Avicenna Journal of Phytomedicine (AJP)

Original Research Article

Investigating the effects of *Viola odorata* hydroalcoholic extract on cognitive function, antioxidant/anti-inflammatory parameters and brain damage in response to unavoidable stress

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Article history:
Received: Jan 05, 2026
Received in revised form: Jul 26, 2026
Accepted: Jul 26, 2026
Epub ahead of print

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Keywords:
Anti-inflammatory activity
Antioxidant activity
Learning and memory
Stress
Viola odorata

Abstract

Objective: The purpose of this investigation was to assess the influence of *Viola odoratas* hydroalcoholic extract on cognitive performance alongside changes in antioxidant activity and inflammation in rats experiencing unavoidable stressors.

Materials and methods: Forty male Wistar rats were divided into five groups: control, stress, stress+*V. odorata* extract, normal saline, and *V. odorata*. The stress and stress+*V. odorata* groups underwent four hours of daily restraint stress for 21 days. The extract-treated groups received *V. odorata* via gavage 30 min before stress. Barnes maze, elevated plus maze (EPM), hematoxylin-eosin staining, and ELISA kit were employed to evaluate memory, anxiety-like behaviors, neuronal density, and inflammatory and antioxidant factors evaluation, respectively .

Results: Administration of *V. odorata* extract to stressed rats significantly improved memory and recall performance (evidenced by reduced distance traveled and fewer errors in the Barnes maze test) and decreased anxiety-like behaviors (in the EPM test) compared to the stress animal. Furthermore, chronic stress caused significant increases in oxidative markers (Malondialdehyde; MDA), pro-inflammatory cytokines (Tumor Necrosis Factor-alpha; TNF-α and Interleukin-1β), and decreased antioxidant enzyme (Gamma-glutamyl transferase; GGT) levels in rats compared to the control. Notably, the *V. odorata* treatment showed neuroprotective effects against stress-induced damage by enhancing antioxidant activity and increasing neuronal density in brain regions including the amygdala, hippocampus, and prefrontal cortex compared to the stressed animal .

Conclusion: Evidence from this study suggests that *V. odorata* may help mitigate the negative effects of stress on cognitive performance, anxiety-like behaviors, and neuronal damage. The antioxidant and anti-inflammatory characteristics of the extract probably mediate these protective effects.

Please cite this paper as:
Sadeghi M, Moghtadaei Khorasgani E, Meftahi Gh.H. Investigating the effects of Viola odorata hydroalcoholic extract on cognitive function, antioxidant/anti-inflammatory parameters and brain damage in response to unavoidable stress. Avicenna J Phytomed, 2026.

Introduction

Stress is a natural and complex response that the body shows to any type of threat or pressure. This pressure can be physical such as illness or injury, or psychological such as relationship problems, work pressures, or financial concerns (James et al. 2023). In reaction to stress, our bodies generate hormones including cortisol and adrenaline that raise the heart rate, intensify blood pressure, and enhance energy. These responses help the person cope with the stressful situation (Deussing and Chen 2018; Tsai et al. 2024). However, if stress continues chronically and for a long time, this can result in harmful repercussions for one's physical condition and emotional state, leading to problems such as heart disease, digestive disorders, depression and anxiety (Ghasemi et al. 2024; Yang et al. 2025).

The presence of stress may hamper cognitive functions related to acquiring knowledge and recall. Under stress condition, stress hormones such as cortisol are released in the brain. These hormones can disrupt the function of the hippocampus, a region of the brain that plays a vital role in memory and learning (Khazani et al. 2024; Kim and kim 2023). Chronic and long-term stress can lead to a reduction in hippocampal volume which results in decreased ability to learn and remember information (Barzi 2025; Hrtanek et al. 2021).

Stress, whether acute or chronic, has significant effects on oxidative changes in the brain. The increased levels of free radicals resulting from stress, following the induction of the sympathetic nervous system and secretion of hormones such as cortisol, lead to increased oxidative stress. This increase, as an intervening factor, can cause extensive structural and functional damage to brain tissue, particularly in sensitive regions like the prefrontal cortex hippocampus and amygdala (Herbet et al. 2017; Lv et al. 2019).

Stress also affects neuronal morphology in the brain. In the hippocampus which plays a key role in learning and memory, chronic stress can lead to reduced hippocampal cell volume, decreased dendritic branching, and reduced neural synapses. In the amygdala which is the center for processing emotions, particularly fear and anxiety, stress can lead to increased amygdala cell volume and increased synaptic activity. This increased activity can result in anxiety disorders and exaggerated stress responses. Serving as a pivotal area for emotional regulation, decision-making, and overcoming obstacles, the prefrontal cortex plays an indispensable role in our mental functions, chronic stress can lead to reduced prefrontal cortex volume, decreased dendritic growth, and changes in synaptic connections (Dalooei et al. 2016; McEwen et al. 2016; McManus et al. 2024).

Plants with medicinal properties are harnessed for their therapeutic uses. The use of medicinal plants has a long history and is employed as a natural treatment method in many cultures. The violet flower, known as *Viola odorata*, is a species from the Violaceae family that is mostly found in temperate regions of the world. This flower has a short stem and its color is violet or a combination of yellow and violet. This plant has therapeutic and medicinal properties, and in the past, it was commonly used to make violet crystals (Feyzabadi et al. 2017). Research revealed that plant extracts containing active compounds can have positive effects on cognitive function by enhancing brain health and preventing oxidative damage caused by stress and harmful factors. In this regard, research by Tayarani-Najaran et al. (2019) on the properties of violet flowers reported that this plant reduces free radicals, thereby decreasing oxidative stress and its consequences. Moghimi Golmakani et al. (2022) also investigated violet flower properties and reported that violet extract and essential oil have protective, anxiolytic, antioxidant, and anti-inflammatory properties. Although *V. odorata* is believed to have a protective impact on brain

neurons linked to anxiety symptoms, cognitive ability, and oxidative processes, its effect on neuron structure in specific brain regions under prolonged immobility stress remains unexamined. Therefore, the aim of this study was to investigate the protective effects of *V. odorata* extract on brain structure, anxiety-related behaviors, cognitive abilities, and oxidative potential under conditions of chronic restraint stress.

Materials and Methods

Animal and experimental groups

The present investigation involved acquiring 40 adult male Wistar rats, ranging in weight from 220 to 250 g, from Baqiyatallah University of Medical Sciences, Tehran, Iran animal breeding center. Once relocated to the animal facility, the rats were grouped in sets of four within clear plastic enclosures sized 15×30×45 cm, and kept under typical lab settings that provided fresh food and water, with 12-hr cycle of light and darkness, and a temperature range of 22-24°C. In order to help the animals adjust to the new laboratory setting, they were moved to the facility a week prior to the commencement of experiments. In this research, animals were randomly divided into the following groups (n=8): 1) The control animals: with no exposure to stress, medication, or saline solutions. 2) Stress animals: Exposed to stress (placed in restrainer for 2 hours daily) for 21 days. 3) Stress + *Viola odorata* animals: For 21 days, received *Viola odorata* extract (150 mg/kg) (Alipanah et al. 2018) via gavage 30 min before stress. 4) *Viola odorata* extract group: For 21 days, received only violet extract (150 mg/kg) via gavage. 5) Group receiving normal saline: For duration of 21 days, they were treated exclusively with normal saline that acted as the solvent for *V. odorata* extract. The Ethics Committee of the Baqiyatallah University of Medical Sciences located in Tehran, Iran authorized the procedures involving experimentation (Protocol approval number: IR.BMSU.AEC.1403.023). Figure 1 displays the timeline for the experiment.

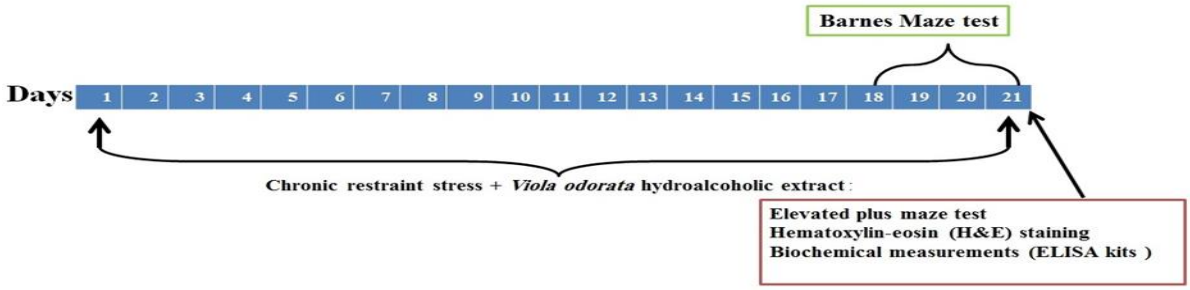

Figure 1. Timeline for the experiment

Extracting hydroalcoholic compounds from the plant *V. odorata*

One kilogram of dehydrated violet foliage was sourced, after which the leaves were ground into a powder and soaked in a mixture of 96% ethanol and distilled water at a 30:70 ratio for a duration of 72 hr. During this period, the mixture was stirred many times. After three days, the liquid was clarified by passing it through a Buchner funnel paired with a vacuum pump. The extracted solution was placed into petri dishes and stored under a laboratory hood for five days to allow the alcohol to evaporate. Finally, the required quantity of extract was mixed into normal saline.

Induction of immobilization stress

Immobilization stress was induced using a restrainer device (Meftahi and Aboutaleb 2023). This device consisted of a plastic tube (cylindrical in shape) with one end partially closed, allowing animals to place their snouts in the semi-open section to breathe, while completely restricting any movement including backward motion or

rotation. The opposite side of the tube had been entirely sealed off with a plug. For 21 consecutive days, the animals were confined in a restraining apparatus for 4 hr each day. While undergoing induction, the animals had their movements confined, as well as being blocked from eating and drinking; however, their breathing remained unaffected. Following their daily restraint, the animals were returned to their enclosures.

Barnes Maze test

A technique known as the Barnes maze is utilized to evaluate spatial memory in both mice and rats. The maze consists of a circular platform made of milky-white plexiglass with a diameter of 92 cm. Surrounding the maze are twelve openings, each with an eight cm diameter, situated 2 cm away from the boundary. The distance between holes is 5 cm. Under one of these holes (target chamber), there is a dark plexiglass chamber measuring 20×20 cm. Perched at a height of 100 cm, the maze is equipped with a low-consumption lamp of 500 watts positioned directly above it, while a camera diligently monitors all rat activities within this setup. For four consecutive days, we utilized the Barnes maze apparatus, spending the initial three on familiarization and training, followed by a memory assessment on the final day. On the opening day, animals had 240 sec of unrestricted movement within the Barnes maze, allowing them to explore and make their way into the box. In cases where the rats failed to locate the box independently, they were directed into it, after which the light was switched off and the lid was shut, leaving them inside for 60 sec before returning them to their cage. The procedures took place over a span of three days, conducted four times daily; however, on day four—dedicated to memory testing—each rat underwent the test just once. Following each trial of the experiment, the maze was sanitized with 70% alcohol to eliminate any lingering olfactory signals from the surface. Prior to each experiment, the target chamber underwent cleaning with alcohol. The analysis conducted through this method looked into several aspects, including error hole counts, traveled distance by the animal, and time spent reaching the target hole.

Elevated plus maze (EPM) test

Used frequently to assess anxiety-like behaviors in rats, the elevated plus maze (EPM) consists of a plus-shaped layout comprising two open arms (0.5×5×25 cm) and two closed arms (16×5×25 cm) situated across from one another, elevated to a height of 50 cm above the floor. To enhance safety and reduce the risk of falling, 1 cm high barriers are placed on the sides and ends of the open arms, converging at a central zone sized 5×5 cm. During testing, each animal was putted in the central area facing an open arm, and its behavior was recorded for 5 minutes using an overhead camera to enable detailed analysis of all behavioral aspects, with the entire maze surface cleaned with 20% alcohol after each trial to eliminate residual olfactory cues. The evaluated parameters included: time spent in central area, number of entries into open arms, number of entries into closed arms, freezing duration (complete immobility), head dipping frequency, grooming duration, rearing duration (standing on hind legs), time spent in open arms, and time spent in closed arms.

Hematoxylin-eosin (H&E) staining

Following the execution of the Barnes maze and EPM assessments, the rats were anesthetized using ketamine (100 mg/kg) in combination with xylazine (10 mg/kg). We then performed ventricular cavity opening and cardiac blood collection, transferring each rat's blood into plain tubes without anticoagulant. Following decapitation, brain tissue was surgically extracted and fixed for histopathological examination. The tissue processes included dehydration through graded alcohols, clearing, and paraffin embedding. Using a microtome

(Cambridge Medical Instruments, UK), we prepared five micrometer thick sections that were mounted on slides and stained with hematoxylin-eosin (H&E) using a standardized protocol: initial hydration (1 min each in 100 and 70% alcohol), distilled water rinse, hematoxylin staining (15 min), running water rinse, acid-alcohol differentiation (15 min), two water rinses, eosin staining (2 min), and final dehydration (1 min each in 70, 95, and 100% alcohol). This comprehensive protocol enabled detailed histological evaluation of brain tissue morphology and inflammatory markers.

Mounting

The mounting was accomplished with the use of Canada balsam and DPX (distyrene, a plasticizer, and xylene) adhesives. The method required adding a droplet of glue onto the tissue sample affixed to the slide, gently applying the coverslip to eliminate any air pockets, and permitting it to air dry at ambient temperature.

Examination

After preparing the pathology slides, examination was performed. Cells in three regions (prefrontal cortex, amygdala, and hippocampus) were counted within a 10×10 microscopic field. Samples were examined under a light microscope at 400× magnification, with eight photomicrographs taken per sample. Three micrographs with at least 40 µm spacing were randomly selected for cell counting using Imaging-Pro-Plus software, and the averages were calculated.

Blood processing

Plain tubes containing samples were left to clot before being centrifuged to extract serum, which was then preserved at -70°C until analysis.

Inflammatory and antioxidant factors evaluation

Quantification of Tumor Necrosis Factor-alpha (TNF-α) and Interleukin-1 (IL-1) was conducted using ELISA kits sourced from Karmania Pars Gene, Kerman, IRAN (IL-1 catalog number CN:KPG-RIL1; TNF-α catalog number CN:KPG-RTNF- α). Utilizing ELISA kits manufactured by Naxifer Navand Salamat Co. of Iran, we precisely measured the concentrations of Malondialdehyde (MDA) and Gamma-glutamyl transferase (GGT) according to the kit's guidelines.

Statistical analysis

Data evaluation was executed using version 27 of SPSS. To determine whether the data distribution was normal, the one-sample Kolmogorov Smirnov test was applied. The analysis employed one-way ANOVA for data evaluation, and to assess mean differences among groups, Tukey's test was applied with a significance level set at $p<0.05$.

Results

Spatial learning and memory

The impact of hydroalcoholic extract from *V. odorata* on spatial memory and learning was explored in this study utilizing the Barnes maze. On the first day of testing, stress levels (223.413 ± 21.573 Sec) resulted in a noteworthy delay in reaching the target hole ($p<0.001$) (Table 1). On both the first and second days (Table 2), the stress group covered a greater distance (cm) to reach the target hole ($p<0.01$). The group receiving *V. odorata* extract with stress showed increased distance on the fourth day ($p<0.01$). The stress animal made more errors (number) on the first, second, and third days, while the *V. odorata* extract + stress group showed this only on the second day ($p<0.01$). No significant differences were found between groups in time to reach

the target hole on the third or fourth days (Table 3, 4). The stress + *Viola odorata* group (50.625 ± 12.188) traveled farther than the control (36.625 ± 4.438 Cm) and normal saline (35.625 ± 4.926 Cm) groups. The group experiencing stress committed a higher number of errors compared to both the control and normal saline groups, with no notable distinctions observed among the stress, *Viola odorata*, and combined stress + *Viola odorata* groups. There were no significant differences in errors in the stress + *Viola odorata* group relative to the other groups.

Table 1. Barnes maze performance on day 1

	Treatments	Time to reach the target hole (Sec)	Number of Error (Number)	Distance traveled to reach the target hole (cm)
First day	Control	37.00 ± 12.535[a]	2.375 ± 0.916[a]	49.875 ± 11.993[a]
	Normal saline	43.25 ± 14.429[a]	2.125 ± 0.640[a]	50.750 ± 14.159[a]
	Stress	223.625 ± 21.573[d]	4.75 ± 1.982[b]	115.375 ± 10.112[b]
	Stress+ Violet	87.00 ± 43967[b]	3.00 ± 1.309[a]	88.50 ± 48.876[b]
	Violet	118.25 ± 7.629[c]	3.00 ± 0.755[a]	96.00 ± 20.225[b]
	p-value	p<0.001	0.001	p<0.001
	DF (Between Groups)	4	4	4
	DF (Within Groups)	35	35	35
	F	81.350	5.635	10.301

[a-c] ***p<0.001, [d]*p<0.05 vs control

Table 2. Barnes maze performance on day 2

	Treatments	Time to reach the target hole (Sec)	Number of Error (Number)	Distance traveled to reach the target hole (cm)
Second day	Control	59.75 ± 53.253[a]	1.125 ± 0.640[a]	42.875 ± 8.374[a]
	Normal saline	61.75 ± 53.917[a]	1.25 ± 0.886[a]	44.250 ± 9.528[a]
	Stress	144.50 ± 51.389[b]	3.50 ± 1.603[c]	104.125 ± 23.086[b]
	Stress+ Violet	60.50 ± 28.948[a]	2.50 ± 0.534[b]	84.00 ± 24.295[b]
	Violet	48.00 ± 24.894[a]	1.50 ± 0.755[a]	56.625 ± 31.190[a]
	P-value	P<0.001	P<0.001	P<0.001
	DF (Between Groups)	4	4	4
	DF (Within Groups)	35	35	35
	F	6.277	8.811	12.727

a-c ***p<0.001, *p<0.05 vs control group

Table 3. Barnes maze performance on day 3

	Treatments	Time to reach the target hole (Sec)	Number of Error (Number)	Distance traveled to reach the target hole (cm)
Third day	Control	51.125 ± 32.202	1.125 ± 0.640[a]	53.125 ± 23.745
	Normal saline	50.00 ± 35.351	1.125 ± 0.640[a]	53.75 ± 29.020
	Stress	47.125 ± 9.128	2.250 ± 0.886[b]	58.25 ± 11.297
	Stress+ Violet	58.00 ± 48.314	1.375 ± 0.916[a]	71.00 ± 33.683
	Violet	51.75 ± 22.933	0.875 ± 0.640[a]	48.25 ±9.617
	P-value	0.974	0.009	0.379
	DF (Between Groups)	4	4	4
	DF (Within Groups)	35	35	35
	F	0.122	3.981	1.085

[a-b] Different superscripts indicate significant differences (p<0.05). Values are mean±SEM.

Table 4. Barnes maze performance on day 4

	Treatments	Time to reach the target hole (Sec)	Number of Error (Number)	Distance traveled to reach the target hole (cm)
Fourth day	Control	27.000 ± 9.739	0.375 ± 0.512[a]	36.625 ± 4.438[a]
	Normal saline	27.750 ± 10.552	0.375 ± 0.517[a]	35.625 ± 4.926[a]
	Stress	40.750 ± 17.094	1.375 ± 1.187[b]	45.00 ± 11.649[ab]
	Stress+ Violet	40.375 ± 17.848	1.25 ± 1.035[ab]	50.625 ± 12.188[b]
	Violet	32.875 ± 17.569	0.75 ± 0.886[ab]	42.50 ± 8.864[ab]
	P-value	0.208	0.043	0.012
	DF (Between Groups)	4	4	4
	DF (Within Groups)	35	35	35
	F	1.557	2.350	3.765

Different superscripts indicate significant differences (p<0.05). Values are mean±SEM.

Effects of immobilization stress on anxiety-like behaviors in EPM test

Freezing behavior analysis revealed significantly reduced freezing in *Viola odorata* + stress (2.25±1.035 N) and *Viola odorata* (2.38±0.518 N) groups compared to control (1.25±0.707 number) and normal saline (1.13±0.835) groups ($p<0.01$). No significant freezing differences were observed between stress (1.00±0.765 N) and other groups. Both *Viola odorata* groups showed significantly ($p<0.001$) more open arm entries than controls, with no differences in closed arm entries (Table 5, 6).

Viola odorata extract groups spent significantly more time in maze center than control group. The *Viola odorata* + stress group exhibited significantly higher rearing (number) than all groups, while stress animals revealed lower rearing than *Viola odorata* + stress. Grooming was significantly higher in *Viola odorata* + stress versus *Viola odorata*-only and normal saline groups ($p<0.01$), with no head dipping differences. The *Viola odorata* group spent more time in open arms than all groups except *Viola odorata* + stress, and less time in closed arms than other groups.

Impacts of violet flower extract on oxidative stress and inflammatory markers in stressed rats

The stress group showed significantly elevated malondialdehyde (MDA) levels (3.8±0.9 pg/ml) compared to control (1.2±0.3 pg/ml) and other experimental groups, indicating increased lipid peroxidation (Figure 2a). Gamma-glutamyl transferase (GGT) activity was significantly reduced in the stress group (22.4±3.1 µl) compared to the control (45.6±5.2 µl). The *Viola odorata* + stress group showed partial restoration of GGT activity (32.7±4.3 µl, $p<0.01$ vs control) (Figure 2b).

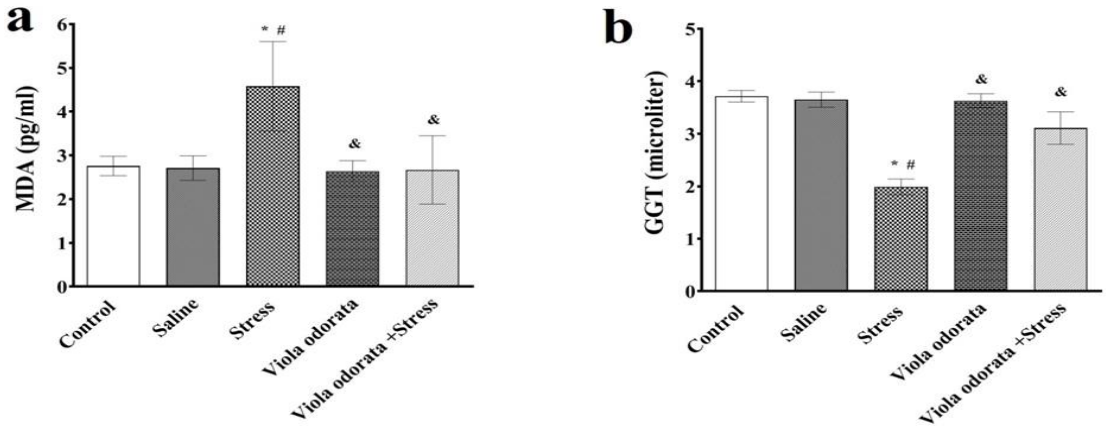

Figure 2. a) Malondialdehyde (MDA) and b) Gamma-glutamyl transferase (GGT) levels assessment in different groups using ELISA kits. $^{*}p<0.01$ as relative to the control group, $^{\#}p<0.01$ as relative to the saline group, $^{\&}p<0.01$ as relative to the stress group.

Table 5. Analysis of freezing behavior, open arm entries, closed arm entries, and time spent in center across experimental groups in EPM

Treatments	Freezing (Sec)	The number of entries into the open arm (Number)	The number of entries into the closed arm (Number)	Time spent in central (Sec)
Control	99.38 ± 74.146^{b}	1.25 ± 0.707^{a}	2.13 ± 1.126	8.38 ± 8.331^{a}
Normal saline	81.37 ± 31.341^{b}	1.13 ± 0.835^{a}	2.50 ± 1.309	12.25 ± 7.126^{a}
Stress	60.38 ± 25.207^{ab}	1.00 ± 0.765^{a}	3.00 ± 0.765	14.25 ± 7.305^{a}
Stress+ Violet	27.75 ± 21.972^{a}	2.25 ± 1.035^{b}	2.88 ± 1.246	40.25 ± 18.235^{b}
Violet	30.50 ± 29.838^{a}	2.38 ± 0.518^{b}	3.13 ± 1.126	52.00 ± 21.961^{b}
P-value	0.004	0.001	0.396	P<0.001
DF (Between Groups)	4	4	4	4
DF (Within Groups)	35	35	35	35
F	4.621	5.572	1.049	15.331

*p<0.05, **p<0.01 vs control group

Table 6. Analysis of head dipping, grooming, rearing, and time spent in open/closed arms across experimental groups in EPM

Treatments	Head dipping (Number)	Grooming (Number)	Rearing (Number)	Time spent in open arm (Sec)	Time spent in closed arm (Sec)
Control	2.63 ± 2.134	30.38 ± 17.679^{ab}	10.75 ± 3.240^{a}	29.88 ± 29.624^{a}	261.50 ± 33.581^{b}
Normal saline	2.63 ± 3.068	27.00 ± 17.909^{a}	12.88 ± 3.643^{a}	24.63 ± 29.135^{a}	263.13 ± 30.540^{b}
Stress	2.63 ± 1.506	32.63 ± 9.546^{ab}	18.13 ± 2.375^{ab}	20.13 ± 13.994^{a}	265.00 ± 15.703^{b}
Stress+ Violet	3.75 ± 1.488	48.63 ± 27.547^{b}	39.63 ± 12.817^{c}	31.75 ± 23.909^{ab}	228.00 ± 33.076^{b}
Violet	3.75 ± 1.488	21.38 ± 8.847^{a}	21.00 ± 8.536^{b}	60.38 ± 39.594^{b}	187.63 ± 61.013^{a}
P-value	0.574	0.039	P<0.001	0.064	0.001
DF (Between Groups)	4	4	4	4	
DF (Within Groups)	35	35	35	35	
F	0.735	2.830	19.698	2.452	6.322

**p<0.01 vs control group

In the stress group, TNF-α concentrations were significantly elevated (p<0.001) at 38.6±6.5 pg/ml, in contrast to the control group, which measured 12.5±2.1 pg/ml. *Viola odorata* extract treatment significantly attenuated this increase (19.2±3.4 pg/ml, p<0.01) relative to the stress group (Figure 3a). Similarly, IL-1 concentrations were markedly (p<0.001) higher in the stress group (25.4±4.2 micromol/L) than control (8.3±1.5 micromol/L). Treatment with *V. odorata* extract before stress reducing IL-1 levels than the stress group (14.6±2.8 micromol/L, p<0.01) (Figure 3b). There were no notable variations in any markers across the control group, normal saline group, and *Viola odorata* extract-only group (p>0.05). Every value is expressed as mean±SEM, with statistical analysis carried out via one-way ANOVA and subsequent Tukey's post-hoc test.

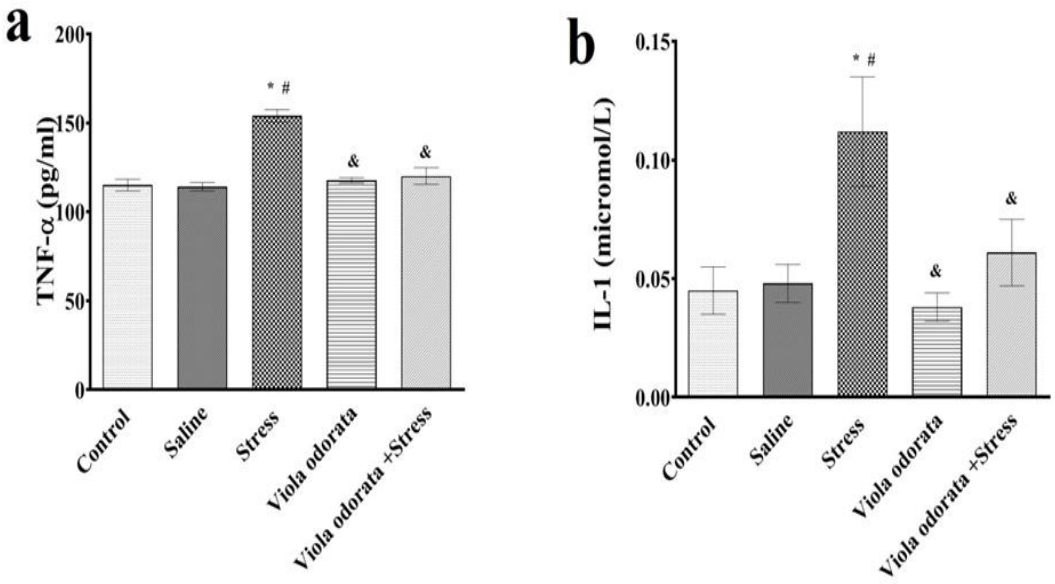

Figure 3. a) Tumor necrosis factor-alpha (TNF-α) and b) Interleukin-1 (IL-1) levels assessment in different groups using ELISA kits. *p<0.01 as relative to the control group, #p<0.01 as relative to the saline group, &p<0.01 as relative to the stress group.

Viola odorata extract reduced stress-induced neuronal damage in the brain

The findings indicated that the group experiencing stress exhibited a markedly reduced neuronal density in the amygdala relative to the control group. The group

receiving *V. odorata* extract alongside stress demonstrated a substantially greater neuronal density than the stress-only group ($p<0.001$), while showing no significant variances from the control and normal saline groups (Figure 4). In the dentate gyrus area of the hippocampus, the stress group showed a 25-32% reduction in viable pyramidal neurons compared to the control animals ($p<0.001$). *Viola odorata* extract treatment provided 68-75% protection against this neuronal loss in the hippocampus (Figure 5). The prefrontal cortex exhibited a 20-28% neuronal loss in the stress group ($p<0.001$), with *V. odorata* extract administration reducing this damage by 60-70% (Figure 6).

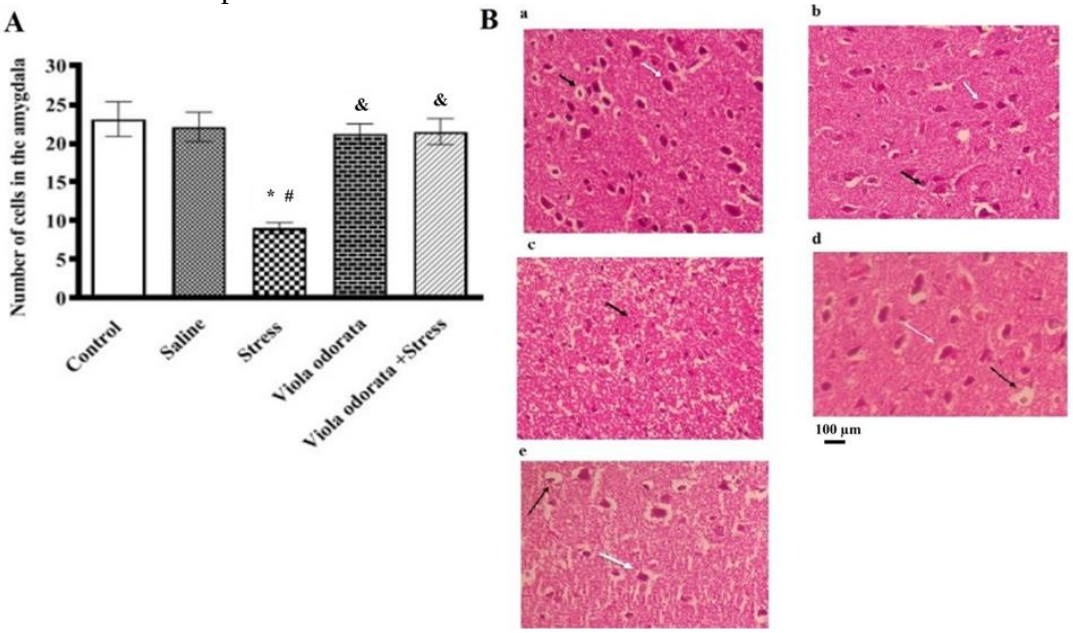

Figure 4. A) A graph to show the comparison in the number of cell in the amygdala area between experimental groups. * in comparison with the control group, # in comparison with the saline group, & in comparison with the stress group. B) Examples of the H&E stained neurons in the amygdala region s in experimental groups (H&E, 400×). a) Control: b) Normal saline: Healthy neurons (black arrows), glial cells (white arrows), c) Stress: Neuronal loss and tissue necrosis, d) Violet extract: Glial cells (white arrows), healthy neurons (black arrows), e) Violet extract+Stress: Healthy neurons (black arrows), glial cells (white arrows). 100µm

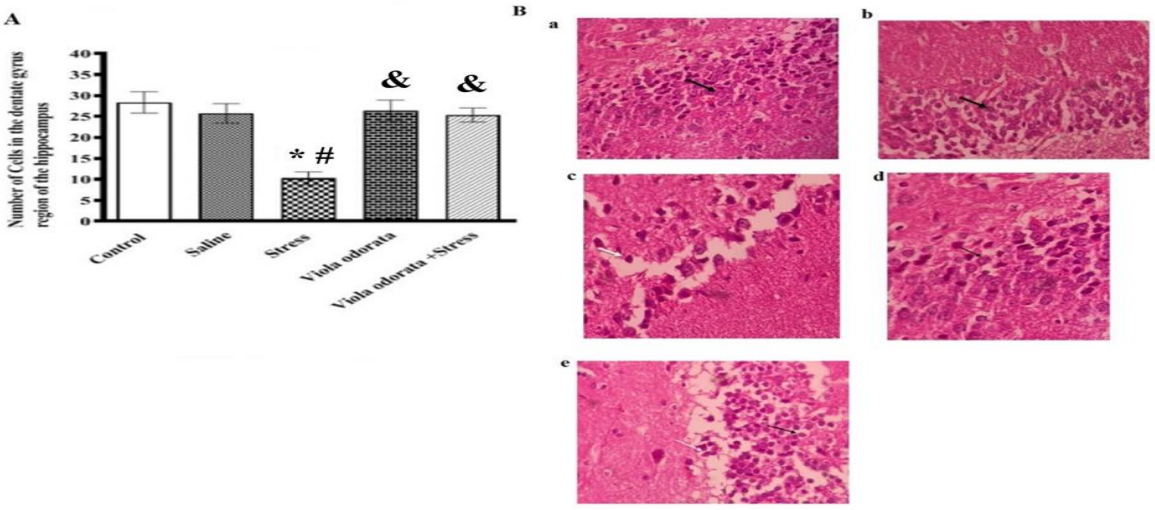

Figure 5. A) A graph to show the comparison in the number of cell in the dentate gyrus region of the hippocampal between experimental groups. * in comparison with the control group, #in comparison with the saline group, & in comparison with the stress group. B) Examples of the H&E stained neurons in the amygdala region s in experimental groups (H&E, 400×). a) Control: Neurons with distinct nuclei (white

arrows), glial cells (black arrows), b) Normal saline: Healthy neurons (black arrows), glial cells (white arrows), c) Stress: Neuronal loss and tissue necrosis, d) Violet extract: Glial cells (white arrows), healthy neurons (black arrows), e) Violet extract+Stress: Healthy neurons (black arrows), glial cells (white arrows), vascular dilation (red arrows).

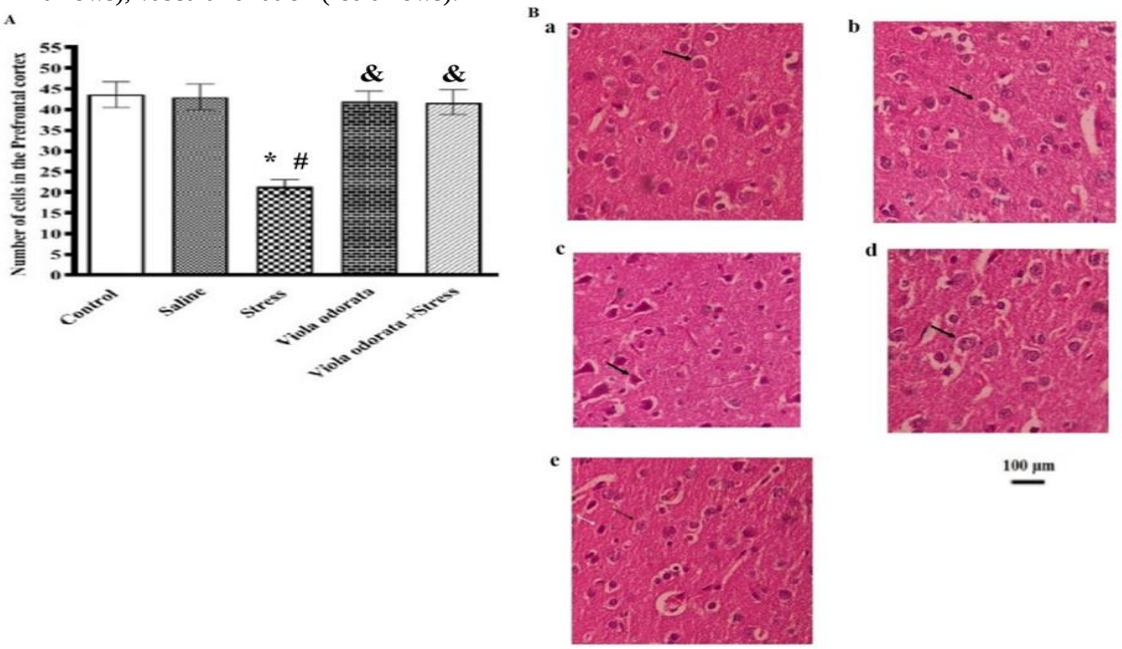

Figure 6. A) A graph to show the comparison in the number of cell in the prefrontal cortex between experimental groups. * in comparison with the control group, # in comparison with the saline group, & in comparison with the stress group. B) Examples of the H&E stained neurons in the prefrontal cortex region s in experimental groups (H&E, 400×). a) Control: Healthy neurons (black arrows), b) Normal saline: Healthy neurons (black arrows), c) Stress: Neuronal loss and tissue necrosis, d) Violet extract: healthy neurons (black arrows), e) Violet extract+Stress: Healthy neurons (black arrows), glial cells (white arrows).

Discussion

This investigation was carried out to investigate the therapeutic potential of the medicinal plant *V. odorata* which has garnered attention due to its bioactive compounds. Thus, the purpose of this study was to assess the impacts of the extract derived from this plant on improving impaired cognitive functions and reducing inflammation and oxidative stress, as the primary mechanisms involved in chronic stress-induced damage. The obtained results demonstrated that *V. odorata*, through its antioxidant properties, exerts protective effects against various stress-induced complications.

The data from this study suggests that administering *V. odorata* extract orally or by gavage significantly boosts rats performance on memory and recall assessments conducted in the Barnes maze. These findings include improvements in the parameters of distance achievement and total errors recorded (incorrect hole entries). It is noteworthy that the reduction in distance traveled by the rat's means they needed less searching and random movement in the Barnes maze. As a result, this strength indicates improvement in the animals ability to identify the correct path and perform spatial memory-related tasks. In other words, animals that consumed violet flower extract were likely able to identify the accurate path faster and more precisely. The decrease in number of errors (incorrect hole entries) indicates increased accuracy in memory recall, meaning the they could well distinguish between the correct and ineffective locations. Increased accuracy in identifying the correct hole in the maze can be considered as an indicator of improvement in spatial learning and memory performance. This improvement may be attributed to changes in memory-

related brain structures such as the hippocampus, which this brain segment contributes significantly to the processes of learning and spatial memory. The positive effect of *V. odorata* extract on memory improvement might be related to its ability to reduce inflammation and provide antioxidant support (Tayarani-Najaran et al. 2019). Findings suggest that extracts from plants, which include specific active ingredients, can enhance mental performance. These compounds might bolster brain wellness and protect against oxidative injury induced by stress and other negative elements (Moghimi Gholmakani et al. 2022). Tayarani-Najaran et al. (2019), in an investigation examining the properties of *V. odorata*, reported that this plant reduces free radicals, thereby decreasing oxidative stress and its subsequent adverse effects, this aligns with the findings of the current research. In another investigation, Moghimi Gholmakani et al. (2022) studied *V. odorata* properties and found that *V. odorata* extract and essential oil possess anti-anxiety, antioxidant, anti-inflammatory, and antimicrobial properties. Additionally, some studies have demonstrated that using *V. odorata* contributes to decreases stress levels, better sleep, alleviates discomfort, and boosts mental tranquility. These results align with the findings of the current study, which can be attributed to the antioxidant and anti-inflammatory components present in the violet plant.

As demonstrated in our study, the EPM test serves as a tool for evaluating anxiety behaviors, *V. odorata*-treated rats displayed fewer anxiety-related behaviors, such as reduced exploration in open areas and increased time spent in safe zones, indicating decreased anxiety in this animal model. It has been shown that *V. odorata* extract possesses significant antioxidant properties, evidenced by reduced free radicals and inflammatory factors in rat blood (Alipanah et al. 2018). It is suggested that lowering inflammation may be a possible way to alleviate anxiety. In other words, *V. odorata* extract may alleviate anxiety by mitigating oxidative stress and inflammation (Rizwan et al. 2019; Tayarani-Najaran et al. 2019).

Histological studies have provided further evidence elucidating the mechanism of action of *V. odorata* extract. The findings demonstrate that *V. odorata* extract administration is associated with increased neuronal density in both the amygdala and hippocampus, which are critically involved in anxiety processing and memory formation. Additionally, a considerable growth in the number of pyramidal cells was noted in the prefrontal cortex, a brain region known to mediate behavioral control, decision-making, and cognitive functions. These histological results collectively indicate that *V. odorata* extract exerts modulatory effects on brain structure and function, consequently leading to improvements in anxiety-related behaviors. The evidence gathered in this investigation illustrates significant effects of stress on biochemical parameters related to inflammation and oxidation in rat brains. As a well-established physiological stimulus, stress can lead to enhanced production of free radicals and subsequent oxidative stress. Elevated levels of MDA, a byproduct of cell membrane oxidation, indicate heightened oxidative stress activity and damage resulting from an imbalance between free radical production and the body's antioxidant systems (Alonso et al. 2005; Amantea et al. 2009). Malondialdehyde serves as a key biomarker for assessing oxidative damage, and its increase clearly reflects the severity of cell membrane damage caused by stress (Kandlur et al. 2020).

As demonstrated in the present study, *V. odorata* plants with their antioxidant properties caused a significant reduction in MDA levels, which represents one of the most important indicators of oxidative stress. It should be noted that the primary damaging factor in oxidative stress involves the formation of reactive compounds known as reactive oxygen species that

readily donate oxygen to other substances. Many of these reactive species include free radicals containing one or two unpaired electrons, which accounts for their instability and high reactivity (Navarro-Yepes et al. 2014). To achieve stability by acquiring additional electrons, free radicals attack nearby molecules, leading to structural and functional damage of these molecules. The formation of free radicals is linked to fundamental metabolic operations within the human organism or from external sources such as pharmaceuticals, pesticides, toxins, and chemical/environmental pollutants (Ghorbani et al. 2014). The maintenance of equilibrium between free radical production and the body's endogenous antioxidant defense system represents a critical physiological balance. Oxidative stress manifests when this equilibrium is disrupted, resulting from excessive generation of reactive oxygen species and free radicals (including hydroperoxyl radicals, hydrogen peroxide, superoxide anions, and hydroxyl radicals) as well as reactive nitrogen species (like nitric oxide synthase and nitric oxide) (Ochiaia et al. 2004).

Concurrently, stress-induced pathways activate inflammatory responses characterized by elevated levels of pro-inflammatory cytokines including TNF-α and IL-1. These cytokines serve as pivotal mediators in inflammatory cascades, where their upregulation can initiate detrimental neuroinflammatory pathways leading to neural tissue damage. Notably, these inflammatory mediators may independently contribute to the pathogenesis of neurological disorders and the impairment of cognitive functions, particularly those related to memory formation and learning processes (Ahmad et al. 2010; Roodbari and Meftahi 2025). This established relationship between psychological stress and elevated cytokine levels underscores the role of stress as a significant exacerbating factor in both inflammatory processes and tissue damage. Furthermore, stress exposure correlates with decreased activity of GGT, an essential enzyme in cellular detoxification and glutathione metabolism that plays a crucial role in maintaining endogenous antioxidant capacity (Longo-Mbenza et al. 2012). The observed reduction in GGT activity reflects a compromised cellular defense system against oxidative stress, thereby potentiating oxidative damage to cellular components. This enzymatic deficiency may serve as a sensitive biomarker for assessing the disruption of antioxidant mechanisms under conditions of chronic stress (Lee et al. 2004). However, *V. odorata* demonstrated significant protective effects against all these adverse outcomes through its bioactive properties. These findings are in agreement with the results of the current investigation. Therefore, the obtained results indicate that stress can significantly negatively affect brain biochemical activities, particularly through alterations in MDA, TNF-α, IL-1, and GGT levels, leading to neuronal damage and consequent cognitive impairments. These findings further emphasize the importance of investigating and identifying herbal therapeutic approaches, such as *V. odorata* extract, that can modulate these adverse effects.

Findings from this study indicate that facing inescapable stressors leads to significant alterations in brain structures associated with memory and stress response. The observed reduction in pyramidal cell count and neuronal density in the amygdala and hippocampus indicates stress-induced structural and functional damage. The amygdala, as the center for processing emotional and fear responses, is particularly vulnerable to chronic stress, and its reduced neuronal density may impair emotional processing and regulation. Similarly, the hippocampus - a crucial structure for spatial and episodic memory and learning - shows decreased neuronal density associated with memory and learning deficits. These findings align

with previous studies demonstrating that chronic stress can cause lasting brain damage (Lonsdorf et al. 2014). This research demonstrated that treatment with *V. odorata* extract markedly ameliorated these stress-induced damages and promoted the normalization of brain structures. These enhancements reveal the restorative capacity of *V. odorata* in addressing the adverse effects that stress has on brain health and operation. However, it should be noted that this normalization process requires more detailed characterization regarding the extent and nature of recovery. For instance, further investigation is needed to determine: (1) the degree of restoration of neuronal density to baseline levels, (2) whether other structural changes are involved in this recovery process, and (3) the precise mechanisms underlying the neuroprotective effects of violet flower extract.

Phytochemical analyses have identified various bioactive compounds in both aqueous extracts and ethanolic of violets aerial components (*Viola* spp), including tannins, flavonoids, cyclotides, vitamins A and C, steroids, saponins, and alkaloids (Akhbari et al. 2012). The outcomes of the present research indicated that enduring stress influences significantly reduced pyramidal cell counts and decreased neuronal density in both the amygdala and hippocampus. Importantly, treatment with *V. odorata* extract significantly ameliorated these stress-induced neurostructural alterations, restoring them to near-normal levels. The findings propose that the neuroprotective benefits associated with *V. odorata* extract are likely driven by its extensive range of phytochemical compounds, which appears to counteract stress-induced neuronal damage in key brain regions involved in emotional regulation and memory processing.

This examination has multiple limitations. The specific molecular interactions responsible for the influence of *V. odorata* have not been closely examined. Employing sophisticated methods like transcriptomics and proteomics can clarify the distinct pathways and gene expression that contribute to the therapeutic effects of *V. odorata*.

Collectively, these findings indicate that *V. odorata* exerts significant protective and therapeutic effects in chronic stress by reducing oxidative stress, improving cognitive function, alleviating anxiety and increasing neuronal density in the prefrontal cortex, amygdala, and hippocampus. Given these promising results, we recommend further studies using different doses and treatment durations of *V. odorata* extract to determine the optimal dosage and treatment period for maximal therapeutic effects. To ensure the efficacy and safety of violet flower extract, conducting clinical trials with human participants is crucial.

Acknowledgment

This study was supported by Neuroscience Sciences Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran.

Conflicts of interest

The authors have no conflicts of interest to declare.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical Considerations

The Ethics Committee of the Baqiyatallah University of Medical Sciences located in Tehran, Iran authorized the procedures involving experimentation (Protocol approval number: IR.BMSU.AEC.1403.023).

Code of Ethics

IR.BMSU.AEC.1403.023

Authors' Contributions

Mohammad Sadeghi: Writing – original draft, Methodology, Formal analysis, Data curation. Elham Moghtadaei Khorasgani: Methodology. Gholam Hossein Meftahi: Writing – review & editing, Supervision, Resources, Project administration, Methodology, Data curation, Conceptualization.

Abbreviations

V. odoratas: Viola odoratas. MDA: Malondialdehyde. TNF-α: Tumor Necrosis Factor-alpha. IL-1β: Interleukin-1β). GGT: Gamma-glutamyl transferase. EPM: Elevated plus maze. H& E: Hematoxylin-eosin.

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