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Neuroprotective impacts of chrysin against clonazepam induced cognitive deficits in male rats

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ABSTRACT

The therapeutic effects of Clonazepam (CZP), a classic anti-anxiety drug, are accompanied by several neurodegenerations and neural disorders in patients. Also, Chrysin is a flavonoid that naturally exists in many plants and honey; it has various pharmacological effects, including anti-cancer, antioxidant, and anti-inflammations, and it has a neuroprotective effect. The purpose of this work is to evaluate the influences of chronic treatments with Chrysin on behavior and neurochemical fluctuations induced by CZP treatment. Forty male rats were classified into four groups with one of them acting as the control group receiving 1% Tween 80; the CZP group was treated with 2 mg $kg^{-1}day^{-1}$; the Chrysin group was treated with 50 mg $kg^{-1}day^{-1}$; the CZP + Chrysin group was treated with the same above-mentioned doses of CZP and Chrysin. All animals were orally treated every day for 6 weeks. Open field and Y maze tasks were performed before decapitation. Then, gamma-aminobutyric acid (GABA), glutamic acid, monoamines (norepinephrine, dopamine, and serotonin) and their metabolites (homovanillic acid, 3,4-dihydroxyphenylacetic acid, and hydroxy indoleacetic acid, respectively), and DNA fragmentation [8-hydroxy-2–deoxyguanosine (8-HdG)] were evaluated in the brain cerebral cortex, hippocampus, and striatum, while brain-derived neurotrophic factor (BDNF) and calcium ATPase (Ca-ATPase) were measured in the whole brain. The results showed that Chrysin treatment improves GABA, glutamic acid, monoamines, and their metabolites in the three brain areas, whereas it inhibits DNA fragmentation 8-HdG and BDNF and modifies downregulation of Ca-ATPase persuaded by CZP treatment at $p < 0.05$. Moreover, Chrysin treatment intensely reverses the consequent behavioral alternations which were elevated by Y maze and open field tests changed by CZP treatment. Overall, results recommended that Chrysin exerts anxiolytic-like effects similar to benzodiazepines and it can produce neuroprotective effects against CZP treatment.

INTRODUCTION

Clonazepam (CZP) is an anti-anxiety drug belonging to the benzodiazepine (BZP) family that affects gamma-aminobutyric acid (GABA) (Mikulecká *et al*., 2014). GABA is a neurotransmitter that obstructs brain function (a chemical used by nerve cells to interact with each other). Excessive brain activity is likely to contribute to anxiety or other psychological disorders (Goddard *et al*., 2004). CZP is used specifically to treat panic disorder and to inhibit some forms of convulsions (Morishita, 2009). On the other hand, it causes disturbance of many brain processes. Neurological impairments like in drug abusers are detected and may signify neural disorders and drug-induced neurotoxicity (Cunha-Oliveira *et al*., 2010). CZP has anxiolytic, sedative, and anticonvulsant effects and is a common drug of abuse due to its euphoric effect (Baselt, 2008). It is supposed that reversing amnesia and weakening memory would have some effect (Baselt, 2008; Storhaug *et al*., 2012).

CZP is utilized mainly to treat epilepticus though it is also commonly suggested for different neurological and psychological disorders containing drug abuse treatment, while CZP is known to be harmless. Addiction medicine specialists established that it is

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often commonly damaged as a drug lane (non-medicinal reasons) (Longo and Johnson, 2000). BZP is hardly the single or chosen medication for misuse (Steentoft and Linnet, 2009). Wilms *et al.* (2003) reported that BZP binding receptors are currently used to assess inflammation in diseases such as Alzheimer, while BZP peripheral receptors are plentiful in several kinds of cells and are stated physiologically merely at small levels in the central nervous system (CNS). Earlier studies indicate equivalent results after experimental injuries in animal models and inflammatory diseases and neurodegeneration in humans (Wilms *et al*., 2003).

Flavonoids are polyphenolic complexes that may be found in food products, brews, and herbs with numerous benefits to health in numerous studies (Li *et al*., 2008). Many flavonoids have antioxidant functions (Lambert and Elias, 2010). Chrysin (5,7-dihydroxyflavone) is a flavonoid that naturally exists and is extracted from various plants, including *Passiflora caerulea* (blue passionflower) and honey (Li *et al*., 2010). Chrysin has various pharmacological effects as other flavonoids, including anti-cancer (Li *et al*., 2010), antioxidant (Pushpavalli *et al*., 2010; Temel *et al*., 2020), antihypertensive (Villar *et al*., 2002), and anti-inflammations effects (Cho *et al*., 2004). Chrysin leaves an effect on the CNS, having an anxiolytic activity (Rodriguez-landa *et al*., 2019); it possesses antidepressant properties which can be beneficial in treating several complaints (Farkhondeh *et al*., 2020; Filho *et al*., 2016a); it improves memory disorders and cognitive deficits in various models of memory loss induction (Souza *et al*., 2015; Taslimi *et al*., 2019), where it affects the neurotransmitter systems like serotonergic, noradrenergic, dopaminergic, and GABAergic systems of brain constructions such as the prefrontal cortex, raphe nucleus amygdala, and hippocampus which are implicated in the pathophysiology of numerous neuropsychiatric disorders (Rodriguez-landa *et al*., 2022). Another work presented that Chrysin confidently reserved tunicamycin which results in the death of neural cells by suppression of mitochondrial apoptosis (Izuta *et al*., 2008). So, it acts as a neuroprotective mediator (Souza *et al*., 2015) and has a neuroprotective effect in chronic cerebral hypoperfusion (He *et al*., 2012). Moreover, Chrysin could be a neuroprotective agent in various models like Parkinson's disease (Goes *et al*., 2018; Krishnamoorthy *et al*., 2019); it decreases neuroinflammation and increases the levels of neurotrophic factor (Filho *et al*., 2015). Additionally, Chrysin has been revealed to possess potential improvements in cerebral ischemia, preventing cognitive impairments and long-term mental potency owing to cerebral ischemia (Sarkaki *et al*., 2019) and managing the PI3K/Akt/mTOR pathway (Li *et al*., 2019). Recently, Chrysin has also been shown to ameliorate lead-acetate-induced toxicity in rats (Kucukler *et al*., 2021). It was also revealed that Chrysin suppresses HT-29 cell death induced by diclofenac through apoptosis and oxidative damage (Özbolat and Ayna, 2021). Additionally, Chrysin alleviates cyclophosphamide-induced toxicities in SH-SY5Y cells (Ayna *et al*., 2020).

This study explained the pathway of neurodegeneration induced by CZP through biochemical and behavioral studies and the impact of Chrysin treatment in the relief of neurodegeneration caused by CZP in male rats in the cortex, hippocampus, and striatum.

MATERIAL AND METHODS

Experimental animals

Forty adult male albino rats (Wister strain) (weighing 120 ± 5 g) were used in this study. The rats were obtained from the animal house of the National Organization for Drug Control and Research (NODCAR) and were housed in plastic cages, 10 rats per cage. Throughout the experiment, animals were preserved under the exact temperature of 25°C ± 2°C and 12 hours of light/12 hours of dark period. A pellet diet billboard was used during the experiment. 1 week before the experiment started, the animals were adapted to the laboratory conditions in compliance with the rules of the Scientific Research Ethics Committee of Ain Shams University and the NODCAR (with the approval number NODCAR/11/22/19) where the experimental procedures were carried out.

Drugs

Chrysin, 5,7-dihydroxyflavone, 98% (C_{15} H_{10} O_4 molecular weight of 254.24) was manufactured by Alfa Aesar, Germany. CZP 2 mg oral tablets are available as generic drugs and the brand name drug is Apetryl. Apetryl tablets are manufactured by Multi-Apex for pharmaceutical industries S.A.E., Badr City, Egypt.

Experimental design

The animals were divided into four groups (10 rats each) as follows.

Group 1, the control group, was treated with a vehicle (1% *w*/*v* Tween 80) according to El Khashab *et al*. (2019). Group 2, CZP group, was treated with 2 mg/kg/day (Mohamed *et al*., 2015) and suspended in 1% *w*/*v* Tween 80 (Socała *et al*., 2018). Group 3, Chrysin group, was treated with 50 mg/kg/day (Mehri *et al*., 2014) suspended in 1% *w*/*v* Tween 80 according to El Khashab *et al*. (2019). Group 4, CZP + Chrysin group, was treated with both CZP (2 mg/kg/day) and Chrysin (50 mg/kg/day). All the animal groups were daily treated for 6 weeks starting on the first day of the experiment by oral gavage. The used doses are human equivalent pharmaceutical doses and were calculated as described previously by Reagan-Shaw *et al*. (2008). The open field and Y maze tests started on the 6th week of the experiment before decapitation.

Biochemical investigation

After 24 hours from the last dose, the rats were sacrificed by fast decapitation. Brains were dissected out and cut into two halves longitudinally. The cortex, hippocampus, and striatum were isolated from the first half and homogenized in 75% methanol high performance liquid chromatography grade for estimation of amino acids (glutamic acid and GABA) and monoamines [norepinephrine (NE), dopamine (DA), and serotonin (5HT)] and their metabolites [homovanillic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC), and hydroxy indoleacetic acid (5-HIAA), respectively] (Pagel *et al*., 2000) and 8-hydroxy-2–deoxyguanosine (8-HdG) (Lodovici *et al*., 1997). The second half was homogenized using a Polytron homogenizer at 40°C with a phosphate buffer of 0.05 M (PH 7). To remove cell waste, unbroken cells, nuclei, erythrocytes, and mitochondria, the homogenate was centrifuged at 10,000 rpm for 20 minutes. The supernatant was utilized by Enzyme-Linked Immuno-Sorbent Assay (ELISA) in conjunction with manual instructions for the determination of brain-derived neurotrophic factor (BDNF) and calcium ATPase (Ca-ATPase). All ELISA kits were measured by an ELISA reader. Color absorbance was read at an objective density range of 490 to 630 nm using an ELISA plate reader (Stat Fax 2200, Awareness Technologies, Palm City, FL).

Behavioral tests

Y maze test

The Y maze involved three identical arms labeled A, B, and C. The angle between the arms is 120 degrees; each arm of the maze has dimensions of 40 × 15 × 30 cm (Roghani *et al*., 2006). Both the floor and sides of all arms are made of wood. The maze is used to assess spontaneous changes. Each rat was placed in one of the arms and permitted to transfer freely among the three arms for 5 minutes. The number of arm entries and the number of trials are stated to evaluate the alteration percentage. The number of maximum random alterations is calculated as the total number of arms entered minus two, and the correct alteration is calculated as the consecutive overlapping entries into the three arms. The percentage of alternation is determined for three groups (i.e., ABC, CBA, and BAC). The percentage of alternation is calculated as [(actual alternations/maximum alternations) × 100], and the percentage of correct alternation is calculated as [(correct alternation/maximum alternations) × 100]. When the four limbs of a rat are in the arm, an entry is determined. The maze is cleaned with 70% ethyl alcohol and permitted to dry among sessions. The Y maze was used to assess the overall stereotypic behavior, locomotor activity, and short-term memory (Roghani *et al*., 2006).

Open field test

This test was utilized in this study to measure the locomotion activity. It was made of white plywood and measured 100 × 100 cm with 45 cm. A marker was used to draw black lines on the floor. The floor was divided into 25 parts with dimensions 20 × 20 cm by the black lines. The central square was drawn in the middle of the maze (20 × 20 cm). In their home cages, the rats were conceded to the practice chamber and were always handled by the base of their tails. Into one of the open field corners, rats were sited and permitted to discover the maze for 5 minutes. Then rats were returned to their cages. The maze was cleaned with 70% ethyl alcohol among trials and permitted to dry among tests. On two consecutive days, rats were exposed to the maze for 5 minutes to assess the process of acclimatization to the newness of the field according to Yang *et al*. (2013). The second-day trials were recorded and used in the statistics.

Statistical analysis

Values reported reflect means ± SEM. Statistical analysis was assessed by one-way ANOVA.

Least significant difference comparisons were conducted to determine the significant differences between different treated groups. SPSS for Windows software, release 23.0, was used with a significant value of $p < 0.05$.

RESULTS AND DISCUSSION

Biochemical investigation

Determination of glutamic acid, GABA, and 8-HdG contents

The data in Table 1 revealed a significant increase in glutamic acid contents in the cortex, hippocampus, and striatum of all treated groups except CZP + Chrysin rats in both brain cortex and hippocampus in comparison with the control rats at $p < 0.05$. Moreover, the GABA contents declined significantly in all treated groups in the cortex, hippocampus, and striatum when compared with the control values. Additionally, GABA content increased significantly in the brain hippocampus while it slightly increased in the brain cortex and striatum in CZP + Chrysin group rats when compared with the Chrysin-treated group at $p < 0.05$. Furthermore, all the CZP-treated groups exhibited a significant increase in the cortex, hippocampus, and striatum 8-HdG contents in comparison with the control group at $p < 0.05$. Otherwise, the group treated with Chrysin exhibited a remarkable decrease in cortex, hippocampus, and striatum 8-HdG contents as compared to CZP-treated rats. Also, CZP + Chrysin group rats demonstrated a significant decrease ($p < 0.05$) in the three brain areas of 8-HdG contents when compared with the CZP-treated group. These results were elucidated by Goddard *et al*. (2004) who revealed a significant decrease in occipital cortex GABA levels after chronic CZP as BZP family treatment and suggest that CZP may be capable of exerting a direct inhibitory effect on the activity of glutamate decarboxylase. CZP is highly potent and a long-acting BZP. It exerts pharmacological effects by acting as a positive allosteric modulator on GABA-A receptors. The GABA-A receptor is a ligand-gated chloride ion-selective channel

Table 1. The effect of CZP and chrysin on glutamic acid, GABA (µg/g tissue), and 8-HdG (pg/g tissue) in brain cortex, hippocampus, and striatum.

Groups/Parameters		Control	CZP	Chrysin	CZP + Chrysin
Brain cortex	Glutamic acid	3.5 ± 0.16	4.6 ± 0.15*	4.4 ± 0.17*	3.8 ± 0.12#+
	GABA	5.4 ± 0.25	3.9 ± 0.09*	4.0 ± 0.13*	4.7 ± 0.18*#+
	8HdG	120.5 ± 3.6	161.0 ± 4.4*	121.0 ± 3.1#	143.2 ± 3.6#+
Brain hippocampus	Glutamic acid	2.9 ± 0.13	3.8 ± 0.13*	3.6 ± 0.16*	3.1 ± 0.09#+
	GABA	4.5 ± 0.20	3.2 ± 0.08*	3.2 ± 0.11*	3.5 ± 0.14*
	8HdG	99.2 ± 2.8	130.2 ± 4.4*	103.0 ± 0.4#	123.5 ± 0.43#+
Brain striatum	Glutamic acid	2.6 ± 0.15	3.4 ± 0.14*	3.4 ± 0.18*	3.1 ± 0.18*
	GABA	4.0 ± 0.24	2.9 ± 0.05*	2.8 ± 0.03*	3.1 ± 0.13*
	8HdG	91.2 ± 2.9	117.3 ± 2.6*	95.8 ± 2.7#	107.8 ± 1.6#+

Values expressed as mean ± SEM of 10 rats/group,* = significant change from control group, # = significant change from CZP-treated group, and + = significant change from chrysin-treated group at $p < 0.05$.

whose endogenous ligand is GABA. BZPs facilitate GABA-A action by increasing the frequency of chloride channel opening, resulting in hyperpolarization of the neurons and decreased firing, thus producing calming effects on the brain by reducing the excitability of neurons (Griffin *et al*., 2013). Alternatively, BZP's influence on other neuronal targets could alter both the excitatory drive for the neuronal activity of GABA and the availability of substrates for GABA synthesis, like glutamate and glutamine. In fact, after BZP administration, glutamic acid levels were slightly elevated, indicating an inhibitory effect of BZP administration on glutamic acid turnover. In addition, BZP enhances the GABA-A receptor-mediated impacts of exogenous and exogenous GABA (Mikulecká *et al*., 2014). This potential consists of an improvement in GABA's apparent affinity for raising chloride conductance without increasing its effectiveness, which may be due to an increased probability of channel opening events at the single chloride channel level. So, recent studies suggest that BZP is a GABA receptor (GABA-R) chloride channel complex site that allosterically modulates the increase of the signal transducer feature of the latter. The exact contact with three groups of ligands is a special characteristic of this medication receptor, which is placed on a neurotransmitter receptor-gated ion channel (Kubová *et al*., 2020).

Rats treated with Chrysin showed a significant elevation in glutamic acid in the three brain areas as compared with the control. These data were supported by El Khashab *et al*. (2019) who found that exposure to Chrysin was followed by Bcl2 elevation and a reduction in both Hsp90 and Bax levels and was also followed by aspartate and glutamate elevation. These records contribute to the understanding of the value of natural flavonoids as neuroprotective agents (Souza *et al*., 2015). Chrysin has been reported to exert neuroprotective effects through different mechanisms, including antioxidant, anti-inflammatory, and anti-apoptotic functions, monoamine oxidase inhibition, and GABA mimetic properties (Mishra *et al*., 2021). In addition, Bortolotto *et al*. (2020) confirmed that Chrysin normalizes the activity of Na^+, K^+- ATPase, and glutamate levels in the hippocampus, which may be related to memory impairment in reverse. The regulation of the glutamic acid level in rats treated with CZP + Chrysin compared to the CZP group can be addressed in this description. Also, the Chrysin-treated group revealed a marked decline in GABA levels in the brain cortex, hippocampus, and striatum, and the detected Chrysin anxiolytic effects in this work looked to be less partially mediated by GABAergic activity according to a previous study by Wang *et al*. (2002) which has revealed that Chrysin possesses a high GABAA receptor affinity. GABA is the most important inhibitory neurotransmitter in the brain. It exhibits a neuroprotective effect by inhibiting brain injury, neuronal damage, autophagy [via the upregulation of Bcl-2/Bax ratio and activating protein kinase B, glycogen synthase kinase-3β, and extracellular signal-regulated kinase 1 (ERK) signaling molecules], and neuronal cell death (Ngo and Vo, 2019). In this way, GABA demonstrates a therapeutic potential in various neurological disorders (Nuss, 2015); Chrysin has been shown to modulate the GABAA receptor and thus abrogate anxiety and depression-like behavior (Cueto-Escobedo *et al*., 2020; Rodriguez-Landa *et al*., 2021). Therefore, it can act as a neuroprotection via the modulation of GABAergic innervation. The chloride ions' conductance increases when GABAergic agonists stimulate these receptors, thus hyperpolarizing the neuron and thereby inhibiting neuronal activity (Majewska *et al*., 1986). The anxiolytic effects of many materials, involving BZP, are associated with this neurophysiological effect, which arises through the chloride ion channels of the GABAA receptor. Moreover, Rashed *et al*. (2016) interpreted the decreased level of the inhibitory neurotransmitter GABA as Chrysin's neuroprotective role.

Moreover, the CZP-treated group showed a significant increase in DNA fragmentation (8-HdG) in all of the brain cortex, hippocampus, and striatum. These results were supported by Girgis *et al*. (2010) who discovered that compared to the control group, CZP-treated rats displayed the highest optical density value of brain DNA degradation and discussed that through several mechanisms CZP can induce apoptosis. The peripheral non-GABAA receptors are one such mechanism. Another alleged mechanism is the oxidative stress caused by the overdose typically achieved in the state of abuse and tolerance, alteration of ion channel influx, and outflux activity modulations in the cell media through GABA-R that cause changes in normal chloride and other ions balance in the neural cells, and finally, activation of the caspase system and eventually apoptosis of cell death (Uren *et al*., 2000). Also, Bittigau *et al*. (2002) stated that at plasma concentrations important to seizure control in humans, CZP induces apoptotic neurodegeneration in the developing rat brain; neuronal death is related to the reduction of neurotrophin expression and decreased brain concentrations of survival-promoting proteins. Anti-epileptic drugs reduced the synthesis of BDNF and NT-3 neurotrophins and decreased levels of c-RAF, ERK12, and AKT active phosphorylated types. These changes indicate the inhibition of signals elevating survival and the difference between neuroprotective and neurodestructive mechanisms in the brain that facilitate apoptotic neurodegeneration during the developmental cycle of ongoing programmed neuronal death (Venters *et al*., 2000). Moreover, rats treated with Chrysin showed a significant reduction in brain cortex, hippocampus, and striatum 8HdG related to CZP-treated rats and showed amelioration impact in co-administration of Chrysin and CZP. These findings were verified by Rashno *et al*. (2019) who discovered that Chrysin prevented neuronal failure, decreased apoptotic index, and increased anti-apoptotic Bcl-2 protein; the expression of proapoptotic Bax protein in the tissues of the cerebral cortex and hippocampus decreased. In addition, Mehri *et al*. (2014) reported that Chrysin has been able to prevent the death of cells and inhibit apoptotic tissue damage by enhancing the activity of caspase 3. Chrysin also strengthened autophagic tissue damage by raising the light chain 3B expression (Kandemir *et al*., 2017). Also, Darendelioglu (2020) found that, through regulations on the mRNA expression of Bcl-2 and Bax protein, Chrysin plays a vital role in preventing apoptosis. Suppression of the activity of caspase 3 and the mitochondrial apoptotic pathway may at least partially involve the protective effects mechanism of Chrysin (Izuta *et al*., 2008).

Determination of monoamines and monoamines metabolites

The results in Table 2 indicated the treatment effect of CZP and Chrysin on monoamines NE, DA, and 5HT contents in brain cortex, hippocampus, and striatum in male rats. In comparison to the control rats, there was a significant elevation

Table 2. The effect of CZP and chrysin on monoamines NE, DA, and 5HT and their metabolites (HVA, DOPAC, and 5-HIAA, respectively) (µg/g tissue) in brain cortex, hippocampus, and striatum.

Groups /Parameters		Control	CZP	Chrysin	CZP + Chrysin
Brain cortex	NE	0.40 ± 0.008	0.55 ± 0.024*	0.55 ± 0.016*	0.46 ± 0.172*#+
	DA	0.78 ± 0.024	1.04 ± 0.033*	0.97 ± 0.032*	0.82 ± 0.047#+
	5HT	0.30 ± 0.021	0.35 ± 0.015*	0.36 ± 0.028*	0.35 ± 0.009*
	HVA	0.28 ± 0.004	0.22 ± 0.009*	0.22 ± 0.006*	0.25 ± 0.008*#+
	DOPAC	0.15 ± 0.012	0.14 ± 0.003*	0.14 ± 0.003*	0.14 ± 0.006*
	5HIAA	0.10 ± 0.003	0.08 ± 0.002*	0.09 ± 0.003*	0.09 ± 0.002*#
Brain hippocampus	NE	0.33 ± 0.008	0.45 ± 0.019*	0.45 ± 0.015*	0.42 ± 0.017*
	DA	0.64 ± 0.018	0.85 ± 0.026*	0.79 ± 0.025*	0.73 ± 0.035 *#
	5HT	0.25 ± 0.019	0.33 ± 0.017*	0.33 ± 0.019*	0.30 ± 0.01*
	HVA	0.24 ± 0.003	0.18 ± 0.007*	0.18 ± 0.006*	0.21 ± 0.005*#+
	DOPAC	0.15 ± 0.012	0.12 ± 0.003*	0.11 ± 0.004*	0.12 ± 0.005*
	5HIAA	0.08 ± 0.002	0.07 ± 0.002*	0.07 ± 0.001*	0.07 ± 0.003*
Brain striatum	NE	0.30 ± 0.004	0.40 ± 0.017*	0.41 ± 0.015*	0.34 ± 0.012*#+
	DA	3.01 ± 0.106	3.99 ± 0.152*	3.60 ± 0.097*	3.02 ± 0.202#
	5HT	0.22 ± 0.012	0.23 ± 0.015*	0.28 ± 0.009*	0.28 ± 0.021*
	HVA	0.22 ± 0.005	0.17 ± 0.006*	0.19 ± 0.009*#	0.16 ± 0.007*+
	DOPAC	0.12 ± 0.007	0.10 ± 0.003	0.10 ± 00003	0.10 ± 0.005
	5HIAA	0.08 ± 0.002	0.06 ± 0.002*	0.06 ± 0.002*	0.07 ± 0.002

Values expressed as mean ± SEM of 10 rats/group. * = significant change from control group, # = significant change from CZP-treated group, and + = significant change from chrysin-treated group at $p < 0.05$.

($p < 0.05$) in NE, DA, and 5HT contents of the three brain areas in CZP- and Chrysin-treated groups. Also, CZP + Chrysin-treated rats showed a significant increment in monoamines contents of the brain areas except for DA contents of the brain cortex and striatum compared to control rats. Furthermore, the treatment with CZP + Chrysin induced a significant reduction in NE and DA contents of the brain areas except for hippocampus NE content when compared with the CZP-treated rats at $p < 0.05$.

The monoamines metabolites, HVA, DOPAC, and 5-HIAA, of the three brain areas, were significantly decreased ($p < 0.05$) when comparing the treated groups with the control except for striatum 5-HIAA and DOPAC contents of CZP + Chrysin-treated groups and striatum DOPAC contents of CZP- and Chrysin-treated rats. Moreover, Chrysin treatment induced a significant elevation in the HVA contents in the brain striatum in comparison with the CZP-treated group. Moreover, there was an increase ($p <0.05$) in the HVA brain cortex and hippocampus content of the CZP + Chrysin-treated group compared with either CZP- or Chrysin-treated groups. However, rats treated with CZP + Chrysin showed a significant reduction in striatum HVA content as compared with the Chrysin-treated group.

CZP therapy demonstrated a significant rise in three forms of monoamines in the brain cortex, hippocampus, and striatum, as confirmed by Frey *et al*. (1991) who found that 5-HT levels were high in the midbrain and hemispheres and showed fluctuations in the cerebellum. Additionally, Inoue *et al*. (2007) proposed that CZP is a special high-power 1,4-BZP derivative and its activities are regulated not only by the GABA sub-type A receptor but also by the regulation of the metabolism of central 5-hydroxytryptamine (5-HT, serotonin). Also, Fennessy and Lee (1972) have reported the rise in DA and NE; many authors suggest that increases in 5-HT, DA, and NE production and improvement of 5-HT and monoamine activity mediated by the GABA system are linked to anti-depressive effects. While CZP increases the synthesis of serotonin, it was found that CZP decreased the release of 5-HIAA from the brain without changing 5-HT synthesis and clearly decreased the usage of 5-HT (Jenner *et al*., 1986). Based on their observations, Kishimoto *et al*. (1988) proposed that the anti-depressive effects might be due to the decrease in 5-HT use or the decline of the sensitivity of its receptors. In addition, it was suggested that GABA-B antagonists of the receptor possess antidepressant properties (Slattery *et al*., 2005). GABA-B receptor activation prevents serotonin production, so it has been proposed that serotonergic transmission plays a role in facilitating the antidepressant properties of the GABA-B receptor antagonist (Chaki *et al*., 2006). CZP reduced the GABA-B portion of inhibitory postsynaptic potential (Huguenard and Prince, 1994). Winkler *et al*. (2003) mentioned that CZP interacts with the GABA system and alters the humor phases. During the development of tolerance, monoamine transformation displayed an overall decline which, however, tended to be the most linked to tolerance in the case of NE in different brain areas and DA in the midbrain. There was also a transient rise in 5-HT turnover accompanied by a decline, except for the cerebellum (Frey *et al*., 1991). Vinkers *et al*. (2009) reported that BZP can produce its anxiolytic effects by stimulating alpha 3 subunits situated on serotonergic neurons. In support of that, serotonergic raphe nuclei obtain a protuberant GABAergic input from remote sources besides interneurons (Varga *et al*., 2001; Vinkers *et al*., 2009) and orders in these arches for novel anxiolytic drugs may be useful for the reaction of the GABA and 5-HT system in anxiety, together with the fact that BZP acutely decreases anxiety (Inoue *et al*., 2007).

Chrysin therapy also indicates a significant improvement in the brain areas of the cortex, hippocampus, and striatum, confirming the serotoninergic system's role in the antidepressant-like impact of Chrysin (Bortolotto *et al*., 2018). Ye *et al*. (2015) mentioned that this finding is significant since both the frontal cortex and the hippocampus are involved in the organization of depression-related emotions, memory, and learning. Also, Filho *et al*. (2016a) related the serotoninergic system to the antidepressant-like consequence of Chrysin in mice exposed to changeable prolonged stress. Moreover, Chrysin has been efficient in normalizing the hippocampal DA level, and it works at the level of cerebral synaptogenesis, returning the levels of serotonin and dopamine in the synaptic cleft and synthesizing or taking up these neurotransmitters. So, the serotonergic and dopaminergic systems are involved in Chrysin antidepressant-like impact (Bortolotto *et al*., 2018). Otherwise, Angelopoulou *et al*. (2020) proved that by mono-amino-oxidase B inhibition, Chrysin increased DA levels in the striatum; this finding was verified by our results showing a significant decrease in the metabolites of monoamines in the three brain regions. Chrysin administration dramatically enhanced dopaminergic neuronal loss, leading to reduced shivering, stiffness, postural instability, rearing activity, and animal memory dysfunctions (Ahmed *et al*., 2018). Furthermore, in the study of Guo *et al*. (2016), in Parkinson's disease mice models, Chrysin might save dopaminergic cell death in the substantia nigra pars compacta. In addition, oral therapy with Chrysin decreased the loss of tyrosine hydroxylase-immunoreactive striatal cells and preserved the morphology of 6-OHDA nigrostriatal neurons (Goes *et al*., 2018).

Determination of BDNF and Ca-ATPase contents

The data illustrated in Figure 1a and b displayed a significant reduction in whole BDNF and Ca-ATPase contents in the CZP group when compared to control rats at $p < 0.05$. Moreover, Chrysin and CZP + Chrysin treatment caused a significant increase in BDNF and Ca-ATPase compared to rats treated with CZP. Neurotrophic factors are the proteins family that is responsible for the development, survival, differentiation, and function of neuronal cells that produce and sustain mature neurons, including BDNF. BZPs are safe medications for the treatment of anxiety, sleeping disorders, and seizures; nevertheless, their usage has often led to unfavorable effects, containing memory dysfunctions and misuse (Licata *et al*., 2013). Huopaniemi *et al*. (2004) reported that BZP (Diazepam) decreases animal BDNF contents. Also, the work carried out by Huang and Hung (2009) revealed a reduction in serum BDNF contents in schizophrenia patients treated with lorazepam. This connection may be related to the unrecognized impact of the BDNF on the GABA neurotransmitter responsible for the relaxing and anxiolytic properties of BZP (Canas *et al*., 2004; Singh *et al*., 2006). The release pool that needs calcium entry via voltage-dependent calcium channels is affected by glutamate release from hippocampal synaptosomes and BDNFs. Moreover, the inhibitory effect of BDNFs on the liberation of potassium-evoked GABA from the synaptosomes of the hippocampus has been revealed to be independent of the admission of calcium into synaptosomes via voltage-sensitive calcium channels. Nevertheless, based on the operation of GABA transporters, it has been observed that BDNF acts as a result of transport reversal in the release pool (Canas *et al*., 2004). Accordingly, BDNF has been shown to inhibit the transport of GABA into synaptosomes (Vaz *et al*., 2008). Furthermore, Brünig *et al*. (2001) demonstrated a quick downregulation by BDNF of GABAA receptor surface expression levels. These results may interpret the significant decrease in whole brain BDNF according to its relationship with decreasing GABA levels in CZP-treated rats compared with the control group. On the other hand, while Chrysin decreased GABA levels in the three brain regions, it increased the whole brain BDNF level, which is confirmed by Sathiavelu *et al*. (2009) that Chrysin may confer a defensive effect by diminishing free radical production. These scientists also recorded that the fifth and seventh positions of the hydroxyl group can contribute to its strong antioxidant impact. Also, Souza

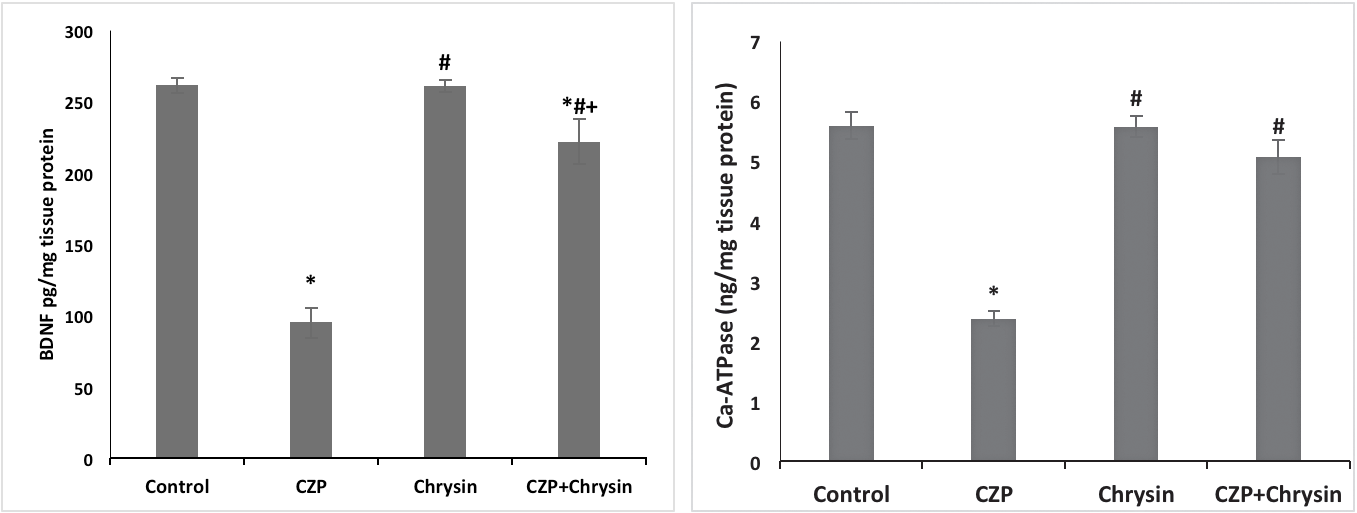

Figure 1. (a): The effect of CZP and chrysin on BDNF (pg/mg tissue protein) in whole brain. Values expressed as mean ± SEM of 10 rats/group. * = significant change from control group, # = significant change from CZP-treated group, and + = significant change from chrysin-treated group at $p < 0.05$. (b): The effect of CZP and chrysin on Ca-ATPase (ng/mg tissue protein) in whole brain. Values expressed as mean ± SEM of 10 rats/group. * = significant change from control group and # = significant change from CZP-treated group at $p < 0.05$.

et al. (2015) proved that the decline in cortical and hippocampal BDNF levels in aged mice was mitigated by Chrysin; these findings showed that the antioxidant compound flavonoid Chrysin was possibly able to inhibit age-related memory deficit through its free radical scavenger activity and BDNF output modulation. Thus, Chrysin may demonstrate a novel pharmacological strategy, performing as an anti-aging agent. Filho *et al*. (2015) reported that treatment with Chrysin caused upregulation of BDNF levels in the hippocampus of female mice. In the other work, they proposed that Chrysin's antidepressant effect might be accompanied by the upregulation of this neurotrophin in the structures of the brain (Filho *et al*., 2016b). Also, Xu *et al*. (2023) suggested that Chrysin restrains the neuroinflammation throughout the signaling pathways of BDNF. Additionally, the flavonoid Chrysin close to the antidepressant fluoxetine increases 5-HT levels and decreases both indoleamine-2,3-dioxygenase and caspases 3 and 9 activities in the prefrontal cortex and hippocampus of mice accompanied by the antidepressant-like effect detected in the cortex and hippocampus of mice that were related to the antidepressant-like effect which was noticed in the suspension test (Filho *et al*., 2016a) with the participation of BDNF.

Mata *et al*. (2011) realized that Ca^{2+}-ATPases existent in the plasma membrane are a strongly expressed brain multi-isoform family of proteins that are involved in maintaining low intraneural Ca^{2+} concentration. The increased concentration of monoamines in brain areas thus represents the increased concentration of intracellular Ca^{2+} and decreased intracellular Ca^{2+} ATPase, which was supported by our CZP-treated rats' results related to the control rats. In addition, Ca^{2+} homeostasis changes are related to neurodegenerative diseases and brain aging (Jiang *et al*., 2012) associated with improved liberation of glutamate (Garside *et al*., 2009) and raised remaining calcium inside the presynaptic end associated with removal of protein misfolding cyclic amplification feature (Jensen *et al*., 2007) which may reflect increasing monoamines in our CZP results. BZPs have been identified as calmodulin inhibitors (Seckin *et al*., 2007) which are involved in Ca pump plasma membrane control (Vincenzi *et al*., 1980). The Ca^{2+}-ATPase inhibition may be due to the collaboration between CZP and calmodulin. Evidence in support of the contribution of intracellular Ca^{2+} concentration changed homeostasis in several events leading to neural cell apoptosis was given in the same pathological settings (Annunziato *et al*., 2003).

Co-administration of Chrysin with CZP showed improvement in Ca-ATPase content, where Chrysin seems to preserve the components of the redox mechanism at close-to-normal standards and avoid the unusual expression of certain apoptotic cascade proteins, thereby avoiding calcium accumulation and cell death (Sundararajan *et al*., 2016). Furthermore, Chrysin can degrade intracellular Ca^{2+} levels by controlling the consumption of Ca^{2+} extracellular intake throughout calcium voltage-operated channels and obstructing the intracellular discharge of Ca^{2+} from the sarcoplasmic reticulum (Tew *et al*., 2023). Moreover, Ontiveros *et al*. (2019) proved that flavonoids modify intracellular Ca^{2+} homeostasis associated with mitochondrial Ca^{2+} channel and Ca^{2+} pump activity alterations. These results suggest the presence of the plasma membrane Ca^{2+}-ATPase, as it actively transfers Ca^{2+} to the extracellular medium attached to ATP hydrolysis, thereby preserving ion cell homeostasis. The main calcium-regulating proteins involved in the calcium homeostasis equilibrium are Ca^{2+}-ATPases, which are affected by oxidative/nitrosative stress and associated disorders or aging. Flavonoids not only work as antioxidants but can also bind directly to Ca^{2+}-ATPases, thus altering their conformation and leading to enzyme activity modulation (Horáková, 2011).

Behavioral tests

Y maze test

The data depicted in Figure 2a revealed a notable upsurge in the number of arm entries/5 minutes and a decrease in correct alternation in the rats treated with CZP as compared to the control after 6 weeks, whereas Chrysin usage induced a reduction in the number of arm entries/5 minutes and a raise in the correct alternation in comparison to CZP-treated group at $p < 0.05$. The rats treated with CZP + Chrysin exhibited a marked increase in the number of arm entries/5 minutes related to the control group. Furthermore, the correct alternation in CZP + Chrysin-treated rats

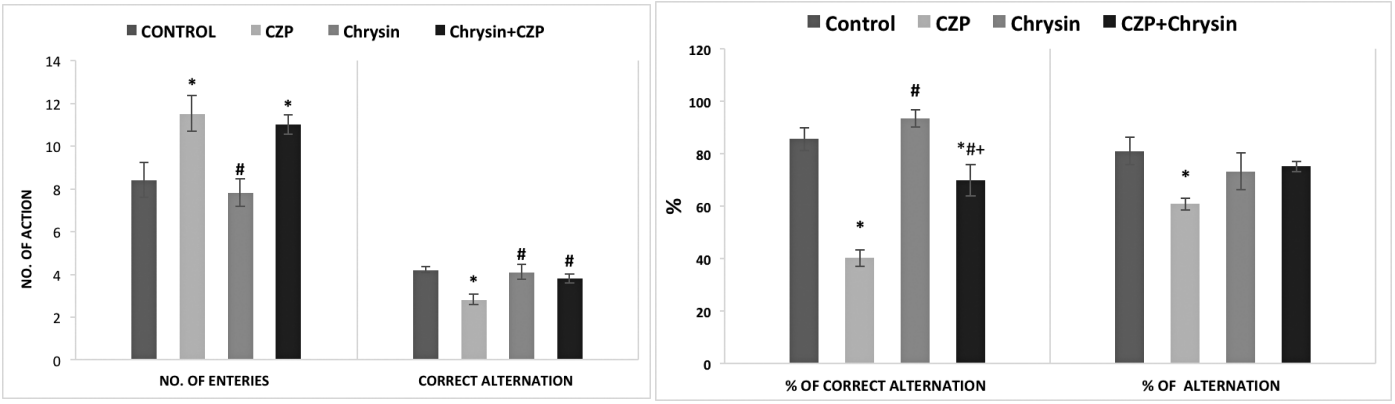

Figure 2. (a): The effect of CZP and chrysin on Y maze (the number of entries and correct alternations/5 minutes) after 6 weeks of treatment. Values expressed as mean ± SEM of 10 rats/group. * = significant change from control group, # = significant change from CZP-treated group at $p < 0.05$. (b): The effect of CZP and chrysin on Y maze (percentage of alternations and correct alternations) after 6 weeks of treatment. Values expressed as mean ± SEM of 10 rats/group. * = significant change from control group, # = significant change from CZP-treated group, and + = significant change from chrysin-treated group at $p < 0.05$.

demonstrated a significant increase as compared to the CZP group after treatment at $p < 0.05$. The data in Figure 2b for the Y maze revealed a decrease in percent alternation and percent of correct alternation in the CZP group when compared to the control after treatment at $p < 0.05$. Although a notable increase was shown in the percent alternation in CZP + Chrysin-treated rats, there was a significant increase in the percent of correct alternation in rats that received Chrysin and CZP + Chrysin in comparison to those which received CZP only. Moreover, the CZP + Chrysin-treated group exhibited a marked decrease in the percent of correct alternation when compared to the Chrysin group ($p < 0.05$). The Y maze test is the general operation of locomotors, short-term memory, and stereotypic actions (Roghani *et al*., 2006).

The data clarified by Mikulecká *et al*. (2014) suggested that chronic administration of CZP contributes to the development of motor action tolerance which is associated with disorganization of the GABA-A receptor, and the stopping of CZP leads to behavior disorder containing augmented activity related to the GABA-A receptor upregulation. CZP thus tends to be comparable to many other BZP in behavioral and neurochemical effects. Additionally, CZP is related to enhanced motor activity and upregulation of receptors after discontinuation (Lopez *et al*., 1990). The decrease in the correct alternation and percent of correct alternation in rats treated with CZP was confirmed by Crowe and Stranks (2018). They discovered that the highest deficiencies were detected in the operational memory regions, speed processing, attention division, vasoconstriction, new memory, and meaningful language not only for patients who used BZP for long periods but also for those who used CZP as a member of the drug class (Sussman, 2006). These results are largely supportive of the studies reported after the previous meta-analysis (Helmes and Østbye, 2015; Pomara *et al*., 2015). Moreover, the same authors found that BZP withdrawal from long-term use was still expressively reduced in all cognition regions except in the field of executive function. Thinking/memory impairment is considered the main side effect of CZP treatment. The way by which CZP caused impairment of memory remains unclear. The possible way may be through reducing brain levels of acetylcholine (Kalachnik *et al*., 2003). Also, Jisham *et al*. (2015) found that CZP administration exhibited significant memory deficiency in mice assessed by Morris Water Maze. On the other

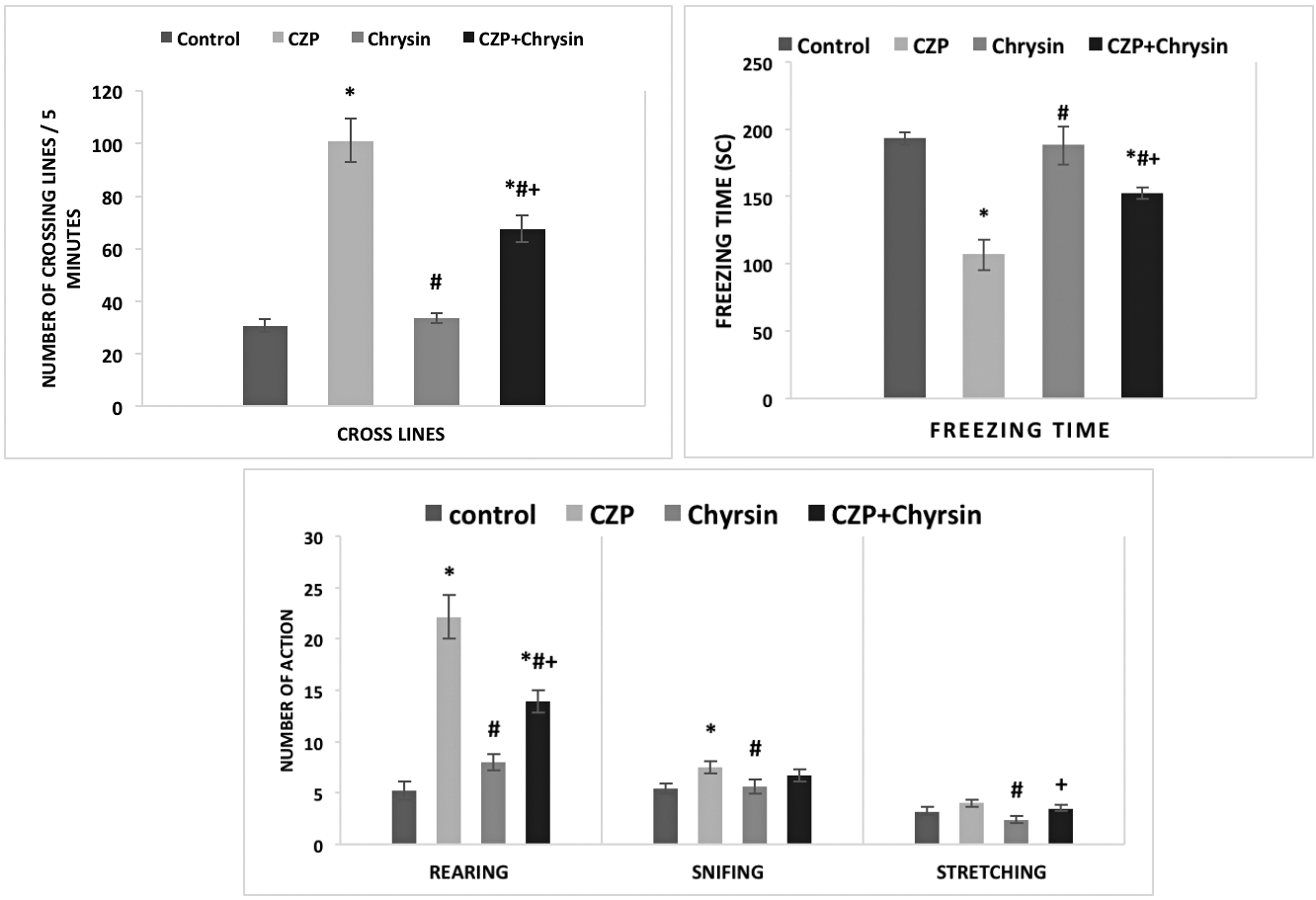

Figure 3. (a): The effect of CZP and chrysin on open field (number of crossing lines movement/5 minutes) after 6 weeks of treatment. Values expressed as mean ± SEM of 10 rats/group. * = significant change from control group, # = significant change from CZP-treated group, and + = significant change from chrysin-treated group at $p < 0.05$. (b): The effect of CZP and chrysin on open field (freezing time (sc)/5 minutes) after 6 weeks of treatment. Values expressed as mean ± SEM of 10 rats/group. * = significant change from control group, # = significant change from CZP-treated group, and + = significant change from chrysin-treated group at $p < 0.05$. (c): The effect of CZP and chrysin on open field (rearing, sniffing, and stretching/5 minutes) after 6 weeks of treatment. Values expressed as mean ± SEM of 10 rats/group. * = significant change from control group, # = significant change from CZP-treated group, and + = significant change from chrysin-treated group at $p < 0.05$.

hand, there was an increase in correct alternation and percent of correct alternation in the Chrysin and CZP + Chrysin groups in comparison with the CZP group. These were discussed by Li *et al*. (2014) who proved that Chrysin improves learning and memory functions. Moreover, Bortolotto *et al*. (2020) have proposed that Chrysin attenuates memory deficiencies by modulating the activities of both glutamate and sodium-potassium ATPase.

Open field test

The data demonstrated in Figure 3a and b showed at 6 weeks of treatment, a marked upsurge at $p < 0.05$ in the number of crossing lines as well as a reduction in freezing time in animals treated with both CZP and CZP + Chrysin when compared with the control. However, Chrysin- and CZP + Chrysin-treated groups exhibited a significant decline in the number of crossing lines and an increase in the freezing time in comparison with the CZP group at $p < 0.05$. Figure 3c showed that the CZP rats exhibited a significant upsurge in the number of rearing and sniffing times in comparison with control rats at $p < 0.05$, whereas the number of rearing and stretching times decreased significantly in the Chrysin-treated group in comparison to the CZP-treated rats at $p < 0.05$. In addition, the Chrysin rats decreased the number of sniffing times significantly compared to the CZP-treated group. Moreover, the rats treated with CZP + Chrysin proved a significant enhancement in the number of rearing and stretching times compared to the Chrysin rats at $p < 0.05$. The open field is a very common animal model of anxiety-like behavior and locomotion (Yang *et al*., 2013). A rise in the movement crossing lines, rearing, sniffing, stretching, and freezing time parameters is seen as a primary index of an anxiolytic-like impact (Germán-Ponciano *et al*., 2020). CZP treatment increased the number of rearing and sniffing times in open field tests which is confirmed by the study of Mikulecká *et al*. (2014) who discovered that higher locomotion was shown by the animals treated with CZP, which stayed more time in the central square of the open field, indicating decreased anxiety, and had impaired intersession habituations. But the administration of Chrysin with CZP showed an improvement in all these factors when compared with CZP treatment. Germán-Ponciano *et al*. (2020) indicated that Chrysin flavonoid therapy induced anxiolytic and protective effects against behavioral alterations triggered by a stressful condition and that Chrysin's effects were partly correlated with cellular variations in the lateral septal nucleus. The behavioral stimulation observed with stimulant exposure can be mediated by different neural pathways. Dopamine is one potential pathway. Many brain regions that receive dopaminergic feedback, such as the dorsal striatum stimulants, increase extracellular dopamine, which in turn stimulates behavior (Anderson, 2014) and is conformed to our monoamines results.

CONCLUSION

This study concluded that Chrysin ameliorated neural behavior by adjusting the neurotransmitters and amino acids. Chrysin modulated the inhibition of BDNF and Ca-ATPase levels in the whole brain, modulated the neurotransmitters and their metabolites, and prevented DNA degradation in the cerebral cortex, hippocampus, and striatum induced with CZP. So, the co-administration of Chrysin with CZP creates pharmacological tolerance, which should be considered to validate or discard this possibility in potential experimental protocols.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

ETHICAL APPROVALS

The animal study's research protocol was approved by the Institutional Animal Care and Use Committee, Ain Shams University, and NODCAR (approval number NODCAR/11/22/19).

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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