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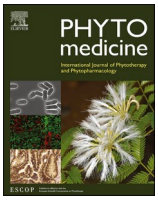

Saffron carotenoids reversed the UCMS-induced depression and anxiety in rats: Behavioral and biochemical parameters, and hippocampal BDNF/ERK/CREB and NR2B signaling markers

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ABSTRACT

Background: Depression is a debilitating condition that affects the mind and the individual's body. The improving effects of saffron on depression and anxiety have long been discussed, with limited information about the molecular mechanism of action.

Hypothesis/Purpose: Investigating the effect of saffron carotenoids, Crocin and Crocetin, on depression and anxiety in rats by emphasizing some signaling pathways involved.

Study Design: Depression and anxiety were induced in rats via unpredictable chronic mild stress (UCMS). Then different rat groups were treated with Crocin, Crocetin, Fluoxetine, and vehicle. Behavioral tests were done before and after treatment.

Methods: The serum Serotonin and Corticosterone and the expression of some hippocampal signaling proteins were studied. Furthermore, bioinformatics tools were used to predict the interactions of Crocin/ Crocetin with the Serotonin transporter and NMDA receptor subunit NR2B. Then, the patch-clamp was used to study the interaction of Crocetin with the NMDA receptor.

Results: Various behavioral tests confirmed the induction of depression and the improvement of depression by these natural carotenoids. In addition, Crocin/ Crocetin significantly increased the decreased serum Serotonin and reduced the increased serum Corticosterone in the depressed groups. They also increased or caused a trend of increase in the CREB, ERK, BAD, BDNF, p11, and 5-HT1B expression in the hippocampus of the depressed groups. In addition, there were an increase or a trend in p-CREB/CREB, p-$ERK_{1/2}$ /$ERK_{1/2}$, and p-BAD/BAD ratios in the Crocin/ Crocetin treated depressed groups. However, the NR2B and FOXO3a expression showed a trend of decrease in depressed groups after treatment. The bioinformatics data indicated that Crocin/ Crocetin could bind to the Serotonin transporter (SLC6A4) and NR2B subunit of the NMDA receptor. Both carotenoids bind to the same site as Fluoxetine in the SLC6A4. However, they bound to different sites on the NR2B. So, Crocetin binds to NR2B at the same site as Ifenprodil. But Crocin bound to another site. The whole cell patch-clamp recording on the normal rat hippocampus revealed a significant decrease in the NMDA peak amplitude after Crocetin treatment, indicating its inhibitory effect on this receptor.

Abbreviations: (±)-2-Amino-5-phosphopentanoic acid, AP5; 5-Hydroxytryptamine, 5HT; 6-Cyano-7-nitroquinoxaline-2–3-dione, CNQX; Analysis of variance, ANOVA; Artificial cerebrospinal fluid, ACSF; B-cell lymphoma 2, Bcl-2; Bcl-2-associated death promoter protein, BAD; Brain-derived neurotrophic factor, BDNF; Control, C; Cyclic adenosine monophosphate, cAMP; cAMP response element-binding, CREB; Depressed group under UCMS, D; Extracellular signal-regulated kinase, ERK; Extrasynaptic NMDA receptor, NR2B; Forkhead box O, FOXO; Mitogen-activated protein kinase, MAPK; N-methyl-D-aspartic acid, NMDA; Normal Saline, NS; No Treatment, NT; Polyvinylidene fluoride, PVDF; Protein Data Bank, PDB; S133-phosphorylated CREB, p-CREB; Saffron aqueous extract, SAE; Selective Serotonin reuptake inhibitors, SSRIs; Sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE.

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Conclusion: The antidepressant activities of Crocin/ Crocetin are possibly due to their effects on Serotonin and Corticosterone serum concentrations, NR2B expression, and the downstream signaling pathways. Furthermore, these natural carotenoids, like Fluoxetine, induced an increasing tendency in p11 and 5HT1B in depressed rats.

Introduction

Depression is a common mental disorder. Globally, it is estimated that 5% of adults suffer from depression. Nowadays, SSRIs that cause an increase in serotonin availability are mainly used to alleviate depression (Chu and Wadhwa, 2022).

Saffron is a valued herb from the *C. Sativus* Linn (Iridaceae) stigmas. The biological and pharmaceutical effects of saffron and its constituents have been extensity reviewed by us (Bathaie et al., 2019, 2014; Bathaie and Mousavi, 2010). The antidepressant and antianxiety effects of saffron have been known since ancient times. It was introduced as an exhilarant spice in Avicenna's "Canon of Medicine" more than 1000 years ago (Hosseinzadeh and Nassiri-Asl, 2013). In addition, some clinical studies indicated SAE's role in reducing depression symptoms, like Fluoxetine (Noorbala et al., 2005) and imipramine (Akhondzadeh et al., 2004).

Now a day, several antidepressant drugs, including Fluoxetine, Imipramine, Citalopram, and Duloxetine, are prescribed for patients. Although they have different molecular targets, their major limitation is side effects (Khawam et al., 2006). Therefore, the search for new drugs or supplements is of particular importance.

Among targets for antidepressant drugs, CREB has been known as a convergence point for multiple classes of antidepressant drugs (Blendy, 2006). It could serve as a target for stress, anxiety, and depression. In addition, CREB-BDNF signaling has been suggested as a critical path in cell survival, synaptic structure, and synaptic plasticity (Karege et al., 2005). MAPK/ERK pathways are also involved in neuronal survival and neuroplasticity signaling pathways in depression. BAD is a downstream signal of this pathway and has a role in controlling apoptosis of the nerve cells (Bonni et al., 1999).

The NMDA receptor, an ionotropic glutamate receptor, is also associated with depression. So, the NMDA receptor antagonists show significant antidepressant activity. Extrasynaptic NMDA receptors (NR2B) overactivation can initiate oxidative stress in neurons. Following dephosphorylation and inactivation of CREB, nuclear import of the pro-death transcription FOXO and inactivation of $ERK_{1/2}$ have been accrued in depression (Burnouf et al., 2013; Hardingham and Bading, 2010). Moreover, the SSRI administration activates a particular type of Serotonin receptor, 5-HT1B, thus reducing serotonin synthesis and release. Furthermore, p11 can interact with the Serotonin receptor and regulate cell surface distribution and function (Svenningsson et al., 2006).

Because of the available antidepressant and antianxiety activity of saffron for a long time and limited data about the molecular mechanism of its action, we decided to study the role of each of the saffron carotenoids, Crocin and Crocetin, on the rat model of depression and anxiety. For this purpose, we first induced depression and anxiety in rats. Then, rats were treated with Crocin, Crocetin, and Fluoxetine (an FDA-approved SSRI drug as a positive control). Some behavioral tests assessed the induction of depression and improvement. The rats' serum concentrations of Serotonin and Corticosterone were also determined. Then some possible signaling pathways were studied in hippocampus tissue using Western blot. Finally, Bioinformatics and patch-clamp studies were used to obtain more information on the possible interaction between saffron carotenoids with the NR2B receptor and the Serotonin transporter. In addition, we investigated the role of these natural carotenoids on p11 and 5HT1B expression in the hippocampus of rats.

Materials and method

According to the previously registered methods (Patents #54,958 and #54,960, Oct. 27, 2008, Iran), Crocin and Crocetin (Fig. 5S) were isolated from Ghaenate's Saffron, Iran.

Animals and treatments groups

Sixty adults male Wistar rats (weight 250–300 g) were ordered from the Animal Laboratory of Tarbiat Modares University and kept under standard conditions. After adaptation, rats were randomly divided into two main groups, control (C) and UCMS (D) groups (# 30 in each). Then the rats in each group were randomly divided into five subgroups, # 6 in each. Schema Supplementary 1A shows the rat groups and the treatment procedure.

The rats in each group were treated with Normal Saline (NS, 1 ml/kg/day) and Fluoxetine (Flu, 10 mg/kg/day). (Li et al., 2017; Pehrson et al., 2015), Crocin (Cro, 30 mg/kg/day) (Dastgerdi et al., 2017; Vahdati Hassani et al., 2014) and Crocetin (Crt, 10 mg/kg/day) (Tashakori-Sabzevar et al., 2013) through intraperitoneal injection (i.p.) for 21 days. Each month rats were anesthetized with a mixed Ketamine (80 mg/kg) and Xylazine (8 mg/kg), and blood samples were collected from the retro-orbital sinus puncture via the medial canthus of the eye. Finally, after seventy-two days, the rats were sacrificed under anesthesia. Procedures involving animals and their care were conducted in conformity with national and international laws and policies (National Institutes of Health Guide for the Care and Use of Laboratory Animals, National Institutes of Health Publication no. 85–23, 1985) and received Institutional approval by the Animal Ethics Committee of Tarbiat Modares University.

UCMS application

To induce depression in rats, we used different chronic stress procedures described in rats (Farooq et al., 2012). Briefly, rats in the depressed groups were exposed to 10 different stressors for three weeks. The stressors were two out of ten each day. They included 24 h food deprivation, 24 h water deprivation, 24 h at 45° tilted cages, damp bedding, lights on overnight, lights off daytime, 5 min rotation on a shaker, 5 min placement at 40 °C environments, isolation, and crowd.

Behavioral tests

Various behavioral tests, as follows, were used to confirm the UCMS induction. After treatment, the same tests were done to confirm the improvement.

Locomotor activity test

Locomotor activity was measured by examining the movement velocity and distance using an open field box, which will be explained in the next section.

Depression

Two following tests were used to evaluate depression.

Sucrose Preference Test (SPT): The SPT was performed using the method described by Liu (Liu et al., 2018). The data were collected for four days before and after treatment. Briefly, after fasting and water deprivation, the rats were exposed to two bottles containing 1% sucrose solution or pure water. Before the experiment and after four hours, the weight of the bottles was measured. Then, the preference value was calculated using Eq. (1).

$$Preference = \frac{Sucrose\ Intake}{Sucrose\ Intake\ +\ Water\ Intake} \quad (1)$$

Forced Swimming Test (FST): The procedure was based on the previously described method (Yankelevitch-Yahav et al., 2015). Briefly, 24 h after a pre-swimming period, rats were individually placed inside a vertical cylinder, 16 × 40 cm, containing water up to 25 cm for 5 min, and their behavior was recorded.

Anxiety tests

Two following tests were used to evaluate anxiety in rats.

Open field test (OFT): The test was performed based on the previously described method (Seibenhener and Wooten, 2015). Briefly, the rat was individually placed in the central apparatus and allowed to explore freely, and they were observed directly and continuously using a video tracking system for 5 min (Ethovision XT 11, Noldus, Netherlands).

Elevated plus-maze test (EPM): The method was performed as previously described (Walf and Frye, 2007). Briefly, each rat was placed in the center of a plus-shaped maze apparatus and allowed for 5 min free movement. Then, the animal behaviors were recorded using a video camera mounted above it.

Weight measurement

All rats were weighed on day one and then every ten days. Then in each group, differences between the weight of rats on 1 and 31 days, and 31 and 71 days were obtained and plotted.

Immunoblotting

ELISA

Serotonin and Corticosterone levels were measured in the serum samples using a Serotonin and a Corticosterone ELISA Kit (Supplementary 2), according to the manufacturer's instructions.

Western blotting

Western blotting was performed based on our previously described method (Ebrahimi et al., 2019) to evaluate the expressions of proteins in the hippocampus of rats. In summary, hippocampus samples were dissected immediately after the rats were sacrificed, frozen in liquid nitrogen, and stored under −80 °C until the experiment. All the normalized samples were separated by 15% SDS-PAGE, transferred onto PVDF membrane, blocked by 3% BSA, and incubated with the desired primary antibody overnight at 4 °C. After washing, the PVDF membrane incubated with the desired secondary antibodies for 2 h at room temperature. The β-Actin expression was used as an internal control. ImageJ (1.53e; Java 1.8.0_60) software was used to evaluate the protein bands to measure protein expression empirically.

The characteristics of all antibodies are shown in Supplementary 2.

Immunohistochemistry (IHC)

Brain samples were collected and immunostained for fluorescence detection in Immunohistochemistry using the free-floating tissue sections (Potts et al., 2020) using materials in Supplementary 2. In addition, Image J was used for analyzing the protein intensity in the CA1 region of the hippocampus. For this purpose, the Paxinos and Watson Rat Brain Atlas was used.

Bioinformatics

To investigate the interaction of Crocin/ Crocetin with 1) Serotonin transporter and 2) NMDA receptor, the *in-silico* analysis was performed using AutoDock Vina (version 1.1.2) based on our previously reported method (Hashemi et al., 2020). For this purpose, the X-ray crystal structure of the ts3 Serotonin transporter (5i6x) and crystal structure of amino-terminal domains of the NMDA receptor subunits GluN1 and GluN2B in complex with Ifenprodil (3qel) in pdb format were obtained from PDB. Furthermore, the binding of Fluoxetine and Ifenprodil (an NR2B antagonist) was used as controls for the binding of Crocin/ Crocetin to SLC6A4 and NR2B, respectively. In addition, the interaction of the first conformer (with the minimum binding energy) with the Serotonin transporter and NMDA receptor was visualized in three-dimensional (3D) views by PyMOL (version 1.7.4.5). To determine the amino acids involved in the interaction of the best conformers of Crocin/ Crocetin with the Serotonin transporter and NMDA receptor, we used LigPlot (version 1.4.5).

Whole-cell patch-clamp recording in brain slices

As explained previously, the Path-clamp recording was run in hippocampal slices to check the effect of extracted Crocetin on NMDA currents (Ghafouri et al., 2019). NMDA currents were recorded in voltage clamp mode after adding bicuculline (GABAA receptor antagonist) and CNQX (AMPA receptor antagonist) into the aCSF. To confirm the NMDA currents, AP5 (NMDA receptor antagonist) was added to the solution to block NMDA receptors. The Axon pClamp 10 acquisition software did the offline signal analysis.

Statistical analyses

Data are expressed as the mean ± SEM of three independent repeats. Data analysis was done using an unpaired *t*-test before treatment and two-way analysis of variance (ANOVA) for after-treatment data, using SPSS Version 16 or Graph Pad Prism Version 9. In addition, Cohen's d was used as an appropriate effect size measure to evaluate the differences between experimental groups Supplement 1B).

Results and discussion

Behavioral tests

Figs. 1A and 1C show the locomotor activity of rats after UCMS induction. Likewise, Figs. 1B and 1D show these results after treatment. So, depression induction and none of the mentioned treatments caused changes in the locomotor activity of rats.

Figs. 1E and 1G show a significant decrease in the SPT and a significant increase in the immobility time in the depressed group. Figs. 1F and 1H show the lowest SPT and the highest immobility time in the depressed group treated with Normal Saline (D-NS). Administration of Crocin, Crocetin, and Fluoxetine in depressed groups significantly increased SPT and decreased immobility time compared to D-NS.

Figs. 1I, 1K, and 1M show a significant decrease in OFT and EPM in the depressed group. Figs. 1J, 1L, and 1N show that Crocin, Crocetin, and Fluoxetine significantly changed OFT and EPM compared to D-NS.

A previous study also indicated that a combination of escitalopram-Crocin was more effective than escitalopram alone on rats' brain function and motor activity (Joodaki et al., 2021). It has also been reported that oral administration of saffron in animals improved anxiety/depression in the FST and SPT tests (Orio et al., 2020). The significant improvement role of Crocetin has also been shown by applying FST and OFT in the depressive symptoms of rats (Farkhondeh et al., 2018). Our results confirmed all these data based on the behavioral test.

Body weight

Fig. 1O shows that in contrast to the increased weight of control rats in the first month before treatment, a significant weight loss happened in the depressed group after twenty days, which was reversed in the next ten days. Fig. 1P indicates a significantly lower body weight difference in the depressed group than in the control group. Similar changes in the body weight of animals under stress have been reported in mice (Zain

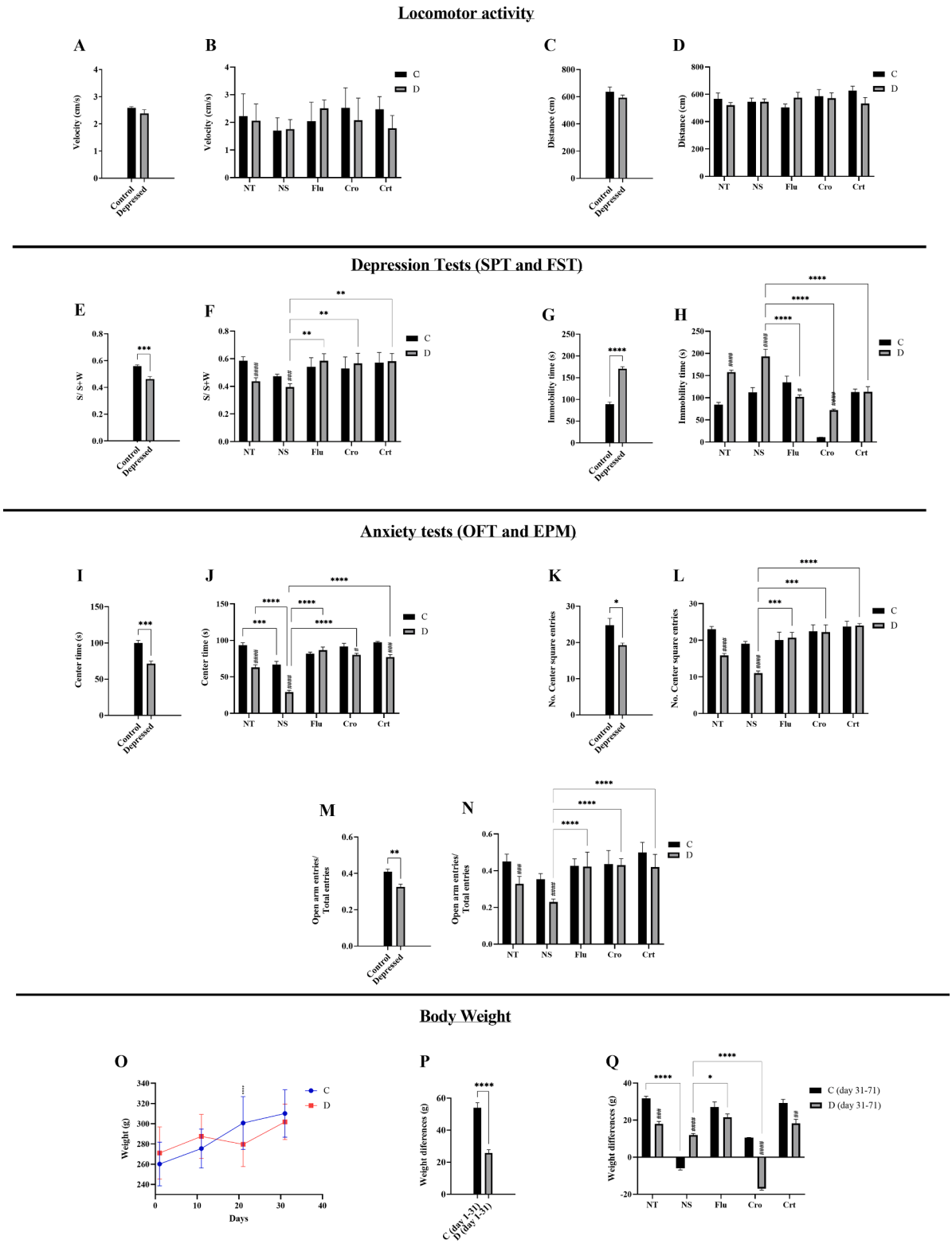

Fig. 1. The results of the Behavioral tests and Body weight.

The Locomotor activity was evaluated with monitoring the velocity and distance traveled in OFT before (A and C) and after treatment (B and D). The depression was evaluated by SPT and FST before (E and G) and after treatment (F and H) respectively. The anxiety was evaluated with open filed test (OFT) and elevated plus maze (EPM). The evaluated parameters in OFT and EPM are center time (I and J), the number of center square entries (K and l), and open arm entries/ total entries (M and N) before and after treatments, respectively. The number of rats in each group was 6.

Body weight was determined every ten days (O). Body weight differences between day 1 and day 31 (P). The body weight differences between days 31 and 71 (after treatment) (Q).

* Show comparison between different depressed groups.

Show comparison between control and depressed groups after each treatment.

and*$p < 0.05$, ## and **$p < 0.01$, ### and ***$p < 0.001$, #### and ****$p < 0.0001$.

et al., 2019). However, after discontinuing the stresses, the pattern of rats' weight gain got close to the control group. Fig. 1Q shows the weight differences of different experimental groups after treatment.

In contrast to other groups, a negative weight gain was observed in the depressed group treated with Crocin. Our previous study on cardiovascular disease patients indicated a decreased dietary intake and reduced appetite in patients who received Crocin (Abedimanesh et al., 2017). Similar data have also been reported about the negative weight gain of rats treated with Crocin (Mashmoul et al., 2014).

Considering the body weight loss and behavioral tests in rats under NS injection, it can be concluded that NS injection is a stress for rats, like the UCMS group. These results are consistent with those previously reported (Izumi et al., 1997).

Serum serotonin and corticosterone

Figs. 2A and 2C show that the serum Serotonin significantly decreased in the depressed group. While the serum Corticosterone levels significantly increased. Figs. 2B and 2D indicate the lowest serum Serotonin level and the highest Corticosterone level in the D-NS group. After treatment, the highest Serotonin level was observed in the control rats treated with Fluoxetine (C-Flu). Crocin/ Crocetin also significantly increased serum Serotonin and decreased serum Corticosterone in depressed rats.

The improvement effect of Crocin/ Crocetin on these parameters is consistent with the effect of saffron pill supplement on Serotonin in young males (Moghadam et al., 2021), and SAE/ Crocin treatment on serum Corticosterone levels of mice (Halataei et al., 2011).

Western blotting

Figs. 3A to 3H, top, show the Western blot results of various signaling proteins and the related β-Actin in different groups. Histograms at the bottom of these figures show the relative expression of the proteins. They indicate that Crocin/ Crocetin administration in the depressed group significantly increased or caused a trend of increase with large effect sizes (Supplementary 1B) in CREB, p-CREB, p-CREB/CREB ratio, $ERK_{1/2}$, p-$ERK_{1/2}$, p-$ERK_{1/2}$ / $ERK_{1/2}$ ratio, BDNF, p11, 5-HT1B, p-BAD, and p-BAD/BAD ratio compared to the depressed group treated with NS. However, BAD, NR2B, and FOXO3a showed a reduction trend in the depressed groups treated with Crocin/ Crocetin. In the depressed group treated with vehicle, the expression of the BDNF, p11, and 5-HT1B, and phosphorylation of CREB, $ERK_{1/2}$, and BAD, therefore the ratios of p-CREB/CREB, p-$ERK_{1/2}$ / $ERK_{1/2}$, and p-BAD/BAD were low.

Some studies have also shown the neuroprotective role and increase in the CREB and BDNF expression in rat brains after Crocin administration (Motaghinejad et al., 2019; Razavi et al., 2017; Ebrahimzadeh et al., 2020). The antidepressant effect of Crocin via increased phosphorylation and expression of ERK protein has also been suggested (Wang et al., 2022; Yang et al., 2018). The neuroprotective role of Crocetin has also been reported, accompanied by changes in MKP-1-$ERK_{1/2}$–CREB signaling (Karimi et al., 2020; Lin et al., 2021). Our results also confirmed these results after the administration of both saffron carotenoids.

Immunohistochemistry

Figs. 4A upper and bottom show the IHC results of the CA1 of the rat's hippocampus. Fig. 4B shows the histograms of NR2B intensity, indicating that the administration of Fluoxetine, and Crocin, in the depressed groups led to significant changes in NR2B intensity. In addition, based on Cohen's *d*, large effect sizes were observed in D-Crt compared to D-NS= 1.82417, indicating a decreased NR2B intensity.

Bioinformatics and whole-cell patch-clamp recording

Figs. 5A to 5I show the interaction of SLC6A4 with Crocin, Crocetin, and Fluoxetine. Figs. 5A, 5D, and 5G represent all amino acids involved in hydrogen bonding and hydrophobic interaction of Fluoxetine, Crocin, and Crocetin with SLC6A4. The complete list of amino acids involved in these interactions and the free energies are shown in Supplementary Table 1C. These data predict the binding of Crocin/ Crocetin to the same site as Fluoxetine in SLC6A4. This binding may have resulted in the inhibition of this transporter. The subject of the subsequent experimental studies.

Figs. 5J to 5R show the interaction of NR2B with Ifenprodil, Crocin, and Crocetin. Figs. 5J, 5M, and 5P represent all amino acids involved in hydrogen bonding and hydrophobic interaction of Ifenprodil, Crocin, and Crocetin with NR2B. The complete list of amino acids involved in these interactions and the Free energies are shown in Supplementary Table 1C. According to the free energy changes, the most favorable bindings are in the following orders: Crocin > Fluoxetine > Crocetin for interaction with SLC6A4, and Ifenprodil > Crocetin > Crocin for interaction with NR2B.

The docking data indicates that Crocetin can bind in the binding site of Ifenprodil. However, Crocin binds to another site in this protein. Therefore, we used the patch-clamp to show the effect of Crocetin on this receptor (Fig. 5T). In typical situations, the amplitude of NMDA current was 246.2 ± 113.6 pA. Applying the vehicle had no significant effect on NMDA current. However, after Crocetin administration, the NMDA peak amplitude was reduced significantly and reached 150.2 ± 95.87 pA. To

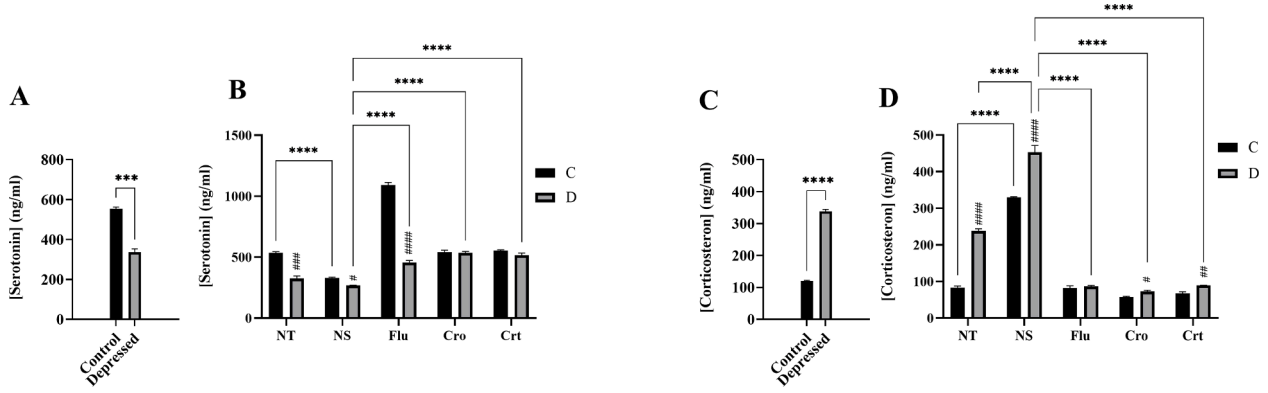

Fig. 2. The serum Serotonin and Corticosterone levels. (A and B) The serum Serotonin levels in different groups under the study, before and after treatment, respectively.
(C and D) The serum Corticosterone levels in different groups under the study, before and after treatment, respectively.
* Show comparison between different depressed groups.
# Show comparison between control and depressed groups after each treatment.
# and*$p < 0.05$, ## and **$p < 0.01$, ### and ***$p < 0.001$, #### and ****$p < 0.0001$.

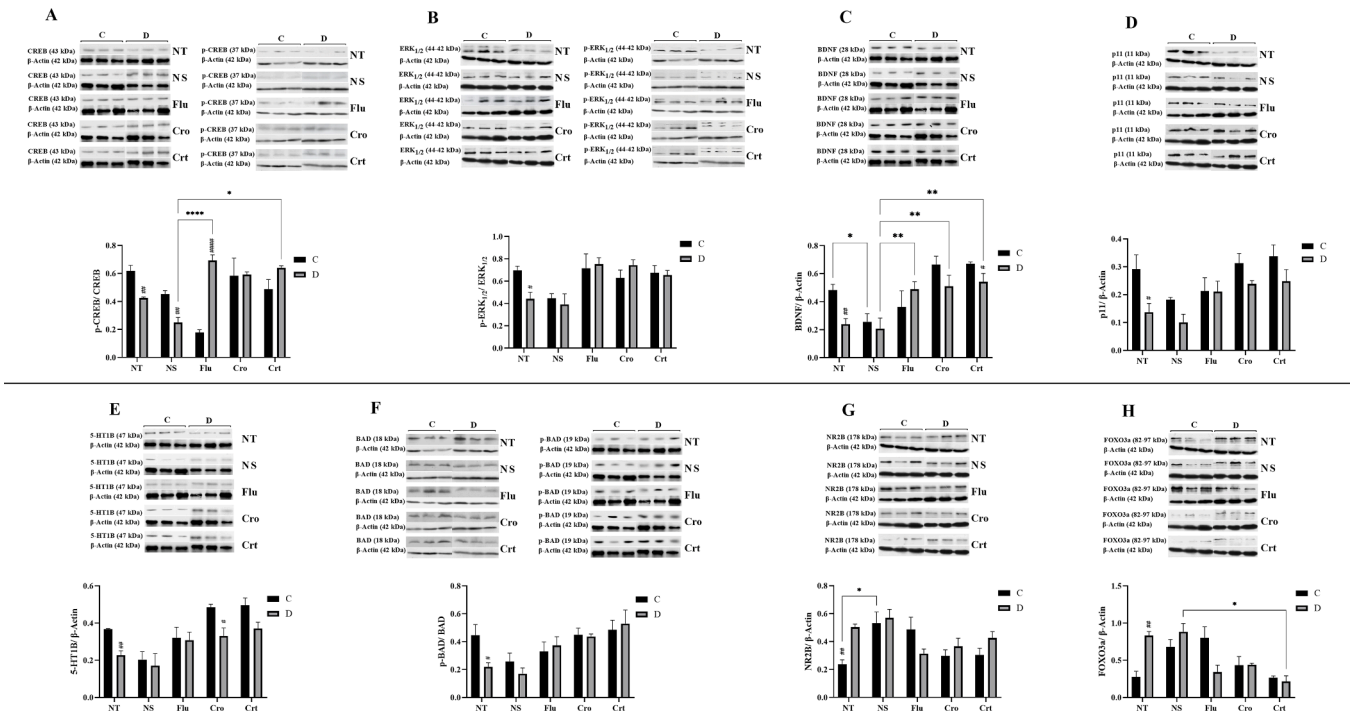

Fig. 3. The Western blot results. (A to H, top) Western blot spots of the expressed proteins and their related β-Actin in hippocampal lysates of different rat groups. (A to H, bottom) The histograms of the relative protein levels. A) p-CREB/CREB ratio, B) p-$ERK_{1/2}$ / $ERK_{1/2}$ ratio, C) BDNF, D) p11, E) 5-HT1B, F) p-BAD/ BAD ratio, G) NR2B, and H) FOXO3a.
* Show comparison between different depressed groups.
# Show comparison between control and depressed groups after each treatment.
# and*$p < 0.05$, ## and **$p < 0.01$, ### and ***$p < 0.001$, #### and ****$p < 0.0001$.

confirm that the recorded current was purely an NMDA current, AP5 was applied to the bath solution, which completely blocked the NMDA current.

These results confirmed the direct effect of Crocetin on this receptor, which significantly antagonizes the postsynaptic NMDA receptors. This effect may reduce the depolarization induced by exogenously applied glutamate. A similar effect of saffron on the NMDA receptors has been reported previously (Berger et al., 2011).

In summary, this study indicated the antidepressant and antianxiety effects of Crocin and Crocetin, similar to Fluoxetine. Crocin/ Crocetin led to an increase in serum Serotonin and a decrease in serum Corticosterone. Based on the observed changes in the expression level of some protein markers, these natural carotenoids, like Fluoxetine, could act as an SSRI and affect the Serotonin pathway. Furthermore, they upregulated CREB. As seen in Fig. 4C, after CREB phosphorylation by $ERK_{1/2}$, the expression of BDNF was induced. The CREB-BDNF signaling was introduced as a critical synaptic plasticity event. In order, BDNF increases (MEK)/ERK phosphorylation and activation. This positive loop that was begun in the presence of these carotenoids indicated the activation of the Serotonin signaling pathway. In addition, the CREB phosphorylation at Ser133 induces the p11 expression. Here we also observed a large effect size in the p11 level after the Crocin/ Crocetin treatment of depressed rats. The p11 has a crucial role in Serotonin receptors' function and distribution. It has been shown that the direct interaction of 5-HT1B (a Serotonin receptor) with p11 causes receptor recruitment to the cell surface and increases its functionality (Svenningsson et al., 2006). Some survival factors, such as BDNF, also induce p11. The hormone-inducible p11 preferentially binds to the unphosphorylated BAD and attenuates its pro-apoptotic activity (Hsu et al., 1997). BAD plays a critical role in apoptotic paths controlled by Bcl-2 family proteins. After the MAPK/ERK pathway activation and BAD phosphorylation, the Bcl-2 expression induces, which activates the antiapoptotic mechanism. Our results indicated the positive effects of saffron carotenoids on BDNF and an increase in the p-BAD/BAD ratio that results in nerve survival.

In the nervous system, high glucocorticoid concentration results in glutamate excitotoxicity (Burnouf et al., 2013). Due to the overactivation of NR2B, a series of cytoplasmic and nuclear processes initiates that induce oxidative stress and lead to cell death (Hardingham and Bading, 2010). These natural Carotenoids decreased the expression of NR2B and its downstream signaling. Our bioinformatic study, confirmed by patch-clamp data, indicated Crocetin's important role in this type of NMDA receptor. In addition to the mentioned changes in CREB and $ERK_{1/2}$, we observed a decrease in the expression of FOXO3a, which was increased due to the depression induction. This is another mechanism that decreases the expression of apoptotic markers.

Conclusions

Crocin and Crocetin induced changes in Serotonin and Corticosterone serum concentrations and decreased hippocampal NR2B expression. Furthermore, Crocetin inhibited the NMDA receptor, accompanied by some changes in its downstream signaling pathways. These changes include a decrease in FOXO3a expression and removing the inhibitory effect on ERK and CREB phosphorylation. In addition, these natural carotenoids led to changes in the downstream signaling pathways of Serotonin that increase BDNF, p11, and 5HT1B expression, as well as CREB and ERK phosphorylation. All these changes indicate the antidepressant activities of Crocin and Crocetin.

Author contributions

Ms. Sahar Mohammadi: Conception and design; Data acquisition, Analysis; Interpretation of data, Drafting the manuscript.

Prof. Mohsen Naseri: Conception and editing.

Dr. Nassim Faridi: Data acquisition (Western Blot) and analysis.

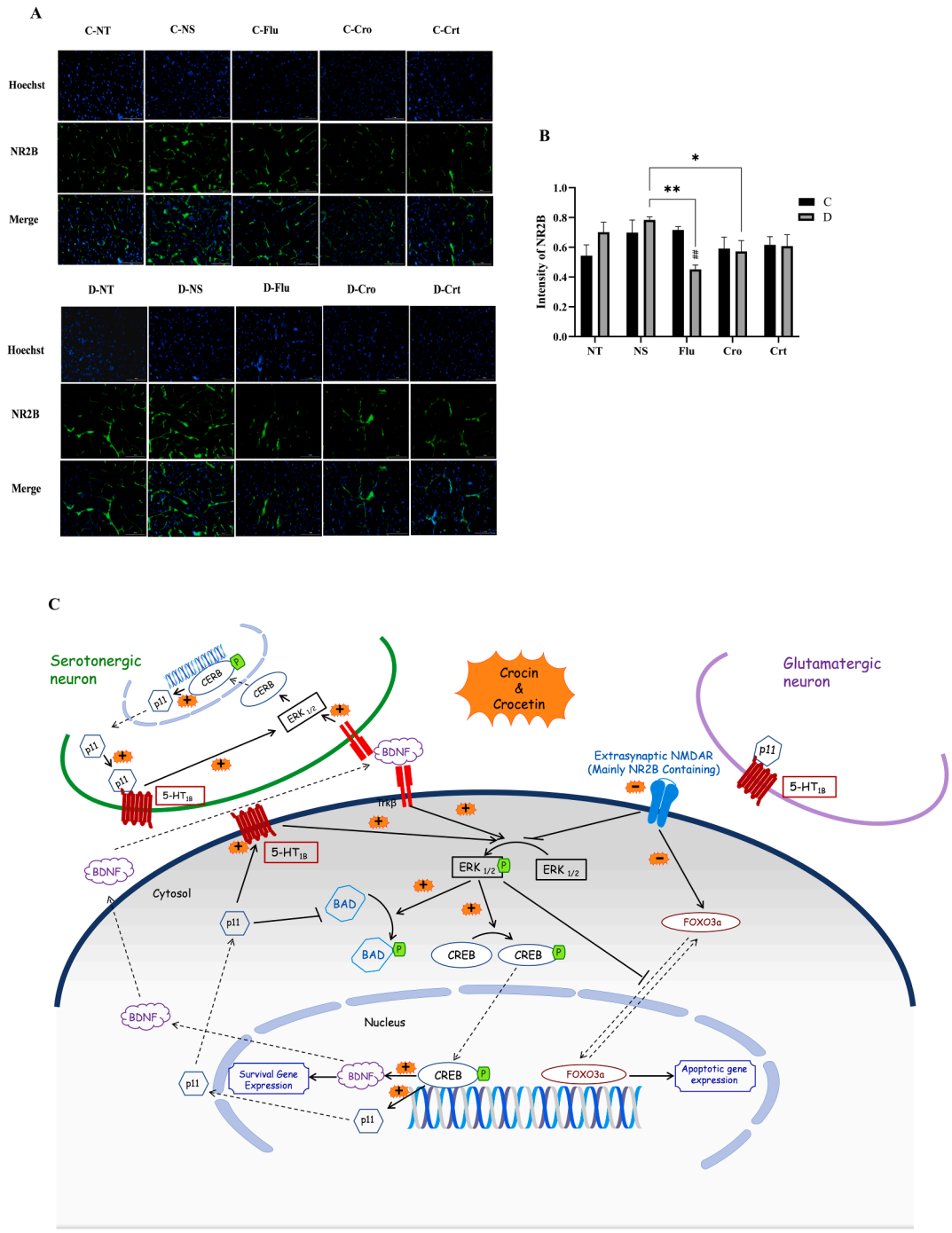

Fig. 4. The immunofluorescence staining of NR2B and effect of Crocetin on NMDA currents. (A) The immunofluorescence images of NR2B (green fluorescent) and Hoechst (blue fluorescence) in CA1 of the rats' hippocampus in control (up) and depressed groups (bottom).
(B) The average fluorescence intensity of NR2B.
(C) Schematic representation of the effect of Crocin and Crocetin on signaling pathways.
* Show comparison between different depressed groups.
# Show comparison between control and depressed groups after each treatment.
# and*$p < 0.05$, ## and **$p < 0.01$, ### and ***$p < 0.001$, #### and ****$p < 0.0001$.

Ms. Parisa Zareie: Data acquisition (patch-clamp recording) and analysis.

Dr. Leila Zare: Data acquisition (immunostaining) and analysis.

Prof. Javad Mirnajafi-Zadeh: Monitoring the behavioral tests; patch-clamp recordings and analysis; Data interpretation; Editing the manuscript.

Prof. Zahra Bathaie: Supervision of the study; Conception and design of the work; Analysis; Interpretation of data; Editing and Revising the manuscript.

All authors approved the last version of the manuscript and agreed with all aspects of the work.

All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

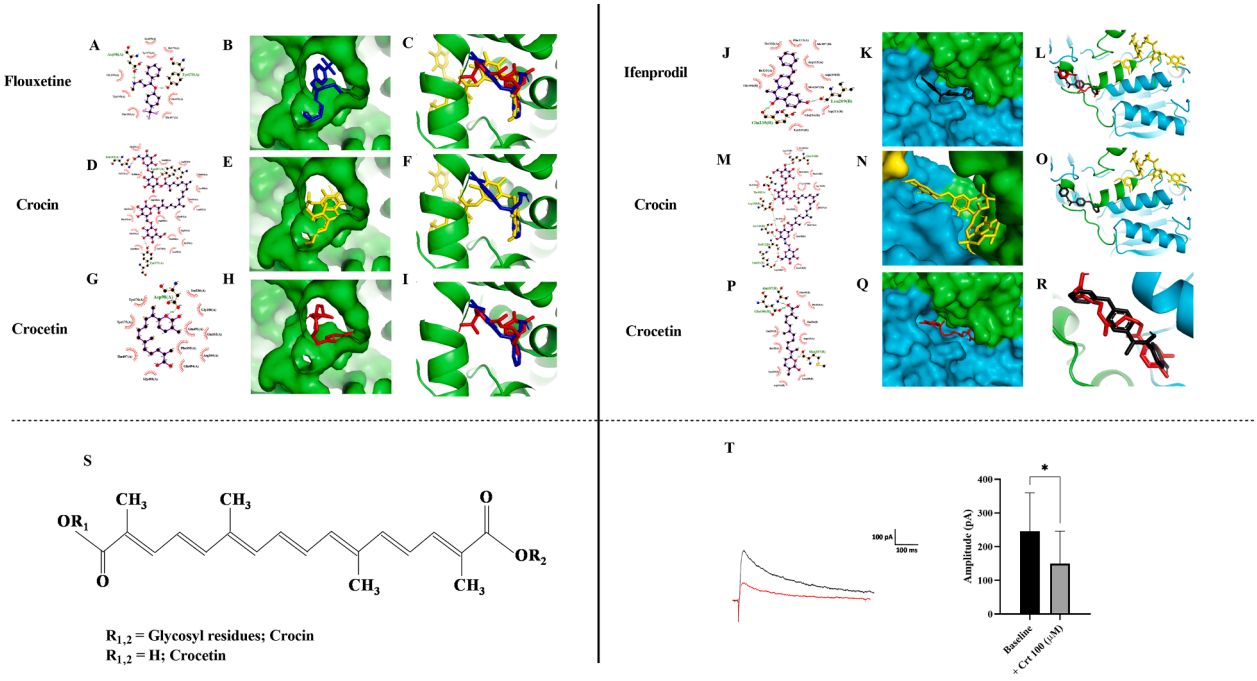

Fig. 5. The docking results of the Crocin and Crocetin interaction with SLC6A4 and NR2B.
The interaction of the named ligands with SLC6A4 are shown in Figs. A to I. The peptide chains of SLC6A4 in 3D views are shown in green.
(A, D, and G) Amino acids and chemical bonds (hydrogen bonds with green dashes and hydrophobic bonds with red semi-circle radial spikes) involved in the interaction of SLC6A4.
(B, E, and H) 3D presentation of the binding site of ligands on SLC6A4.
(C, F, and I) The overlap between the mentioned ligands in SLC6A4.
The interaction of the named ligands with NR2B are shown in Figs. J to R. The peptide chains of NR2B in 3D views are shown in green and blue.
(J, M and P) Amino acids and chemical bonds (hydrogen bonds with green dashes and hydrophobic bonds with red semi-circle radial spikes) involved in the interaction of NR2B.
(K, N, and Q) 3D presentation of the binding site of ligands.
(l, O, and R) The overlap between the mentioned ligands in NR2B.
In all Figs. Crocin, Crocetin, Fluoxetine, and Ifenprodil are shown yellow, red, blue, and black sticks respectively.
(S) Chemical structures of Crocin(s) and Crocetin.
(T) Sample traces show the average amplitude of NMDA currents in 5 consecutive records in baseline (black) and after adding Crocetin (100 μM) to the bath solution (red). (*$p < 0.05$).

Supplementary Material

Supplementary 1. (A) The rat groups in this study. (B) Table of statistical analysis data and Cohen's *d* effect size values of proteins expression and their ratios. (C) Table of Docking results.

Supplementary 2. The list and characteristics of the materials used in different experiments of this study.

Declaration of Competing Interest

I hereby declare that the disclosed information is correct and that no other situation of real, potential or apparent conflict of interest is known to me and other authors. I undertake to inform you of any change in these circumstances, including if an issue arises during the course of the submission or work itself.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2023.154989.

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