

Full Length Research Paper

Effect of aqueous, ethanolic and acetonitrile *Crocus sativus* L. extracts on stress biomarkers in male rats

Jalal Izadi Mobarakeh¹, Nahid Fakhraei¹, Zeinab Abedini Sadr^{1,3}, Assiyeh Hamidipour², Ehsan Mostafavi⁴, Reza Haji Hosseini³, Soroush Sardari^{2*}

¹Physiology and Pharmacology Department, Pasteur Institute, Tehran, Iran

²Medical Biotechnology Department, Biotechnology Research Center, Pasteur Institute, Tehran, Iran

³Biology Department, Faculty of Basic Sciences, Payame Noor University, Tehran, Iran

⁴Epidemiology Department, Pasteur Institute, Tehran, Iran

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In this study, antianxiety-like behavior of aqueous, ethanolic and acetonitrile *Crocus sativus* L. extracts have been investigated in forced-swimming stress in rats. In addition, main metabolites crocin and safranal were quantified in all extracts using HPLC. Different doses of extracts (10, 30, 60 mg/kg) were injected intraperitoneally (i.p.) in a 9-day period, meanwhile, swimming stress was performed for 15 minutes in four sessions (days 3, 5, 7 and 9). The time performing the followings was measured: immobility, swimming and struggling. Moreover, free fatty acids, glucose, corticosterone and HSP70 were measured. The outcomes demonstrated saffron decreased stress significantly by prolonging immobility and decreasing the active behavior swimming, without much effect on struggling. The extracts also showed significant reduction in levels of the stress biomarkers. With having the highest amount of safranal and the lowest amount of crocin, comparing the other extracts, acetonitrile has been identified as the most effective extract in reducing anxiety. The saffron extracts probably proved anti-stress and sedative properties, partly due to distinct proportion and synergistic impact of the active constituents. On the other hand, crocin and safranal have anti-oxidant and anti-inflammatory powers that may aid to mediate this protective central impact. Regarding these information, saffron may have the potential to be employed in clinical practice.

Key words: HPLC method, antianxiety-like behavior, *Crocus sativus* L. (saffron), forced-swimming stress, rat, stress biomarkers.

INTRODUCTION

Anxiety is considered to cause the release of several stress hormones, primarily glucocorticoids by activation of the hypothalamic–pituitary–adrenal (HPA) axis and catecholamines through the sympathetic nervous system (Marketon and Glaster, 2008). Chronic stress exposure may lead to excessive plasma glucocorticoid levels and is involved in depression and other disorders (Chrousos and Gold, 1998), related to general susceptibility to disease (Bale, 2005), alterations in other neurochemical pathways and biological and behavioral changes (Harro

and Oreland, 2001). The important biochemical changes in plasma under stressful conditions, that is, elevated corticosterone is necessary to maintain the energy balance which include increased plasma glucose, triglyceride and creatine kinase levels (Rai et al., 2003). Astonishingly, improvements in understanding of stress and anxiety reveal they are accompanied with increased oxidative stress in brain cells (Bouayed et al., 2009). Protein damage and misfolded protein structure are common presentations of various types of stress. These

*Corresponding author. E-mail: ssardari@hotmail.com.

states bring about the activation of Hsp chaperons, for example, heat shock protein 70 (HSP 70) that constitute the major stress-induced chaperonin mammals (Hightower and Hendershot, 1997). Inducible HSPs are produced in large quantities under conditions of stress and stabilize other proteins, prevent denaturation during stress (Maunder, 2000).

Volatile agents (example, safranal), bitter principals (example, picrocrocin) and dye materials (example, crocetin and its glycoside, crocin) are compounds considered pharmacologically active and essential in *Crocus sativus* L., (Iridaceae), saffron, (Rios et al., 1996). Saffron-colored compounds are crocins, which are water-soluble carotenoids. Safranal is the unstable oil responsible for the saffron smell and aroma. Crocin, crocetin and safranal are considered the major active constituents of saffron (Hensel et al. 2006). Saffron extract, crocin and safranal exhibited profound radical scavenging and thereby anti-oxidant activity (Assimopoulou et al., 2005). Daily intake of 100 mg of saffron in milk for six weeks led to an improvement of the anti-oxidative status of patients with coronary heart disease (Verma and Bordia, 1998). The aqueous saffron extract decreased products of lipid peroxidation. Moreover, crocin and the extract pretreatment increased anti-oxidant power as well (Hosseinzadeh and Sadeghnia, 2005). Active extracts of saffron raise long-term potentiation (LTP), the mechanism underlying learning and memory (He et al., 2009). Memory enhancing effects of saffron in aged mice has shown to be correlated with antioxidant protection, especially crocetin (Papandreou et al., 2011). Crocin exerts a variety of pharmacological impacts in mouse including, prevention of LTP inhibition caused by ethanol in rat and improvement of ethanol-impaired learning behavior (Abe and Saito, 2000), anti-hyperlipidemic (Lee et al., 2005). DFT approach showed sugar moiety in crocin molecule has a considerable contribution to the polarizability of molecule and can influence crocin antioxidant behavior (Akhtari et al., 2013). The anti-depressant action of *C. sativus* petal as well as aqueous, ethanolic extracts of stigma, safranal and crocin has been shown in mice (Karimi et al., 2001). In a clinical double-blind study, patients with mild to moderate depression were treated either with saffron or imipramine for six weeks. The effects were found to be equivalent, with a better tolerability of saffron (Akhondzadeh et al., 2004). Saffron extracts diminished depression and anxiety that can lead to emotional eating (Pitsikas and Skellardis, 2006) and there is evidence that this capability may be associated with their anti-oxidant capacity (Behl et al., 2010).

Abe et al. (1999) indicated special interactions occur between saffron and its constituents and CNS activity. Several studies have concentrated on functional role of saffron constituents in the brain. Studies have proved that saffron extract or its active constituents have anti-convulsant (Hosseinzadeh and Khosravan, 2002) and anti-inflammatory effect (Hosseinzadeh and Younesi, 2002), as well as learning and memory improving properties

(Abe et al., 1999). Saffron extracts protect brain cells in culture from inflammatory damage and death induced by cytokines (Soeda et al., 2001). Saffron extract and crocin have protective effect on ischemia/reperfusion injury (IRI)-induced oxidative stress in rat's kidney, that at least is partially due to anti-oxidant properties of saffron (Hosseinzadeh et al., 2005) and protects from genotoxins-induced oxidative stress in mice as well (Premkumar et al., 2003). Safranal has an overall protective effect against cerebral IRI-induced oxidative stress in rat (Hosseinzadeh and Sadeghnia, 2005).

On account of increased physical and psychological demands in the present day life and emergence of various anxiety-related disorders, there is an urgent need to develop agents which overcome or at least minimize these abnormalities. Although treating anxiety with benzodiazepines may be temporarily effective, it produces various side effects, among them impaired memory (Uzun et al., 2010). The drugs obtained from plant origin are gaining importance and being investigated in a number of disorders including stress. Assuming that in the forced-swimming stress, immobility is reduced and active behaviors are increased (Harro and Oreland, 2001; Häidkind et al., 2003) and that both swimming and climbing behaviors may reflect coping responses to stress (Lucki, 1997) and in the light of above information, this study is focusing on potential antianxiety-like behavior of aqueous, ethanolic and acetonitrile saffron extracts. Furthermore, different stress biomarkers including HSP70, corticosterone, glucose and free fatty acids in forced-swimming stress rats were determined. Furthermore, amounts of two pharmacologically active compounds, crocin and safranal in the extracts and their relations with the level of antianxiety-like behavior were compared.

MATERIALS AND METHODS

Drugs

Saffron stigmas were purchased from Saharkhiz Co. (Mashhad, Iran); The safranal (Product no. W33, 890-7-k) with purity of 88% was supplied by SAFC (Germany) and crocin (Product no. 17304, purity?) obtained from Fluka. Diazepam was obtained from Sigma Co. Methanol (HPLC grade), acetonitrile, ethanol and acetic acid were purchased from Merck (Germany).

Preparation of plant extract

The stigmas, after being powdered for 10 min with the use of mortar and pestle, was extracted by applying maceration technique with acetonitrile (22,727 mg of powder using 227 ml of solvent), ethanol (80% v/v) (2,652 mg of powder using 265 ml of solvent) and water (16,666 mg of powder using 166 ml of solvent) and then shaken by stirrer (Heidolph MR 3001, Germany) at 350 rpm overnight in cold room (Hadizadeh et al., 2010; Hosseinzadeh et al., 2009). After centrifugation (Eppendorf 5810 R, Germany) at 4000 rpm (1.341 g) for 30 min at 4°C, the supernatant separated. The same volume solvent was added to the sediment and extraction was repeated five times. The extracts were passed through a 0.45 µm plastic filter

with pore size of 0.45 μm (Sartolon polyamide, Germany). The crude extract was evaporated with Rotary evaporator (IKA RV 05 Basic, Germany) to dryness at 40°C. The yield (w/w) of aqueous, ethanolic and acetonitrile extracts of stigmas was 27.6%, 86% and 20.2%, respectively.

Sample preparation for HPLC analysis

The stock solutions of safranal and crocin were prepared in methanol (5 $\mu\text{l/ml}$; 1 mg/ml) individually, and stored at 4°C in darkness until they used. Standard solutions were prepared in methanol to build the calibration curves.

Chromatographic conditions

The HPLC analysis was performed with a Pharmacia LKB (Uppsala, Sweden) HPLC system, consisting of Pharmacia LKB solvent mixing and KNAUER (No: 63573) degasser (Berlin, Germany), dual wavelength UV/VIS Pharmacia LKB 2141 detector and Pharmacia LKB 2248 Pump and the analytical column employed was a Nucleosil 100 C18, 3 μm Pore size, (250 \times 4.6 mm), Part No: n13.9eS2546, (Dr. Maisch GmbH; Berlin, Germany), a KNAUER (Part No: 7725i) sample injection valve (Berlin, Germany) to inject 20 μl of the sample from a 25 μl Hamilton straight-edge needle syringe (Switzerland) onto the column was used. All data were recorded and analyzed on HPLC Manager chromatography software (Uppsala, Sweden). A gradient method was used for chromatographic determination of crocin (λ_{max} = 440 nm) and safranal (λ_{max} = 310 nm) in all compounds. For the mobile phase, solvent B (1% [v/v] aqueous acetic acid in water) and solvent C (acetonitrile) were used. The mixing of the gradient solvent eluting system was as follows: initial 80% B and 20% C; 0 to 7 min, linear change to 20% B; 7 to 11 min, change to 80% B; 11 to 12 min, change to 80% B; 12 to 14 min, at a flow rate of 1.0 ml/min at 14 min. Separation was accomplished at 25°C.

Animals

Seventy two adult male Wistar rats (225 \pm 25 grams) were employed. Animals were housed in twelve groups of six and maintained under standard conditions with free access to standard rodent feed and water. Three groups were allocated to aqueous extracts (10, 30, 60 mg/kg) and three to ethanolic (10, 30, 60 mg/kg). Acetonitrile extract (10, 30, 60 mg/kg) also included three groups. One group received only saline (0.9%) as a vehicle and the last group was given diazepam as a reference drug (0.3 mg/kg, in saline) (i.p.). All experiments were performed in accordance with the institutional animal care and use. This study was approved by the guidelines of the Ethics Committee of Department of Physiology and Pharmacology, Pasteur Institute, Tehran, Iran.

Measurement of forced swimming stress test in rats

We employed the swimming stress protocol based on that previously described by Bispo and Pereira (1992) with some modifications. In brief, rats were submitted individually to a chronic forced-swimming stress procedure (15 min) within vertical glass cylinder (diameter 22.5 cm, height 60 cm) containing 35 cm of water maintained at 25°C. Water was changed after testing each subject. Behavior was analyzed along the categories of immobility and two active behaviors, swimming and struggling, within five minutes (Armario et al., 1988; Häidkind et al., 2004). Swimming was defined as movement of the fore and hind limbs without the front paws breaking the surface of the water.

A rat was judged to be struggling when it was making active movements with its forepaws in and out of the water, usually

directed against the walls. Immobility recorded when there was an absence of any movement other than those necessary to keep the head and nose above the water (e.g., when rats were floating in a vertical position) and extremely mild swimming was also regarded as immobility. Animals were subjected to i.p. injection of various saffron extracts (10, 30, 60 mg/kg) for nine consecutive days, while the chronic swimming stress was given to rats (15 min) in four trials as follows, on days 3, 5, 7 and 9 after the administration of the extracts (Twenty minutes before the test). The time spent in three behaviors in control groups and the extract-treated rats were considered.

Hormonal and biochemical measurements in chronic swimming stressed rats

On day nine, ten minutes after performing the last swimming trial following deep anesthesia with ether, blood was taken from the heart and collected into pre-chilled EDTA-coated blood collection tubes (before 11:00 am) and centrifuged (4000 rpm, 5 min, 2 to 4°C). Serum was isolated and immediately stored at -80°C until analytical days. Corticosterone hormone, were measured using an ELISA kit, with a sensitivity less than 27 pg/ml, (R&D Systems Europe Ltd., UK). Serum lipid concentrations (cholesterol, HDL and LDL) were determined using enzymatic kits for free fatty acids (Wako NEFA C kit; Trichem Aps, Frederikssund, Denmark), triglycerides (GPO-TRINDER; Sigma) and total cholesterol (CHOD-PAP; Roche Molecular Biochemicals). Low-density lipoprotein-cholesterol (LDLC) and high-density lipoprotein-cholesterol (HDL-C) levels were determined using diagnostic kits from Boehringer Mannheim (Mannheim, Germany). Serum glucose was measured by enzymatic colorimetric methods with commercial kits (Pars Azmone, IRI) on an automatic analyzer (Abbott, model Alcyon 300, USA).

Hippocampus extraction and HSP70 evaluation

On day nine, following the last swim exposure and the blood sampling, the animals were decapitated and their hippocampus removed. To make an assessment of HSP70 production, the levels of endogenous HSP70 were measured by HSP70 ELISA kit (Abnova co.). Hippocampus was washed with PBS (Phosphate-buffered saline) and homogenized with a sonicator at 4°C. The homogenates were centrifuged for ten minutes at 27,000 g. The supernatants were dissolved in PBS having a cocktail of protease inhibitors (1 μl to 20 mg of tissue according to the manufacturer's protocol). In brief, 100 μl of the sample were incubated in each well of a 96-well plate for two hours at room temperature. After the plate was washed six times with washing buffer, 100 μl biotin-conjugated anti-HSP70 antibody solution were poured to each well. Following incubation for one hour, once again the plate was washed six times with the buffer. After that, the avidin-conjugated goat anti-rat IgG antibody was added to each well, and the microtiter plate incubated for one hour at room temperature. After washing six times with the buffer, the substrate solution (100 μl) was added to each well. The solution (10 μl) contained 8 μg of ortho-phenylenediamine and 4 μl of 30% H_2O_2 in citrate phosphate buffer (pH 5.0). After incubation for ten minutes, the reaction was ended with 25 μl of 4N sulfuric acid. The absorbance was measured at 450 nm using an ELISA plate reader (model 3550; Bio-Red, Hercules, Calif., USA). Recombinant HSP70 protein was used to obtain the standard curve in our experiment.

Statistical analysis

All values are expressed as means \pm S.E. SPSS (version 18.0,

Chicago, USA) was applied for the analyses. One-way ANOVA was employed for analyzing the data, followed by Tukey's test for multiple comparisons. Differences were considered significant at P -value < 0.05 .

RESULTS

Effects of various *C. sativus* L. extracts in forced swimming-stressed rats

In this study, we examined anti-anxiety-like effect of *C. sativus* L. extracts (10, 30, 60 mg/kg) on duration (s) of swimming, struggling and immobility behaviors in forced swimming-stressed rats (Figure 1 to 3). As can be seen in Figure 1a, in the first trial (day 3), rats received aqueous extract (60 mg/kg) and diazepam (0.3 mg/kg), spent significantly less time in swimming ($P < 0.01$) and ($P < 0.01$), respectively. Immobility increased by both the extract (60 mg/kg) ($P < 0.01$) and diazepam ($P < 0.001$) (Figure 1c). In the second trial (day 5) (Figure 1a), aqueous extracts 10, 30, 60 mg/kg decreased swimming behavior ($P < 0.001$), ($P < 0.001$), ($P < 0.05$), respectively. The animals in diazepam group also showed significantly less swimming ($P < 0.001$). Struggling declined significantly in the extract (60 mg/kg) ($P < 0.05$) (Figure 1b). The extracts (30 mg/kg) and (60 mg/kg) increased the immobility, ($P < 0.001$) and ($P < 0.001$), respectively. Also, diazepam elevated immobility considerably ($P < 0.001$) (Figure 1c). In the third trial (day 7) (Figure 1a), the extract (60 mg/kg) ($P < 0.05$) and diazepam ($P < 0.01$) decreased the struggling action (Figure 1b). Moreover, the extract (60 mg/kg) ($P < 0.01$) and diazepam ($P < 0.001$) increased the immobility behavior (Figure 1c). From Figure 1a, it can be considered that in the last trial (day 9) aqueous extracts (10 mg/kg) ($P < 0.05$) and (60 mg/kg) ($P < 0.05$) showed decrease in swimming. Diazepam reduced struggling measure significantly ($P < 0.05$) (Figure 1b). The extract (60 mg/kg) ($P < 0.01$) and diazepam increased the immobility ($P < 0.05$) (Figure 1c).

As illustrated in Figure 2a, in the first trial (day 3), ethanolic extract (30 mg/kg) decreased swimming ($P < 0.01$). Diazepam reduced this activity ($P < 0.01$) as well and reduced struggling in a significant manner ($P < 0.05$) (Figure 1b). Diazepam prolonged immobility significantly ($P < 0.001$). In the second trial, swimming showed profound decrease by the extracts (10, 30, 60 mg/kg) ($P < 0.001$) and diazepam ($P < 0.001$) (Figure 2a). Immobility was increased in the extracts 10 ($P < 0.01$), 30 ($P < 0.01$), 60 ($P < 0.05$) mg/kg and diazepam ($P < 0.001$) (Figure 2c). In the third trial (day 7) (Figure 2a), ethanolic extract (30 mg/kg) exerted a significant reducing impact on swimming ($P < 0.01$) Figure 2a. Diazepam reduced struggling markedly ($P < 0.05$) (Figure 2b). Diazepam elevated immobility measure ($P < 0.01$) (Figure 2c). In the last trial (day 9), swimming rate decreased by the extracts 10 mg/kg ($P < 0.001$), 30 mg/kg ($P < 0.001$), 60

mg/kg) ($P < 0.01$) (Figure 2a). Ethanolic-treated animals (10, 30 mg/kg) indicated an increase in immobility ($P < 0.05$) (Figure 2c).

In the first trial (day 3), acetonitrile and diazepam treated rats (10, 30, 60 mg/kg) significantly spent less time swimming ($P < 0.001$) (Figure 3a). The extracts (10, 30, 60 mg/kg) increased immobility considerably ($P < 0.001$), ($P < 0.01$), ($P < 0.01$), respectively. Diazepam was also able to elevate immobility time ($P < 0.001$) (Figure 3c). In the second trial (day 5), acetonitrile (10, 30, 60 mg/kg) and diazepam treated rats decreased the swimming measure considerably ($P < 0.001$) (Figure 3a). The immobility duration was improved markedly in both the extracts 10 ($P < 0.001$), 30 ($P < 0.01$), 60 ($P < 0.001$) mg/kg and diazepam treated groups ($P < 0.001$) (Figure 3c). In the third trial (day 7), with acetonitrile, the extracts (10, 30, 60 mg/kg) noticeably shortened swimming ($P < 0.001$). Diazepam reduced this figure as well ($P < 0.01$) (Figure 3a). Only diazepam changed struggling significantly ($P < 0.05$) (Figure 3b). The immobility time demonstrated an overall increase regarding both the extracts 10, 30, 60 mg/kg and diazepam, ($P < 0.001$), ($P < 0.01$), ($P < 0.001$), ($P < 0.001$), respectively (Figure 3c). In the last trial (day 9), the extracts (10, 30, 60 mg/kg) reduced swimming significantly ($P < 0.05$), ($P < 0.05$), ($P < 0.01$), respectively (Figure 3a). The extracts 10 ($P < 0.001$), 30 ($P < 0.05$), 60 ($P < 0.01$) mg/kg prolonged the immobility in a significant manner (Figure 3c).

Effects of saffron extracts on chronic stress biomarkers in the rats

Plasma corticosterone concentrations were found to be significantly increased following chronic forced swimming stress sessions. In acetonitrile extract rats, corticosterone went down in a significant manner ($P < 0.001$). In 30 mg/kg dose (Figure 4b), the aqueous extract could reduce elevated corticosterone level ($P < 0.001$). On the other hand, both ethanolic and acetonitrile extracts were also able to decrease corticosterone markedly ($P < 0.001$) though not as well as diazepam. In 60 mg/kg dose (Figure 4c), the aqueous extract could reduce elevated corticosterone level ($P < 0.001$). Ethanolic extract was also able to decrease corticosterone markedly ($P < 0.05$) while it was not as good as diazepam and significantly had higher level ($P < 0.001$). Similarly, in acetonitrile extract rats, corticosterone went down in a significant manner ($P < 0.001$); however, it showed significantly more corticosterone concentrations comparing diazepam ($P < 0.001$).

Plasma LDL concentrations were found to be significantly increased following chronic forced swimming stress sessions. In dose 10 mg/kg (Figure 4a), aqueous extract could reduce LDL ($P < 0.05$), ethanolic extract was also able to decrease LDL markedly ($P < 0.01$), moreover, it had lower value compared to diazepam ($P < 0.05$). In acetonitrile extract rats, LDL went down in a significant manner ($P < 0.001$) as well; in addition, it showed significant

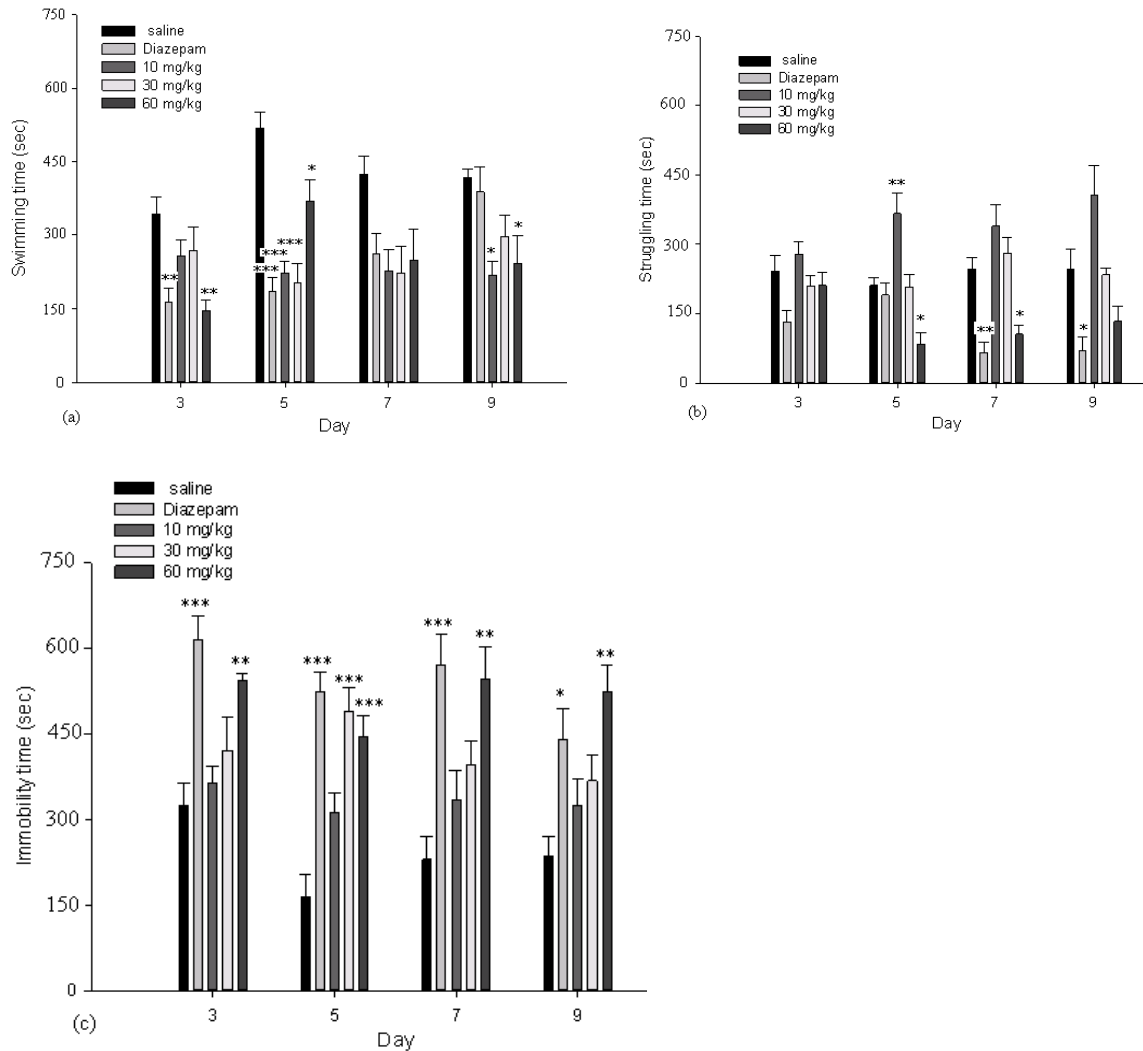


Figure 1. Effects of aqueous extracts of *C. sativus* (10, 30, 60 mg/kg) on swimming, struggling and immobility times in the forced-swimming stressed rats on days 3, 5, 7, 9 (after i.p. administration of *C. sativus* extracts). (a) Swimming. (b) Struggling. (c) Immobility. Values are means \pm S.E. of 6–8 rats. * Statistically significant difference from negative control group (saline) ($P < 0.05$).

significant difference with diazepam ($P < 0.05$). In dose 30 mg/kg (Figure 4b), aqueous extract could reduce elevated LDL level ($P < 0.01$), on the other hand, ethanolic extract was also able to decrease LDL markedly compared with both saline ($P < 0.001$) and diazepam ($P < 0.01$). Furthermore, in acetonitrile extract rats, LDL went down in a significant manner, comparing either saline ($P < 0.001$) or diazepam ($P < 0.01$). In dose 60 mg/kg (Figure 4c), aqueous extract could reduce elevated LDL level ($P < 0.01$), on the other hand, ethanolic extract was also able to decrease LDL markedly ($P < 0.001$), in addition, it demonstrated even lower value compared with diazepam ($P < 0.05$). Similarly, in acetonitrile extract rats, LDL went down in a significant manner ($P < 0.001$), it also showed significantly less LDL concentrations comparing diazepam ($P < 0.05$).

Plasma HDL concentrations were found to be significantly decreased following chronic forced-swimming stress sessions. In dose 10 mg/kg (Figure 4a), ethanolic extract, on the other hand was able to increase HDL markedly, compared with both saline ($P < 0.001$) and diazepam ($P < 0.001$). In acetonitrile extract rats, HDL also went up in a significant manner compared with both saline ($P < 0.001$) and diazepam ($P < 0.001$). The extracts in doses 30 and 60 mg/kg also demonstrated the same results as dose 10 mg/kg (Figure 4b and 4c).

Following administration of the extracts in dose 60 mg/kg (Figure 4c), aqueous extract could reduce elevated cholesterol level ($P < 0.001$), on the other hand, ethanolic extract was able to decrease cholesterol markedly ($P < 0.01$). Furthermore, in acetonitrile extract rats, cholesterol showed significantly less concentrations

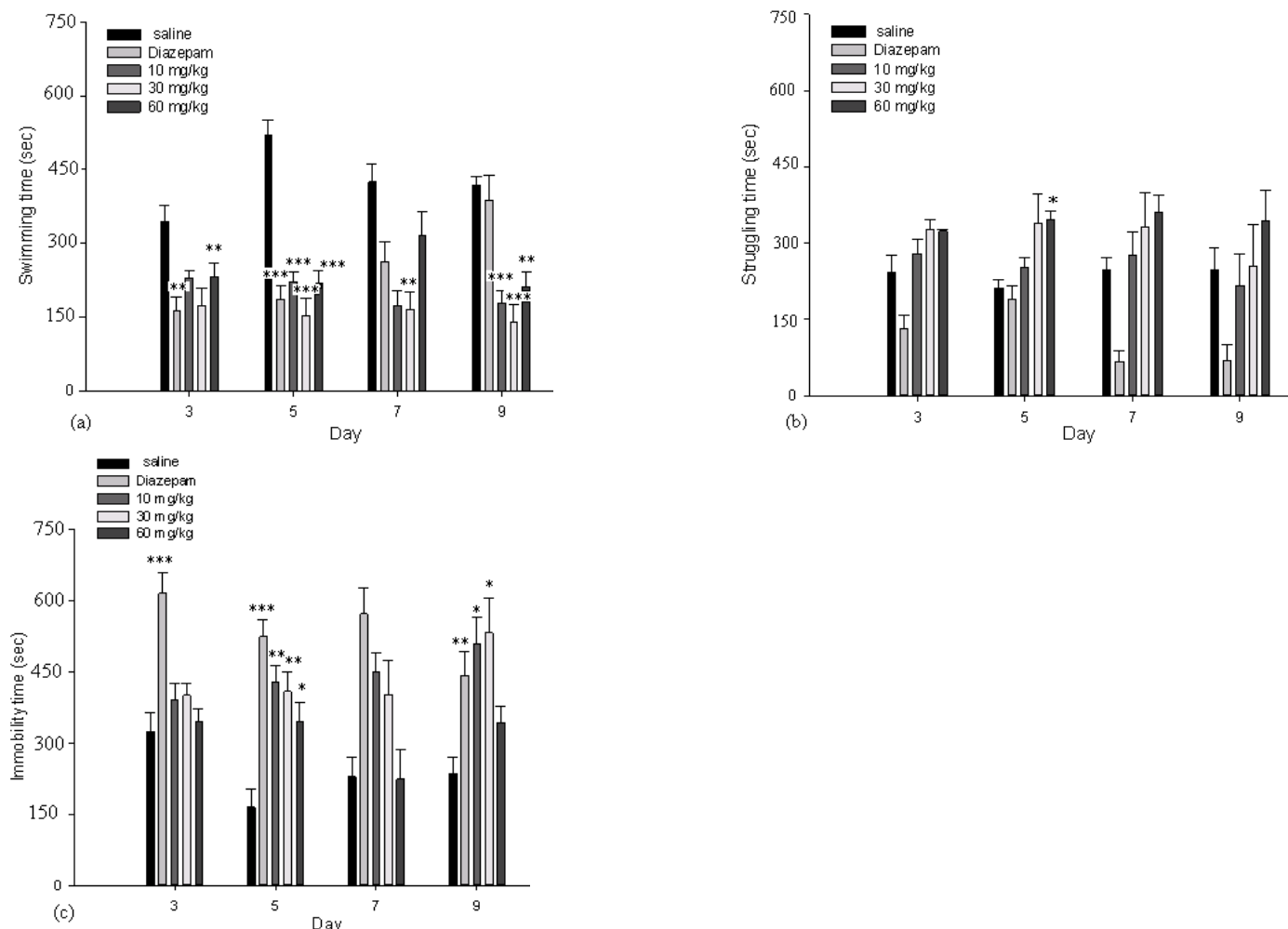


Figure 2. Effects of ethanolic extracts of *C. sativus* (10, 30, 60 mg/kg) on swimming, struggling and immobility times in the forced-swimming stressed rats on days 3, 5, 7, 9 (after i.p. administration of *C. sativus* extracts). (a) Swimming. (b) Struggling. (c) Immobility. Values are means \pm S.E. of 6–8 rats. * Statistically significant difference from negative control group (saline) ($P < 0.05$).

comparing diazepam ($P < 0.001$).

Plasma glucose concentrations were determined to be significantly increase compared following chronic stress sessions. In dose 10 mg/kg (Figure 4a), in acetonitrile-treated rats, glucose went down in a significant manner, compared with both saline ($P < 0.05$) and diazepam ($P < 0.01$). In dose 30 mg/kg (Figure 4b), aqueous extract could reduce elevated glucose level ($P < 0.001$). Furthermore, in acetonitrile extract rats, glucose went down in a significant manner comparing either saline or diazepam ($P < 0.05$). In dose 60 mg/kg (Figure 4c), aqueous extract could reduce elevated glucose level compared with negative control ($P < 0.001$). Furthermore, in acetonitrile extract rats, glucose went down in a significant manner ($P < 0.01$), it also indicated significantly lower glucose concentrations comparing diazepam ($P < 0.001$).

HSP70 factor were proved to be significantly increased as a stress marker following chronic stress sessions. In dose 10 mg/kg (Figure 4a), ethanolic extract was able to decrease HSP70 markedly ($P < 0.01$) while it was not as

well as diazepam and significantly had higher level ($P < 0.001$). In acetonitrile extract rats, HSP70 went down in a significant manner ($P < 0.001$). In dose 30 mg/kg (Figure 4b), aqueous extract could reduce elevated HSP70 level ($P < 0.001$), on the other hand ethanolic extract (30 mg/kg) was also able to decrease HSP70 markedly ($P < 0.001$). Furthermore, in acetonitrile extract rats, HSP70 went down in a significant manner ($P < 0.001$). In dose 60 mg/kg (Figure 4c), aqueous extract could reduce elevated HSP70 level ($P < 0.001$), on the other hand, ethanolic extract was also able to decrease HSP70 markedly ($P < 0.05$). Furthermore, in acetonitrile extract rats, HSP70 went down in a significant manner ($P < 0.001$).

HPLC results

Five distinct concentrations of crocin and safranal solutions were prepared for determination of the

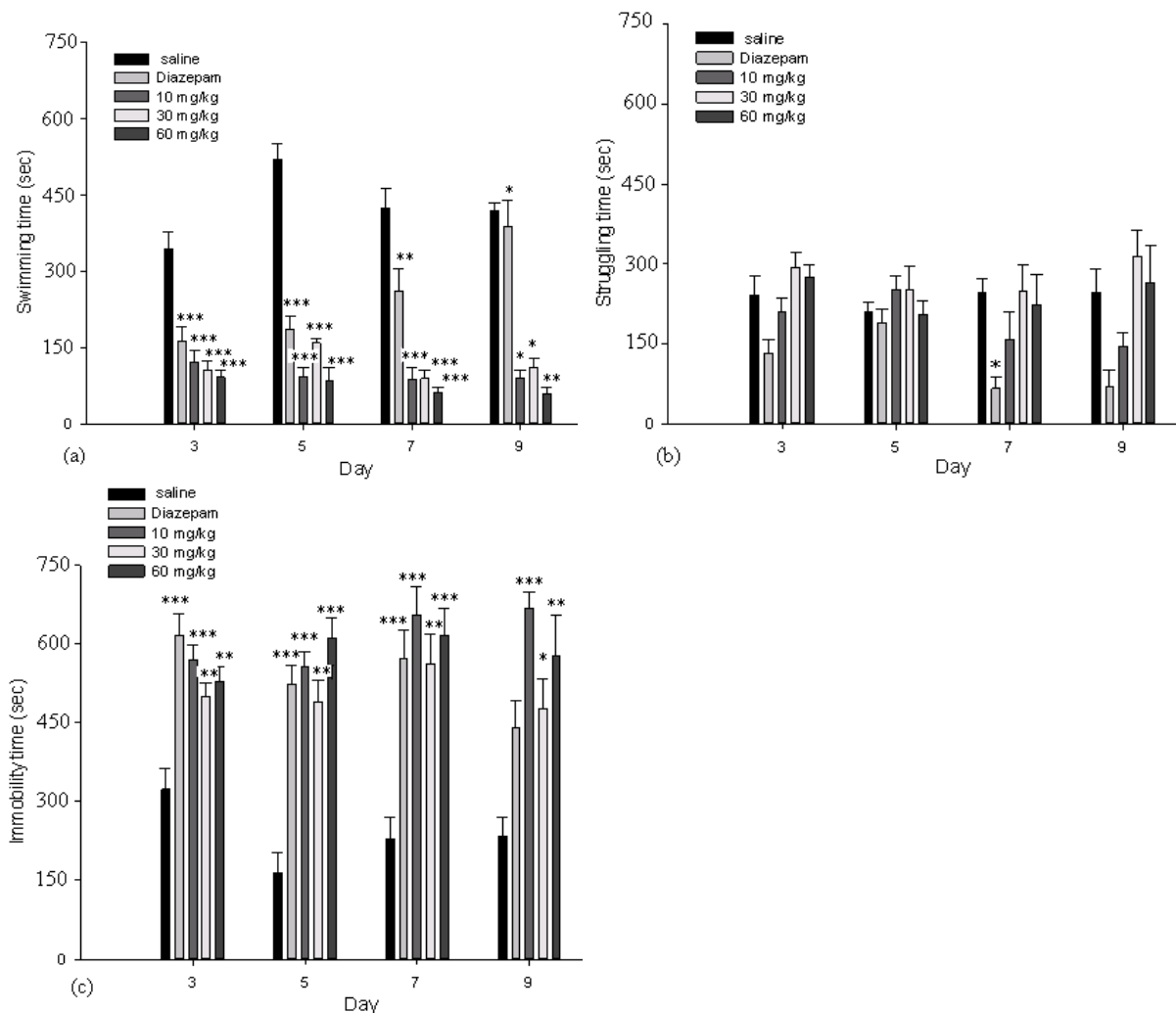


Figure 3. Effects of acetonitrile extracts of *C. sativus* (10, 30, 60 mg/kg) on the swimming, struggling and immobility times in forced-swimming stressed rats on days 3, 5, 7, 9 (after i.p. administration of *C. sativus* extracts). (a) Swimming. (b) Struggling. (c) Immobility. Values are means \pm S.E. of 6–8 rats. * Statistically significant difference from negative control group (saline) ($P < 0.05$).

calibration curve. The calibration curve was constructed with crocin and safranal content versus peak area $Y = 3586x + 8395$, $R_2 = 0.999$; $Y = 50467x + 25385$, $R_2 = 0.999$, respectively and each injection repeated three times. The method used in this work for quality determination of saffron is reverse-phase high-performance liquid chromatography (HPLC) with UV/Visible detector. This technique is the most efficient analytical method for the analysis of sensitive compounds in complex extracts of natural products.

At 440 nm, seven types of crocins were detected in all three chromatograms. In spite of our expectation, Fluka product was not a pure crocin. AUCs and percent of crocin types in chromatogram of Fluka sample at 440 nm in a gradient method were shown in Table 1. AUCs and percent of crocin in chromatogram of samples obtained by HPLC at 440 nm in a gradient method. The area under curve (AUC) of crocin in fluka sample was 68.40% of total

area of crocin types. Only the main corresponding crocin constituent at Rt 6.183 min was used in the measurement of plant ingredient, since many of these degradation products do not exist in plant extracts. According to the HPLC method, the highest amount of crocin was found in ethanolic extract ($W = 8.338$ mg/ml) and the lowest amount in acetonitrile extract ($W = 0.178$ mg/ml).

At 310 nm, safranal was seen in time = 10.10 minutes in chromatogram in SAFC sample. Our results indicated the highest amount of safranal among of all analyzed extracts was found in acetonitrile ($W = 7.647$ μ g/ml) and the lowest one was in aqueous extract ($W = 0.357$ μ g/ml) (Table 2).

DISCUSSION

We examined the capability of saffron extracts on

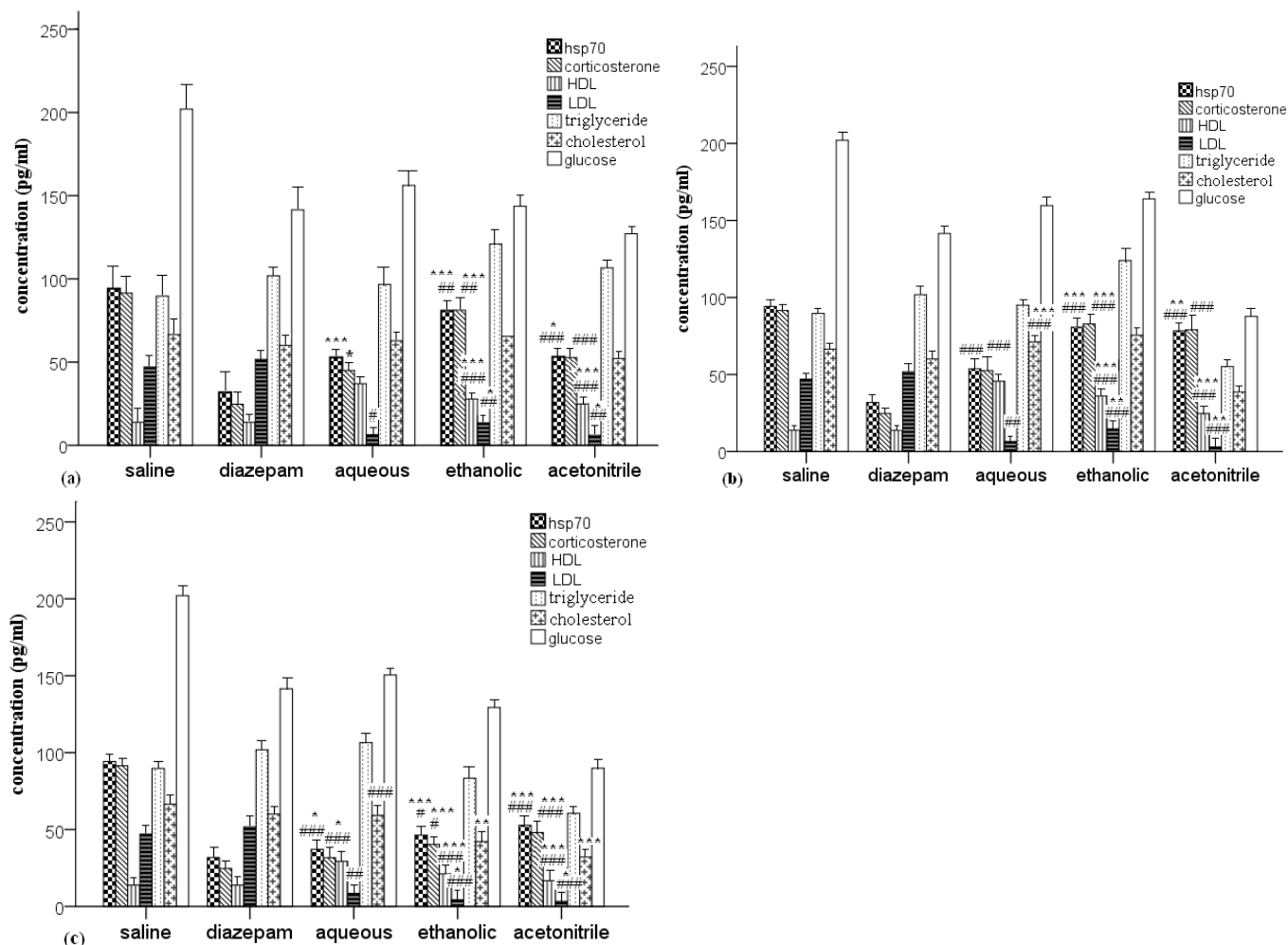


Figure 4. Effects of aqueous, ethanolic and acetonitrile extracts of *C. sativus* (10 mg/kg) (a), (30 mg/kg) (b) and (60 mg/kg) (c) on HSP70, corticosterone, LDL, HDL, cholesterol and glucose in the forced-swimming stressed rats. Values are means \pm S.E. of 6–8 rats. * Statistically significant difference from negative control group (saline) ($P < 0.05$).

Table 1. AUCs and percent of crocin in chromatogram of samples obtained by HPLC at 440 nm in a gradient method.

Compound	Retention Time (min)	Area peak	Area (%)	Peak height
Crocin 1	3.400	20363	1.0094	2706
Crocin 2	6.183	1379904	68.4026	154106
Crocin 3	6.633	118920	5.8949	15163
Crocin 4	7.117	220997	10.9550	24494
Crocin 5	7.500	30667	1.5202	3045
Crocin 6	7.800	188635	9.3507	20535
Crocin 7	8.433	28913	1.4333	2183

Table 2. The amount of crocin and safranal obtained from HPLC analyses.

Extract	Crocin (mg/ml)	Safranal (μ g/ml)
Aqueous	3.145	0.357
Ethanolic	8.338	1.423
Acetonitrile	0.178	7.647

probable protective alterations in metabolic, hormonal and behavioral responses of rat submitted to forced-swimming stress and demonstrated their sedative and antianxiety-like properties. Our findings are consistent with previous studies in terms of stress reduced immobility and increased active behaviors in the forced-swimming test (Harro and Orelund, 2001; Häidkind et al., 2003). The present investigation suggests saffron has an antianxiety-like behavior mediated by raising the time spending immobile and lowering the time performing the active behavior swimming, without affecting struggling figure. In addition, reducing the stress biomarkers, virtually identical to diazepam. On the whole, there was not an obvious dose-response manner in our results and some times lower doses demonstrated even better outcome. Diazepam is known to prevent environmental stress (Gesi et al., 1999). We achieved antianxiety-like behavior results in our experiments and employed diazepam as reference drug and our results were found to be comparable and in some concentrations equivalent, with a better tolerability of saffron, suggesting the effect of the extracts on swimming behavior is possibly linked to an anti-stress property.

Swimming exercise has been considered an important emotional stress stimulus, capable of increasing corticosterone and catecholamine plasma concentrations (Yang et al., 1993). Forced swimming stress can cause an acute release of ACTH and corticosterone from the hypothalamus and this has been confirmed in our serum analysis. The effects of stress on plasma corticosterone levels and the effectiveness of saffron extracts in inhibiting these effects have been achieved in our experiment.

In the present study, plasma corticosterone was elevated after chronic exposure to swim stress, supporting the idea that a stress inducing component of forced swimming is present throughout the training period. In agreement with our data, saffron extract and crocin inhibited corticosterone secretion in stressed rats (Lechtenberg et al., 2008). Moreover, saffron ethanolic extract and crocin might also reduce adrenocorticotrophic hormone release from the pituitary gland. These effects can be mediated by brain stress systems involving the amygdale (Gallagher et al., 2008) and hypothalamus (Adam and Epel, 2007).

It has been suggested that the effects of saffron extract and crocin may also be mediated by central mechanisms. The extracts may interact with NMDA and sigma-opioid receptors, and these interactions reduced the effects of stress on the HPA axis. The other possible mechanism might be the interaction of one or more constituent of the extracts with other brain or periphery structures to diminish side effects of stress (Sahraei et al., 2012).

Virtually consistent with our study, Sahraei et al. (2012) showed ethanolic extract as well as crocin reduced stress induced by electric foot-shock test, they examined effects of them on chronic stress-induced metabolic and

hormonal changes in dopamine-related behavior in rats. They emphasized plasma corticosterone level elevation, anorexia, induced by stress in rats were reversed by ethanolic extract and crocin. In another study, saffron water extract and safranal could reduce the metabolic and hormonal disturbances induced by electric foot-shock in rats (Hooshmandi, 2011).

In the light/dark test, either crocins (50 mg/kg), or diazepam (1.5mg/kg), significantly increased the latency to enter the dark compartment and prolonged the time spent in the lit chamber in the rats. The results indicate that treatment with these active constituents of *Crocus sativus* L. induce antianxiety-like effects in the rat (Pitsikasa et al., 2008). Our findings appear to be in agreement with these observations. The pharmacological mechanism(s) that might account for the anti-anxiety effect of saffron has yet to be determined. Further studies will be required to assess the generality of the present findings to other species and behavioral paradigms.

However, we compared different kind of extracts in a different stress model and measured another stress biomarkers as well as corticosterone. The analyzed biomarkers in our experiment showed changes in the rats submitted to chronic swim stress. Therefore, these biomarkers seem to be suitable to more accurately interfere in the stress levels of rats exercised in swimming. We indicated reduction in serum glucose, LDL, cholesterol levels and HSP70 marker in hippocampus, and also increase in HDL after treating with the extracts. Eighty percent of the cholesterol used in steroid synthesis is derived from the plasma LDL (Mello et al., 2004). Therefore, it is possible that the higher synthesis of corticosterone in our stress group had been supported by the plasma cholesterol (LDL). In our measurements HSP70 has shown a significant reduction following extracts treatment.

It has been shown that forced swim stress activates rat hippocampal serotonergic neurotransmission involving a corticotrophin-releasing hormone receptor-dependent mechanism. Forced swimming evoked a marked increase in hippocampal 5-HT levels (Linthorst et al. 2002). It has stated the swimming behavior is related with serotonin system and that increase in this neurotransmitter leads to an increase in swimming measure (Häidkind et al., 2004). As our extract decreased swimming measure significantly, it is probable that they influenced the serotonergic system. Crocin and safranal inhibit reuptake of dopamine, norepinephrine and serotonin as well (Karimi et al., 2001).

For qualitative and quantitative determination of saffron components in plant extracts, a variety of techniques including HPLC have been used over the last ten years (Castellar et al., 1993). However, according to the method of extraction, and HPLC technique applied, evaluated concentrations of secondary metabolites in saffron's stigma tissues varies to a large degree. As a result, we compared three different kinds of extract.

Consistently, we proved discrepancy in our distinct extracts and achieved different proportion of crocin and safranal in each and accordingly different level of stress inhibition. Using HPLC method, we indicated crocin is the most abundant metabolite in our saffron stigma extracts (mg/ml), especially in ethanolic one and the lowest in acetonitrile extract.

Our results also indicated the highest amount of safranal among of all the analyzed extracts in acetonitrile and the lowest in aqueous extract. Although all the extracts exhibited sedative properties, having the highest amount of safranal and lowest amount of crocin, acetonitrile extract was proved the most effective anti-stress action in our experiments. It also prolonged the immobility time, most strongly. These findings were in agreement with our behavioral, biochemical and hormonal achievements in current experiment.

Aqueous, ethanolic and acetonitrile extracts of *C. sativus* L. showed stress-reducing actions in different degrees by reducing the active behavior, swimming, without marked changes in struggling. While prolonging immobility and at the same time reducing serum glucose, corticosterone, free fatty acids and HSP70 protein which elevated during stressful situations.

Conclusions

Having significantly less side effect compared with chemical drugs, saffron and its active constituents have the potential to be employed in clinical trial. Saffron is a promising herb due to its diverse medicinal properties. The results are encouraging to pursue further studies on the other bioactivity guided fractionation of these extracts to isolate and describe probable bioactive molecules. Together, our data revealed the extracts may have peripheral and central impacts. However, further studies are needed to extend these results and clarify the underlying mechanisms.

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