Research Article

Probable antioxidant therapy of Saffron Crocin in patients with multiple sclerosis: A randomized controlled trial

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ABSTRACT

Introduction and Aim: Multiple Multiple sclerosis (MS) is a complex neurological condition that might emerge as a result of complex combination of genetic risk factors with environmental triggers, including oxidative stress. In this study, we aimed to evaluate the effects of oral Crocin on oxidative stress in patients with MS.

Materials and Methods: Adjunct to standard treatment, the Crocin group (20 patients) received 30-mg/day (15 mg twice daily) dose of Crocin and placebo group (20 patients) received for 4 weeks. Saliva and urine samples were collected to determine the levels of total antioxidant capacity (TAC), catalase activity (CAT), total thiol groups (TTG), lipid peroxidation (LPO), and homeostasis systems and balance between the endogenous ROS (1) and antioxidant defense (2), the basal level of ROS play a main role in cell signaling, immune system function, and homeostasis (1). Oxidative stress is a consequence of failure in the balance between the endogenous ROS generation and endogenous antioxidant defense systems (2). It is well-known that overproduced ROS and oxidative stress has a pathological role in various diseases such as diabetic Mellitus (DM), neurodegenerative disease, and multiple sclerosis (MS) (3). Recently, it has been reported that oxidative stress cause an excessive immune response by altering the auto-antibodies structure which could result in autoimmune disease in the CNS, the overactive immune system promotes the activation of myelin-specific T cells, followed by the activation of microglia causing lesions in the brain (4). Free radicals disturb the blood-brain barrier (BBB) function as well as impacts on the pathogenesis of the CNS diseases, which enhances monocyte penetration and inflammation, thereby increasing the rate of disease progression such as MS (5). In the attenuated antioxidants levels along with high amounts of polyunsaturated fatty acids, the CNS is more vulnerable to free radicals production from oxygen metabolism (6, 7).

MS as a complex neurodegenerative disease caused by demyelination of neurons in CNS and thereby resulting in non-traumatic disabilities in young adults (8, 9). Symptoms in patients with MS are widely varied and include fatigue, muscle weakness, ataxia, cognitive impairment, and depression (10). Although there is no study with detailed explanation of genesis involved in the disease, it has been revealed that complex combination of autoimmune processes with genetic predisposition and environmental factors might be the main cause of MS pathogenesis (11-13). Previously, it has been reported that oxidative stress could play a critical role in MS pathogenesis, also antioxidants have been considered as preventative, curative, and most outstandingly, oxidative stress counteracting factors (14). Antioxidants agents as scavengers remove the ROS and reactive nitrogen species (RNS) and substitute them with less harmful substances (15).

INTRODUCTION

Reactive Oxygen Species (ROS) is commonly produced under normal cellular metabolism. In the physiological condition, the basal level of ROS play a main role in cell signaling, immune system function, and homeostasis (1). Oxidative stress is a consequence of failure in the balance between the endogenous ROS generation and endogenous antioxidant defense systems (2). It is well-known that overproduced ROS and oxidative stress has a pathological role in various diseases such as diabetic Mellitus (DM), neurodegenerative disease, and multiple sclerosis (MS) (3). Recently, it has been reported that oxidative stress cause an excessive immune response by altering the auto-antibodies structure which could result in autoimmune disease in the CNS, the overactive immune system promotes the activation of myelin-specific T cells, followed by the activation of microglia causing lesions in the brain (4). Free radicals disturb the blood-brain barrier (BBB) function as well as impacts on the pathogenesis of the CNS diseases, which enhances monocyte penetration and inflammation, thereby increasing the rate of disease progression such as MS (5). In the attenuated antioxidants levels along with high amounts of polyunsaturated fatty acids, the CNS is more vulnerable to free radicals production from oxygen metabolism (6, 7).

MS as a complex neurodegenerative disease caused by demyelination of neurons in CNS and thereby resulting in non-traumatic disabilities in young adults (8, 9). Symptoms in patients with MS are widely varied and include fatigue, muscle weakness, ataxia, cognitive impairment, and depression (10). Although there is no study with detailed explanation of genesis involved in the disease, it has been revealed that complex combination of autoimmune processes with genetic predisposition and environmental factors might be the main cause of MS pathogenesis (11-13). Previously, it has been reported that oxidative stress could play a critical role in MS pathogenesis, also antioxidants have been considered as preventative, curative, and most outstandingly, oxidative stress counteracting factors (14). Antioxidants agents as scavengers remove the ROS and reactive nitrogen species (RNS) and substitute them with less harmful substances (15).
Saffron is an effective natural antioxidant, which is widely used in traditional medicine as analgesic, antidepressant, antispasmodic, anti-tumor, and sedative medicine (16, 17). Chemical composition study of saffron has shown various biological active constituent, Crocin is the main active component of saffron which acts as an effective antioxidant agent by removing free radicals (18, 19). Our previous study suggested that crocin has desirable effects against inflammation and DNA damage in MS patients, Crocin as an antiinflammary and antioxidant agent play critical role in regulating inflammatory processes in the immune system in autoimmune disease. However the precise mechanism by which Crocin has a protective role in patients remains unclear. Therefore the purpose of this investigation was to find the role of Crocin on the oxidative stress markers in urine and saliva in patients with MS.

MATERIALS AND METHODS

Participants
This study included 40 patients with MS who were recruited from the Farshchian Hospital, Hamadan University of Medical Sciences. The diagnosis of MS patients was clinically evaluated for disability using the Expanded Disability Status Scale (EDSS). The inclusion criteria: all patients with an initial diagnosis of MS based on history & EDSS, and exclusion criteria were: EDSS>4, pregnancy, underlying diseases (diabetes, heart failure, valvular heart diseases and chronic liver and kidney disorders),usage of immunomodulator drugs such as interferons and sensitivity to Saffron. This randomized double-blinded clinical trial was conducted in a specialty clinic affiliated to Hamadan University of Medical Sciences, Hamadan, Iran in 2017. This trial was approved by the Ethics Committee of Hamadan University of Medical Sciences and registered in the Iranian Registry of Clinical Trials (IRCT) with registration code of IRCT2016122013194N3. All participants signed informed consent before being enrolled in the study and this study approved with Hamadan University of Medical Sciences and Health Services (NO: IR.UMSHA.REC.1395.375). 40 new patients with relapsing-remitting MS, between 20 and 40 years old were included in this clinical trial study. Patients randomly divided into two control and case groups and assigned the cases with Crocin and the others with similarly looking placebo. Patients took capsules twice a day for 4 weeks (20). After the treatment period, we collected the urine and saliva samples of the groups for oxidative stress biomarkers analysis.

Oxidative stress biomarkers
Measurement of catalase activity (CAT) activity
CAT activity was measured off the absorbance reduction at 240 nm in a medium with amounts of H2O2 (10 mM) and sodium phosphate buffer (50 mM, pH = 7.0). The enzyme activity unit is 1 mol of H2O2 consumed as a substrate per minute, and the specific activity is units/ml plasma (21).

Measurement of total thiol groups (TTG)
TTG in the urine and saliva samples were estimated with di-thio-nitro-benzoic acid (DTNB) as a reagent. A yellowish complex made from thiol molecules and DTNB absorbs maximally at 412 nm in a spectrophotometer (22).

Measurement of total antioxidant capacity (TAC)
Urine and saliva samples TAC was measured with ferric reducing ability of plasma (FRAP) method (measuring the ability to reduce Fe3+ to Fe2+ when TPTZ is present). Fe2+ and TPTZ together have blue color with maximal absorbance at 593 nm in a spectrophotometer (22).

Measurement of lipid peroxidation (LPO)
Detecting the LPO was done by the reaction of TBA with MDA, which is the end product of LPO. For a short time, urine and saliva samples and trichloroacetic acid (20%) were mixed along with dispersing the precipitate in H2SO4 (0.05 M). Then TBA (0.2% in 2M sodium sulfate) was added and the solution was heated for approximately 30 min in 100°C water bath. The absorbance of LPO adducts exploited with n-butanol was measured at 532 nm in a spectrophotometer (22).

Statistical analysis
All data analyses were performed using the statistical package for social sciences version 16 (SPSS Inc, Chicago, USA). The Kolmogorov-Smirnov test was used to evaluate whether the variables were normally distributed or not. The data were analyzed using independent and paired sample t-test. If assumptions of parametric tests were not met, nonparametric tests were used instead. A value of p < 0.05 was considered as indicative of significance.

RESULTS
Table 1 shows the demographic, and clinical characteristics of the 40 MS patients and control included in the study.

Table 1: Demographic characteristics of patients

| Age EDSS Male/ female (No) | Crocin Group Mean ± SD | Placebo Group Mean ± SD |
|---------------------------|------------------------|-------------------------|
| 29 ± 4.99                 | 31.47 ± 5.31           |
| 2.98 ± 0.78               | 3.01 ± 0.89            |
| 3/17                      | 2/18                   |

Effect of Crocin on urinary and salivary CAT activity
The catalase activity of urine sample was presented in fig. 1. There was no significant difference in the urine catalase activity between the controls and cases (p>0.05) at the beginning of the study, as well as we
also found that 4 weeks Crocin therapy of cases showed no notable change in catalase activity (p>0.05). Moreover, fig. 1 indicates that no notable increase in catalase enzyme levels was detected during the intervention in controls and cases (p>0.05) (Fig. 1). As presented in the fig. 2, the catalase enzyme activity in saliva at the beginning of the study had no significant difference between cases and controls (p>0.05). After 4 weeks of treatment with Crocin, a notable increase in the catalase activity was detected in the cases (p<0.05), however, there was no significant change in controls after 4 weeks of crocin trapy (p>0.05).

Fig. 1: The CAT activity in urine of MS patients in Crocin and placebo groups at the beginning and four weeks after treatment. p<0.05 was considered as a significant difference.

Fig. 2: The CAT activity in saliva of MS patients in Crocin and placebo groups at the beginning and four weeks after treatment. p<0.05 was considered as a significant difference.

Effect of Crocin on urinary and salivary TTG level

According to the analysis which is presented in Fig. 3, at the beginning of the study, the levels of TTG in urine samples were not notably different in the case and the control groups (p>0.05). however, After 4 weeks of treatment with Crocin, The urinary TTG levels in the case group increased significantly in comparison to the control group (p>0.05). Moreover, no significant differences were observed in the level of TTG between the beginning in the control group (p>0.05) Furthermore, fig. 3 indicates that the case group showed a notable increase in the level of urine TTG between the beginning and the end of the administration (p<0.05) (Fig. 3).

As represented in fig. 4, there was a considerable difference in the TTG level of saliva between the case and the control (p<0.05). we also found that Crocin administration resulted in a significant increase in the saliva TTG level in the case group compared to the control group (p<0.05). However, no notable differences were seen in the saliva TTG level of the control group between the beginning and the end of the intervention (p>0.05). Furthermore, saliva TTG determination demonstrated that there was a notable difference in the TTG level of the case group between the beginning and the end of the intervention. (p<0.05).

Fig. 3: The TTG level in urine of MS patients in Crocin and placebo groups at the beginning and four weeks after treatment. p<0.05 was considered as a significant difference.

Fig. 4: The TTG level in saliva of MS patients in Crocin and placebo groups at the beginning and four weeks after treatment. p<0.05 was considered as a significant difference.

Effect of Crocin on urinary and salivary TAC level

Determination of TAC levels in urine samples revealed that there was no significant difference between the case and control groups at the beginning of the study (p>0.05). As well as, we found that 4 weeks of treatment with Crocin had no effects on urine TAC levels as compared to the control group (p<0.05). However, we found that urine TAC levels of the case and the control groups were significantly increased at the end of the intervention (p<0.05) (Fig. 5).

The TAC levels of the saliva was presented in Fig. 6. there was no notable difference between the TAC levels of saliva in the case and the control group at the beginning of the study (p>0.05). 4 weeks treatment with Crocin showed that There was no significant changes in the case group’s TAC level compared with
the control group (p>0.05). Additionally, we showed that the saliva TAC levels was same at the beginning and the end of the intervention in the control group (p>0.05). Fig 6 indicates that the level of TAC in the case group at the beginning and the end of the intervention increased significantly. (p<0.05).

![Fig. 5: The TAC level in urine of MS patients](image)

**Fig. 5: The TAC level in urine of MS patients** in Crocin and placebo groups at the beginning and four weeks after treatment. p<0.05 was considered as a significant difference.

**Effect of Crocin on urinary and salivary LPO level**

The LPO levels of urine sample was showed in Fig. 7. There was no significant difference between the urine LPO levels of the case and control groups at the beginning of the study (p>0.05). Our study demonstrated that 4 weeks administration of Crocin resulted in a notable decrease in the level of LPO of the case group in comparison with the control group (p<0.05). We also found that the LPO levels of the case group was significantly lower at the end of the intervention as compared to the beginning (p<0.05).

As shown in Fig. 8, there was no notable difference between the LPO levels of saliva samples in the case and control groups at the beginning of the study (p>0.05). Following 4 weeks administration of Crocin, there was no significant changes in the LPO levels of the case group in comparison with the control group. Furthermore, no significant difference was seen in the LPO level of the control group between the beginning and the end of the intervention (p>0.05). Furthermore, Fig 8 indicates that the level of LPO in the case group between the beginning and the end of the intervention decreased significantly (p<0.05).

![Fig. 6: The TAC level in saliva of MS patients](image)

**Fig. 6: The TAC level in saliva of MS patients** in Crocin and placebo groups at the beginning and four weeks after treatment. p<0.05 was considered as a significant difference.

**DISCUSSION**

The results of our study demonstrated that a 4-week treatment with Crocin along with standard treatment could lead to a decrease in several oxidative stress factors in the urine and saliva samples of the patients suffering from MS. Urine analysis results showed no significant changes in LPO, TAC, CAT activity, and TTG between the control and the case group at the beginning of the assessment. Moreover, no significant difference in TTG and the amount of CAT activity was seen between the beginning and the end of the intervention in the control group, but there was a notable decrease in the level of LPO and a prominent increase in the level of TAC and TTG at the end of the intervention after 4 weeks of Crocin therapy. A significant increase were shown in the levels of LPO and TTG at the end of the intervention but there was no notable difference in the CAT activity after 4 weeks of Crocin therapy. The LPO levels was significantly decreased at the end of the intervention, which confirms the antioxidant effects of Crocin in patients with MS. Recent study carried out by Boussabbeh et al. revealed that crocin attenuates the oxidative stress by producing patulin in the liver and kidney (23). In line with our findings, it was also found that Crocin reduced the amount of LPO and protein oxidation (24). Premkumar et al. have demonstrated that saffron...
administration resulted in a significant decrease at the level of LPO with the increase of antioxidants in the mice livers at the same time (25), which was consistent with present study results that indicating the effects of Crocin as free radical scavenger and antioxidant.

Investigation of the total antioxidant capacity at the beginning and the end of the intervention indicated that TAC increased significantly at the end of the study. In previous study, using of ethanolic extract of Saffron (Crocus sativus L.) on oxidative stress markers in the hippocampus of experimental models of MS resulted in oxidative stress attenuation in animal model (26). Moreover, other studies have shown that Crocin has the potential to increase total antioxidant capacity and also reduce LPO, which is consistent with our findings (27). Also, the previous studies showed the beneficial effect of Crocin on the activity of antioxidant enzymes, free radicals, and apoptosis in cells exposed to dichlorvos (28), our findings showed that Crocin was able to change the level of CAT activity, Whereas no notable difference was spotted in the activity level of CAT in the urine of MS patients between the beginning and at the end of the study. Notably, observed data showed that in the presence of ROS, Oligodendrocytes are selectively damaged by superoxide radicals in cell cultures, while there was no effect on Astrocytes, however, brain macrophages and catalase showed that can fully protect this cytotoxic effect of oxidative stress (29). Therefore the previous study showed that anti-inflammatory and antioxidant compounds like curcumin can reduce oxidative stress in some tissues (30). According to the findings of present study and previous study, it can be suggested that the neurons can be protected from the process of demyelination by both extracellular and intracellular superoxide dismutase (SOD) and CAT activities and also the blood-brain barrier damage can be reduced about 60-70% (31).

Saliva analysis showed that there was no notable difference in the LPO, TAC, CAT activity, and TTG between the beginning and the end of the intervention in the control group, while after 4 weeks of treatment with Crocin, a significant drop was noted in LPO and a significant increase in TTG and TAC and CAT activity observed at the end of the intervention. Notably, treatment with Crocin resulted in a considerable increase in the activity of CAT in the case group compared with the control group. In a study conducted by Karlik et al. in 2015 on plasma and saliva of MS patients has shown that treatment with corticosteroids lead to a significant increase in LPO and glycation level in patients with MS (32). In line with our findings the results of this study showed reduced antioxidant capacity and elevated oxidative stress in MS patients. Previously, it has been reported that oral administration of Crocin potentially protects macromolecules against glycation and oxidative damages (33) which confirms our findings that crocin had antioxidant effects in MS patients. Additionally, previous reports showed that crocin can decrease intracellular production of free radicals of ROS and H2O2 and prevented apoptosis and platelet aggregation in a dose-dependently manner (34) which we have discussed in our previous study (20).

In conclusion, our study demonstrated that Crocin can decrease several oxidative stress biomarkers in saliva and urine patients with MS. Therefore, the results of this study suggest that Crocin due to potent antioxidant properties, safety, can be a favorable therapeutic choice to be used as an adjuvant treatment in MS. However, this study had several limitations, and further studies are needed to confirm our results by antioxidant therapy in MS patients in the future.

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CONFLICT OF INTEREST

No potential conflicts of interest were disclosed.

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