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Effect of Ethanol Extract of Saffron (*Crocus sativus* L.) on the Inhibition of Experimental Autoimmune Encephalomyelitis in C57bl/6 Mice

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Abstract: In this study, effect of ethanol extract of Saffron (*Crocus sativus* L.) in the treatment of Experimental Autoimmune Encephalomyelitis (EAE) in C57BL/6 mice was evaluated. EAE was induced by immunization of 8 week old mice with MOG₃₅₋₅₅ with complete Freund's adjuvant. Therapy with saffron was started on day the immunization. Total Antioxidant Capacity (TAC) was assessed by Ferric Reducing-Antioxidant Power (FRAP) method. Nitric oxide (NO) production was also estimated by Griess reaction. For histological analysis, mice brain was harvested and sections were stained with Hematoxylin-Eosin. After daily oral dosage the saffron significantly reduced the clinical symptoms in C57BL/6 mice with EAE. Also, treated mice displayed a delayed disease onset compared with control mice. TAC production was significantly elevated in saffron treated mice. Effect of saffron on serum NO production was not significant. Typical spinal cord leukocyte infiltration was observed in control mice compared with saffron treated mice. These results suggest for the first time that saffron is effective in the prevention of symptomatic EAE by inhibition of oxidative stress and leukocyte infiltration to CNS and may be potentially useful for the treatment of Multiple Sclerosis (MS).

Key words: Saffron (*Crocus sativus* L.), experimental autoimmune encephalomyelitis, nitric oxide, total antioxidant capacity, C57BL/6 mouse

INTRODUCTION

Multiple Sclerosis (MS) is an inflammatory demyelinating disease of the Central Nervous System (CNS) that afflicts more than million people worldwide (Dean, 1994). There is no medical treatment available so far than can cure MS (Clegg and Bryant, 2001). Experimental Autoimmune Encephalomyelitis (EAE) is the most useful model of MS (Gold *et al.*, 2000). The pathogenesis of EAE comprises immunization with encephalitogenic myelin antigens in the presence of adjuvant, presentation of such antigens to T cells, migration of activated T cells to the CNS and development of inflammation and/or demyelination upon recognition of the same antigens (Owens and Sriram, 1995). An important aspect of the pathogenesis of EAE, with potential for therapeutic manipulation, is the role of free radicals such as nitric oxide (NO) in the inflammatory process (Staykova *et al.*, 2006; Teixeira *et al.*, 2005; Dalton and Wittmer, 2005). Thus a scavenger of Reactive Oxygen Species (ROS) is expected to prevent these free radical mediated EAE. There is increasing interest in the protective biological function of natural antioxidants

contained in dietary plants, which are candidates for the prevention of oxidative damage (Aniya *et al.*, 2005).

Saffron is obtained from the flowers (dried, dark red stigmata) of *Crocus sativus* L. (Iridaceae) and is cultivated widely in Iran, Spain, France, Italy, Greece, Turkey, India and China (Evans, 2002). Saffron is employed mainly to give color and flavor to foods and also is a valuable herbal drug that is used in traditional medicine for treating numerous diseases such as amenorrhea, bronchitis, sore throat, vomiting and fever and also as an analgesic, antispasmodic, sedative, stomachic and abortifacient (Ody, 2000; Dyle *et al.*, 2001). Modern pharmacological studies have demonstrated that saffron has cancer chemopreventive, tumoricidal, anti-arthritis, antihypertensive, hypolipidemic, antioxidant, radical scavenger, antinociceptive and anti-inflammatory effects (Abdullaev, 2002; Hosseinzadeh and Younesi, 2002).

The chief constituents of saffron are the carotenoids crocin and crocetin and the monoterpene aldehydes picrocrocin and safranal (Jafarova *et al.*, 2002). The carotenoids are the main components responsible for the pharmacological effects of saffron (Rock, 1997; Handelman, 2001).

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However, no earlier study has examined the use of saffron in the treatment of MS or other TH1 cell-mediated inflammatory diseases of the CNS. In the present study, was examined the effect of saffron on the day of onset and severity of EAE, NO production, total antioxidant capacity and leukocyte infiltration into the brain.

MATERIALS AND METHODS

Mice: Inbred male C57BL/6 mice were obtained from the Pasture Institute of Iran, at 8 weeks age. The mice (weighting about 20 ± 2 g) were randomly divided into two groups, each with 10 mice. The mice were housed at the animal house at Immunologic research center in accordance with all Institutional Ethical and Health guidelines. All studies were performed with approval from the Animal Ethics Committee of University of Medical sciences. This study was conducted in Arak University of Medical Sciences from August 2007 to November 2008.

Induction of Experimental Autoimmune Encephalomyelitis (EAE): Male C57BL/6 mice (8 weeks age and 20 ± 2 g weight) were inoculated subcutaneous in the flank with 100 μ L of an emulsion containing 200 μ g of the encephalitogenic peptide MOG35-55(MEVGWYRSPFSRVVHLYRNGK; Diapharm Ltd., Russia) and equal volume of complete Freund's adjuvant (Sigma, St. Louis, MO) supplemented with 4 mg mL⁻¹ *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI, USA). Mice were then injected intraperitoneally with 400 ng of pertussis toxin (Sigma) on the day of immunization and 2 days later (Mosayebi *et al.*, 2007).

Preparation of the extracts: Stigmas of *Crocus sativus* that had been collected in December 2006 in Ghaen (Khorasan Province, Northeast of Iran) were used and were identified by the Department of Biology of Arak University, Arak, Iran. Saffron extract was prepared by percolation method of Phrompittayarat *et al.* (2007). The stigma's extract was prepared as follow: the stigma was dried under shade and 5 g of dried and milled stigmas were extracted with 200 mL ethanol (80%) by percolation procedure in three steps then the ethanolic extract was dried by evaporation in temperature 35°C. The extract was stored in -4°C from where it was used when required. This extract was suspended in Double Distilled Water (DDW) and the soluble fraction of the extract determined. The concentration was adjusted by addition of vehicle if necessary before injecting the mice.

Saffron treatment: All mice were fed for the duration of the experiment with tap water and mouse fodder. In the experimental group (n = 10), mice were given 100 μ L saffron extract by oral gavage (500 mg kg⁻¹ per day) (Abdullaev, 2002). Control mice (n = 10) were given 100 μ L DDW by oral gavage. Saffron and DDW gavage were started on day the immunization.

Clinical evaluation of EAE: Following the encephalitogenic challenge, mice were monitored daily and neurological impairment was scored on an arbitrary clinical score as follows: (0) no clinical sign; (1) partial loss of tail tonicity; (2) complete loss of tail tonicity; (3) flaccid tail and abnormal gait; (4) hind leg paralysis; (5) hind leg paralysis with hind body paresis; (6) hind and foreleg paralysis and (7) moribund or death. The relapse was defined when a mouse developed an increase of the clinical score (more than 1) accompanied by weight loss. For each animal a Cumulative Disease Index (CDI) was calculated from the sum of the daily clinical scores observed between day 7 and day 21. Under recommendation of the animal ethics committee, mice were killed on 21day post immunization (Niino *et al.*, 2001).

Brain histology: On day 21 (sacrifice day) the brain were taken from saffron-treated and control EAE mice, fixed in 10% buffered formalin and embedded in paraffin. Sections (5 μ M thick) were cut and mononuclear cell infiltration was visualized by Hematoxylin-Eosin (H and E). Briefly, H and E staining sections were air-dried, fixed in 10% formaldehyde, dehydrated, stained for 1 min with hematoxylin, then 2 min with eosin (Sigma), then dehydrated and mounted in Permount. Semi-quantitative histological evaluation was performed using the following scored based on the severity of inflammation; 0: no inflammation; 1: cellular infiltrates only in the perivascular areas; 2: mild cellular infiltrates in parenchyma (1-10/section); 3: moderate cellular infiltrates in parenchyma (11-100/section) and 4: marked cellular infiltrates in parenchyma (>100/section) (Okuda *et al.*, 2002).

Total antioxidant capacity: TAC was measured by FRAP (Ferric reducing antioxidant power) method (Benzie and Strain, 1996). In brief, three reagents were used: (1) sodium acetate, acetic acid buffer (pH 3.6); (2) 10 mM solution of 2, 4, 6-tripyridyl-s-triazine in a 40 mM solution of hydrochloric acid (Sigma, St. Louis, MO) and (3) 20 mM solution of ferric chloride hexahydrate prepared in deionized water. The FRAP reagent was prepared daily with 25 mL of reagent one, 2.5 mL reagent two and three that were heated to 37°C before using. Sample was added

to reagent in cuvettes with an autosampler and then read on a spectrophotometer at 593 nm at 4 min FeSO₄ was used as a standard and antioxidant power expressed as μM FRAP.

Nitric oxide (NO) production: NO production was measured by determining the increase in nitrite concentration in serum using the Griess reaction adapted to microliter plates (Kayhan *et al.*, 2003). Briefly, 100 μL serum was mixed with 100 μL Griess reagent (equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthylethylenediamine-HCl), in flat bottomed 96 well microliter plates and incubate at room temperature for 10 min and then the absorbance at 550 nm was measured using a microplate reader (Stat-Fax, 2001). The amount of nitrite in the samples (in micro molar units) was calculated from a sodium nitrite standard curve freshly prepared in distilled water. Nitrite concentration expressed as μM.

Statistical analysis: Statistical analysis was performed with the Student's t-test for the mean maximal score, mean onset day, histological score and level of nitrite and TAC in serum. All data are expressed as Mean±SD. A p<0.05 was considered significant.

RESULTS

In the current study, we investigated the effect of saffron administration on EAE. Immunization with 150 μg of MOG₃₅₋₅₅ peptide induced severe EAE in control mice. Figure 1a and b shows the effect of saffron on body weight and clinical scores in saffron-treated and control EAE mice. The Cumulative Disease Index (CDI) for saffron-treated mice was significantly lower than that of control EAE mice (22±1.8 and 29.2±2.4, respectively) (Table 1).

Histological findings: To examine the effect of saffron on amelioration of EAE, we histologically analyzed the brain of control and saffron-treated EAE mice. For this study, ten mice in each group were administrated with either saffron (500 mg/kg/day) or DDW from on day the first MOG immunization up to the sacrificed day (day 21 after immunization). As shown in Fig. 2a-c, the brain of mice treated with saffron showed less cell infiltrates than that in control mice. To evaluate quantitatively the inflammatory lesions, the mean lesion area was calculated as described in materials and methods. Table 2 shows that a significant reduction in the mean lesion area in saffron-treated group is demonstrated compared to that in control group (p<0.05).

TAC and NO concentration: Sera obtained from normal mice and both control and saffron -treated EAE mice at day 21 after immunization were analyzed for nitrite oxide production and TAC (Table 2). The results show that the level of nitrite in saffron-treated mice was more than that of control mice, although this difference was not significant. The mean TAC of the saffron-treated group was significantly more than that of control group (p<0.05).

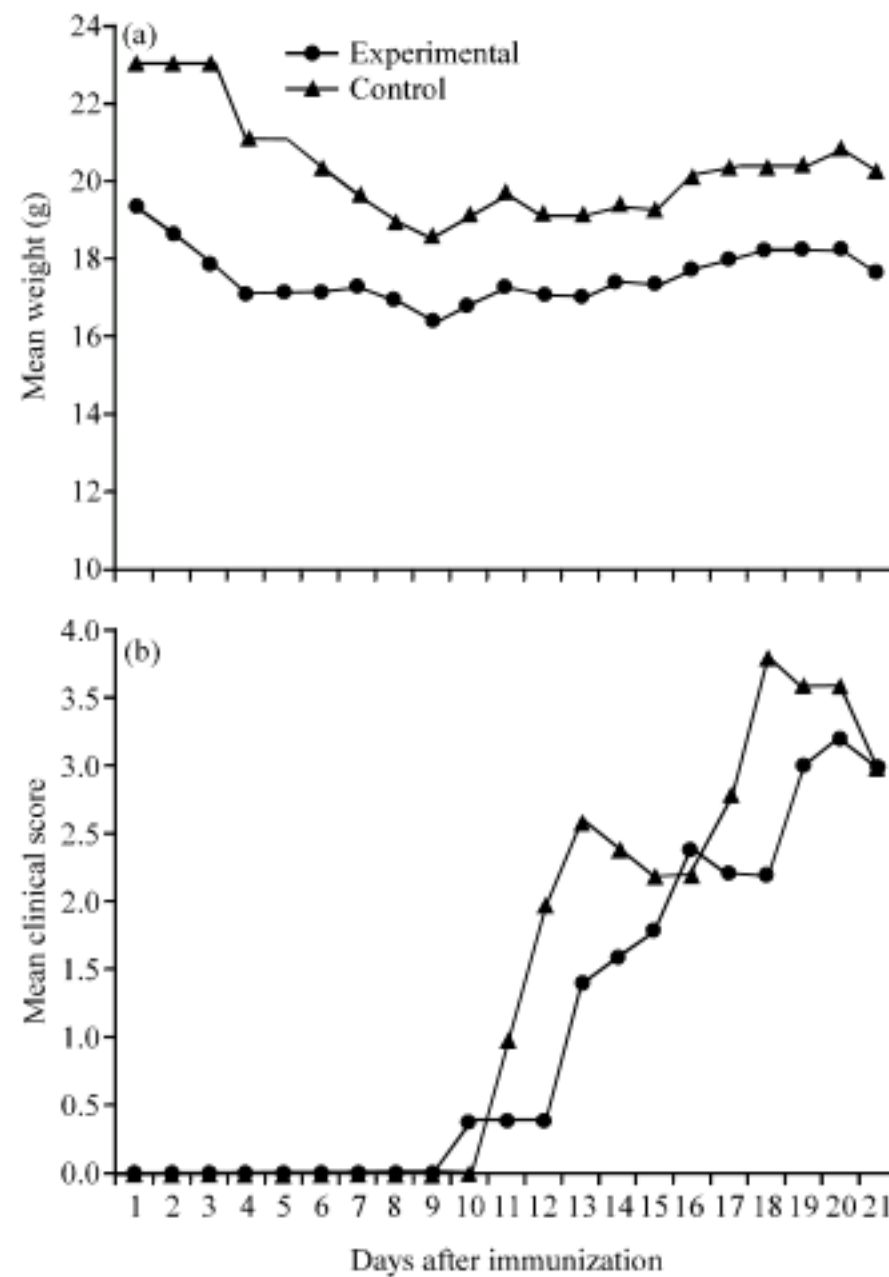


Fig. 1: (a) Mean body weight and (b) clinical course of MOG₃₅₋₅₅-induced EAE in C57BL/6 mice administrated saffron or vehicle. Mice were monitored daily for symptom and scored as described in materials and methods. Values are expressed as Mean±SD for the 10 mice tested daily in each group

Table 1: Clinical features of EAE in control and saffron treated mice

Immunization	No. of mice	Incidence of EAE (%)	Day of onset	Maximum clinical score	Cumulative Disease Index (CDI)
MOG-EAE+ DDW(Control)	10	100	11±1	3.8±0.7	29.2±2.4*
MOG-EAE+ saffron (experimental)	10	100	13±1	3.2±0.6	22.0±1.8

*Significantly different than control, p<0.05. Data are expressed as Mean±SD

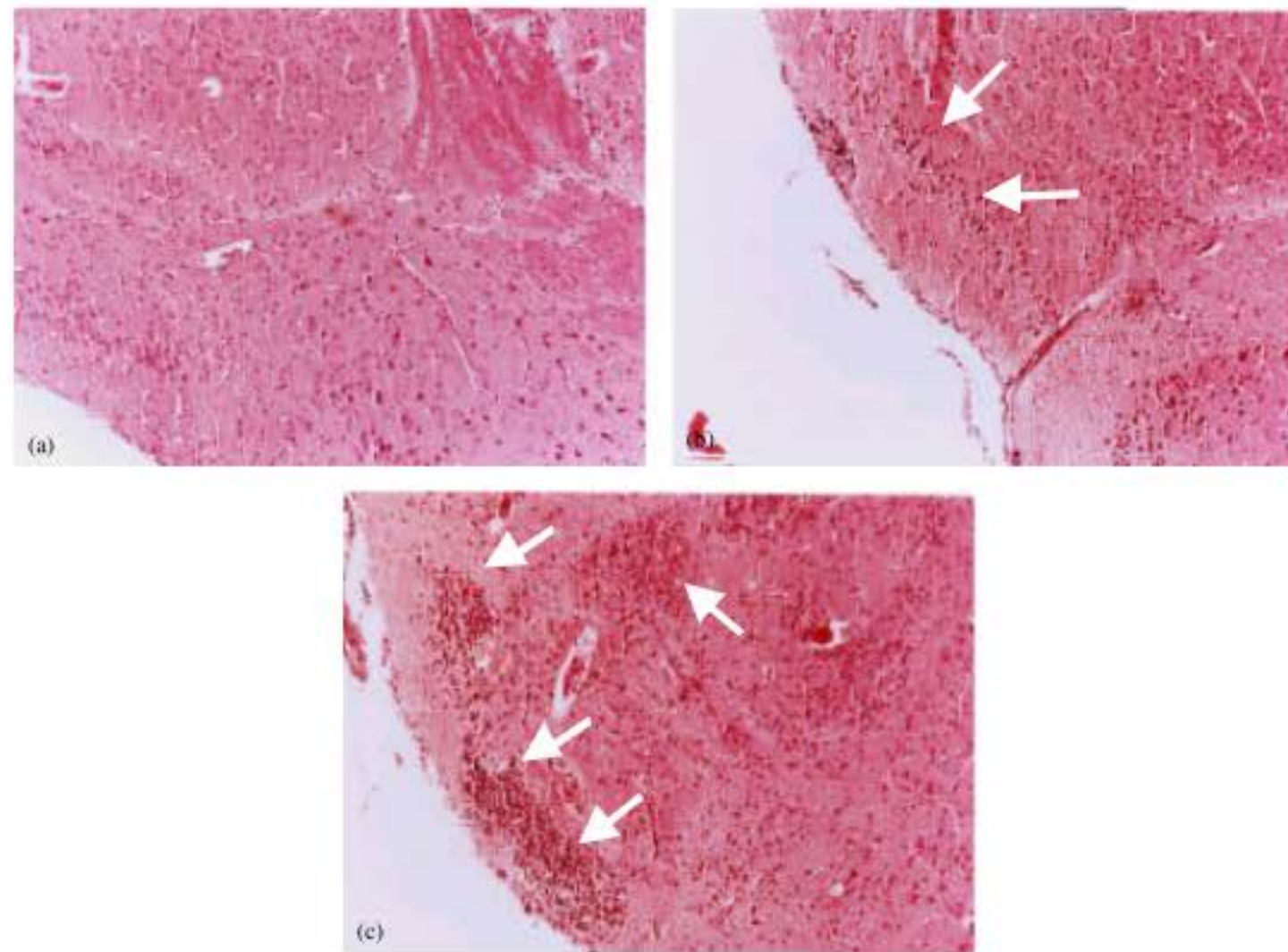


Fig. 2: Brain sections from (a) normal mice and saffron and (b) MOG-induced EAE mice treated with DDW (c) were stained with Hematoxylin and Eosin (H and E) for leukocyte infiltration. Leukocyte infiltrates are less evident in the saffron-treated mice (c) in comparison with control mice (b)

Table 2: Histological score, nitrite and TAC levels in control and saffron treated mice

Parameters	Saffron-treated mice	Control mice
Histological score of the brain	2.4±1.02*	3.4±1.34
Nitrite concentration ($\mu\text{mol L}^{-1}$)	24.0±5.1	20.0±4.4
Total antioxidant capacity (TAC) ($\mu\text{mol L}^{-1}$)	590.0±20*	360.0±40

Data are expressed as Mean±SD (n = 10), *Significantly different than control, p<0.05

DISCUSSION

The search for new and more effective therapeutic agents includes the study of plants used in traditional medicine systems to treat neurological disorders (Richelson, 1994).

In this study, we have demonstrated a protective effect of saffron on EAE induced in C57BL/6 mice. Saffron delayed the disease onset of EAE and reduced the maximum clinical scores. The Mean cumulative disease index of saffron-treated mice was significantly lower than that of control mice. The overall course of EAE was significantly reduced in saffron-treated mice compared to controls. The mechanism by which saffron exhibits its observed beneficial effect is still unknown, but some studies showed that saffron has potent anti-inflammatory and antioxidant effects.

Free radicals such as nitric oxide (NO) may be important in the pathogenesis of EAE and MS. NO is an

important mediator implicated in pathophysiological processes. Various investigations have indicated the high expression of iNOS and excessive amount of NO production in the pathogenesis of murine inflammatory CNS demyelination (Okuda *et al.*, 1995). Furthermore, an excessive increase of NO production was found in both the CSF and the peripheral blood of MS patients (Yuceyar *et al.*, 2001). In contrast, protective role has been demonstrated for NO in EAE (Willenborg *et al.*, 1999). NO has been shown to inhibit the cytotoxic effects of superoxide and hydrogen peroxide to neuronal cells. NO may also react with peroxy radicals such as lipid peroxy radicals and inhibit free radical chain reactions such as lipid peroxidation. Lipid peroxidation plays an important role in pathogenesis of EAE and MS (Hogg, 1995).

Because of the pathogenic role of oxygen and nitrogen free radicals in MS pathology, antioxidants might prevent free radical-mediated tissue destruction and inhibit some of the early pro-inflammatory events, such as T cell trafficking into the CNS, that lead to inflammation and tissue destruction in EAE and MS (Mosayebi *et al.*, 2007).

Thus, with consider of increase stress oxidative in multiple sclerosis and EAE, saffron as an antioxidant agent may suppress clinical score of EAE by affecting nitric oxide production and total antioxidant capacity

(TAC) of serum. Present results showed that saffron significantly increases TAC in serum of EAE mice but serum nitrite production was unaffected.

Also, histological examination of brain tissues demonstrated reduced levels of infiltration of leukocytes in the saffron-treated EAE mice. One mechanism that might lead to reduced severity of induced EAE in saffron-treated mice is reduced leukocyte infiltration into the CNS. Saffron may exert beneficial effects as it may interfere with the inflammatory cell recruitment to the infiltration of encephalitogenic T cells and may protect blood-brain barrier breakdown. Saffron has anti-inflammatory effect and it has been suggested that saffron or constituents of saffron induces growth arrest and apoptosis of cancer.

Rink *et al.* (1998) demonstrated that consumption of carotenoids in adults reduces the secretion of pro-inflammatory cytokines such as tumor necrosis factor- α (Rink *et al.*, 1998). It is clear that the role of the Th cells in these effects of saffron should not be disregarded. Future studies about the effects of saffron on the pattern of cytokines secreted by the Th cells may disclose the mechanisms of the effects of saffron.

To best of the knowledge, this study is the first clinical trial of saffron in the treatment of EAE induced in C57BL/6 mice so it is not possible to draw any comparisons with others trials. The limitation of the present study, including using only a fixed dose of saffron should be considered so further research in this area is needed.

CONCLUSION

The results of this study indicate the efficacy of *Crocus sativus* L. in the treatment of EAE induced in C57BL/6 mice. These properties of saffron may be mediated, at least in part, by its inhibiting oxidative stress and leukocyte infiltration to CNS. According to these findings, saffron may be potentially useful for the treatment of MS.

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