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Original article

Effects of saffron and estrogen on cognition and glial cells in a menopausal demyelination model

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Abstract

Objective: Crocus sativus (Saffron) is a spice that has neuroprotective properties. The present study aimed to investigate how saffron and estrogen might affect behavior, the number of glial fibrillary acidic protein (GFAP)-positive astrocytes and the number of oligodendrocyte transcription factor 2 (Olig2)-positive cells in menopausal, demyelinated rats.

Materials and methods: Seventy-five female Sprague-Dawley rats weighing 200-250 g were divided to five group; Control, Treatment Control 1, Treatment Control 2, Saffron treatment and Estrogen Treatment. Demyelination was induced by a single injection of 3 µl of 0.01% ethidium bromide (EB) into the dentate gyrus (DG). Saffron aquatic extract dose was 60 mg/kg/day given orally for 21 days and estrogen dose was 5 µg/kg/day given for 7 days. By ovariectomy (OVX), both ovaries were removed. Cognitive behaviors were assessed using the Morris water maze (MWM) test. Immunohistochemistry and ImageJ software quantified the numbers of GFAP- and Olig2-positive cells.

Results: Demyelination resulted in impaired hippocampal histopathology. It also caused deficits in MWM performance. The extent of blood-brain barrier (BBB) disruption was significantly greater ($p<0.05$) in the treatment control groups compared to the other groups. The numbers of GFAP-positive astrocytes and Olig2-positive oligodendrocytes were significantly reduced ($p<0.01$) in both treatment groups relative to the other groups

Conclusion: Results indicate that saffron and estrogen have similar effects on reducing demyelination impairment. They improve histopathological defects, MWM performance, and BBB dysfunction. This may be related to the changes in the number of GFAP- and Olig2-positive cells.

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Introduction

Multiple sclerosis (MS) is frequently diagnosed in women during their reproductive years, with many patients entering the menopausal transition while living with the disease. Estrogen and progesterone levels reduction in menopause, lead to symptoms such as hot flushes, sleep disturbances, mood changes, cognitive impairment, and fatigue. These manifestations often overlap with or exacerbate MS-related symptoms, particularly fatigue and cognitive dysfunction, making clinical differentiation challenging. Approximately 30% of the MS population consists of peri- or postmenopausal women (Bove et al. 2022).

The higher prevalence of MS in women and its progression further underscore the role of hormonal influences in the disease pathophysiology. Consequently, hormone-based interventions have been explored as potential therapeutic strategies. Menopausal hormone therapy has been shown to alleviate vasomotor symptoms, mood disturbances, and sleep disorders, which may indirectly improve MS-related symptom burden (Juutinen et al. 2022).

A shared pathological mechanism linking menopause and MS is increased oxidative stress. Oxidative stress damages proteins, lipids, and DNA within neurons and glial cells, ultimately impairing cellular function and promoting neurodegeneration (Maldonado et al. 2023; Pinar-Morales *et al*. 2025). Therefore, interventions aimed at reducing oxidative stress may offer therapeutic benefits in both conditions. In this context, medicinal plants have attracted attention due to their antioxidant properties. *Crocus sativus* (saffron) and its bioactive compounds—crocin, crocetin, and safranal—exhibit potent antioxidant, anti-inflammatory, and neuroprotective effects (Golpour- Hamedani et al. 2024). Saffron and its bioactive compounds have demonstrated therapeutic potential in a wide range of disorders including neurodegenerative diseases, psychological disorders, and cardiovascular conditions (Bian et al. 2020). In MS models, saffron has been shown to enhance total antioxidant capacity and reduce inflammatory damage (Zha et al. 2022), while clinical studies indicate its beneficial effects on mood and well-being in postmenopausal women (Delam et al. 2023).

Cognitive dysfunction is a prominent feature of both MS and menopause and significantly impacts quality of life. The Morris Water Maze (MWM) is a test for assessing spatial learning and memory, functions critically dependent on hippocampal integrity (Othman et al. 2022). Declining estrogen levels during menopause are associated with hippocampal dysfunction and cognitive complaints, and estrogen-based hormone therapy is commonly used to mitigate these effects (Conde et al. 2021). In MS, early detection of cognitive impairment is essential for timely intervention and disease management (Portaccio and Amato 2022).

At the cellular level, astrocyte activation and oligodendrocyte dysfunction play central roles in MS pathology. Glial fibrillary acidic protein (GFAP) is a key marker of astrocytes and has emerged as a reliable biomarker of diffuse white matter damage in MS (Saraste et al. 2021). Brain injury and neuroinflammation lead to astrocyte activation, making GFAP a valuable indicator of disease progression. Recent findings indicate that reduced estradiol levels in women with MS are associated with increased brain atrophy and elevated serum GFAP levels, reflecting enhanced astrogliosis and possibly explaining the accelerated disease progression observed after menopause (Juutinen et al. 2024).

In parallel, oligodendrocytes are responsible for myelination in the central nervous system, and their loss is a hallmark of MS. Oligodendrocyte transcription factor 2 (Olig2) is critical for remyelination (Wegener et al. 2015). Experimental evidence suggests that saffron extract can increase Olig2 expression, thereby promoting remyelination (Azari et al. 2018). Conversely, reduced Olig2 expression has been reported in menopause models, potentially contributing to impaired myelination and cognitive decline. Given the regulatory influence of estrogen on Olig2 expression, hormonal disruption during menopause may heighten susceptibility to demyelination and neurodegenerative processes in women (Gannon et al. 2023).

To model MS-like demyelination experimentally, ethidium bromide (EB) is commonly used due to its ability to induce oligodendrocyte apoptosis, and subsequent neuroinflammation. This model provides a valuable platform for investigating the combined effects of demyelination and menopause, as well as evaluating potential therapeutic interventions (Goudarzvand et al. 2016).

Accordingly, the present study aimed to investigate the effects of saffron and estrogen on spatial learning and memory performance in the MWM, as well as on the number of GFAP-positive astrocytes and Olig2-positive cells in the hippocampus of rats with menopause-associated demyelination. By examining saffron and estrogen separately, this study sought to clarify their individual contributions to cognitive function, glial activation, and remyelination in the context of the combined pathology of menopause and MS.

Materials and Methods

Materials

Ethidium bromide (Sigma-Aldrich, E2129), Saffron (Ghaen in Khorasan province, Iran), Estrogen (Sigma-Aldrich, E8875), ketamine (Alfasan, Netherlands), and xylazine (Alfasan, Netherlands).

Animals and housing conditions

Adult female Sprague–Dawley rats (200–250 g) were obtained from the Laboratory Animal Center of Shiraz University of Medical Sciences, Iran, and housed under standard conditions. The animals had free access to food and water. Animal procedures were reviewed and approved by the institutional ethics committee of the School of Veterinary Medicine, Shiraz (98INT1M1755).

Study design

Rats were randomly divided into five groups, n=15 in each group (Each 5 rats were used for histology, GFAP immunohistochemistry, and Olig2 immunohistochemistry):

1- Control group; rats received 3 µl of artificial cerebrospinal fluid in the dentate gyrus (DG) + 1% ethanol with the same volume as saffron for 21 days.

2- Treatment Control 1; received 3 µl 0.01% EB injection in the DG for a single time without treatment.

3- Treatment Control 2; rats received 3 µl 0.01% EB injection into the DG for single time + ovariectomy (OVX) without treatment.

4- Saffron treatment; rats received 3 µl 0.01% EB injection into the DG one time + OVX + saffron (60 mg/kg/day for 21 days).

5- Estrogen Treatment; rats received 3 µl 0.01% EB injection into the DG for one time + OVX + the estrogen (5 µg/kg/day for 7 days (Samantaray et al. 2011)).

Saffron and water were given by gavage. The estrogen was administered intraperitoneally (IP injection). Study design is depicted in Figure 1. OVX was performed according to a reference (Taherianfard et al. 2012).

Preparation of the saffron extract

In the present study, high-quality saffron stigmas were collected from Ghaen in Khorasan province of northeastern Iran (voucher number 55130). To prepare saffron extract, 1 g of dried saffron stigma powder was combined with 25 ml of distilled water and was soaked in a shaker jar for 3 days in a dark, cool environment. After filtration and centrifugation, 5 ml of the supernatant was frozen in universal jars and then freeze-dried to obtain powder. These powders were stored in a freezer until further use. At the time of administration, the saffron extract 60 mg/kg was administered by gavage.

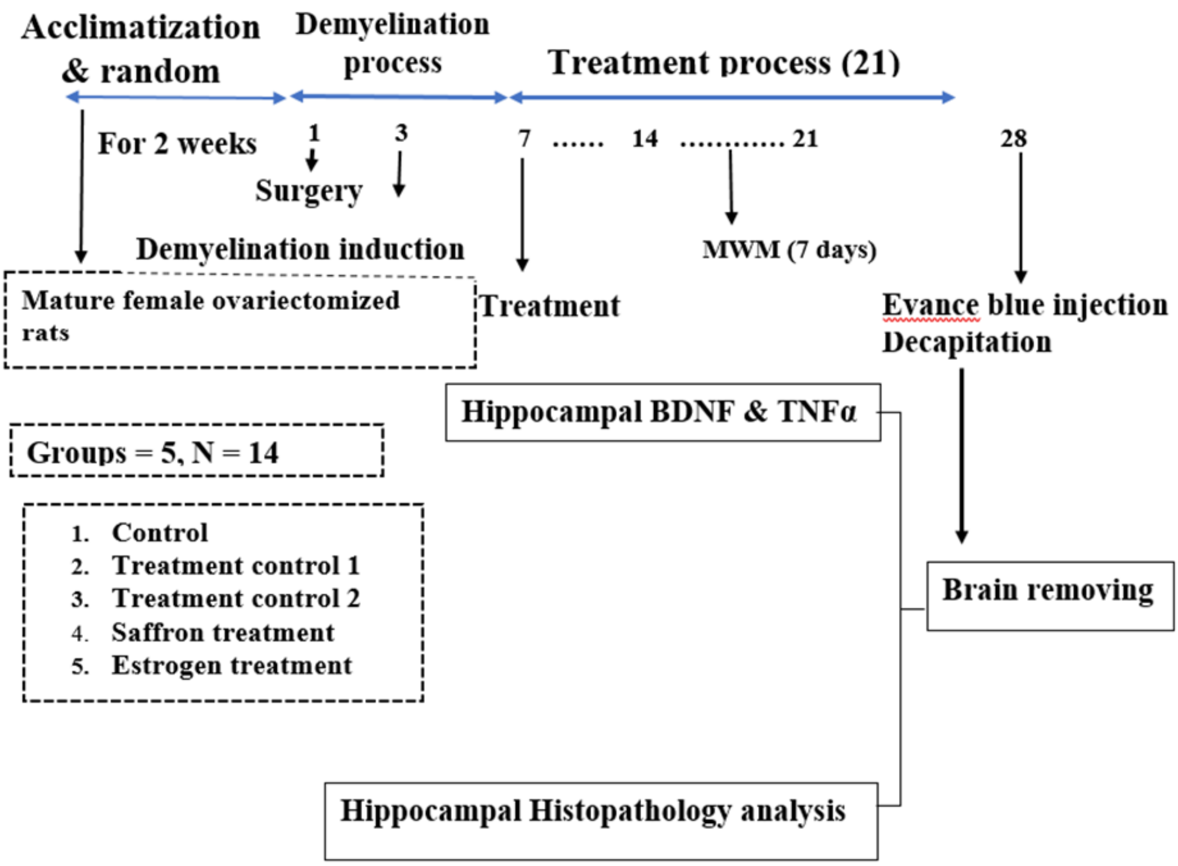


Figure 1. A schematic study design

Ovariectomy procedure

For ovariectomy (OVX), rats were anesthetized via intraperitoneal (IP) injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). The abdominal area was cleaned and disinfected with a scrub solution. Under sterile conditions, a 1 to 1.5 cm midline abdominal incision was made with a surgical blade 10. The abdominal muscles were retracted, and after locating the uterine bifurcation, the ovaries were carefully separated from the uterus with minimal incision. Following OVX, the inner abdominal area and skin were sutured using 3-0 suture thread.

Stereotactic surgery and induction of demyelination

Rats were anesthetized by ketamine (100 mg/kg) and xylazine (10 mg/kg) (Alfasan, Netherlands). For cannula implantation into the DG, the rats were placed in a stereotaxic apparatus (Stoelting; Wood Dale, IL, USA). The stereotaxic coordinates according to the rat brain atlas were AP = -3.96 mm; ML = -2 mm; DV = -4.3 mm from the cortex (Paxinos and Watson 2007). Here, 3 µl of 0.01% EB was injected into the right DG for induction of demyelination (Goudarzvand et al. 2010). The rats were allowed to recover from the surgery for one week.

MWM apparatus

The water maze used in the present study is a dark, circular pool (150 cm diameter and 60 cm height) filled with water to a depth of 45 cm. A black metal platform (10×10 cm^2) was placed in the center of one of the arbitrarily determined quadrants in the northeast (NE), southeast (SE), southwest (SW), or northwest (NW) directions. The platform was the only means of escape from the water. There were four clues on the walls of the water maze. The cues were fixed in position during the study so that all rats could use the same visual cues. A Charge-Coupled Device camera (Panasonic Inc., Japan) was suspended from the ceiling above the MWM tank. It recorded swimming and movement patterns which were measured by a video tracking system for automatic analysis of animal behavior using Neuro-Vision software.

MWM test

The first three consecutive days are considered visible platform sessions. The platform was visible and located in the center of one of the fourth quadrants. On the first day, each rat was allowed to swim for 90 sec to locate the platform. If the rat could not reach the platform within 90 sec, it had to be led to the platform by the experimenter. After finding the platform, the rats remained on the platform for 60 sec. On the second three days, the platform is placed in the center of one of the four quadrants. The time for finding the platform and resting on the platform was 60 and 30 sec, respectively. On the seventh day, the probe test was conducted in the absence of the platform. However, the position of the platform is fixed in the neuro-vision program. For the recorded traces, the latency to reach the target quadrants was calculated for subsequent analysis. Each training session consisted of 4 trials per day (trial interval: 2 min). On each training trial, cognitive performance was scored so that higher cognitive strategies received a higher score. The scaling was as follows: thigmotaxis = 1, random = 2, chaining = 3, focused = 4, corrected = 5, and direct = 6 (Illouz et al. 2016).

Histological evaluation

Hematoxylin-eosin and Luxol fast blue staining: At the end of the experiment, rats were deeply anesthetized with a high dose of ketamine (150 mg/kg) based on, in accordance with ethical guidelines for humane endpoint for histological examination. The heart was then perfused with 20 ml of 4% formaldehyde as a fixative solution. After initial fixation, the animals' brains were carefully removed from the skull and processed into tissue blocks. Sections 3–5 µm thick were then prepared using a microtome. The sections were stained with hematoxylin and eosin (H&E) and Luxol Fast Blue. Histometric analysis was performed using a light microscope equipped with a Dino-Lite digital objective and DinoCapture 2 software (AnMo Electronics Corp., Taiwan). GFAP density in astrocytes and Olig2 density in oligodendrocytes of DG were measured using the Image Analyzer software (version 1.33), which determines the density according to three factors, including hue, saturation, and intensity, and displays a number that has an inverse relationship with the protein (GFAP) density. In other words, a lower number in the program represents a higher protein density and vice versa. ImageJ software was used to assess the extent of demyelination (Goudarzvand et al., 2010; Negah et al., 2015).

Immunohistochemistry procedure

Tissue sections from the hippocampal cortex (3 µ) were deparaffinized in xylene, rehydrated through a graded alcohol series, and washed with Phosphate-buffered saline PBS. Following these procedures, the appropriate antigen retrieval procedure was performed to detect GFAP and Olig-2 factors in oligodendrocytes and astrocytes.

Statistical analysis

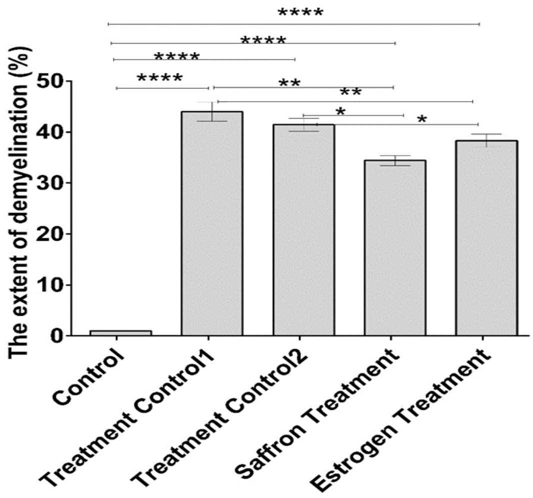
SPSS (version 26) was used for statistical analysis. Normality was assessed using the Kolmogorov–Smirnov test. Data were analyzed using one-way analysis of variance (ANOVA) and repeated-measures ANOVA, followed by Tukey's post hoc test. Statistical significance was set at $p<0.05$. All results are presented as mean ± SEM.

Results

Effect of saffron and estrogen on demyelination induced by EB

Figure 2 shows that the percentage of demyelination in the control is close to zero. Demyelination in the other groups was 44±2%, 41.4±1.3%, 34.4±1%, and 38.3±1.3% in the treatment controls 1 and 2, saffron, and estrogen treatments, respectively. Demyelination in the treatment control groups was significantly (p=0.0035) higher than the other groups.

a



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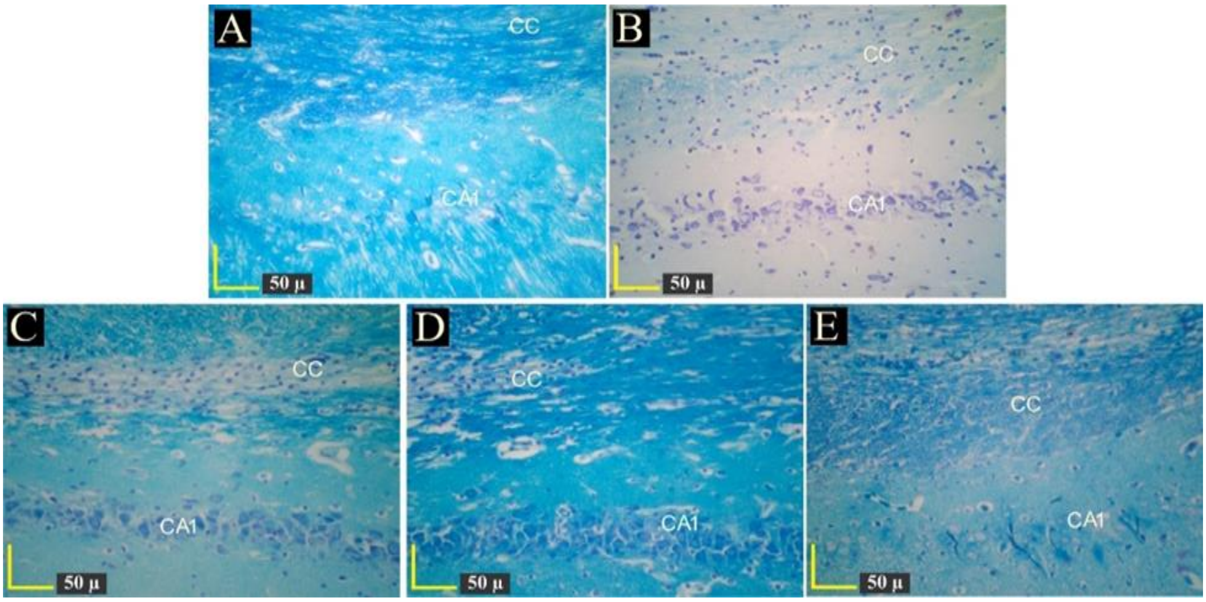


Figure 2. a; The extent of demyelination in CA1 and CC, different characters represent significant differences between the groups. b; Photomicrograph of CA1 and CC in all groups. A (Control); B (Treatment Control1); C (Treatment Control2); D (Saffron Treatment); and E (Estrogen Treatment). Data were represented as mean±SEM; Magnification 10×10 and n=5.

Effect of saffron and estrogen on gliosis and GFAP-positive astrocytes

Figure 3 shows that after EB injection into the DG, the most significant changes were gliosis, characterized by an increase in astrocyte numbers and reactive changes. Immunohistochemical staining for GFAP revealed that the number of GFAP-positive astrocytes in the DG was significantly lower in the both treatment control compared to the control (p<0.001) and the saffron and estrogen treatment (p=0.035). The number of GFAP-positive CA1 astrocytes in the both treatment control, and estrogen treatment, significantly was lower than in the control (p=0.0058) and saffron treatment (p=0.018) (Figure 4).

Effect of saffron and estrogen on Olig2-positive oligodendrocytes

Immunohistochemical staining for Olig2 showed that the number of Olig2-positive oligodendrocytes in CA1 was significantly (p=0.0009) lower in both treatment control than the other groups. The number of Olig2-positive oligodendrocytes in the DG was significantly higher (p=0.0008) in the estrogen treatment compared to the other groups. The number of Olig2-positive oligodendrocytes of the corpus callosum (CC) in the both treatment control was significantly lower than the estrogen (p=0.006) and saffron treatment (p=0.025) (Figure 5).

a

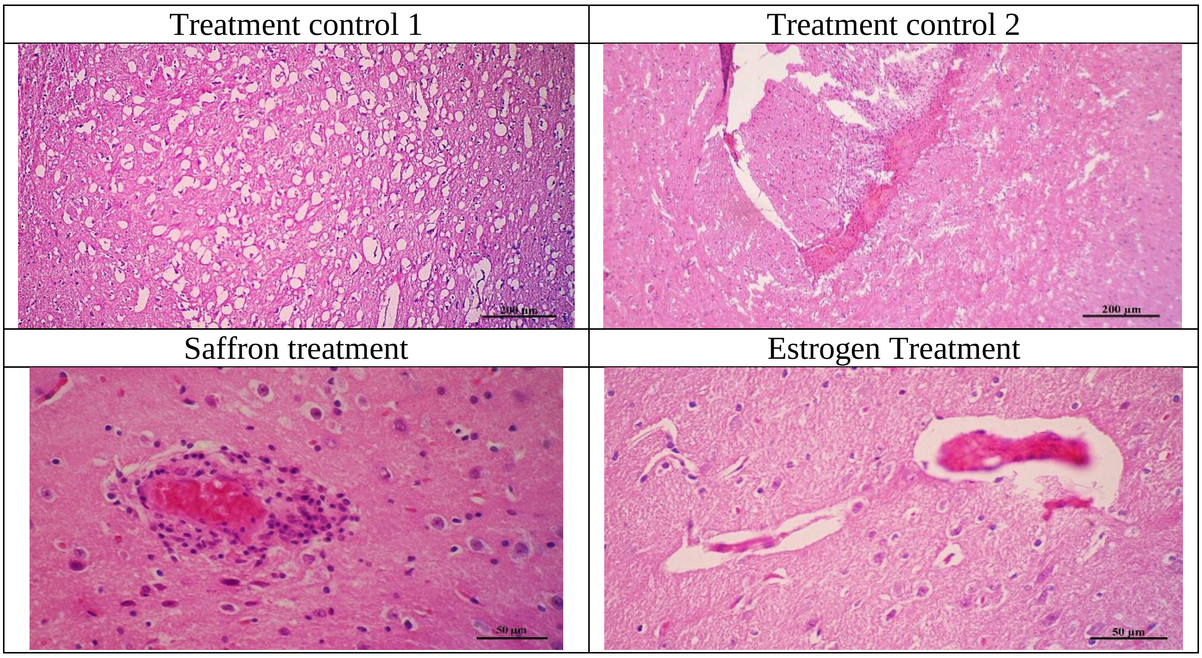
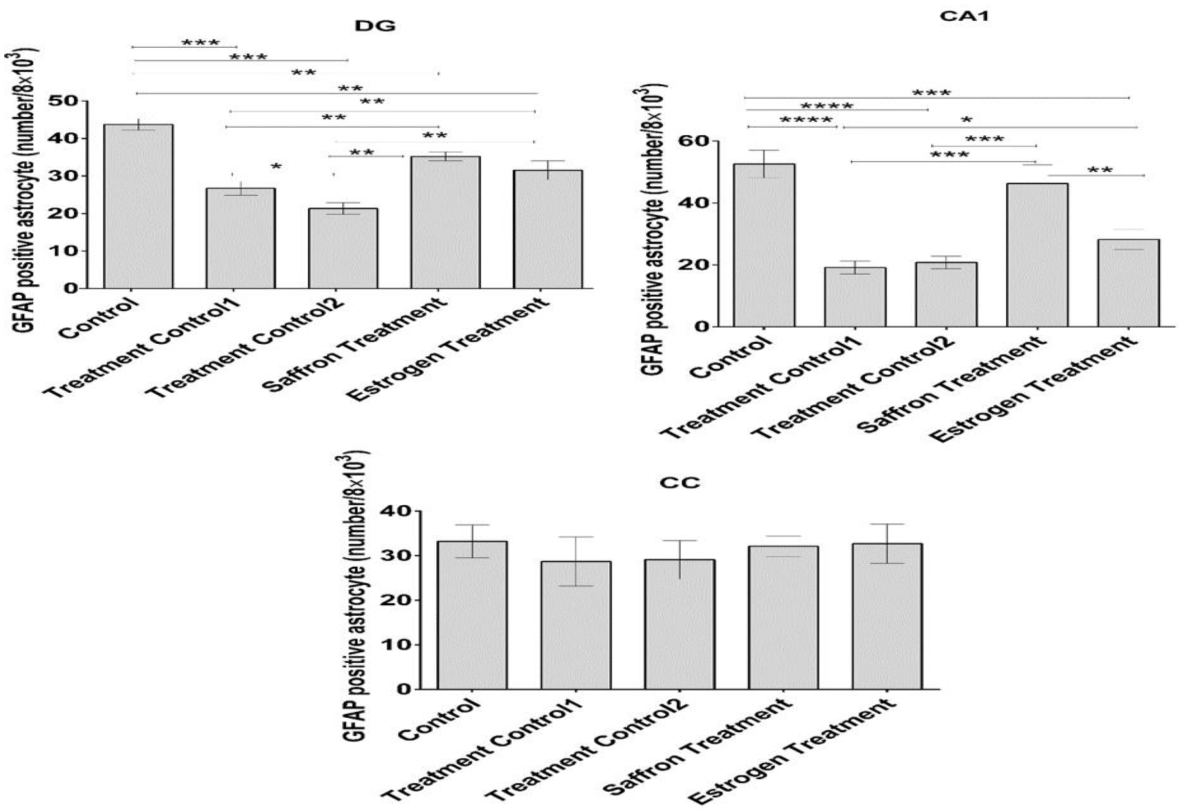


Figure 3. Effect of saffron and estrogen on histopathology of the brain in MS induced by EB. Magnification for up column photo10×10 and for down column photo 10×40, n=5.

a



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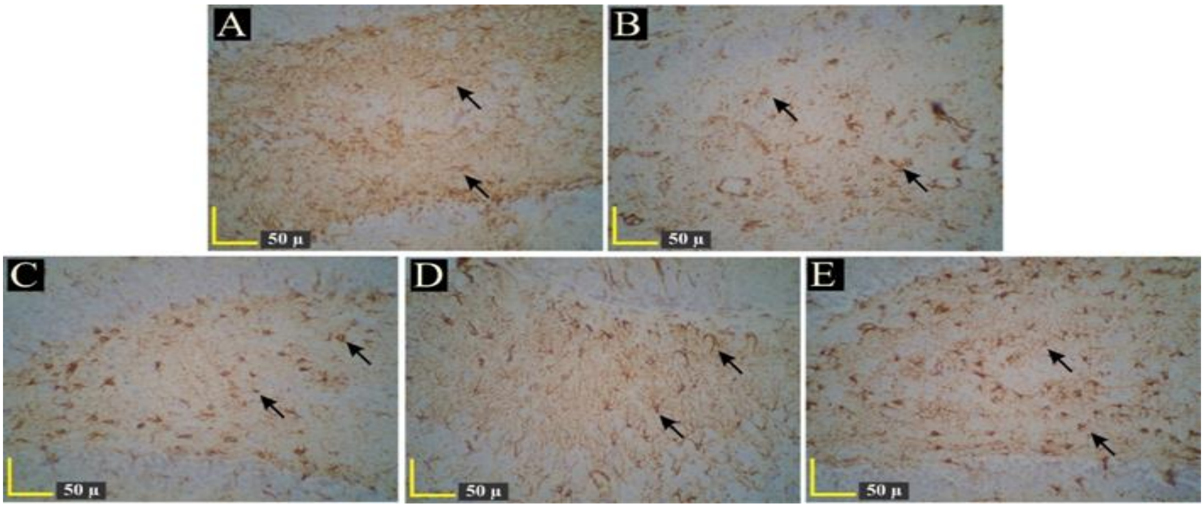
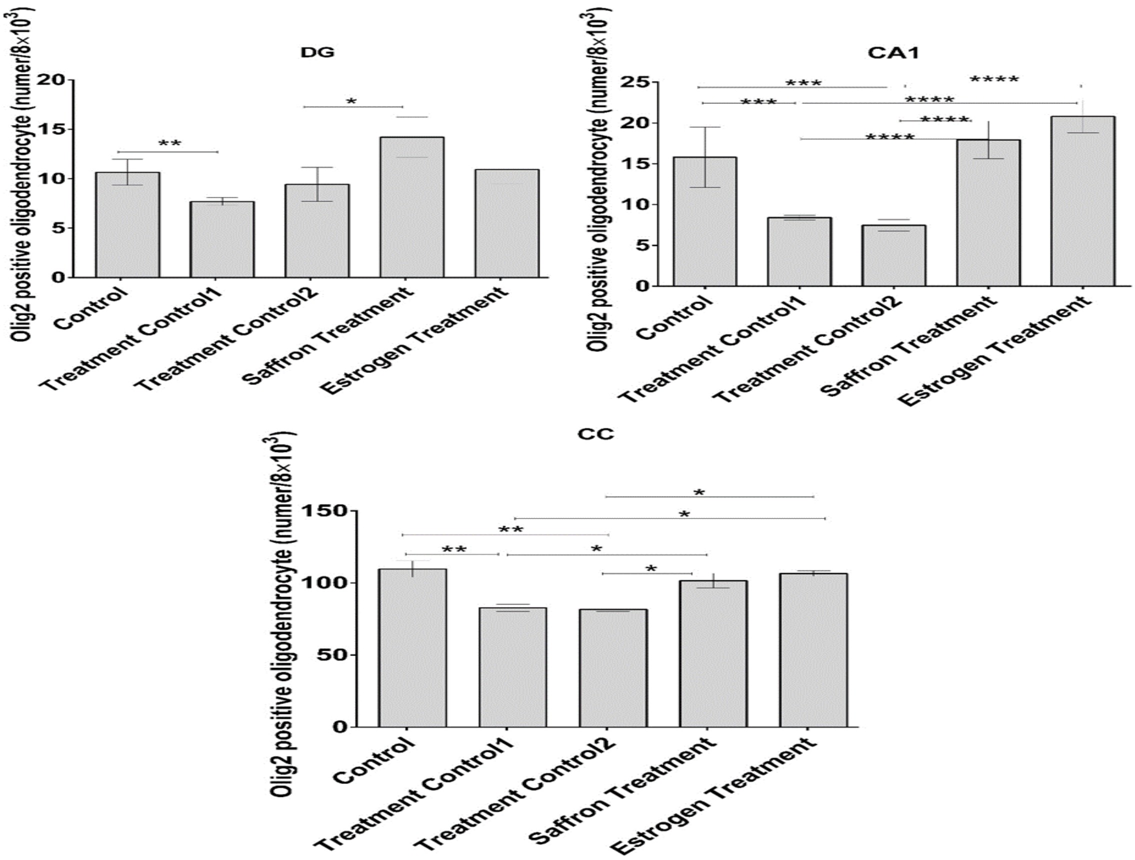


Figure 4. a; Effect of saffron and estrogen on GFAP-positive astrocytes of the DG, CA1, and CC in EB-induced MS. Different characters represent significant differences between the groups. b; Photomicrograph of GFAP-positive astrocytes in the DG of all groups. A (Control); B (Treatment Control1*); C (Treatment Control2**); D (Saffron Treatment); and E (Estrogen Treatment). * Received 3 µl 0.01% EB injection in the DG for a single time without treatment. ** Rats received 3 µl 0.01% EB injection into the DG for single time + ovariectomy (OVX) without treatment. Data were represented as mean±SEM; Magnification 10×10, n=5.



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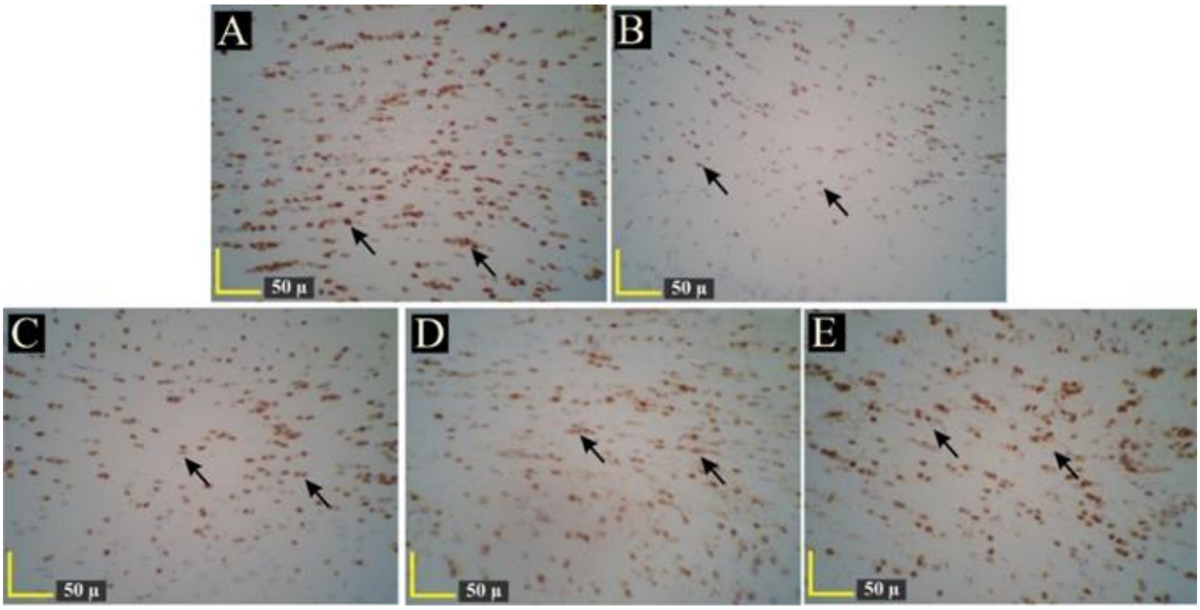

Figure 5. a; Effect of saffron and estrogen on Olig2-positive oligodendrocytes of the DG, CA1, and CC in EB-induced MS. Different characters represent significant differences between the groups. b; Photomicrograph of Olig2-positive oligodendrocytes in DG. A (Control); B (Treatment Control1*); C (Treatment Control2**); D (Saffron Treatment); and E (Estrogen Treatment). * Received 3 µl 0.01% EB injection in the DG for a single time without treatment. ** rats received 3 µl 0.01% EB injection into the DG for single time + ovariectomy (OVX) without treatment. Data were represented as mean±SEM; Magnification 10×10, n=5.

Effect of saffron and estrogen on the density of GFAP and Olig2

Figure 6 shows the density of GFAP in astrocytes and Olig2 in oligodendrocytes. It shows that the density of GFAP and Olig2 was significantly (p=0.000) lower in two treatment control groups compared to other groups.

Effect of saffron and estrogen on latency time and cognitive behaviors

Figure 7a shows that the MWM latency time score during the seven days of the MWM test was significantly (p=0.007 or p=0.0009) higher in the treatment control groups compared to other groups.

Figure 7b shows that the treatment control groups exhibited a higher rate of "thigmotaxis" behaviors and a lower rate of "correct" and "direct" behaviors, whereas the other groups showed the opposite.

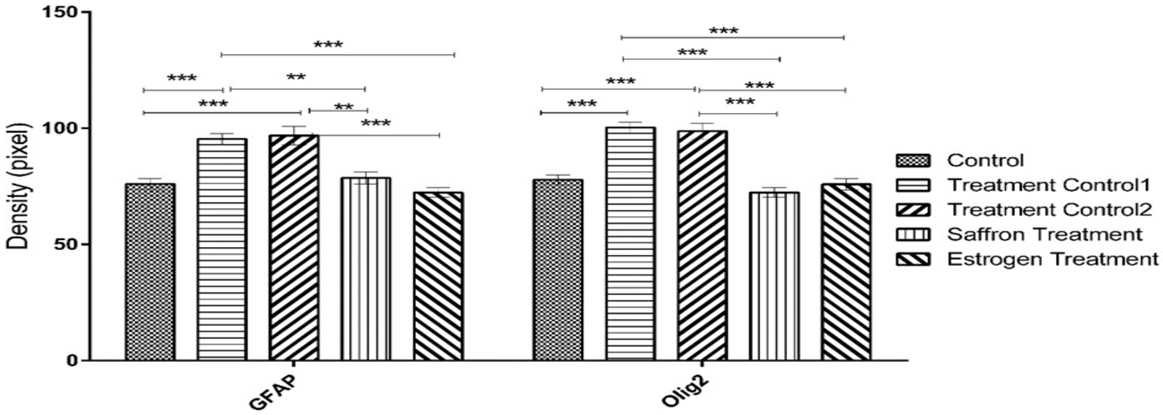

Figure 6. Effect of saffron and estrogen on GFAP density in astrocyte and Olig2 density in oligodendrocyte of the DG in EB - induced MS. Treatment Control1; received 3 µl 0.01% EB injection in the DG for a single time without treatment. Treatment Control2; rats received 3 µl 0.01% EB injection into the DG for single time + ovariectomy (OVX) without treatment. Data were represented as mean±SEM, n=5.

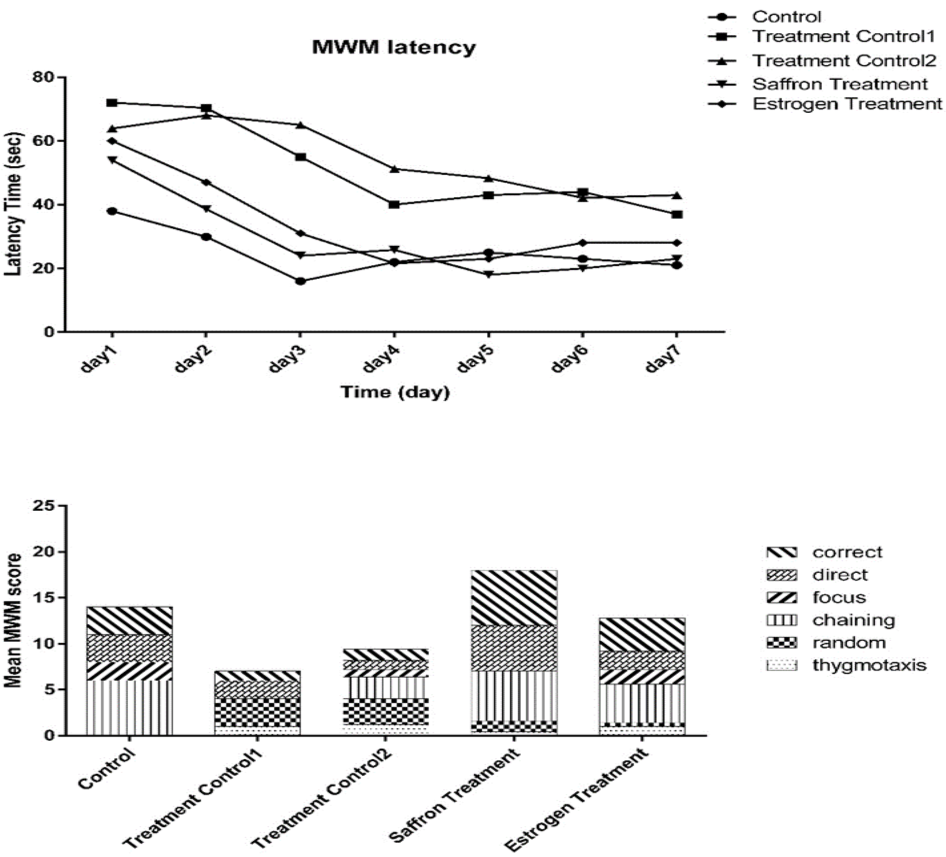

Figure 7. Effect of saffron and estrogen on latency time (non-cognitive response) and mean MWM score (cognitive behavior) in EB - induced MS rats. Treatment Control1; received 3 µl 0.01% EB injection in the DG for a single time without treatment. Treatment Control2; rats received 3 µl 0.01% EB injection into the DG for single time + ovariectomy (OVX) without treatment. Data were represented as mean±SEM, n=5.

Discussion

Women with MS undergoing the menopausal transition may simultaneously experience symptoms of menopause-associated physiological changes, progression of MS disability, and age-related comorbidities, making it important to proactively consider multifactorial causes of worsening symptoms or function at menopause (Bove et al. 2021). Understanding the impact of menopause on MS is essential for providing optimal care to women as they age and for informing treatment decisions aimed at minimizing relapses, reducing disease progression, and improving quality of life (Bridge et al. 2023). According to the experimental and clinical studies, since saffron has few negligible side effects and is generally well tolerated, it could be considered an alternative treatment for specific conditions such as postmenopausal symptoms (Bagheri and Kashani 2021).

According to present findings, saffron and estrogen reduced the extent of demyelination in the EB-induced MS. 17-β-Estradiol, the most potent estrogen, has numerous physiological functions in both male and female animals through its two nuclear estrogen receptors, ERα and ERβ (Breton et al. 2021). Estrogen replacement therapy may also play a role in protecting middle-aged women from the adverse effects of menopause, including myelin abnormalities and cognitive impairment. (He et al. 2018).

Crocin exhibits significant neuroprotective effects in multiple sclerosis through its antioxidant, anti-inflammatory, and anti-apoptotic properties. These effects are mediated by its interactions with various transcription factors, enzymes, inflammatory cytokines, proteins, growth factors, and receptors involved in neuroprotection and immune modulation (Fatemi et al. 2025).

The findings of the present study indicate that gliosis caused by demyelination is reduced in the saffron and estrogen-treated groups, suggesting the efficacy of saffron and estrogen in treating demyelination. Saffron may help prevent harmful gliosis, supporting tissue repair and neuroprotection in various neurological disease models (Zhong et al. 2020). Estrogen acts to attenuate gliosis and neuroinflammation via receptor-mediated pathways, contributing to neuroprotection and improved outcomes in neurological injury or disease models (Lu et al. 2020).

According to the present results, saffron and estrogen improved cognitive and non-cognitive behavior in the EB-induced MS. A study has shown an interaction between hippocampal-related structural and functional networks in explaining spatial learning and memory in the early stages of the MS model (Nooraei et al. 2022). Based on the search results, there is evidence that estrogen can have beneficial effects on performance in the MWM test, which is commonly used to assess spatial learning and memory in rodents. Studies have demonstrated that administration of 17β-estradiol enhances spatial learning and memory performance in the MWM in both male and female rodents (Prakapenka et al. 2020). Estrogen can promote dendritic spine formation and synaptic plasticity in the hippocampus.

Studies have shown that saffron treatment in demyelinated rats improves performance in the MWM by enhancing spatial learning and memory, likely through its antioxidant and neuroprotective effects. Specifically, saffron extract administered at doses of 5 and 10 µg/rat over a week improved escape latency, swim distance, and other MWM parameters, indicating better cognitive function (Hamedani et al. 2024).

The present study showed, saffron increased the number of GFAP-positive astrocytes in CA1 in the EB-induced MS, but estrogen had no effect. The present results indicated that saffron and estrogen increased the number of GFAP-positive astrocytes and the level of GFAP expression in DG astrocytes in EB-induced MS. In the context of MS, GFAP levels reflect the degree of astrocytic activation and damage, which is particularly pronounced during the progressive stages of the disease. The level of GFAP expression reflects the continuum of morphological modifications of reactive astrocytes. Initial studies defined a dual astrocytic response upon CNS injury with “mild” astrogliosis (referred to as activation) associated with CNS repair and protection, whereas “severe” astrogliosis (or reactivity) was associated with the prevention of CNS repair and glial scar formation (Traiffort et al. 2020). Reduced GFAP immunoreactivity and damage to the astrocytic end-feet vasculature coverage have also been reported in MS patients. These combined processes suggest that loss of astrocyte function at the vascular interface is a component of the Experimental Autoimmune Encephalomyelitis EAE/MS disease mechanism, resulting in blood-brain barrier (BBB) breakdown and massive immune cell infiltration into the CNS (Aharoni et al. 2021).

The number of activated astrocytes, glial scar thickness, and GFAP expression in cerebral ischemia decreased after saffron (Zhong et al. 2020). Ma et al. used different physiological concentrations of Estradiol E2 to stimulate primary hippocampal astrocytes and found that treatment with E2 significantly increased GFAP protein expression. They found numerous GFAP-positive astrocytes and high levels of GFAP protein expression in the hippocampus of normal adult female mice, whereas few GFAP-positive cells and minimal GFAP expression were observed in OVX mice, which have very low serum estrogen levels. They also found that treatment of OVX mice with 50 µg/kg E2 for 3 weeks not only maintained the serum estrogen levels, but also significantly increased the number of GFAP-positive astrocytes and the level of GFAP protein expression compared to the OVX group (Ma et al. 2016).

In the present study, saffron and estrogen increased the number of Olig2-positive oligodendrocytes in the DG, CC, and CA1, as well as Olig2 expression levels in DG oligodendrocytes in EB-induced MS. Olig2-positive oligodendrocytes play a crucial role in MS pathology, particularly in the context of remyelination. Their presence, density, and turnover are key factors in understanding disease progression and developing effective therapeutic interventions (Gacem and Nait-Oumesmar 2021). Saffron and crocin have been shown to increase the number of Olig2-positive oligodendrocytes by meliorating the differentiation of Neural stem cells (NSCs) into OPCs, which may have therapeutic implications in demyelinating diseases (Azari et al. 2018).

Menopause reduces the number of Olig2-positive oligodendrocytes and basic protein expression, which suggests that menopause may affect myelination processes (Gannon et al. 2023). Estrogen plays a crucial role in promoting the differentiation and maturation of oligodendrocytes, including those expressing Olig2, thereby promoting myelin production and potentially aiding in remyelination processes (Khalaj et al. 2013).

Conclusion, limitations, and future directions

The present results showed that saffron and estrogen ameliorated pathological damage and cognitive and non-cognitive behaviors in the MWM test, and modulated the reduction in the number of GFAP-positive astrocytes and Olig2-positive oligodendrocytes. Further research is needed to clarify the direct relationship between menopause, MS, and the dynamics of GFAP-positive astrocytes and Olig2-positive oligodendrocytes. Saffron and its bioactive compound crocin, as well as estrogen, have demonstrated potential to promote myelin regeneration (remyelination) by facilitating oligodendrocyte differentiation and exerting neuroprotective effects in models of MS and other neurodegenerative diseases. However, further research is required to fully elucidate the underlying mechanisms and to translate these findings into clinical applications. In this regard, advanced techniques such as Western blotting and hippocampal electrophysiology should be employed to better characterize the molecular functions and mechanisms involved in demyelination and remyelination.

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Conflicts of interest

The author declares no conflicts of interest.

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Ethical Considerations

The animal handling was conducted according to the Ethical Committee for Animal Experiments at Shiraz University

Code of Ethics

98INT1M1755

Author contributions

M.T. Conceptualization, Experimental design, Methodology, revising the final draft of the manuscript, Z.J. Experimental design, methodology. J.S. Methodology, investigation, and data analysis. K.A. Pathological Methodology.

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