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Viola tricolor Hydroalcoholic Extract Improves Behavioral Deficiencies in Rats Exposed to Chronic Immobilization Stress

Khadijeh Mohammadi[1]
https://orcid.org/0000-0002-0566-5162

Reza Mohammadi[2]
https://orcid.org/0000-0002-5858-8826

Masoumeh Asle-Rousta[1]*
https://orcid.org/0000-0002-2087-5483

Mehdi Rahnema[1]
https://orcid.org/0000-0001-9176-5153

Sanaz Mahmazi[2]
https://orcid.org/0000-0003-2022-4354

[1]Islamic Azad University, Department of Physiology, Zanjan Branch, Zanjan, Iran; [2] Islamic Azad University, Department of Genetics, Zanjan Branch, Zanjan, Iran.

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*Correspondence: mrousta@iauz.ac.ir; Tel.: +98-9125606327 (M.A.).

HIGHLIGHTS

- *V. tricolor* ameliorates memory impairment caused by immobilization in rats.
- *V. tricolor* diminishes anxiety- and depression-like behaviors in immobilized rats.
- *V. tricolor* decreases chronic immobilization-induced neuronal loss in the hippocampus.
- *V. tricolor* reduces expression of IL-6 in the hippocampus of immobilized rats.

Abstract: This study aims to investigate the effect of *Viola tricolor* extract on hippocampal neuronal death, interleukin (IL) -6 and IL-10 expression, spatial memory, anxiety, and depression in rats exposed to chronic immobilization stress. Rats were divided into groups Control, Viola300, Viola600, Stress, Stress-Viola300, and Stress-Viola600. Animals were placed in a restrainer (6 h / 21 days) to stress exposure. *V. tricolor* hydro-alcoholic extract was also administrated at doses of 300 and 600 mg/kg by gavage. The extract caused immobilized animals to spend more time in the target quadrant in the Morris water maze test. It also increased the percentage of entries into the open arm and the percentage of time spent in the open arm of the elevated plus-maze in immobilized rats. Treatment with the *V. tricolor* extract significantly reduced the immobility time of stressed rats in the forced swimming test. Furthermore, it significantly reduced neuronal death and expression of IL-6 in the hippocampus of immobilized animals but could not prevent the decrease of IL-10

expression. We concluded that *V. tricolor* protects rats from stress-induced behavioral damages, at least in part, by suppressing neuronal death and decreasing IL-6 expression.

Keywords: *Viola tricolor*; Memory; Anxiety; Interleukin-6.

INTRODUCTION

Immobilization is used in several studies to induce stress. In the long period, immobilization causes the production of free radicals, weakens the antioxidant system, and ultimately leads to oxidative stress in the prefrontal cortex and hippocampus. Also, it decreases interleukin-10 (IL-10; an anti-inflammatory cytokine) in the brain and increases the expression of pro-inflammatory cytokines such as interleukin-6 (IL-6) and pro-apoptotic factors, and eventually results in neuronal death [1-3]. Additionally, immobilization affects the neurotransmitters signaling. For example, stress stimulates dendritic retraction in the frontal cortex by activation of N-methyl-D-aspartate receptors. Immobilization enhances glutamate-induced toxicity and provokes apoptosis in the hippocampus. Chronic immobilization attenuates acetylcholine neurotransmission and reduces noradrenaline, serotonin, and dopamine levels in the brain. These molecular, biochemical, and synaptic alterations cause the emergence of behavioral disorders such as memory deficit, anxiety-like behavior, and depression [4-8]. Since immobilization is now a part of people's lifestyles, it is necessary to find a way to reduce its detrimental effects. Antioxidants are one of the most effective compounds to decrease the harmful effects of immobilization stress. These compounds overcome oxidative stress in rats exposed to immobilization by strengthening the antioxidant system [9].

Viola tricolor is a plant from the Violaceae family. The methanol extract of its flowers has an abundant amount of flavonoids (such as rutin and violantin) and carotenoids (such as lutein and β-carotene). *V. tricolor* possesses an antioxidant capacity, and this property is mainly related to rutin [10-12]. Furthermore, it has anti-inflammatory [13] and neuroprotective effects. *V. tricolor* inhibits serum/glucose deprivation-induced reactive oxygen species (ROS) production in PC12 cells and rescues them from apoptosis caused by ROS [14]. Therefore, we hypothesized that *V. tricolor* alleviates the chronic immobilization-induced behavioral disorders in rats by reducing neuroinflammation and neuronal death. Thus, this study aimed to evaluate the effects of *V. tricolor* hydro-alcoholic extract on memory impairment, anxiety, and depression created by chronic immobilization. Moreover, we examined molecular and histological changes in the hippocampus of stressed rats.

MATERIAL AND METHODS

Animals and study design

60 male Wistar rats (7–8 weeks of age, weight 200–220 g) were kept in laboratory standard conditions (temperature 23-25 ^{o}C, 12h/12h light and dark, with free access to water and food). Experiments were conducted by the standard guidelines for the care and use of laboratory animals and were approved by the Ethics Committee of Islamic Azad University (IR.IAU.Z.REC.1396.23).

Animals were randomly divided into 6 groups (10 rats per group) as follow:

- Control (C): rats received no treatment.
- Viola300 & Viola600: they received a hydro-alcoholic extract of *V. tricolor* in doses of 300 or 600 mg/kg by gavage for 21 consecutive days [15].
- Stress: These animals were placed in a restrainer for 6 hours every day for 21 days [8].
- Stress-Viola300 and Stress-Viola600: rats that received the extract in addition to exposure to immobilization stress.

The time interval for exposure to stress and gavage of the extract was 21 days. Spatial learning and memory were studied by Morris water maze (MWM) on days 17 to 21. On day 22, anxiety-like behaviors were assessed through the elevated plus maze (EPM) in the morning. Depression behavior also was evaluated by a forced swimming test (FST) in the afternoon of the same day. After behavioral examinations, animals were anesthetized by intraperitoneal injection of chloral hydrate (400 mg/kg) and were sacrificed by decapitation. The brains were removed for histological study and to determine mRNA expression of IL-6 and IL-10.

Extraction

V. tricolor was collected from the Iranian province of Zanjan and was verified by Biology Research Center (Zanjan, Iran) botanists. The *V. tricolor* flowers were dried in the fresh air and were powdered. Then the powder (500 g) was soaked in methanol 70% (5 liters, for 3 days) and was shaken. After the filtration of plant material through muslin cloth and Whatman paper, the filtrate was concentrated by an evaporator (at 35-40 °C) to obtain the extract with a yield of 17% (w/w). Then the extract was diluted with distilled water for gavage administration. Here, to determine the dose of the extract, we used the Saqib and coauthors study on the cardioprotective effect of the *V. tricolor* extract [15]. The volume of each oral gavage was 2 mL/kg body weight.

Behavioral tests

Morris water maze test

Spatial learning and memory were evaluated by the MWM test. This test consists of two steps. The first stage, which lasts four days, is dedicated to training. Animals swam in the water maze for 90 seconds, 4 times a day, to find the hidden platform by signs around the tank. The elapsed time (s) and swimming distance (cm) to reach the hidden platform were recorded and analyzed by MazeRouter (Tabriz, Iran). When the animal learns the platform position, it swims less time and distance to find it. The platform was removed on day 21 (second phase), and each rat swam in the tank for 60 s. The length of the time spent in the target quadrant (platform location on training days) was recorded [8,16]. The rat with the better memory swam longer in the target quadrant.

Elevated plus maze test

After 21 days, anxiety was assessed using the EPM test. This experiment lasted 5 minutes for each rat. Open arm entrance (OAE) and time spent in the open arm (OAT) were recorded. Increasing the percentage of OAE and OAT means reducing anxiety-like behaviors in the animal [8,16].

Forced swimming test

FST was used to examine depression. Each rat was placed in the cylinder (40 × 18 cm i.d.) containing water for six minutes. The immobility time of the animals was calculated in the last 4 minutes of the test. Lowering the immobility time means decreasing the depression [16].

Tissue preparation

5-µm sections of the paraffin-embedded left hemisphere (between 2.7 and 3.7 mm posterior to the bregma) were stained with cresyl violet (nissl staining). The number of survived pyramidal neurons was counted at 1 mm midline in the CA1 region of the hippocampus by optical microscope (×400), and the cell death percentage was calculated [8].

Real time PCR

The right hippocampus of 4 rats of each group was removed and was stored at -80 °C for evaluation of mRNA expression by real-time PCR. The total RNA of hippocampi was extracted according to instructions of the RNA extraction kit (CinnaGen, Iran), and its concentration and purity were determined by spectrophotometer at 260 nm and 280 nm (A260/A280 ratio). Then, cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, USA). The Primers were synthesized by CinnaGen (Iran). The primer sequences are listed in Table 1. RealQ Plus 2x Master Mix Green (Ampliqon, Denmark) was used to carry out real-time PCR. The fold changes of IL-6 and IL-10 mRNA levels were determined by $2^{-\Delta\Delta ct}$ method [17] and HPRT1 was used as a housekeeping gene.

Table 1. Sequences of primers used for real-time PCR

Gene	Primers	Sequence (5´- 3´)
IL-6	Forward	TCC ATC CAG TTG CCT TCT TG
	Reverse	TTC CAC GAT TTC CCA GAG AAC
IL-10	Forward	GCT CTT ACT GAC TGG CAT GAG
	Reverse	CGC AGC TCT AGG AGC ATG TG
HPRT1	Forward	AAAGGACCCC ACGAAGTGTT
	Reverse	TCAAG GGCATATCCTACAACAA

Statistical analysis

Data are shown as mean ± S.E.M. The differences among groups were determined by one-way ANOVA followed by the Tukey HSD post hoc. In each group, the learning task between 4 days in the MWM test was analyzed by repeated measures. *P* value of less than 0.05 was defined as statistically significant.

RESULTS

V. tricolor extract improved learning and spatial memory in rats exposed to chronic immobilization stress

Statistical analysis (repeated-measures two-way ANOVA) showed a significant effect of training on time and distance traveled to reach the hidden platform ($F_{1, 59}$ = 2633.108, *P* = 0.000 and $F_{1, 59}$ = 3217.243, *P* = 0.000 respectively). On the fourth day of training, the Stress group spent more time and distance to reach the platform than the Control group (*P* = 0.002) (Figure 1A, B). Consuming doses of 300 and 600mg/kg *V. tricolor* extract caused a meaningful reduction of these factors in stressed rats (*P* = 0.009 and *P* = 0.002), returning them to almost Control values (Figure 1A, B). Also, according to the results of the probe test, the Stress group spent less time in the target quadrant than the Control group (*P* = 0.001), while swimming time in the target quadrant significantly increased in groups Stress-Viola300 and Stress-Viola600 compared with the group Stress (*P* = 0.001 and *P* = 0.005) (Figure 1C).

V. tricolor extract decreased anxiety-like behavior in stressed rats

The results of the EPM test showed that chronic immobilization stress caused anxiety-like behaviors in rats. The percentage of OAT and OAE in the Stress group was significantly lower compared with the Control group (*P* = 0.015 and *P* = 0.003). Both doses of *V. tricolor* extract increased OAT (*P* = 0.039 and *P* = 0.013) and OAE (*P* = 0.044 and *P* = 0.039) percentages in rats exposed to immobilization (Table 2) indicating attenuation of anxiety-like behavior in stressed rats. However, although treatment with *V. tricolor* extract returned the percentage of OAT to values determined in non-stressed rats, OAE values in these groups were slightly lower than controls. Consumption of the extract by non-stressed animals did not affect the percentage of OAE and OAT.

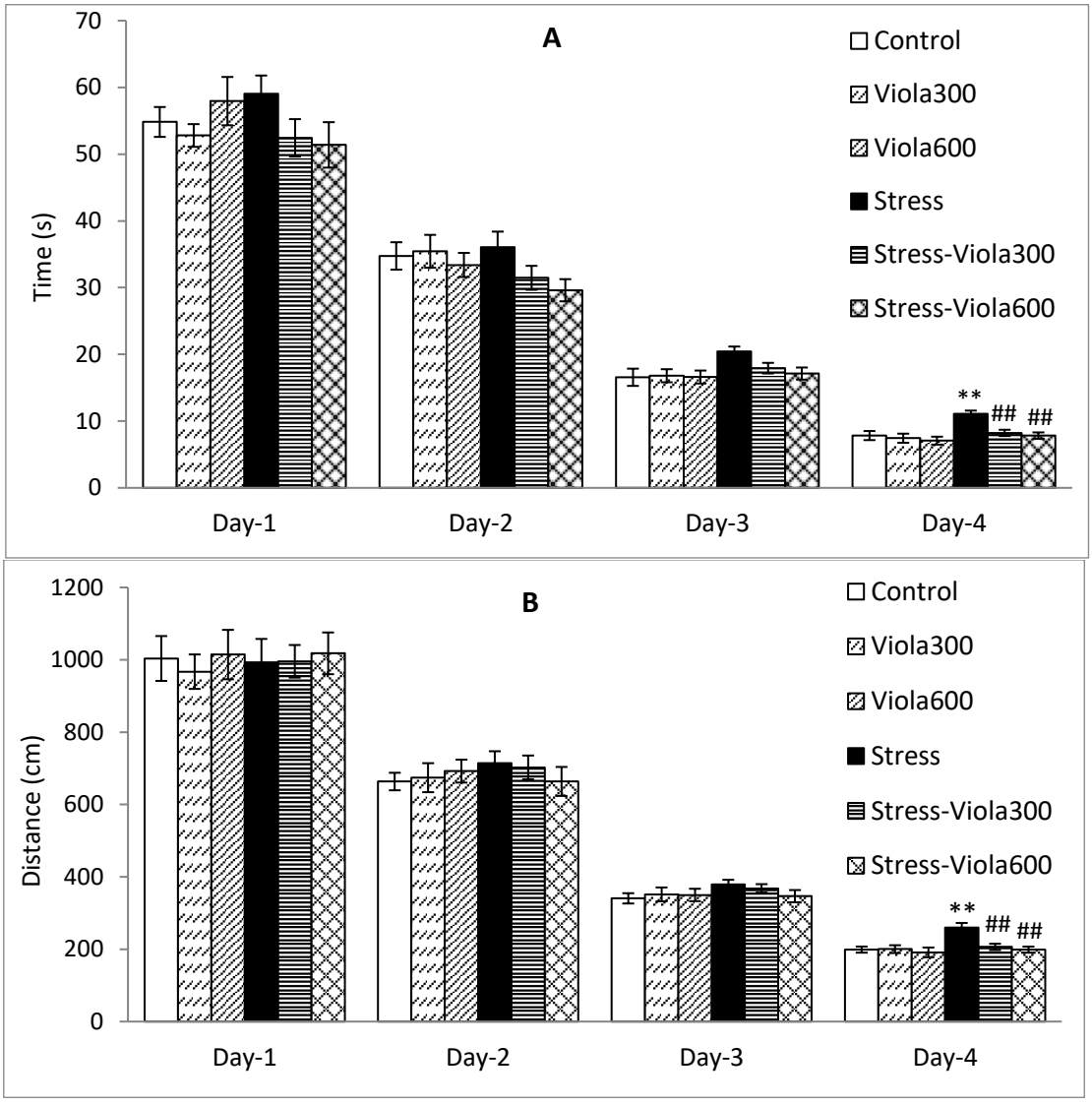
V. tricolor extract decreased depression in rats exposed to chronic immobilization stress

Chronic immobilization generated depression in rats. Immobilization notably increased the total time of immobility in these animals compared with the Control group in the FST (*P* = 0.000). Both doses of *V. tricolor* extract decreased this factor in stressed rats (*P* = 0.001) (Table 2). Extract consumption in normal rats did not cause a significant difference in immobility time compared with controls.

Table 2. Effect of *V. tricolor* extract on chronic immobilization induced anxiety-like behaviors and depression in rats which were evaluated by elevated plus maze (EPM) test and forced swimming test (FST) respectively.

Group	OAT %	OAE %	Immobility time (s)
Control	41.36 ± 3.55	49.55 ± 1.60	56.5 ± 6.59
Viola300	41.59 ± 3.08	59.81 ± 2.42	52.8 ± 9.70
Viola600	37.86 ± 1.66	52.61 ± 2.91	45.1 ± 8.72
Stress	29.0 ± 1.90 *	35.50 ± 2.16 **	117.1 ± 10.74 ***
Stress-Viola300	40.08 ± 1.72 #	46.11 ± 3.18 #	63.6 ± 7.95 ##
Stress-Viola600	41.60 ± 2.82 #	46.24 ± 2.34 #	65.5 ± 6.11 ##

The percentage of open arm entrance (OAE) and open arm time (OAT) were calculated in EPM test. Total immobility time was also determined in forced swimming test. The results are shown as mean ± SEM. Each group contains 10 rats. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. group Control and # $P < 0.05$, ## $P < 0.01$ vs. group Stress.



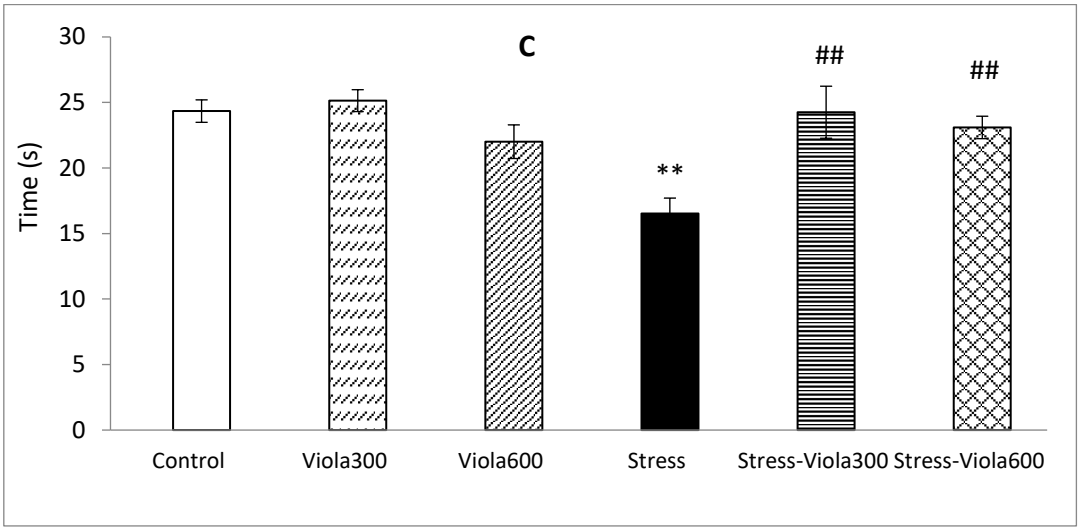


Figure 1. Effect of *V. tricolor* extract on chronic immobilization induced learning and memory impairment in rats. Time elapsed (A) and distance traveled (B) to reach the hidden platform in training days, and time spent in the target quadrant in the probe test (C) in the MWM test were recorded. Results are shown as mean ± SEM. Each group contains 10 rats. ** $P < 0.01$ vs. group control and $^{\#\#}$ $P < 0.01$, vs. group stress.

V. tricolor extract ameliorates neuroinflammation in the hippocampus of rats exposed to chronic immobilization stress

Immobilization significantly increased IL-6 mRNA expression in the hippocampus of rats ($P = 0.000$). Consumption of 300 and 600 mg/kg doses of the extract reduced the expression of this proinflammatory cytokine in stressed rats ($P = 0.010$ and $P = 0.000$, respectively) (Figure 2). IL-6 mRNA expression in Viola300 and Viola600 groups was lower than in the Control group (didn't have a significant difference). In contrast, the level of IL-10 mRNA declined in the Stress group, which was statistically significant ($P = 0.021$) (Figure 2). Expression of this anti-inflammatory cytokine was enhanced in the Stress-Viola300 and Stress-Viola600 groups compared with the Stress group but was not significant ($P = 0.984$ and $P = 0.925$, respectively). The extract dose of 600 mg/kg increased IL-10 mRNA level in non-stressed rats compared with controls ($P = 0.003$) (Figure 2).

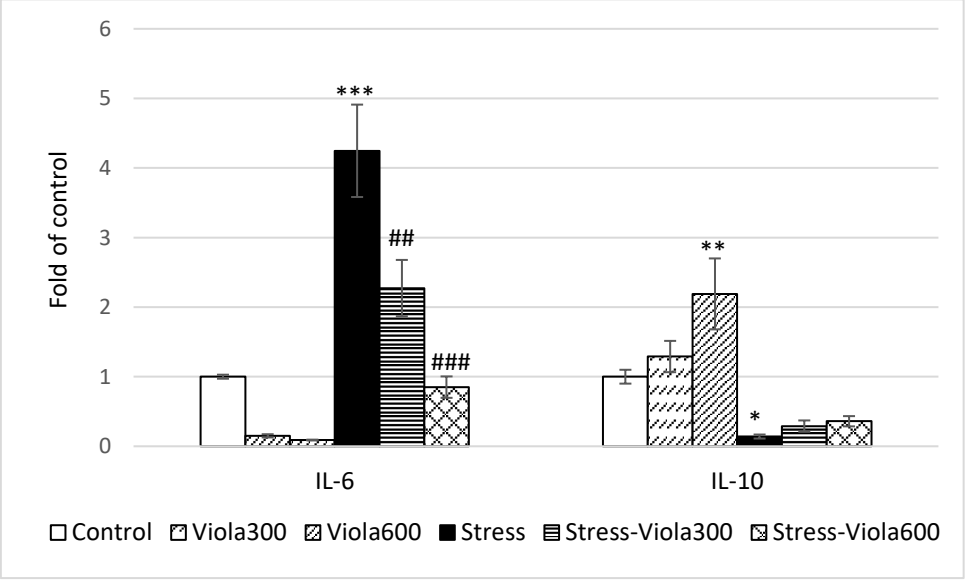


Figure 2. Effect of *V. tricolor* extract on mRNA expression of IL-6 and IL-10 in the hippocampus of rats exposed to chronic immobilization. Results are presented as means ± SEM. Each group contains 4 rats. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. group control and $^{\#\#}$ $P < 0.01$, $^{\#\#\#}$ $P < 0.001$ vs. group stress.

V. tricolor extract inhibits neuronal death in the hippocampus of stressed rats

Chronic immobilization caused extensive neuronal death in the hippocampal CA1 region and thinned the neuronal layer. The percentage of pyramidal neuronal death in this area was significantly different from that in Control group (P = 0.000) (Table 3 and Figure 3). The extract treatment largely prevented neuronal death so that the percentage of neuronal loss in the Stress-Viola300 and Stress-Viola600 groups showed a meaningful decrease compared with the Stress group (P = 0.000). However, the percentage of neuronal death in the Stress-Viola300 and Stress-Viola600 groups was significantly different from the control group (P = 0.000).

Table 3. Effect of *Viola tricolor* extract on immobilization stress-induced neuronal loss in CA1 region of hippocampus.

Group	Percentage of neuronal loss in CA1 area (mean ± SEM)
Control	0
Viola300	1.22 ± 0.13
Viola600	1.25 ± 0.14
Stress	11.56± 0.62 ***
Stress-Viola300	4.97 ± 0.27 ### ***
Stress-Viola600	4.14 ± 0.24 ### ***

The percentage of neuronal loss was determined by [(average neuron number in control sections − neuron number in treated group sections)/ average neuron number in control sections ×100] in all groups. Each group contains 5 rats. Data are shown as mean ± SEM. *** P < 0.001 vs. group control; ### P < 0.001 vs. group stress.

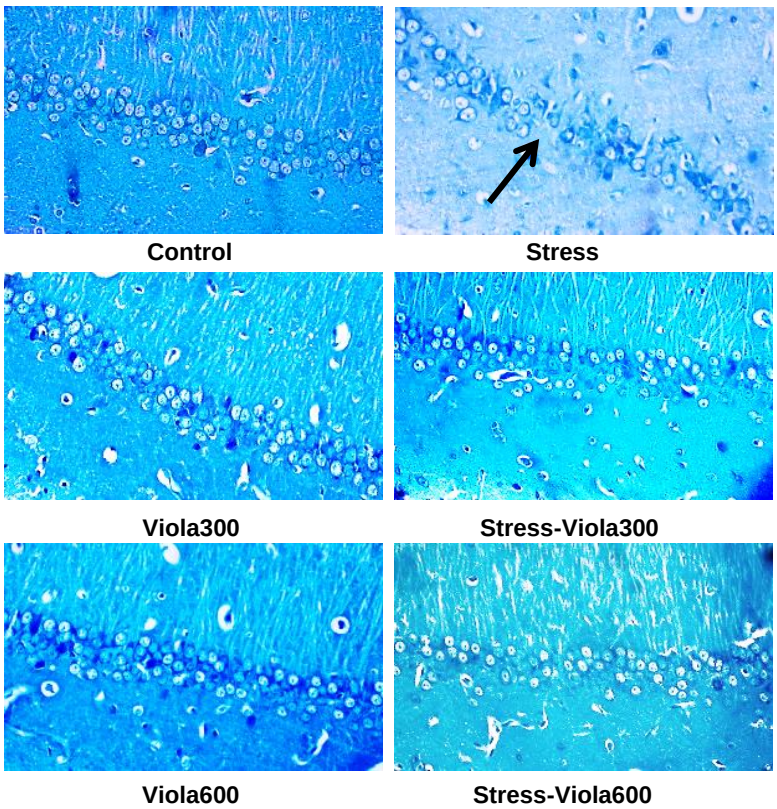


Figure 3. Chronic immobilization reduced the number of pyramidal neurons and the thickness of the neuronal layer in the CA1 subfield of the hippocampus. The location of the damage is indicated by an arrow. *V. tricolor* extract prevented neuronal depletion in this area of the brains in immobilized rats.

DISCUSSION

Here adult male rats were exposed to immobilization stress for 6 hours every day for 21 consecutive days. Immobilization increased the expression of IL-6, decreased the expression of IL-10, reduced the number of pyramidal neurons in the CA1 area, and led to behavioral disorders such as spatial memory impairment, anxiety-like behaviors, and depression in animals. These results are consistent with previous studies [2,3,8].

Histological examinations showed that both doses of *V. tricolor* could prevent hippocampal neuronal depletion in stressed rats. Mousavi and coauthors [14] demonstrated that, through reducing oxidative stress, the hydro-alcoholic extract of *V. tricolor* inhibits neurotoxicity induced by serum/glucose deprivation in PC12 cells. The anti-apoptotic effect of viola extract is related to its compounds, such as rutin, beta-carotene, and lutein. These components rescue cells from chemicals-induced toxicity by preventing the expression of pro-inflammatory and pro-apoptotic factors and inhibiting mitochondrial dysfunction [18-20].

Inflammation is one of the most important causes of neuronal death, and IL-6 plays an essential role in this process. Exposure to IL-6 causes neuronal death in the hippocampus and results in neurodegeneration [21]. Accordingly, suppressing IL-6 expression may be an appropriate strategy to inhibit neurodegeneration. Both doses of *V. tricolor* extract reduced the mRNA expression of this pro-inflammatory factor in the hippocampus of immobilized rats. This effect is probably due to its compounds, such as rutin, lutein, and β-carotene in *V. tricolor* [22-24]. The anti-inflammatory effect of *V. tricolor* has been confirmed by in vitro and in vivo several studies [15,25]. IL-10 is an anti-inflammatory cytokine, unlike IL-6 [26]. Although the dose of 600 mg/kg of the extract increased the expression of IL-10 in non-stressed rats, but could not significantly enhance it to reach the control level. However, if the treatment with extract continues for a more prolonged period, the hippocampal expression of IL-10 in stressed rats will probably not be different from the controls. Due to the essential role of IL-10 in inhibiting apoptosis, we suggest that increasing the expression of IL-10 in the hippocampus of stressed rats may be able to minimize neuronal death in the hippocampal CA1 area [27].

The results of the MWM test showed that *V. tricolor* extract improved learning and spatial memory in stressed rats. Currently, there is no report of memory improvement by this plant, but one month of consumption of *V. odorata* oil enhances memory in patients with chronic amnesia [28]. Some viola compounds improve memory performance. For example, Xu and coauthors (2014) showed that rutin has the potential to treat Alzheimer's disease (AD). It inhibits beta-amyloid accumulation, reduces oxidative stress and neuroinflammation in AD model mice, and ultimately enhances memory in these animals [22]. Also, lutein reduces cognitive deficits in ethanol-receiving rats by reducing acetylcholinesterase activity in the cortex and hippocampus [29]. Oxidative stress, neuroinflammation, and neuronal death in the brain, especially in the hippocampus, leading to memory deficit [30,31]. Accordingly, *V. tricolor* may ameliorate memory by strengthening the antioxidant system, reducing the expression of IL-6, and suppressing neuronal death in the hippocampus of stressed rats.

Results of the EPM test confirmed that *V. tricolor* extract reduced anxiety-like behaviors in rats exposed to chronic immobilization. Earlier, Harati and coauthors [32] also confirmed the anxiolytic effect of this plant in asthmatic mice. Some of the compounds in *V. tricolor* affect neurotransmission of γ-aminobutyric acid (GABA). For example, rutin reduces anxiety by stimulating the GABAergic system in the basolateral amygdala [33]. Hence, the extract may also strengthen the GABAergic system. To confirm this hypothesis, it is necessary to examine the GABA neurotransmission in the brains of the Stress-Viola300 and Stress-Viola600 groups. In addition, neuroinflammation also plays a chief role in the development of anxiety-like behaviors under stress conditions. There is a close relationship between increased levels of IL-6 and anxiety [34]. Therefore, the anxiolyticeffect of viola in stressed rats is also related to the reduced expression of this pro-inflammatory factor. On the other hand, considering the role of oxidative stress in the etiology of anxiety [35], we assumed that the antioxidant property of *V. tricolor* has also been effective in reducing anxiety in the stressed rats.

The results of FST also revealed a reduction in depression in immobilized rats consuming *V. tricolor* extract. The antidepressant effect of this plant has not been studied before, but some of its constituents decrease depression-like behavior. For instance, rutin, which is one of the major compounds of this plant, reduces depression by increasing access to serotonin and noradrenaline [36]. The antidepressant effect of lutein is also related to diminishing oxidative and nitrosative stress and protecting hippocampal neurons [37]. There is a close correlation between neuronal death in the hippocampus and the occurrence of depression in stressed humans. Stress stimulates neuronal death in this region of the brain by increasing the secretion of glucocorticoids, thus leading to hippocampal atrophy over time [38]. By suppressing apoptosis in hippocampal neurons, geniposide could reduce depression in chronically immobilized rats [39]. Inflammation also contributes to the occurrence of depression, so that increasing the expression of IL-6 plays a substantial role in the development of depressive-like behaviors [40]. Hence, the reduction of depression in the Stress-Viola300 and Stress-Viola600 groups is partially related to *V. tricolor's* success in reducing inflammation and neuronal death. It is necessary to measure the level of monoamines in the brains of Stress-Viola300 and Stress-Viola600 rats to confirm the hypothesis that *V. tricolor* possesses antidepressant effects.

CONCLUSION

Overall, we concluded that the hydro-alcoholic extract of *Viola tricolor* enhanced learning and memory and reduced anxiety-like behaviors and depression in rats exposed to chronic immobilization stress. These effects were partly mediated by reducing IL-6 expression and inhibiting neuronal death in the hippocampus. *Viola tricolor* is likely to be beneficial in the prevention and treatment of neurodegeneration.

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Conflicts of Interest: The authors have no conflict of interests.

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