therefore, if an imbalance appears between pro-oxidants and antioxidants, an oxidative stress derives from this imbalance.[6] The imbalance itself may be either resulted from a lack of antioxidant capacity caused by disturbances in production and distribution or by an excessive amount of reactive oxygen species (ROS).[7] Oxidative stress is supposed to be an essential factor in the development of diseases such as neurodegenerative diseases,[8] hepatic inflammation,[9] hepatic cirrhosis, acute and chronic alcoholic liver, hypercholesterolemia, chronic kidney disease, etc.[10]

Currently, people's attention has been increasingly drawn to complementary or alternative medicine because of the supposedly less side effects of botanicals.[11] Crocus is a genus of flowering plants in the iris family comprising 90 species of perennials growing from corms. *Crocus sativus L*, commonly known as saffron, is originally grown in Iran and Spain.[12] Saffron has a long medicinal history as part of traditional.[13] Some recent studies have shown that saffron has possible anti-carcinogenic, anti-mutagenic,[12] and immune-modulating effects. These effects mainly resulted from antioxidant-like agents of saffron such as volatile agents (e.g., safranal), bitter principles (e.g., picrocrocin), and dye materials (e.g., crocetin and its glycoside, crocin).[13] Both saffron and crocin have free radical scavenging and antioxidant activities. Moreover, these constituents

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protect mice DNA from genotoxin-induced oxidative stress and methyl methane sulfate-induced damages as well.[14] Saffron extract has been shown to have protective effects against genotoxins-induced oxidative stress in Swiss albino mice.[15] In a recent study, we found that saffron and its main constitute crocin prevented chronic stress induced impairment of learning and memory and oxidative damage of the hippocampus.

The aim of this study was to investigate whether saffron and crocin would protect the brain, liver and kidney against chronic stress induced oxidative stress in rats.

Materials and Methods

Animals

Adult male Wistar rats (230-240g, n = 42) were obtained from breeding colony of Semnan University of Medical Sciences, Semnan, Iran. They were housed in groups of seven in cages in a 12-h light/dark cycle at 22-24 °C, with food and water *ad libitum*. All procedures were conducted in agreement with the National Institutes of Health Guide for care and use of laboratory animals.

Drugs

Pure red saffron powder was kindly supplied by the Pharmaceutical Research Center, Mashhad University of Medical Sciences, Mashhad, Iran (The specimen number of the plant is 134-0319-1). Crocin was purchased from Sigma Aldrich. Both substances were dissolved in a physiological saline and injected intraperitonealy (IP) in a volume of 2 ml/kg. Saffron extract at a dose of 30 mg/kg and crocin at dose of 30 mg/kg were injected for 21 days in stressed and control groups. These doses were chosen on the basis of our pilot studies and previous reports.[16,17]

Chronic restraint stress

Animals of the stress group were restrained daily for six hours (from 10:00 am - 16:00pm) for a total of 21 days in well-ventilated Plexiglas tubes (20 cm length, 6.5 cm diameter) without access to food and water. There was a 1-cm hole in one far end for breathing. During restraint, control animals stayed in their home cage but food and water were not accessible for the same period to match access with the stress group. After the restraint procedure, the stressed rats were placed back in their home cage. During restraint, animals were not physically compressed and did not experience pain. Body weights were recorded daily prior to the onset and during the entire period of daily restraint.

Experimental groups

Rats were randomly divided into six experimental groups (7 rats per group) as follows: 1) saline (SAL) + no-stress (NS) (SAL-NS); 2) saffron extract (30 mg/kg, IP) + no-stress (SE-NS), 3) crocin (30 mg/kg, IP) + no-stress (C30-NS); 4) saline + stress (SAL-S), 5) saffron extract (30 mg/kg, IP) + stress (SE-S), 6) crocin (30 mg/kg, IP) + stress (C30-S). The first three groups received systemic administration of saline or saffron extract or crocin daily for 21 days. The last three groups were stressed in Plexiglas tubes, 6 hrs/ day, for 21 days and received (60 min before the application of stress) systemic injections of saline or saffron extract or crocin. Immediately after the last day of stress, animals in each group were decapitated and laparotomy and removed brain, liver and kidneys for oxidative stress markers measurements.

Measurements of oxidative stress markers in the tissues

Preparation of tissue homogenates

After the removal of tissues, it was washed in cold 0.9% saline and kept at - 70°C until used for preparation of homogenates with a homogenizer (Polytron PT 2100, KINEMATICA AG, Switzerland). For ferric reducing antioxidant power (FRAP) and malondialdehyde (MDA) assays, a fraction of tissue was homogenized (1:10 w/v) in cold 1.15% KCl. Homogenates for SOD, GPx, and GR measurements were prepared in ratio of 100 mg tissue in 1 ml phosphate buffer (50 mmol/l; pH 7.5) containing 1 mM EDTA. The supernatants obtained after centrifugation at 20,000×g for 10 min at 4°C were used for biochemical analyses. The level of total protein in supernatants was determined by the Bradford method.[18]

Lipid peroxidation assay

MDA results from degradation of polyunsaturated lipids. The production of this substance is used as a biomarker to measure the level of lipid peroxidation. MDA reacts with thiobarbituric acid (TBA) as a thiobarbituric acid reactive substances (TBARS) to form a 1:2 MDA-TBA adduct, which absorbs at 532 nm. Thus, the quantity of TBARS is proportionate to the amount of MDA. Concentration of TBARS is determined according to a method of Mihara and Uchiyama.[19] The concentration of TBARS was calculated using MDA standard curve and was expressed as nmol/mg of protein.

Total antioxidant activity assay

Total antioxidant activity is measured by FRAP according to method of Benzie and Strain.[20] Briefly, 1.5 ml of working FRAP reagent (25 ml 0.3 M sodium acetate buffer, pH 3.6; 2.5 ml 0.01 M TPTZ (2, 4, 6-tri (2-pyridyl)-1, 3, 5-triazine) in 0.04 M HCl; 2.5 ml 0.02 M $FeCl_3 \cdot 6H_2O$; preheated to 37°C) was mixed with 50 µl of supernatant. The mixture was incubated at 37°C for 5 min, and the absorbance was determined at 593 nm. $FeSO_4$ solutions from 0.2 to 1.2 mM in 1.15% KCl were used for calibration. FRAP value was expressed as µmol/mg of protein.

Enzymes activity assay

The activity of antioxidant enzymes were measured photometrically (STAT FAX 3300, Awareness Technologies) using commercially available kits supplied by Randox Laboratories Ltd (Randox Laboratories, Crumlin, UK).

SOD activity was determined in the supernatant using the nitroblue tetrazolium (NBT) by method of Goldberg et al.[21] This method employs xanhine and xanthie

oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenole)-5-phenyltetrazolium chloride (INT) to form a red formazen dye. The SOD activity is then measured by the degree of inhibition of this reacton. One unit of SOD is that which causes a 50% inhibition of the rate of reduction of INT under the conditions of the assay. The level of SOD was expressed as U/mg protein. The activity of GPx was evaluated by the method of Paglia and Valentine.[22] GPx catalysis the oxidation of Glutathione by Cumene Hydroperoxide. In the presence of GR and NADPH the oxidized Glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP. The decrease in absorbance at 340 nm is measured. GR catalyses the reduction of glutathione in the presence of NADPH, which is oxidized to NADP. The decrease in absorbance at 340 nm is measured. The levels of GPx and GR were expressed as U/g protein. Detailed procedures for the above measurements were performed according to the kits' protocol.

Statistical analysis

Data were analyzed by two-way analyses of variance (ANOVA) followed by Tukey's test for multiple comparisons. Statistical differences were considered significant when $P<0.05$.

Results

TBARS levels

Two-way ANOVA on the brain TBARS levels data revealed a significant effects of stress ($F_{1,36}$ = 64.11, P<0.0001), of treatments ($F_{2,36}$ = 18.78, P<0.0001) and a significant interaction between both factors ($F_{2,36}$ = 32.10, P<0.0001). Two-way ANOVA on the liver TBARS levels also showed a significant effects of stress ($F_{1,36}$ = 49.73, P<0.0001), of treatments ($F_{2,36}$ = 13.70, P<0.0001) and a significant interaction between both factors ($F_{2,36}$ = 12.23 P<0.0001). In a similar manner, two-way ANOVA on the kidney TBARS levels demonstrated a significant effects of stress ($F_{1,36}$ = 19.55, P<0.0001), of treatments ($F_{2,36}$ = 13.11, P<0.0001) and a significant interaction between both factors ($F_{2,36}$ = 3.80, P=0.03). Between group comparisons indicated that the TBARS levels of the Sal-S group in all tissues were significantly higher than those of all other groups (all, P<0.01). The TBARS levels in the C30-S (P<0.01), and the SE-S (P<0.01) were significantly lower than those of the Sal-S group (Table 1).

FRAP levels

Two-way ANOVA on the brain FRAP levels data revealed a significant effects of stress ($F_{1,36}$ = 36.09, P<0.0001), of treatments ($F_{2,36}$ = 6.51, P=0.003) and a significant interaction between both factors ($F_{2,36}$ = 16.70 P<0.0001). Two-way ANOVA on the liver FRAP levels showed a significant effects of stress ($F_{1,36}$ = 34.38, P<0.0001), of treatment ($F_{2,36}$ = 6.99, P = 0.002) and a significant interaction between both factors ($F_{2,36}$ = 14.53 P<0.0001). Two-way ANOVA on the kidney FRAP levels showed a significant effects of stress ($F_{1,36}$ = 29.16, P<0.0001), but no significant effects of treatments ($F_{2,36}$ = 2.37, P = 0.1) and no significant interaction between both factors ($F_{2,36}$ = 3.01 P = 0.06). Between group comparisons indicated that FRAP levels of the Sal-S group was significantly higher than that of all other groups (all, P<0.01). FRAP levels in the C30-S (P<0.05) and SE30-S (P<0.01) groups were significantly higher than those of the Sal-S group (Table 1).

SOD activity

Two-way ANOVA on the brain SOD activity revealed a significant effects of stress ($F_{1,36}$ = 33.39, P<0.0001), of treatments ($F_{2,36}$ = 5.54, P<0.008) and a significant interaction between both factors ($F_{2,36}$ = 3.92 P<0.02). Two-way ANOVA on the liver SOD activity a significant effects of stress ($F_{1,36}$=22.37, P<0.0001), of treatments ($F_{2,36}$=9.26, P<0.0006) and a significant interaction between both factors ($F_{2,36}$ = 3.43 P < 0.04). Similarly, two-way ANOVA on the kidney SOD activity showed a significant effects of stress ($F_{1,36}$ = 30.67, P<0.0001), of treatments ($F_{2,36}$ = 7.89, P = 0.001) and a significant interaction between both factors ($F_{2,36}$ = 5.98 P = 0.005). Between group comparisons indicated that SOD activity in the Sal-S group in all tissues was significantly higher than that of all other groups (all, P<0.01). SOD activity in the C30-S, and SE-S groups was significantly lower than that of the Sal-S group (both, P<0.01) (Table 1).

GPx activity

Two-way ANOVA on GPx activity in the brain revealed a significant effects of stress ($F_{1,36}$ = 13.45, P = 0.0008), of treatments ($F_{2,36}$ = 5.04, P = 0.01) and no significant interaction between both factors ($F_{2,36}$ = 0.14 P<0.8). Two-way ANOVA on GPx activity in the liver revealed a significant effects of stress ($F_{1,36}$ = 55.88, P<0.0001), of treatments ($F_{2,36}$ = 22.78, P<0.0001) and a significant interaction between both factors ($F_{2,36}$ = 19.90 P<0.0001). Two-way ANOVA on GPx activity in the kidney revealed a significant effects of stress ($F_{1,36}$ = 10.71, P = 0.002), no significant effects of treatments ($F_{2,36}$ = 1.53, P = 0.22) and no significant interaction between both factors ($F_{2,36}$ = 0.87 P = 0.42). Between group comparisons indicated that GPx activity in the Sal-S group in all tissues was significantly higher than that of all other groups (all, P<0.01). GPx activity of the liver in the C30-S (P<0.01), and all tissues in the SE-S (P<0.05) was significantly lower than that of the Sal-S group (Table 1).

GR activity

Two-way ANOVA on GR activity of the brain revealed a significant effects of stress ($F_{1,36}$ = 16.88, P = 0.0002), of treatments ($F_{2,36}$ = 9.24, P=0.0006) and a significant interaction between both factors ($F_{2,36}$ = 3.32 P = 0.047). Two-way ANOVA of GR data in the liver showed significant effects of stress ($F_{1,36}$ = 25.33, P<0.0001), of treatments ($F_{2,36}$ = 14.17, P<0.0001) and a significant

interaction between both factors ($F_{2,36}$ = 8.36 P < 0.001). Similarly, Two-way ANOVA on GR activity of the kidney demonstrated significant effects of stress ($F_{1,36}$ = 28.30, P<0.0001), of treatments ($F_{2,36}$ = 4.83, P = 0.01) and a significant interaction between both factors ($F_{2,36}$ = 4.07 P = 0.02). Between group comparisons indicated that GR activity in all tissues in the Sal-S group was significantly higher than that of all other groups (all, P<0.01). GR activity in the C30-S (P<0.01), and the SE-S (P<0.01) groups were significantly lower than those of the Sal-S group (Table 1).

Table 1. Antioxidant enzyme activities superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reduced (GR) and Antioxidant power (FRAP) thiobarbituric acid reactive substances (TBARS) levels in brain, liver and kidney of immobilization stress and control groups, (amount/mg protein).

Tissue	Group	Treatment	Lipid peroxidation (TBARS nmol/mg Pr)	Antioxidant power (FRAP µmol/mg Pr)	Superoxide dismutase (SOD U/mg Pr)	Glutathione peroxidase (GPx U/mg Pr)	Glutathione reductase (GR U/mg Pr)
Brain	NS	Saline	2.76±0.05	0.33±0.011	3.35±0.05	0.271±0.002	0.082±0.001
		Crocin	2.85±0.07	0.32±0.01	3.28±012	0.26±0.003	0.080±0.001
		Saffron	2.89±0.06	0.31±0.01	3.25±0.11	0.259±0.003	0.080±0.002
	S	Saline	3.83±0.07**	0.217±0.006**	4.8±0.12**	0.29±0.007**	0.090±0.0008**
		Crocin	3.02±0.04♦♦	0.29±0.01♦	3.87±0.28♦♦	0.27±0.006♦♦	0.083±0.001♦♦
		Saffron	3.01±0.09♦♦	0.30±0.008♦♦	3.8±0.27♦♦	0.27±0.004♦♦	0.082±0.001♦♦
Liver	NS	Saline	1.73±0.08	1.54±0.01	0.64±0.039	12.42±0.17	2.14±0.06
		Crocin	1.71±0.1	1.50±0.04	0.58±0.035	12.35±0.15	2.16±0.05
		Saffron	1.68±0.11	1.48±0.03	0.59±0.031	12.34±0.18	2.02±0.05
	S	Saline	3.11±0.04**	1.13±0.04**	0.87±0.027**	14.9±0.2**	2.60±0.05**
		Crocin	2.09±0.2♦♦	1.41±0.03♦	0.69±0.042♦♦	12.8±0.17♦♦	2.22±0.047♦♦
		Saffron	2±0.12♦♦	1.43±0.05♦♦	0.65±0.031♦♦	12.7±0.21♦	2.16±0.06♦♦
Kidney	NS	Saline	2.24±0.03	1.82±0.06	4.8±0.1	15.63±0.14	3.46±0.08
		Crocin	2.01±0.12	1.78±0.11	4.73±0.24	15.66±0.14	3.46±0.07
		Saffron	1.98±0.16	1.81±0.09	4.69±0.18	15.51±0.15	3.45±0.08
	S	Saline	3.03±0.06**	1.31±0.03**	6.57±0.17**	16.55±0.4**	4.15±0.06**
		Crocin	2.38±0.13♦♦	1.56±0.026♦	5.26±0.25♦♦	16.12±0.2♦♦	3.72±0.1♦♦
		Saffron	2.11±0.14♦♦	1.62±0.06♦♦	5.20±0.23♦♦	15.90±0.16♦	3.67±0.13♦♦

Values represent mean ± SEM of seven rats per group in brain, liver and kidney. Two-way analyses of variance were performed to compare the parameters of control, non-stress (NS) and immobilization stress (S) groups. The details of the experiments are described in the Materials and methods section.
**P<0.01 as compared with the control group (NS); *P<0.05 as compared with the SAL group (NS), ♦P<0.01 as compared with the SAL group (S), ♦♦P<0.01 as compared with the SAL group (S).

Discussion

The main findings of the present study are chronic restraint stress induces oxidative stress in brain, liver and kidney and these harmful effects could be reversed by saffron and crocin pretreatment. These findings indicate that saffron and its most active constituent crocin have potential therapeutic effects against harmful effects of chronic stress in the vital organs. These findings extends our recent findings showing a protective effect of saffron and crocin on chronic-stress induced impairment of spatial learning and memory and oxidative stress in the hippocampus.[23]

Chronic restraint stress induces oxidative stress damage in the vital tissues

Generally, when an imbalance occurs between the production of ROS and the biological detoxification of its reactive intermediates, an oxidative stress appears.[8] Our data show that restraint or immobilization stress produced by 6h/d/21d causes oxidative stress damage in

brain, liver and kidney. These findings support the prior findings indicating that various stress models are associated with enhanced free radical generation and altered antioxidant enzyme activities.[24] Stress probably enhances the pathways leading to increase production of the oxygen free radicals mostly formed in both physiological and pathological conditions in cytosol, mitochondria, lysosomes, peroxisomes, and the plasma membrane in human body.[4,25] Since brain has a high oxygen turnover, brain cells are at high risk of being damaged by free radicals because of their polyunsaturated fatty acid rich neuronal membranes which are open to peroxidation.[26] Lipid peroxidation increases remarkably in correlation with MDA in the cortex, cerebellum, midbrain, and hippocampus in animal brain.[27] Lipid peroxidation is a major injury of the hepatocytes[28] and it is resulted from an imbalance between pro-oxidant and antioxidant systems.[29] Stress also can cause aggravating liver diseases such as hepatic fibrosis and cirrhosis.[30]

Parallel approaches indicate that SOD activity resulted from immobilization stress in animals was higher in brain, liver and kidney rather than in controls. The TBARS levels increased in all tissues especially in brain.[3] Davydov et al. (2004) suggested that immobilization stress stimulates free radical generation in the liver of rats.[31] Additionally, we found that oxidative stress is able to induce oxidative damage in kidney, supporting previous results that in clinical experimentations of renal damages, levels of malondialdehyde and F_2-isoprostanes, two products of lipid peroxidation, increased.[32] The structure and function of the glomerulus may change due to the effect of ROS on mesangial and endothelial cells.[33]

We measured plasma corticosterone levels in the half animals of each group immediately after chronic stress. Consistent with our recent findings,[23] chronic stress increased plasma corticosterone and saffron and crocin significantly decreased plasma levels of corticosterone (data not shown). Glucocorticoids may play an important role in chronic-stress induced oxidative damage.[34] Glucocorticoids appear to increase the liver TBARS in stressed rats and these is a positive correlation between plasma corticosterone and the liver TBARS levels.[35] Patel et al. (2002) reported that the adrenal steroids secreted during stress reduce glutathione levels and disrupt the antioxidant capacity of hippocampal neurons.[36] Behl et al. (1997) also suggested that glucocorticoids cause cell death induced by oxidative stressors in rat brain.[37] Free radical species disrupt the CNS by attacking to the neurons and Schwann cells[38] and the peripheral nerves as well.[39] Increased levels of glucocorticoids during stress may also affect the animal antioxidant capacity as a whole.[3] The mechanisms pointed out above might be involved in tissue alterations induced by oxidative stress damage in our approach.

Saffron extracts and crocin reverse chronic-stress inducing oxidative stress damage

The present study shows that the harmful effects of chronic stress could be reversed by saffron and crocin pretreatment, suggesting a potential therapeutic effect of these agents against chronic stress. These findings confirm other findings showing the protective effects of aqueous extract of saffron and crocin on tissues ischemia-reperfusion-induced oxidative damage in rats.[40] Crocin is the main constituents of saffron that has antioxidant activities.[41] In our study, saffron was more efficient than crocin probably due to synergistic action of many constituents such as crocin, dimethyl crocetin, safranal, and flavonoids that have antioxidant effects. This may have a role in protective effect of saffron on hyperlipidemic stress.[41] The observation above shows that the aqueous extract of *Crocus Sativus* plant has got antioxidant activity which may resist against pathological alterations caused by the free radicals.[42] Crocin is able to suppress the generation of ROS by various oxidative stresses.[43] Our study is along with a recent work introducing a negative correlation between lipid peroxidation products and saffron in some pathological conditions.[44]

The carotenoids in saffron extracts may protect tissues from oxidative damages due to their antioxidant effect.[45] Data show remarkably modulation in the levels of oxidative markers in the brain, liver and kidney caused by crocin. As it was mentioned previously, the brain tissue is highly vulnerable to oxidative stress.[26] When polyunsaturated fatty acids are exposed to ROS, lipid peroxides are formed and the accumulation of end-products of lipid peroxidation may result in brain damage.[46] We observed chronic stress increased lipid oxidation in the hippocampus and this effect was blocked by both saffron extract and crocin treatment. The total antioxidant reactivity was examined by using FRAP assay of the brain of all experimental groups. Then, a significant decrease in antioxidant power in the stressed animals occurred. Saffron extract and crocin treatment reversed the decrease of the antioxidant power. Therefore, it can be concluded that they may possess antioxidant roles in chronic stress.

Organisms have got protective antioxidant defense enzymes including SOD which converts superoxide radicals into H_2O_2, GPx which breaks down peroxides, and those derived from the oxidation of membrane phospholipids. Oxidative stress conditions increase the activity of these enzymes.[47] An increase in the activities of these enzymes in the brain of stressed animals is showed here. Different effects on antioxidant enzymes were shown in a previous study by glucocorticoids administration or an exposure to chronic stress. Administration of glucocorticoids decreased levels of brain SOD and GPx activities[48] and glucocorticoids prevented induction of antioxidant enzymes after kainic acid administration in the hippocampus.[49] Thus, an interaction with HPA might be another mechanism for the protective effects of saffron and crocin on chronic

stress damage. Interestingly, we found that crocin can decrease the corticosterone response to chronic restraint stress.

Conclusion

In conclusion, the present work demonstrates that saffron and crocin can prevent chronic-stress induced oxidative damage of the brain, liver and kidneys. Thus, these substances should be useful as new pharmacological tools for alleviating chronic stress -induced oxidative damages.

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Conflict of Interest

There is no conflict of interest to be reported.

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