RESEARCH PAPER

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Comparative effects of the alcoholic extract of *Terminalia chebula* and crocin on stress-induced anxiety-like behavior and memory impairment in male rats

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Crocin and *Terminalia chebula* (*T. chebula*) were proven to have neuroprotective effects. In this study, we evaluated the preventive effects of crocin and alcoholic extract of the *T. chebula* alone and in combination to examine their efficacy against chronic restraint stress (CRS)-induced cognitive impairment, anxiety-like behaviors, hippocampal synaptic plasticity deficit as well as neuronal arborization damage in the hippocampal CA1 neurons. Over 14 consecutive days, animals received crocin, *T. chebula*, or their combination (5 min before CRS). The elevated plus-maze results showed that crocin and *T. chebula* alone and in combination treatment significantly increased the time spent in open arms, percentage of open arm entries, and head dipping as compared with the CRS group. Barnes maze results showed that administration of crocin and *T. chebula* alone and their combination significantly improves spatial memory indicators such as distance traveled, latency time to achieving the target hole, and the number of errors when compared to the CRS group. These learning deficits in CRS animals correlated with a reduction of long-term potentiation (LTP) in hippocampal CA1 synapses, which both *T. chebula* and crocin treatment improved field excitatory postsynaptic potentials (fEPSP) amplitude and fEPSP slope reduction induced by CRS. Golgi-Cox staining showed that *T. chebula* and crocin treatment increased the number of dendrites and soma arbors in the CA1 neurons compared with the CRS group. Our results suggest that both *T. chebula* and crocin attenuated CRS-induced anxiety-like behaviors, memory impairment, and synaptic plasticity loss in hippocampal CA1 neurons. We found no significant difference between single treatments of *T. chebula* or crocin and their combination in protecting CRS-induced anxiety-like behaviors, memory impairment, and synaptic plasticity loss in hippocampal CA1 neurons.

Key words: crocin, *Terminalia chebula,* chronic restraint stress, field potential recording, Golgi-Cox staining

INTRODUCTION

Stress is a phenomenon that is common in daily life and requires a coordinated interaction between the nervous and hormonal systems to create appropriate behaviors and physiological responses (Gunn and Baram, 2017). In biological systems, any condition that seriously disrupts an organism's physiological/psychological homeostasis is called stress (Kim and Diamond, 2002). An important line of neuroscience research has shown that stressful experiences can harm certain aspects of brain function in recent decades. Uncontrollable stress can have adverse side effects such as impaired learning and memory capacity, exacerbated the

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aging-related decline in cognition, anxious behaviors, decrease reproductive activity and increased predisposition of hippocampal neurons to atrophy or necrosis (Ehteram et al., 2017; Surget and Belzung, 2021).

One of the brain structures that play an important role in memory and the regulation of neuroendocrine stress hormones is the hippocampus (Goldfarb et al., 2020). The hippocampus and related structures are essential for the formation, reorganization, and stabilization of memory (Dong et al., 2021). The hippocampus is highly sensitive to stress and plays an important role in mediating behavioral/functional responses and the neuroendocrine system. This structure is also one of the targets of stress hormones and has the highest concentration of receptors for corticosteroids in the mammalian brain. Prolonged or damaging stressors have been shown to cause morphological changes in the hippocampus. Chronic stress reduces the number of dendritic spines and branches of pyramidal neurons in the hippocampus (McEwen et al., 2016).

Stress can have damaging effects on learning and memory and is implicated in the development of anxiety disorders. Today, therapeutic options for the treatment of stress produce only partial relief, and treatment of stress is still lacking. Thus, it appears essential to use more tolerable and less toxic drugs with more favorable effects to inhibit learning and impairment and reduce anxiety-like behaviors and neuronal structural damage in stress. In recent years, there has been evidence to suggest that crocin (one of the active ingredients in saffron) may prevent learning and memory damage. Saffron is one of the plants that have been used as a medicinal plant in different parts of the world for a long time. This plant has been used as an antispasmodic, sedative, digestive aid, anti-flatulence, diaphoretic, expectorant, stimulant of sexual desire and pain reliever. Crocin has improved memory by acting on NMDA receptors (Abe and Saito, 2000).

Researchers also emphasize the effect of crocin in improving memory and inducing long-term potentiation (LTP) in male rats (Hadipour et al., 2021a).

In addition, *Terminalia chebula* (*T. chebula*) is one of the medicinal plants in traditional Indian and South Asian medicine. In traditional Iranian and Indian medicine, the properties of *T. chebula* are used to treat many diseases such as diabetes, eye infections, diarrhea, and bleeding. Also, studies have shown that *T. chebula* has analgesic, anti-inflammatory, antioxidant effects, and protective effects on cells (Rao et al., 2012).

Most studies have investigated crocin or *T. chebula* in different neural lesions or disease models. However, little is known about the synergistic potential of these therapies on anxiety, and cognitive deficits in chronic stress. On the other hand, many reports based on animal and clinical studies propose that the single treatment of crocin or *T. chebula* has little neuroprotective effects. In this regard, combined therapy with different effective modalities may help prevent chronic stress-induced anxiety and cognitive deficits. Based on these studies, we hypothesized that crocin combined with *T. chebula* might be more beneficial for preventing anxiety, cognitive deficit, and synaptic plasticity impairment in the CA1 hippocampal neurons induced by chronic stress.

METHODS

Animals

Healthy male Wistar rats (weighing 170–200 g and 8-weeks-old on arrival in the animal facility of the Baqiyatallah University of Medical Science, Tehran, Iran) were used in the study. Animals were assigned unique identification numbers and group-housed (2–3 rats per cage) in Plexiglas cages (67 cm × 40 cm × 22 cm). All rats were maintained at a temperature between 22°C and 25°C with 50% humidity and light-controlled (12/12 h = light/dark; lights on 07:00–19:00). Testing was performed during the light part of the cycle. Food and water were available *ad libitum*. The animal ethics committee of the Baqiyatallah University of Medical Science, Tehran, Iran approved all experiments according to ethical guidelines (Protocol approval number: IR.BMSU.REC.1400.115).

Study design

Animals were divided into five groups (n=8 in each) control, chronic restraint stress (CRS), CRS + crocin, CRS + *T. chebula*, and CRS + crocin + *T. chebula*. Rat in the control group received no treatment. Animals in the CRS group were subjected to restraint stress for 14 days for two hours each day. Rat in the CRS+crocin group received 30 mg/kg of crocin (Sigma-Aldrich) that was dissolved in normal saline and intraperitoneally (IP) injected. The dose of crocin was chosen based on our pilot studies and previous reports (Hadipour et al., 2021a). Rat in the CRS + *T. chebula* group received 200 mg/kg alcoholic extract of *T. chebula* that was dissolved in normal saline and orally administered. The dose of *T. chebula* was determined based on a previous report (Ahmadi-Naji et al., 2017). Rat in the CRS + crocin + *T. chebula* group received both crocin and alcoholic extract of *T. chebula*. Rats received crocin, *T. chebula* or crocin + *T. chebula* five minutes before restraint stress, for 14 consecutive days. All solutions were freshly pre-

pared before administration (Fig. 1). To avoid the effect of circadian rhythms on the behavioral test, all rats were tested at the same time frame between 10-13 A.M.

Stress paradigms

The restrainers comprised a plastic cylinder (height, 5 cm; width, 5.5 cm; length, 22 cm). Male Wistar rats were subjected to 2 h of restraint stress daily for 14 days, during which they were deprived of food and water, without physical suppression.

Elevated plus maze test

The elevated plus maze (EPM) test was performed to investigate the neuroprotective effect of *T. chebula* and crocin on anxiety-like behaviors induced by restraint stress. All animals were tested in the EPM test twenty-four h after the 14 consecutive day's restraint stress sessions. The EPM test was based on our previous studies (Hadipour et al., 2021b). Briefly, the maze is a cross-shaped platform and consisted of 2 open and 2 closed arms. All arms communicate by a central zone.

Animals were placed in the central zone of the maze, facing an open arm for five min. Their movements in the maze were monitored for five min with a camera. The number of head-dipping behavior was calculated for five min. Also, the number of open arms entries and time spent in the open-arm were calculated.

Barnes maze test

The Barnes test is used to assess spatial memory in mice and rats (Gawel et al., 2019). The maze is made of a black Plexiglass round table 120 cm in diameter. The maze contains 12 uniform holes (8 cm in diameter) in its periphery. The distance between the holes is 5 cm. One dark Plexiglas box was under the plate (target hole or escape hole) with dimensions of 20 × 20 cm, which the animal could hide. One bright light was placed 110 cm above the maze, which was used as an aversive stimulus. Four proximal visual cues were placed in the room, 80 cm away from the platform. In the present study a protocol based on four days of acquisition trials (or learning; day 1 to day 4), followed by a probe trial (or memory retention day) on the last day was used. On adaptation day (one day before the

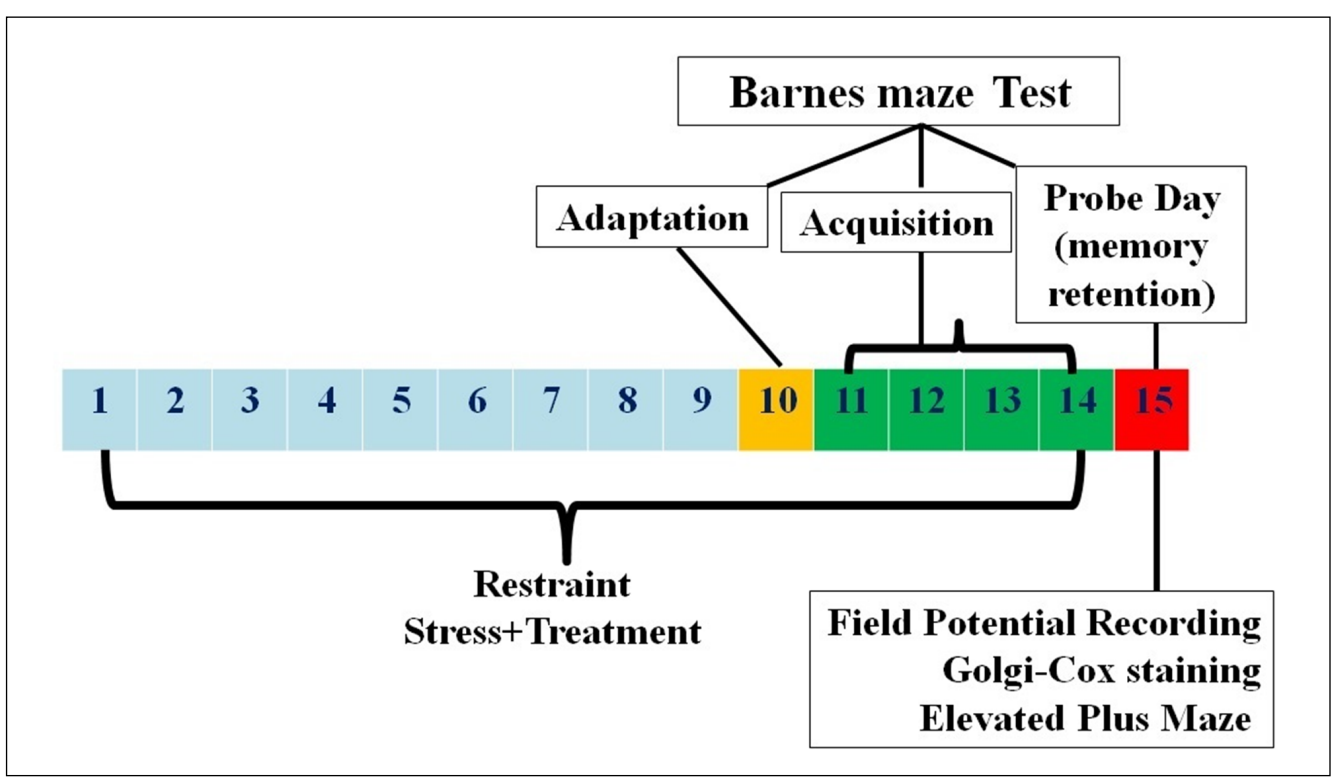

Fig. 1. Schematic timeline to show the design and order of behavioral, electrophysiological, and histological studies. Each column shows a day during the study.

first trial), the animals were put in the center of the maze and moved freely in all directions for 5 min. An acquisition trial consisted of placing a rat in the center of the maze and being permitted to freely move in the maze for 180 s to explore the escape hole and enter it. The animals were tested with the escape box four times per day (10-minute intervals between trials) for four continuous days (from day 11 of stress induction until 14). On the fifth day (one day after 14 days of restraint stress), the animals were submitted to a probe trial that was placed in the center of the maze, and the number of errors, distance traveled, and time to reach the target hole was measured. After testing each rat to eliminate odor the surfaces of the maze and box were cleaned, using 70% ethanol.

In vivo electrophysiology procedure

One day after 14 days of restraint stress, for assessment of LTP in the hippocampal CA1 neurons field potential recording, was performed. Male Wistar rats were anesthetized with urethane (1.5 g/kg; Sigma) and the head was placed in a stereotaxic apparatus. The body temperature of the animal was kept constant by a small animal thermoregulation device. A stimulating electrode (PFA-Coated Stainless Steel, Diameter: 0.005 in.; A-M system, USA) was inserted into the Schaffer collateral pathway region, on the right sides (AP 3.1 mm, ml 3.1 mm; DV 3–3.5 mm). The extracellular stainless steel recording electrode was placed in the CA1 region of the hippocampus (AP 2.8 mm, ml 1.8 mm; DV 2.5 to 3.5 mm), according to the atlas of Paxinos and Watson (2006). Standard procedures for *in vivo* input-output and LTP recording from the CA1 area of anesthetized rats were used as described previously. In brief, signals were passed *via* an analog-to-digital interface to a computer, and the data were analyzed by Potentialize software (ScienceBeam, Tehran, Iran). Extracellular evoked responses were acquired from the CA1 region of the hippocampus. Extracellular field potentials were amplified and filtered (×1000 and 1-3000 Hz bandpass, respectively). After confirming a suitable evoked response, an input/output (I/O) curve was obtained by averaging 10 population spikes induced by variation of the stimulus current (100-1200 µA). The intensity needed to evoke 40-50% of the maximum response of the fEPSP was determined from this I/O curve. Then, this current intensity was used for the paired-pulse analysis and LTP induction. Baseline recordings were taken for 20 min (100 trains) before delivering high-frequency train stimulation (HFS). To induction of LTP, the HFS (10 bursts of 20 pulses; duration 200 µs; burst frequency 400 Hz; intraburst pulse frequency every 2 s) was delivered to the Schaffer collateral pathway. We analyzed the mean of evoked responses (slope and amplitude of fEPSP) during 1 h after HFS (360 trains at 60 min). LTP was induced using HFS protocols (10 bursts of 20 pulses; duration 200 µs; burst frequency 400 Hz; intraburst pulse frequency every 2 s) in the Schaffer collateral pathway. For each animal, the average fEPSP slope and amplitude before the HFS was normalized to 100% (baseline average), and the baseline normalized slope was measured for one hour.

The Golgi-Cox staining

One day after the last stress, all experimental groups underwent Golgi-Cox staining (Kandimalla et al., 2018). The animals were anesthetized, and their brains were removed. The brain was immersed in fresh Golgi-Cox solution (10 parts of dd-H2O + 5 parts of 5% potassium dichromate + 5 parts of 5% mercuric chloride + 4 parts of 5% potassium chromate) for 7-10 days and kept in the dark at room temperature. After that, brains were cut into 100-200 µm coronal sections and loaded on albumin-coated slides for two days. In the developing stage, the sections were first immersed in distilled water for five min, and then immersed for five min in 50% ethanol, eight min in 3:1 ammonia, dd-H2O twice for five min each, ten min in 5% sodium thiosulfate, dd-H2O twice for one min each, 70%, 95%, and 100% ethanol for six min each, and xylene for six min.

Then, slides were mounted with glass coverslips and histological glue. The images were taken using the Nikon Eclipse 50i optical microscope (with Nikon DS-Fi1/DS camera) at different magnifications. Representative images from the hippocampal CA1 neurons were taken from 4 to 6 cells per brain slice. The numbers of branches of the dendrites (dendritic arborization), and soma (soma arborization) were manually counted upon the pyramidal cells. With the microscope set at a magnification of 400×, if the neuron cell body fell within the sampling frame, the neuron would be accepted and analyzed.

Statistical analysis

Data are shown as the mean±SEM for eight animals. All data were analyzed using IBM SPSS statistics (version 24). All data were analyzed using one-way ANOVA, followed by the Tukey *post hoc* test. All data have been reported as mean ± SEM and $p<0.05$ is considered statistically significant.

RESULTS

The effects of crocin and *T. chebula* on the anxiety-like behaviors by EPM

One day after the final CRS episode, all experimental animals underwent an elevated plus-maze to evaluate anxiety-like behaviors. Data analysis showed that CRS significantly decreased the open arms spent time and also decreased open arms entrance percentage in the CRS group as compared to the control rats ($F_{4,20}$ (open time spent time) = 0.8236; $F_{4,20}$ (open arms entrance percentage) = 0.76; p=0.001 by one-way ANOVA followed by Tukey's test) (Fig. 2).

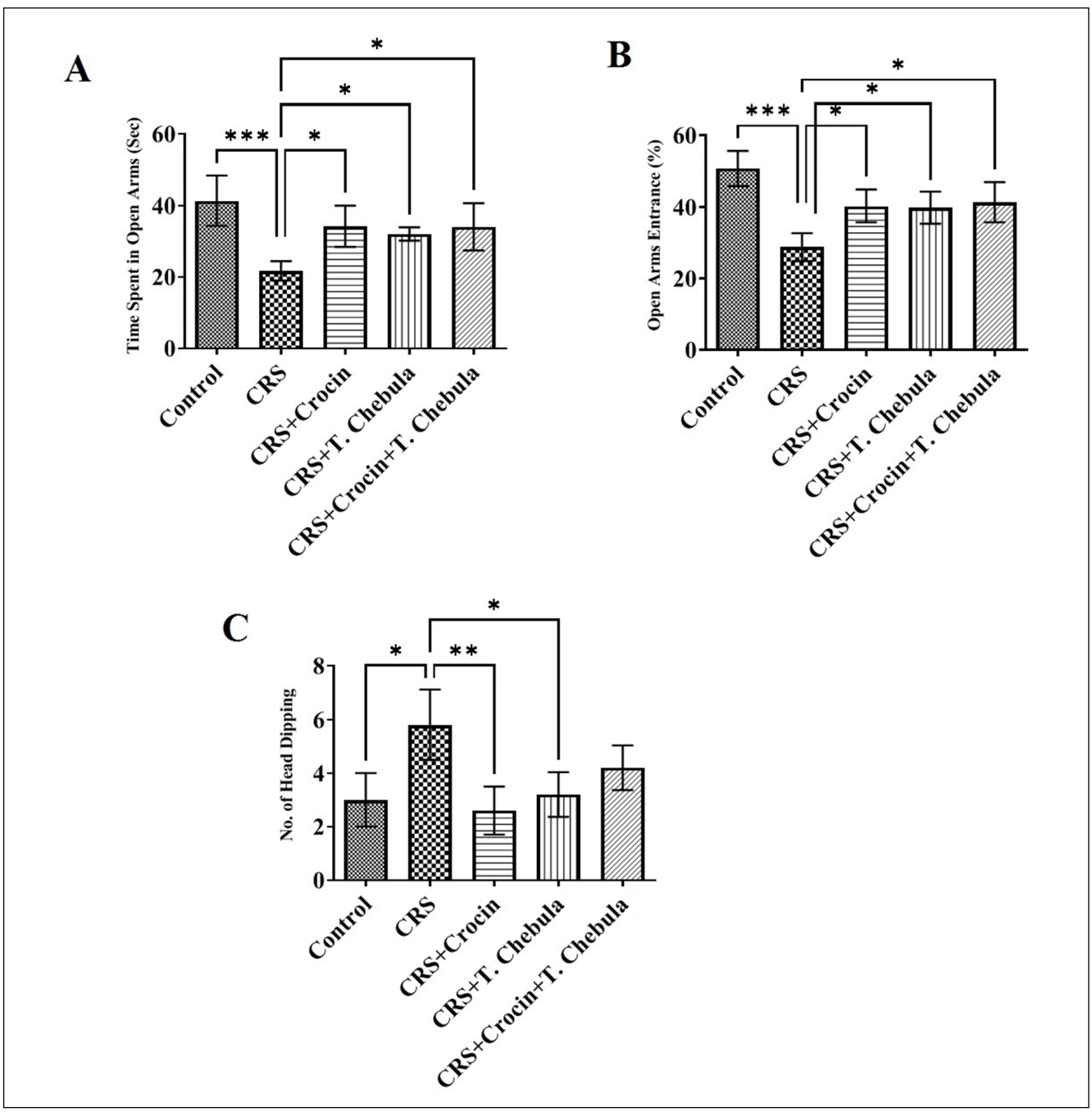

Fig. 2. Effects of *T. chebula* and crocin on CRS-induced anxiety-like behaviors by EPM test. Results revealed significant increases in the time spent into open arms (A), % of the open arms entries (B), and decreased the number of head dipping (C), by the rats within five minutes in the CRS + crocin, CRS + *T. chebula,* and CRS + *T. chebula* + crocin when compared with the CRS group. Data are presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

Additionally, CRS significantly increased the number of head dipping than the control group ($F_{4,20}$=0.09091). Treatment with the *T. chebula* or crocin before CRS for 14 consecutive days significantly increased the percentage of time that animals spent in open arms, open arms entrance percentage (p<0.05) and head dipping (p<0.05) compared to the CRS group. Also, pretreatment with *T. chebula* and crocin significantly increased the percentage of time that animals spent in open arms and open arms entrance percentage compared to the CRS group.

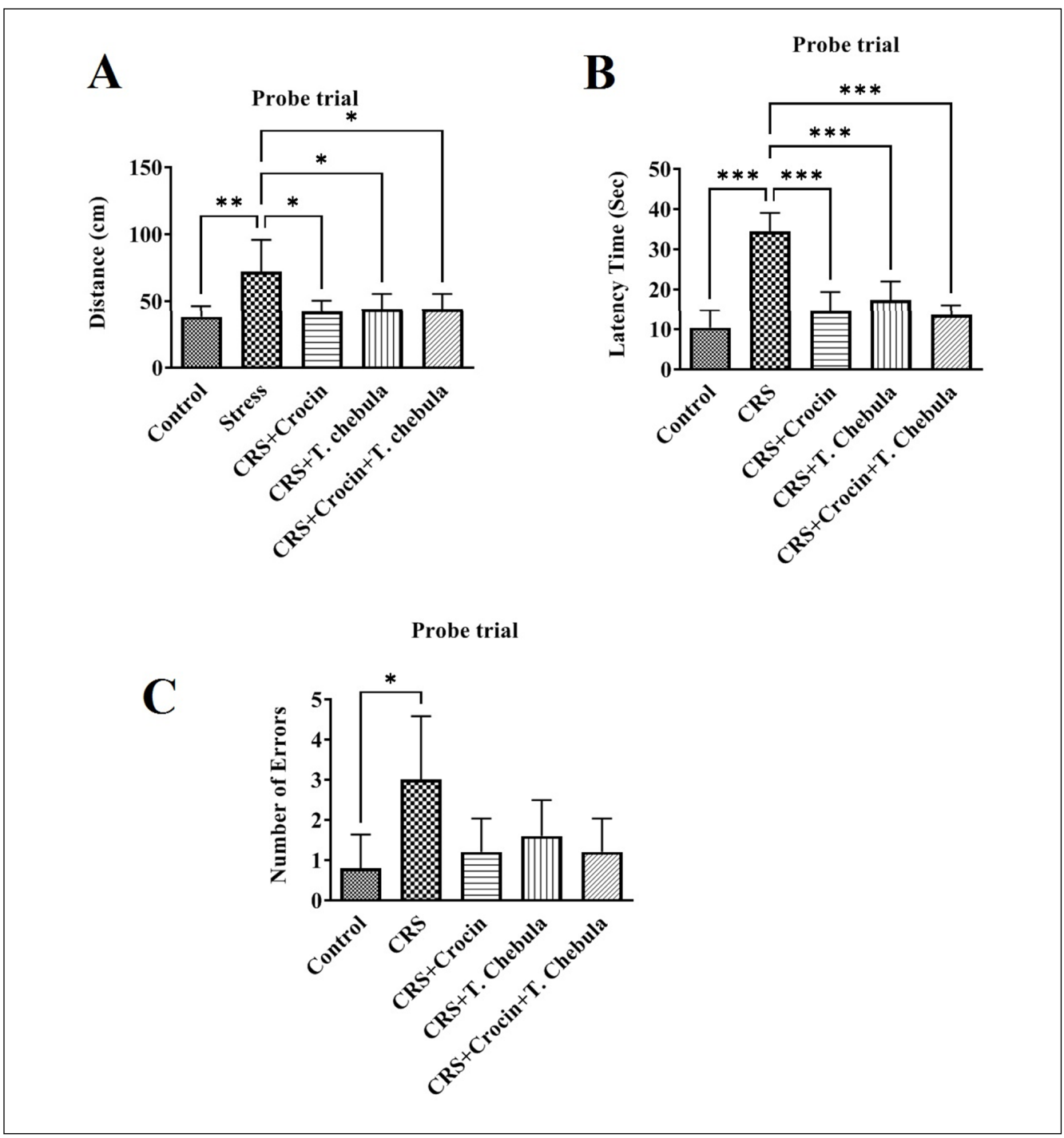

Fig. 3. Effects of *T. chebula* and crocin on spatial memory by Barnes test. Mean of the (A) distance, (B) latency time to achieving the target hole (seconds) and (C) the number of errors reported in all experimental groups. Data are presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

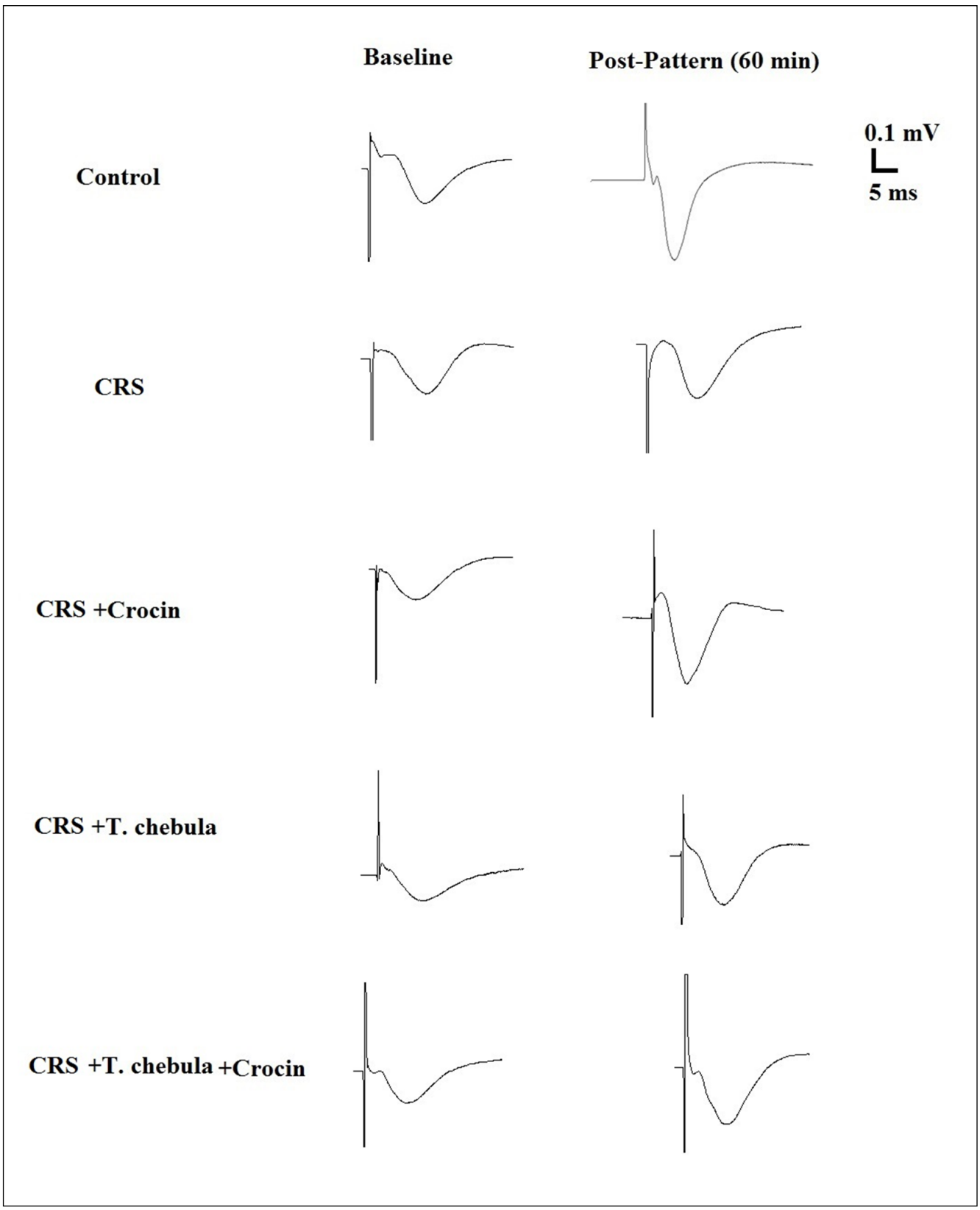

Fig. 4. Evoked potentials were recorded from the CA1 cell layer in the control, CRS, CRS + crocin, CRS + *T. chebula* and CRS + *T. chebula* + crocin group. Long-term plasticity was investigated for 60 min after a theta burst pattern of tetanic stimulation in the CA1 neurons. Sample traces were taken immediately before (baseline) and 60 min after HFS stimulation (post-pattern).

The effect of *T. chebula* and crocin on spatial memory (Barnes maze)

As shown in Fig. 3A, rats in the CRS group had a significantly higher distance traveling to achieve the target hole in the Barnes maze task on the 5th day (probe day) compared with the control group ($F_{4,20}$=1.70, p=0.01; by one-way ANOVA followed by Tukey's test). Treatment with *T. chebula*, crocin, or their combined treatment before CRS for 14 consecutive days significantly increased the distance traveled to achieve the target hole (p<0.05). In addition, as shown in Fig. 3B both crocin and *T. chebula* significantly reduced latency time to achieve the target hole on the probe day test day compared to the CRS animals ($F_{4,20}$=0.33, p=0.001; by one-way ANOVA followed by Tukey's test). Subjecting the animals to CRS increased the mean value of the number of errors on the probe day (p<0.05) compared with the control group ($F_{4,20}$=0.75, p<0.05; by one-way ANOVA followed by Tukey's test). The statistical analysis of the results revealed that treatment with crocin or *T. chebula* does not show any significant difference in the number of errors on the probe day (p<0.05, for both days) compared with the CRS group (Fig. 3C).

Crocin and *T. chebula* administration improved the LTP induction in the hippocampal CA1 region in CRS rats

Examples of the effects of CRS and administering *T. chebula*, crocin or their combined treatment before CRS for 14 consecutive days on LTP have been shown in Fig. 4. Evoked potentials were recorded from the CA1 region of the hippocampus in all experimental groups. The statistical analysis of the results revealed that the ability of synapses for LTP induction in the CRS animals was impaired. The mean of 60 min fEPSP amplitude after HFS in the CRS group was significantly (P<0.001) lower than the value of the control group ($F_{4,20}$=1.255, p=0.001; by one-way ANOVA followed by Tukey's test). A single treatment with crocin, or *T. chebula* showed a significant degree of LTP recovery and considerable increment in the fEPSP amplitude in the crocin + CRS group and *T. chebula* + CRS compared to the CRS group (P<0.001). Co-treatment of *T. chebula* and crocin significantly increased the fEPSP amplitude compared to the CRS group (Fig. 5A). In hippocampal CA1 neurons from all groups, HFS of the Schaffer collaterals reliably increased fEPSP slope that lasted for one-hour. Stress significantly (p<0.001) reduced the fEPSP slope during

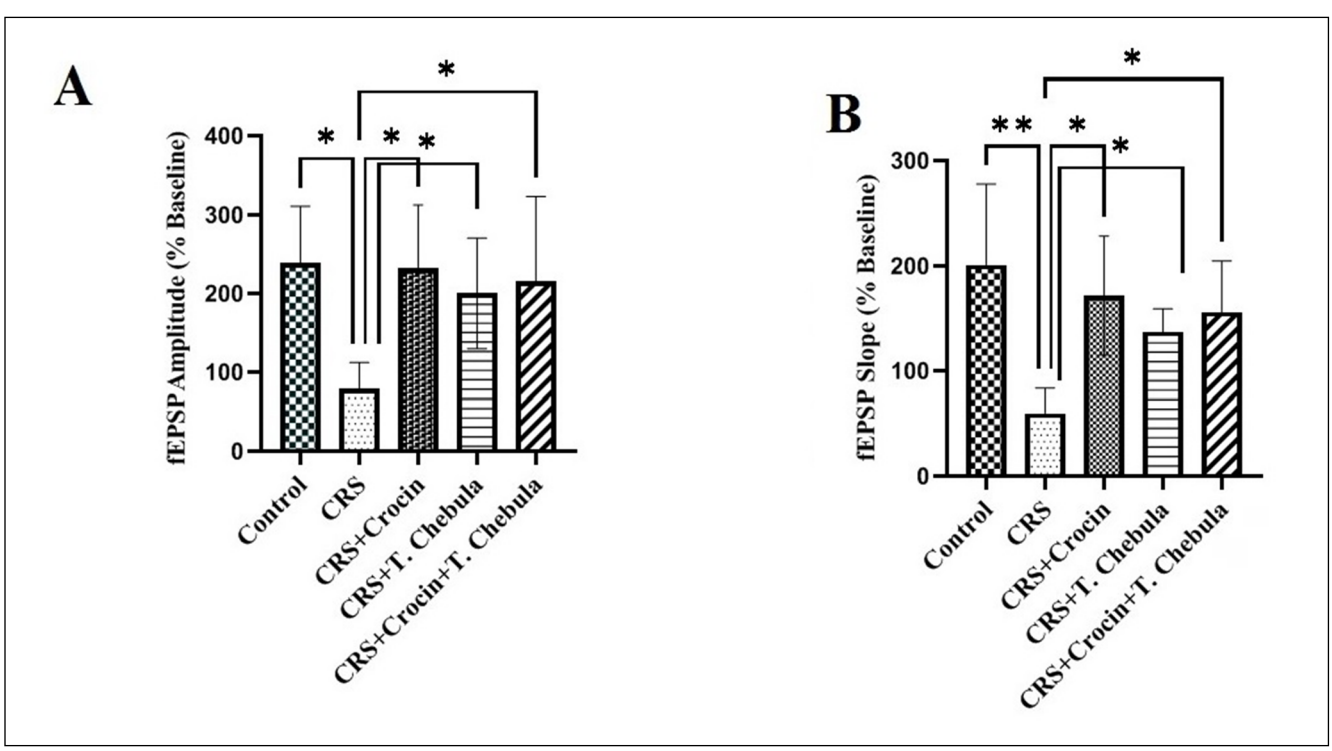

Fig. 5. Effect of administration of *T. chebula* and crocin on CRS-induced impairment on the induction of CA1 LTP. (A) fEPSP amplitude and (B) the fEPSP slope in the CA1 area of the hippocampus was reported in all experimental groups. Data are presented as mean ± SEM. *p<0.05, **p<0.01.

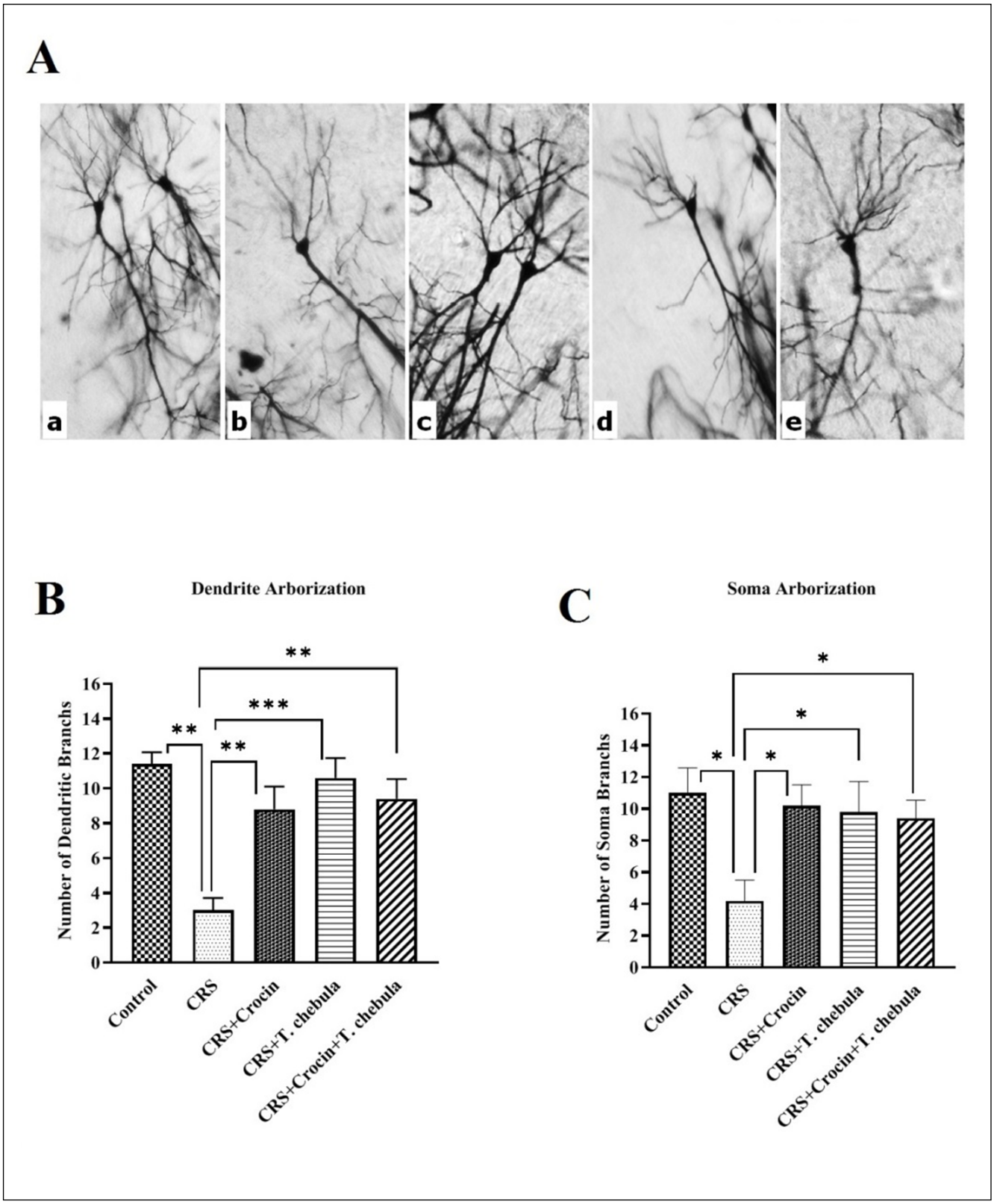

Fig. 6. The neuroprotective effect of *T. chebula* and crocin in the neuronal arborization in the CA1 neurons on CRS-induced neuronal arborization deficit by Golgi-Cox staining. (A) Representative images of Golgi-Cox stained neurons in the hippocampus CA1 neurons. Control (a), CRS (b), CRS+crocin (c), CRS + *T. chebula* (d) and CRS + *T. chebula*+crocin (e) groups. The scale bars indicate 100 μm. Quantitative analysis of neuronal arborization in the dendritic (B) and soma (C) in the CA1 neurons in the different groups. Data are presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

one-hour extracellular recording than the control group ($F_{4,20}$=0.76, p=0.001; by one-way ANOVA followed by Tukey's test).

The statistical analysis of data reveals that there has been a significant (P<0.05) difference between the fEPSP slope before and after delivery of HFS in the crocin + CRS group and *T. chebula* + CRS compared to the CRS group (Fig. 5B). Combined treatment of *T. chebula* and crocin, significantly enhanced the fEPSP amplitude (p<0.05) compared to the CRS animals. The results indicated no significant difference between single treatments of *T. chebula* or crocin and combined treatment of *T. chebula* + crocin animals in fEPSP amplitude.

Effect of and crocin on CRS-induced neuronal arborization reduction within hippocampal CA1 neurons

Ample evidence supports the involvement of chronic stress in the loss of soma and dendritic spines in the hippocampus. Hence, the ability of *T. chebula* and crocin to inhibit the neuronal arborization loss induced by CRS in the pyramidal cells of the CA1 region of the hippocampus was investigated by Golgi-Cox staining. Representative examples of arborization of CA1 hippocampal pyramidal neurons are shown in Fig. 6A. The results of the Golgi-Cox staining of the CA1 area of the hippocampus showed that the CRS significantly decreased the number of branches along the dendrites in the pyramidal neurons from the CA1 region as compared with the control group ($F_{4,20}$=0.61, p=0.001; by one-way ANOVA followed by Tukey's test).

Administration of crocin (CRS + crocin group), *T. chebula* (CRS + *T. chebula*), or their combination (CRS + crocin + *T. chebula*) for 14 consecutive days significantly (P<0.01) increased the number of the dendritic branches in the CA1 area as compared with the CRS group (Fig. 6B). Also, the number of soma arbors (branched of the soma) in the CRS animal significantly (P<0.05) decreased compared to the control animals. Treatment with crocin and *T. chebula* separately and also combined with each other was capable of significantly (P<0.05) increasing the number of the branches of soma compared to the CRS group (Fig. 6C).

DISCUSSION

This study showed that CRS led to rat's spatial memory impairments (assessment of data from the Barnes maze exhibited CRS increased latency time, error, and distance traveling). Additionally, results revealed that the CRS produced significant alterations in animals' anxiety-like behaviors, including a decrease in the time spent in the open arm and the number of open arms entries and an increased head dipping in the EPM test (anxiogenic effect). It was also found that the synaptic plasticity in the CA1 region of the hippocampus is impaired by CRS. CRS has been widely used as a model of chronic psychoemotional stress (Huang et al., 2015). The physiological effects of stress are mainly mediated by stress hormones (cortisol in humans and corticosterone in rodents) (de Abreu et al., 2021).

EPM results after 14 days of CRS showed a decrease in time spent in open arms, the percentage of entries into open arms, and an increase in head dipping. Indeed, the present results indicated anxiety-like behaviors after CRS. A relation between CRS and anxiety was found in several studies. For instance, consistent with our results Chiba et al. (2012) showed CRS induces anxiety-like behaviors in rats, accompanied by down-regulation of glucocorticoid receptors and damaged BDNF-induced glutamate release in the prefrontal cortex. In addition, in male Wistar rats a significant relationship was found between CRS and excessive activation of the HPA (hypothalamic-pituitary-adrenal) axis, 5-HT (5-hydroxytryptamine) system disorders and abnormal alterations in cAMP-PKA-CREB-BDNF signaling and anxiety-like behavior (Geng et al., 2021). Also, a recent study by Moreno-Martínez et al. (2022) revealed that CRS-induced anxiety-like behavior might be accounted for by a reduction in synaptic connectivity of the central nucleus of the amygdala. We next used a crocin, *T. chebula*, or both combinations to determine their role in anxiety-like behaviors in CRS conditions. Treatment with crocin or *T. chebula* produced significant alterations in anxiety-like behaviors in CRS animals. Indeed, both crocin and *T. chebula* markedly decreased anxiety-like behaviors in the EPM test (increased time spent in the open arm and open arm entries, and decreased head dipping). In this study, the combination treatment (CRS+crocin+*T. chebula*) improved anxiety-like behaviors. However, statistical analysis did not show any difference between crocin and *T. chebula* alone or their combination. Therefore, in the present study, crocin and *T. chebula* treatment produced an antianxiety effect induced by CRS.

T. chebula contains rich sources of different chemicals, such as phenolic and tannin. Studies revealed that tannins from *T. chebula* possessed high antioxidant activity as well as neuroprotective agents. Also, it has shown beneficial effects on a variety of neurological disorders, including neurodegenerative disorders (Chang and Lin, 2012). In this regard, Chandrasekhar et al. (2018) in the picrotoxin-induced

anxiety model in mice demonstrated that supplementation of the tannin-rich extract from *T. chebula* remarkably decreased the serum cortisol levels and elevated the monoamine neurotransmitters such as 5-HT, dopamine, and norepinephrine levels in brain tissues. They showed that tannin-rich extract from *T.chebula* up-regulated the BDNF, cAMP response element-binding protein (CREB), GABAA, and 5-HT gene, which is lined with facilitating various mood disorders such as anxiolytic. Recently, another study by Mani et al. (2021) indicated an elevation of monoamine neurotransmitters by suppression of brain monoamine oxidase-A (MAO-A) levels of the *T. chebula* extract treatment. Thus, this beneficial anxiolytic activity may correlate with the presence of tannin-rich compounds in *T. chebula*. Moreover, the present results demonstrated that administering crocin improved the anxiety-like behaviors induced by CRS. Many studies have examined the anxiolytic-like effect of crocin. For instance, Talaei and colleagues (2015) investigated the effect of crocin tablets in patients with major depressive disorder. They mentioned that crocin tablets given with selective serotonin reuptake inhibitors (SSRI) cause a significant improvement in symptoms of anxiety when compared to SSRIs alone. Also, Zhang et al. (2018) found that crocin inhibited lipopolysaccharide-induced anxiety-like behaviors by suppressing NF-kB and NLR family pyrin domain-containing protein 3 (NLRP3) signaling pathways. They showed that crocin could inhibit the effect of increased cytokine expression, including interleukin-1β, interleukin-18, caspase-1, and tumor necrosis factor (TNF-α) in the hippocampus of lipopolysaccharide- injected mice. On the other hand, the results of the present study showed that the crocin had a suppressive effect on CRS-induced anxiety-like behaviors in rats. The mechanism of the anxiolytic effect of crocin has not yet been completely determined, further work is needed to examine this assumption.

Large bodies of evidence reveal that CRS can induce cognitive dysfunction, which involves hippocampal-dependent spatial learning and memory damage (Jangra et al., 2017; Olave et al., 2022). The results of the present study showed that synaptic plasticity and neuronal arborization in the CA1 region of the hippocampus are damaged by CRS. Consistent with our results, several studies revealed that stress changes the hippocampal neural activity, synaptic plasticity, and decreases neuronal cell survival and neurogenesis (Leschik et al., 2021).

The results of the Barnes maze test revealed that the spatial learning and memory of CRS animals were impaired compared to the control group, and the crocin, *T. chebula* and combined crocin + *T. chebula* treatment was beneficial for improving these impairments. Various studies using different hippocampus-dependent tasks such as the Morris water maze task (Ghadrdoost et al., 2011), object recognition task and radial water maze (Pitsikas et al., 2008) have demonstrated that crocin has adverse effects on memory impairment in rodents. Therefore, crocin seems to prevent memory impairment induced by CRS. Various mechanisms may be proposed for the neuroprotective properties of crocin such as enhancement of anti-oxidants and free radical scavenging (Kermanshahi et al., 2020), modulation of NMDA receptor functions (Abe and Saito, 2000), anti-inflammatory and anti-apoptosis properties (Yuan et al., 2020).

Also, administration of an alcoholic extract of *T. chebula* before CRS (*T. chebula*+CRS group) for 14 consecutive days reduced memory impairment (decreased latency time, distance traveling, and the number of errors in the Barnes maze) compared to the CRS group. Kim et al. (2018) showed that *T. chebula* exerts potent anti-amnesic effects through cholinergic modulation and anti-oxidant activity in a scopolamine-induced murine model of memory impairment. *T. chebula* with a high content of phenolic constituents revealed strong antioxidant and antiaging properties (Sancheti et al., 2010). Also, the administration of *T. chebula* reduced quinolinic acid-induced cell death and suppressed oxidative damage by reducing intracellular free radicals, lipid peroxidation, and oxidative DNA damage, describing its antioxidant potential (Sadeghnia et al., 2017). The strong antioxidant and anti-inflammatory of the alcoholic extract of *T. chebula* may play a role in reducing CRS-induced memory impairment.

To better understand how alcoholic extract of the *T. chebula* and crocin exerts neuroprotective effects, we also investigated the LTP (by field potential recording and analysis of LTP markers, including fEPSP slope and fEPSP amplitude) as the most widely suggested mechanism of memory storage. It is well-accepted that synaptic plasticity is the cellular basis of learning and memory; therefore, memory impairment in CRS results from the loss of synaptic plasticity (Sun et al., 2020). The present results are in agreement with other studies that showed a cognitive deficit in CRS is associated with decreases in basal synaptic transmission, which confirmed that LTP induction is impaired in the CRS rat (Bobula et al., 2015). Several factors, such as hippocampal cell loss and volume (Khan et al., 2020), the deficit in basal synaptic transmission (Zelek-Molik et al., 2021), and neurotransmitter release probability (Popoli et al., 2011) are known to be related to the impairment of LTP in CRS animals. However, according to these data, it can, thus, be sug-

gested that the administration of alcoholic extract of the *T. chebula* and crocin may improve LTP by increasing the fEPSP slope and PS amplitude in the CA1 pyramidal neurons.

The combination of crocin and *T. chebula* can act through different mechanisms to improve the symptoms of CRS. The expression levels of cholinergic signaling genes were down-regulated in a CRS rat model (Han et al., 2017), and the cholinergic effect on LTP is well described as a matter of fact (Yang et al., 2021). It has been suggested that crocin increases the acetylcholine level in the hippocampus and restores cholinergic cognitive deficits (Yuan et al., 2020). Also, numerous studies have reported the anticholinesterase properties of *T. chebula*. For instance, Sancheti et al. (2010) study reported the suppressive effects of *T. chebula* on acetylcholinesterase and butyrylcholinesterase. Also, *T. chebula* showed relatively significant acetylcholinesterase inhibitory activity (Khan et al., 2020). Thus, improvement of cholinergic function by crocin or *T. chebula* might preserve the synaptic function in CRS rats and improve LTP impairment in our study. Postsynaptic activation of N-methyl-D-aspartate receptors (NMDARs) and calcium/calmodulin-dependent protein kinase II (CaMKII) is needed to induce LTP in the hippocampus (Shibata et al., 2021). Studies have reported that chronic stress is associated with decreased CaMKII levels (Shen et al., 2021) and a selectively reduced AMPARs-mediated synaptic excitation (Kallarackal et al., 2013). In accordance with our results, Georgiadou et al. (2014) revealed that crocin decreased recognition memory impairment induced by the ketamine (noncompetitive NMDA receptor antagonist) in rats, therefore could improve schizophrenia-related cognitive deficits.

It has been suggested that chebulinic acid (isolated from the extracts of the *Terminalia chebula*) is a potent protectant against glutamate-induced neuronal cell death through suppressing reactive oxygen species (ROS) production, Ca^{2+} influx, and phosphorylation of mitogen-activated protein kinases (MAPKs), as well as decreasing the ratio of Bax to Bcl-2 by glutamate, which contributes to oxidative stress-mediated neuronal cell death (Song et al., 2018). Overall, these reports propose a functional interaction between crocin or *T. chebula* and the glutamatergic system that might be pivotal to the beneficial action exerted by crocin on CRS-induced LTP deficits. Further studies, with more focus on the association of crocin or *T. chebula* on the improvement of LTP *via* the cholinergic and glutamatergic system, are needed to be undertaken.

Chronic stress, synaptic loss, and hippocampal neuronal cell death cause neuropsychiatric symptoms such as cognitive impairment and anxiety. On the other hand, the hippocampus is a region of the brain that plays an important role in processing emotions, cognition and controlling behavior in response to anxiety, depression, and fear (Krugers et al., 2010; Koskinen et al., 2020). Death and atrophy of hippocampal neurons have been reported in rats during CRS (Takuma et al., 2007). It is well-established that structural and functional modifications in the hippocampal neurons during chronic stress are associated with dendritic reorganization as well as alterations in spine density (Patel et al., 2018).

To investigate the neuroprotective effects of *T. chebula* and crocin, Golgi-Cox staining was performed. In this study, the Golgi-Cox staining test showed a significantly reduced number of neuronal branches in the dendrite and soma in hippocampus CA1 pyramidal neurons in CRS rats. These results indicated the synaptic loss of the hippocampus CA1 neurons that were relative to the cognitive deficit and anxiety-like behaviors in rats and are in line with other studies demonstrating synaptic loss in CRS (McEwen, 2016). Also, previous studies have reported that chronic stress causes impaired dendritic arborization, shrinkage in hippocampal neurons, dendritic remodeling, changes in spine shape and density, and damage to plasticity (Patel et al., 2018).

Results of Golgi-Cox staining demonstrated that administration of both *T. chebula* and crocin for 14 consecutive days significantly increased the number of dendritic branches in the hippocampus CA1 pyramidal neurons in the rats that were under CRS. These results indicated the neuroprotective effects of both *T. chebula* and crocin and their preventive effects on the synaptic loss of hippocampus CA1 pyramidal neurons induced by CRS. The neuroprotective effects of crocin have been demonstrated in many studies. For instance, Sadoughi (2019) in the model of Alzheimer's suffering rats induced with trimethyltin chloride suggested that crocin can inhibit the loss of neuronal density of CA1, CA2, and CA3 regions in the hippocampus. Reduced synaptic functions were impaired in chronic stress with the downregulation of synaptic markers postsynaptic density protein 95 (PSD-95), AMPA receptor subunit 1 (GluR-A), and the neurotrophic factor BDNF (Wu et al., 2021). In this regard, Batarseh et al. (2017) reported that *Crocus sativus* extract elevated the synaptic markers and then decreased inflammation. They showed that *Crocus sativus* extract induced the expression of both PSD-95 and synaptosomal- associated protein 25 (SNAP-25) by 36% and 26%, respectively. Also, crocin treatment only increased SNAP-25 by 15%; however, it had a significant effect on PSD-95 expressions (Batarseh et al., 2017). Therefore, it appears likely that, these neurotrophic actors mediate the observed beneficial effects of crocin on CRS-induced synaptic

loss. To our knowledge, there are no studies evaluating the effect of *T. chebula* neuronal arborization. However, several lines of evidence have indicated that the *T. chebula* application shows a neuroprotective effect in rats. For instance, Park and colleagues investigated the neuroprotective effects of *T. chebula* against ischemic damage in the hippocampal CA1 region. They showed that the neuroprotective effects were associated with a decrease in glial activation as well as increases in superoxide dismutase (SODs) and BDNF levels (Park et al., 2011). Also, Gaire et al. (2013) *in vitro* study reported the protective efficacy of the *T. chebula* extract against oxygen-glucose deprivation followed by reoxygenation (OGD-R) ischemia-induced PC12 cell death and the inhibition of activated microglia must be due to the antioxidant and anti-inflammatory potential of *T. chebula* extract and its polyphenols. However, more studies are needed to examine this assumption.

CONCLUSION

Our study showed that treatment with *T. chebula* or crocin separately or their combination was effective in protecting the brain from CRS-induced cognitive and synaptic plasticity impairments, anxiety-like behavior, and neuronal arborization deficit in the pyramidal neurons of the CA1 region of the hippocampus. The results indicated no significant difference between single treatments of *T. chebula* or crocin and combined treatment of *T. chebula* + crocin animals in these parameters. These results represent that crocin or *T.chebula* would be an alternative lead for the treatment of numerous central nervous system disorders such as neurodegenerative disease, antidepressants, and anxiety. However, these results are preliminary to reach the therapeutic application and need further complementary studies about safety profile, the exact mechanism of action, and the development of clinical trials.

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