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Ashwagandha enhances behavior and brain neurotransmitters in Tramadol treated and withdrawal rats.

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Background
Although tramadol (TRE) was designed to be a safer alternative to more potent prescription analgesics, TRE abuse raises the risk of its toxic consequences on the brain.
Objective
The present study aims to reveal the defensive outcome of *Withania somnifera,* (Ashwagandha, ASH), an adaptogen plant with antioxidant performance, on the TRE misuse side-effects in the cortex and brainstem neurotransmitters and behavior and hippocampus cytokines.
Materials and Methods
The rats were divided into two main groups. The treatment group (A) was divided into control, ASH (200 mg/kg), TRE (40 mg/kg) and TRE+ASH treated with the same doses for six weeks. The withdrawal group (B) treated for ten weeks was divided into control, ASH was treated with vehicle for six weeks before being treated with 200 mg/kg for the next four weeks. TRE was treated by successive doses every two weeks (40, 80, 120 mg/kg) for the first six weeks before being treated by vehicle for the next four weeks, and TRE+ASH received the same ASH and TRE doses as the ASH and TRE groups. All the groups were treated daily p.o. The open-field and Y mazes were performed before the end of the experiment. After decapitation, the cortex and brainstem AChE activity were detected calorimetrically, and neurotransmitters were detected by HPLC technique. Also, hippocampus TNF-α and IL-1β contents were assessed by IHC.
Results and conclusion
The TRE+ASH groups showed a significant improvement in open fields and Y maze tasks after the adverse effects of TRE treatment. Also, an improvement in the cortex and brainstem neurotransmitter contents (glutamate, aspartate, gamma amino butyric acid, glycine, norepinephrine, dopamine, serotonin, and β-endorphin) in TRE+ASH groups compared with the TRE groups. Furthermore, ASH reduced the elevated acetylcholinesterase and hippocampal TNF-α and IL-1β contents that were elevated in TRE treated groups. In conclusion, ASH has a valuable neurotherapeutic impact through the modulation of different neurotransmitters and suppression of proinflammatory cytokines, which is reflected on the behavior of the treated rats.

Keywords: Brain, neurotransmitters, behavior, proinflammatory cytokines.

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Introduction

Tramadol (TRE) is a synthesized drug that typically belongs to the familiar opioid painkillers like codeine. TRE causes pain control through serotonin (5-HT) and norepinephrine (NE) reuptake inhibition (SNIR). Also, the O-demethyltramadol metabolite together with TRE stimulates the μ-opioid receptor [1,2]. TRE is approximately 10% as potent as morphine [3]. Long-term use of TRE is linked with several neurodegenerative conditionssuch as neuroinflammation, 5-HT and NE dysregulation and seizure risk. Although TRE was designed to be a safer alternative to more potent prescription analgesics, TRE addiction is still an issue. As an individual's tolerance to TRE increases, the need to increase the dosage also rises [4]. Due to the high frequency of TRE-associated overdose and death, it has been classified as a prohibited material in numerous countries [5]. Long-term TRE abuse can extremely change the brain's constitution and functions. Tramadol misuse

disrupts brain tissues involving the cortex, and TRE abuse signs are produced by neuro-deterioration of prefrontal cortex [6].

Withania somnifera - Ashwagandha (ASH) name comes from the Sanskrit language. It means "the horse smell". It is a small plant classified under the family *Solanaceae*. Ashwagandha has been used as Ayurvedic in the last 3000 years in India for therapy of several neural illnesses such as memory and cognitive function, anxiety, Parkinson's disease, and sleep disorders [7]. Ashwagandha comprises over 35 substances such as flavonoids, alkaloids, steroidal lactones, saponins and glycosides [8]. The capability of ASH to modulate the neurotransmitters in the brain resulted in reducing anxiety and stress, improving sleep quality, and improving mood. It also has immunomodulatory, anti-inflammatory, antioxidant, anti-cancer, antibacterial, anti-diabetic, aphrodisiac, and anti-neurodegenerative properties [9].

The current work aims to explore the ameliorative role of ASH against TRE-treated (A) and withdrawal (B) changes on the cognitive functions of the brain through investigation of the behavior tasks (open field test and Y maze) and the physiological activities of the cortex and brainstem neurotransmitters and acetylcholinesterase. Also, the hippocampus cytokines (TNF-α and IL-1β) were evaluated.

Material and methods

Experimental animals

This study was conducted using 80 Wistar strain adult rats. They weighed 150 g were bought from "Your Research Animal Facility (URAF)" animal house. The rats were housed in URAF in groups in visible standard cages including rice strew wood bedding material. The animal house has a reversed 12 h light/dark cycle, temperature (25±2 C^{O}) and 50% humidity. The rats were fed standard rat chow and water *ad libitum*.

Drugs

Tramadol hydrochloride (TRE) was bought from Memphis company for pharmaceuticals and chemical industries under the supervision of the Egyptian Drug Authority (EDA). *Withania somnifera, (*Ashwagandha, *WS,* ASH) was bought from NOW FOODS, as tablets (450mg) from dry powdered root and leaf (2.5% total withanoides-11 mg). Carboxymethylcellulose (CMC) was provided by Sigma Aldrich as a white powder, CMC (code: 21902 medium viscosity) prepared by adding 5 g CMC to 1000 ml of distilled water. All HPLC standards were purchased from Sigma-Aldrich Chemi GmBH with highest purity as follows: NE, assay ≥ 98% (A5785-250MG); dopamine-HCl, assay ≥ 98% (H8502-5g); 5-HT-HCl, assay ≥ 98% (H9523-250MG); GABA, assay ≥ 98%) A5835-10MG); β- endorphin, assay ≥ 98% HPLC, 61K49581(E 1142-250UG) ; ASP, assay ≥98%(MFCD00002724); GLU, assay ≥98% (MFCD00144405) and GLY, assay ≥ 98%(MFCD00036223).

Experimental design

Rats were divided into 2 groups and left for 5 days for adaptation before the beginning of experiments. The first group (A) (treatment group) was divided into four subgroups (n=10) treated orally every day for 6 weeks as follows:

1- Control: received (CMC, 0.5 % as 0.5 ml/100 g bw).
2- ASH (Ashwagandha): Administrated ashwagandha (200 mg /kg/day) [10]**.**
3- TRE (Tramadol): Administrated TRE (40 mg/kg/day) at a dose equivalent to the largest human therapeutic dose [11]**.**
4- TRE +ASH: Administrated Tramadol (40 mg/kg/day) first then after 1 hour they received ashwagandha (200 mg /kg/day).

The second group (B) (withdrawal group) was divided into four subgroups (n=10) and was treated orally every day for ten weeks as follows:

1- Control: Administrated (CMC, 0.5 % as 0.5 ml/100 g bw).
2- ASH: Administrated CMC for 6 weeks followed by ASH (200 mg/kg) for 4 weeks.
3- TRE withdrawal subgroup: Administrated TRE for 6 weeks (with ascending doses every two weeks in order of (40-80-120 mg/kg/day) [12]**,** followed by CMC for the rest 4 weeks.
4- TRE+ASH: Administrated TRE successive doses as subgroup 3 for 6 weeks followed by ASH as subgroup (2) for the rest of 4 weeks.

At the end of each experiment, the animals in each group were quickly decapitated and their brains were collected for investigation. Cortex and brainstem tissues were extracted from each rat brain, and cortex and the brainstem tissues were homogenized in phosphate buffer saline (PBS; 7.2 PH) as (10% w/v), then centrifuged and the supernatant of homogenates served at -20 to be used for the neurotransmitter's estimations, (n=6) and acetylcholinesterase (AChE). The other four brains were preserved in 10% neutral formalin and embedded in paraffin for immunohistochemical investigation.

Behavioral study

The behavior tests were performed after 6 consecutive weeks of daily oral treatment for group A, and after 10 consecutive weeks for group B. Open field and Y-maze were supplied from (URAF). In between training, the mazes were cleaned with 70% alcohol.

The open field test (OFT)

The OFT was sustained to assess the exploration, anxiety, and movement activities of the considered groups**.** A wood square with dimensions

(100×100×40cm), within the floor split into (20cm×20cm) square grids. It was used in a quiet condition. Each rat was examined for 5 minutes by placing it on the square bottom in the central quadrangular. The locomotive exploration, and anxiety activities of rats were recorded by camera. The number of elongations, rearing number (number of hind limbs standing with forelimbs off the ground), self-grooming number and the number of crossing lines were counted, as well as freezing time, and movement time[13].

The Y-maze

The Y-maze is one of the simplest mazes and has been applied broadly in learning and memory assessments for rat models**.** It includes three identical wood branches, specified A, B, and C. The angle between the branches is 120, and the dimensions of each branch are 40 x 15 x 30 cm. The labyrinth was manipulated to assess spontaneous alterations. Each rat was put in one of the three sections (A-arm) and offered 3 min to liberally move among them. The percentage of alternating arms, and the number of arms entered are recorded for each rat. The maximum number of spontaneous alternations is calculated by subtracting two from the total number of arms entered, and the correct alternation with successive arms entries on the triplet overlay (i.e. ABC, CBA, BAC) is calculated using the percentage of alternation calculated in (actual alternations/maximum alternations) X100 and the percentage of correct alternative calculation. The entry to the branch is recorded when the 4 limbs of the rat are inside it [14].

Determination of neurotransmitters by High Performance Liquid Chromatography (HPLC) analysis

HPLC conditions: AGILENT, 2000, quaternary pump; a column oven, a Rheodine injector and 20µl loop and a UV variable wavelength detector.

An AQUA column 150x4.6 mm 5µ C18, bought from Phenomenex, USA, mobile phase 97/3 20Mm potassium phosphate/methanol, pH 3.0, flow rate 1.5ml/min and UV 270 nm for determination of the cortex and brainstem monoamine neurotransmitters. NE, DA, and 5-HT were split after 10 minutes [15]**.** Determination of the cortex and brainstem amino acids (glutamate (GLU), aspartate (ASP), glycine (GLY) and γ-amino butyric acid (GABA)) concentrations by HPLC was recorded by the following technique. The four amino acids were detected by HPLC [16]. PICO- TAG column (Waters) for free-amino acid analysis 3.9 ´, 30 cm.; Eluent (1) and Eluent (2). Phenylisothiocyanate (PITC), Triethylamine, wavelength 254 nm; flow rate: 1ml/min.

The cortex and brainstem β-endorphin (β-END) were detected by HPLC on a Vydac C4 column) waters USA), using a linear gradient from 0.1% trifluoroacetic acid in 95% water/5% acetonitrile to 0.1% trifluoroacetic acid in 50% water/50% acetonitrile. Peptide was detected at 214 nm [17].

Biochemical investigations

Acetylcholinesterase (AChE) activity is measured with the modified *Ellman assay* [18]**.** The AChE reacted with an acetyl-thiocholine at 37^{o}C for (10 minutes), the reaction was stopped with a dithiobis nitrobenzoic acid (DTNB) reagent. The free thiol group of thiocholine reacted with DTNB for developing the yellow color. The absorbance of the yellow color was measured at 412 nm as µmol of thiocholine / g/ minute using Humalyzer Junior spectrophotometer.

Immunohistochemical investigations

Sections (5 mm) from brains were sliced from the typical paraffin block. Primary antibodies tumor necrosis factor-alpha (TNF- α, Rabbit pAb, ABclonal Technology (Wuhan China), Catalog No- A0277) and interleukin-1beta (IL-1β, Rabbit pAb, ABclonal Technology Co. Catalog No- A16288) at a concentration of 1:150 in phosphate buffer saline (PBS) the slides were inserted and kept at 4 °C for 24 hrs., after the washing with PBS. Applied 3,3-N-Diaminobenzidine Tetrahydrochloride solution (DBA), immunohistochemistry DBA developing Color Kit was put into the antibody investigated slides, then the slides incubated for 15 min afterward washed in water. Next, sections were inserted into the hematoxylin dye stain for 10 min. Then dehydration assessed by alcohol was used and Canda balsam was added to the sections. The slides were examined at 400X by an Olympus BX43 microscope connected to a camera [19]. Six non-overlapping fields were picked at random and examined from hippocampus tissue to assess the expression of TNF-α and IL-1β as the brown color density. The colorimetric analysis of images as the density of the brown color was found by Trigit program [20].

Statistical analysis

The data were analyzed using version (23) of the statistical package for social sciences IBM SPSS software package. For normally distributed data, a comparison of more than two populations must be performed (ANOVA), with LSD as a Post Hoc test. At the 5% significance level, the derived results were considered significant. The data presented as means ± standard error. Percentage of TRE group values change from their corresponding control (TRE %) = ((TRE-control)/control)*100. The percentage of TRE+ASH values change from the

TRE corresponding group values (TRE+ASH%) =((TRE+ASH)-TRE)/TRE)*100.

Results

Neurotransmitters

The cortex GLU, and ASP contents were significantly higher in TRE treated group (A) by 45.16%, and 29.41%, respectively, than in the control. Also, an alleviation in cortex GABA, GLY, NE, DA, 5-HT, and β-END contents in TRE group (A) by -26.95%, -26.67%, -30.23%, -28.89%, -28.57%, and -30%, respectively when compared with the control-treated group as shown in Table 1. Whereas, the TRE group (B) showed a marked increase in cortex GLU, and ASP contents by 34.29%, and 28.57%, compared with the corresponding control. There was a significant decline in cortex GLY, GABA, NE, DA, 5-HT, and β-END by -30.96%, -25%, -17.5%, -27.27%, -23.08%, and -30%, respectively in comparison with the corresponding control. Moreover, the percentage change in TRE+ASH treated group (A) from TRE group (A) was -20%, -20.45%, 22.67%, 22.73%, -16.67%, 15.63%, 5%, and 7.14% in cortex GLU, ASP, GABA, GLY, NE, DA, 5-HT, and β-END contents, respectively. Whereas the percentage change in TRE+ASH withdrawal group (B) from TRE group (B) was -6.38%, -6.89%, 16.59%, 16.67%, 3.3%, 12.5%, 5%, and 14.29% in cortex GLU, ASP, GABA, GLY, NE, DA, 5-HT, and β-END contents, respectively.

Table 1 The cortex neurotransmitters (µg/g) in treated (A) and withdrawal (B) groups.

	group	Control	ASH	TRE	TRE+ASH	TRE%	TRE+ASH%
GLU	**A**	0.31±0.01	0.31±0.004	0.45±0.01^{ab}	0.36±0.01^{abc}	45.16%	-20%
	B	0.35±0.02	0.30±0.003	0.47±0.03^{ab}	0.44±^{ab*}	34.29%	-6.38%
ASP	**A**	0.34±0.01	0.31±0.01	0.44±0.003^{ab}	0.35±0.01^{c}	29.41%	-20.45%
	B	0.35±0.02	0.33±0.004	0.45±0.023^{ab}	0.41±0.03^{ab*}	28.57%	-8.89%
GABA	**A**	3.08±0.13	3.23±0.10	2.25±0.07^{ab}	2.76±0.05^{abc}	-26.95%	22.67%
	B	3.23±0.10	3.31±0.10	2.23±0.13^{ab}	2.60±0.15^{abc}	-30.96%	16.59%
GLY	**A**	0.30±0.002	0.28±0.003	0.22±0.02^{ab}	0.27±0.01^{ac}	-26.67%	22.73%
	B	0.32±0.004	0.31±0.002	0.24±0.002^{ab}	0.28±0.01^{abc}	-25%	16.67%
NE	**A**	0.43±0.0070	0.41±0.0065	0.30±0.009^{ab}	0.35±0.007^{abc}	-30.23%	16.67%
	B	0.40±0.002	0.40±0.011	0.33±0.006^{ab*}	0.34±0.012^{ab}	-17.5%	3.03%
DA	**A**	0.45±0.09	0.46±0.01	0.32±0.01^{ab}	0.37±0.01^{abc}	-28.89%	15.63%
	B	0.44±0.01	0.45±0.02	0.32±0.01^{ab}	0.36±0.01^{abc}	-27.27%	12.5%
5-HT	**A**	0.28±0.01	0.27±0.01	0.20±0.1^{ab}	0.21±0.003^{ab}	-28.57%	5%
	B	0.26±0.08	0.27±0.004	0.20±0.004^{ab}	0.21±0.01^{ab}	-23.08%	5%
β -END	**A**	0.20±0.01	0.21±0.01	0.14±0.004^{ab}	0.15±0.003^{ab}	-30%	7.14%
	B	0.20±0.18	0.20±0.01	0.14±0.004^{ab}	0.16±0.003^{ab}	-30%	14.29%

Glutamate (GLU), Aspartate (ASP), Gamma amino butyric acid (GABA), Glycine (GLY), Norepinephrine (NE), Dopamine (DA), Serotonin (5-HT), and Beta-endorphin (β -END) contents in treatment and withdrawal groups. Values are means± SE (n=6), ASH (Ashwagandha), TRE (tramadol), a=significant from control, b=significant from ASH, and c= significant from TRE, *=significant from the same treatment group at p≤0.05 TRE %=percentage of TRE group values change from their corresponding control. TRE+ASH% = percentage of TRE+ASH values change from the TRE corresponding group values.

Data presented in Table 2 revealed that the brainstem GLU, and ASP contents percentage were elevated in TRE treated group (A) by 62.07%, and 120%, respectively related to the control accompanied by alleviation in brainstem GLY, GABA, NE, DA, 5-HT, and β-END contents percentage by -37.26%, -31.25%, -29.27%, -24.44%, -24%, and -27.78%, respectively when compared with the control treated group. Also, The TRE group (B) showed a marked higher percentage in cortex GLU, and ASP contents by 32.35%, and 35.14% than the corresponding control. While there was a significant decline in cortex GLY, GABA, NE, DA, 5-HT, and β-END percentage by -35.4%, -37.14%, -25%, -26.67%, -26.92%, and -22.22%, respectively in comparison with the corresponding control. Moreover, the percentage change in TRE+ASH treated group (A) from TRE group (A) was -23.04%, -20.45%, 26.4%, 18.18%, 20.69%, 14.7%, 10.53%, and 15.38% in brainstem GLU, ASP, GABA, GLY, NE, DA, 5-HT, and β-END contents, respectively. Also, the percentage change in TRE+ASH withdrawal group (B) from TRE group (B) was 13.33%, -16%, 30.59%, 13.64%, 20%, 15.15%, 15.78%, and 14.29% in brainstem GLU, ASP, GABA, GLY, NE, DA, 5-HT, and β-END contents, respectively

Table 2 The brain stem neurotransmitters (µg/g) in treated (A) and withdrawal (B) groups

	Group	Control	ASH	TRE	TRE+ASH	TRE%	TRE+ASH%
GLU	**A**	0.29±0.02	0.32±0.013	0.47±0.023^{ab}	0.36±0.032^{ac}	62.07%	-23.40%
	B	0.34±0.01	0.31±0.01	0.45±0.01^{ab}	0.51±0.03^{abc*}	32.35%	13.33%
ASP	**A**	0.2±0.02	0.28±0.01	0.44±0.03^{ab}	0.35±0.01^{c}	120%	-20.45%
	B	0.37±0.04	0.32±0.02	0.50±0.02^{ab}	0.42±0.04^{bc}	35.14%	-16%
GABA	**A**	3.14±0.08	3.35±0.16	1.97±0.11^{ab}	2.49±0.03^{abc}	-37.26%	26.40%
	B	3.39±0.07	3.47±0.11	2.19±0.22^{ab}	2.86±0.24^{abc}	-35.40%	30.59%
GLY	**A**	0.32±0.005	0.32±0.008	0.22±0.010^{ab}	0.26±0.011^{abc}	-31.25%	18.18%
	B	0.35±0.007	0.28±0.003	0.22±0.12^{ab}	0.252±0.006^{abc}	-37.14%	13.64%
NE	**A**	0.41±0.01	0.40±0.004	0.29±0.009^{ab}	0.35±0.003^{abc}	-29.27%	20.69%
	B	0.40±0.01	0.41±0.007	0.30±0.007^{ab}	0.36±0.002^{abc}	-25%	20%
DA	**A**	0.45±0.14	0.44±0.01	0.34±0.003^{ab}	0.39±0.01^{abc}	-24.44%	14.7%
	B	0.45±0.13	0.44±0.14	0.33±0.01^{ab}	0.38±0.004^{abc}	-26.67%	15.15%
5-HT	**A**	0.25±0.01	0.26±0.01	0.19±0.02^{ab}	0.21±0.004^{abc}	-24%	10.53%
	B	0.26±0.01	0.25±0.004	0.19±0.01^{ab}	0.22±0.005^{ab}c	-26.92%	15.78%
β -END	**A**	0.18±0.002	0.19±0.004	0.13±0.004^{ab}	0.15±0.001^{abc}	-27.78%	15.38%
	B	0.18±0.003	0.19±0.07	0.14±0.004^{ab}	0.16±0.005^{abc}	-22.22%	14.29%

Glutamate (GLU), Aspartate (ASP), Gamma amino butyric acid (GABA), Glycine (GLY), Norepinephrine (NE), Dopamine (DA), Serotonin (5-HT), and Beta-endorphin (β-END) contents in treatment and withdrawal groups. Values are means± SE (n=6), ASH (Ashwagandha), TRE (tramadol), [a] significant from control, [b] significant from ASH, and [c] significant from TRE, *=significant from the same treatment group at p≤0.05. TRE %=percentage of TRE group values change from their corresponding control. TRE+ASH% = percentage of TRE+ASH values change from the TRE corresponding group values.

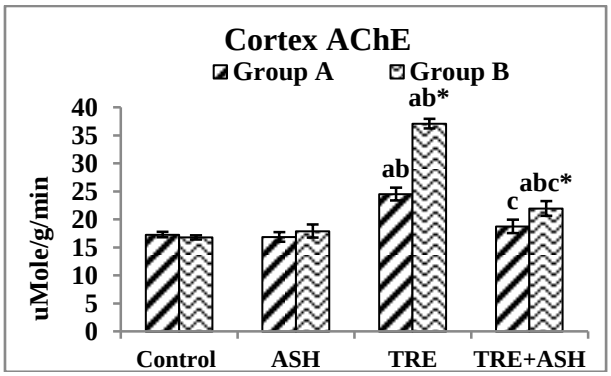
Acetylcholinesterase (AChE)

The percentage change in acetylcholinesterase (AChE) activities in the cortex increased significantly in TRE groups (A&B) by 41.85% and 120.82% respectively from the control groups (Fig. 1, A & B). The brainstem TRE groups showed a percentage increase in AChE activities by 36.07% in treated (A) and 68.56% in withdrawal (B) when compared to the corresponding control group. Whereas the TRE+ASH treated and withdrawal group showed a substantial lower percentage in cortex AChE activity by -23.43% and -40.84% than the TRE treated and withdrawal groups, respectively. Moreover, TRE+ASH (A&B) groups showed a percentage decline in brainstem AChE activities by -9.88% and -38.80% respectively than the corresponding TRE groups.

Proinflammatory cytokines

The hippocampus tumor necrosis factor-alpha (TNF-α) expressed by the color intensity was significantly increased when comparing the TRE-treated and withdrawal groups with the control and ASH corresponding groups at p≤0.05 as shown in Fig. 2. Whereas significant lower hippocampus TNF-α contents were noticed in TRE+ASH treated and withdrawal groups than in the TRE-treated and withdrawal groups.

The hippocampus interleukin-1 beta (IL-1β) contents expressed by the color intensity, was significantly increased in TRE-treated group (A) as compared to the control and ASH-corresponding groups (Fig. 3). Furthermore, the TRE+ASH treated group showed a substantial alleviation when compared with the TRE-corresponding groups at P≤0.05. Also, the TRE withdrawal group showed considerable higher hippocampus IL-1β contents than the ASH and control corresponding groups. Moreover, the TRE+ASH withdrawal group showed a noteworthy lower hippocampus IL-1β contents than the TRE group (B) at P≤0.05.



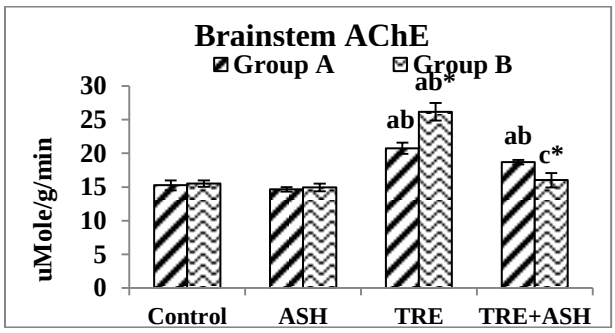
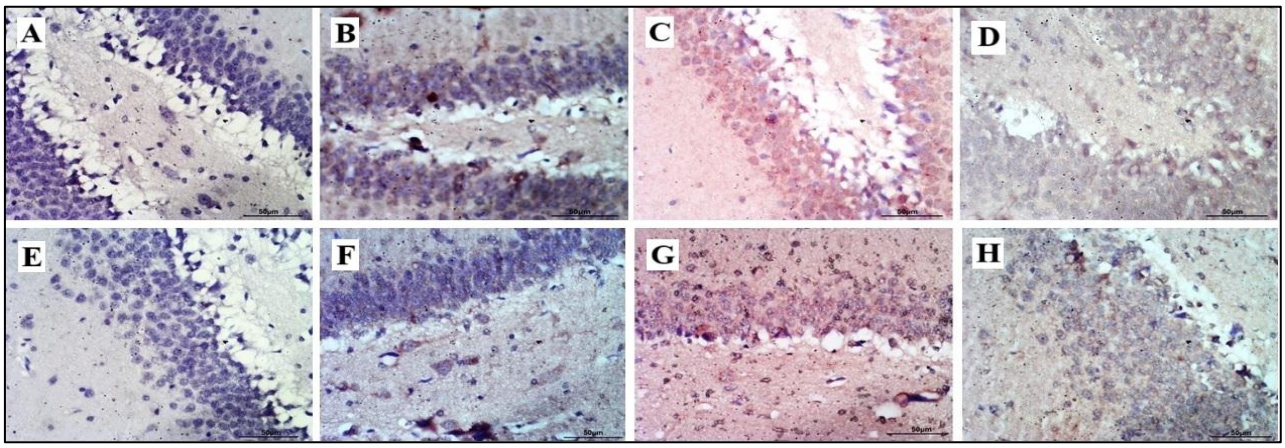


Fig. 1 The cortex (Fig. 1, A) and brainstem (Fig. 1, B) Acetylcholinesterase (AChE) activity in treatment and withdrawal groups values are means± SE (n=6), ASH (Ashwagandha), TRE (tramadol), [a] significant from control, [b] significant from ASH and [c] significant from TRE, and * significant from the same treatment group at p≤0.05



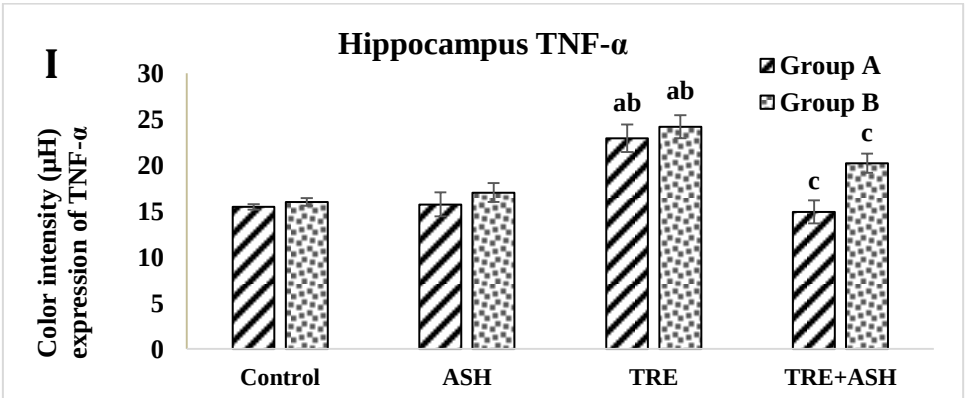


Fig. 2 The photomicrograph of the immunohistochemistry stain of hippocampus tissue TNF-α contents in control group (Fig. 2, A& E); Ashwagandha (ASH) groups (Fig. 2, B&F); Tramadol (TRE, A) group (Fig. 2, C); TRE+ASH, A group (Fig. 2, D); TRE, B group (Fig. 2, G) and TRE+ ASH, B group (Fig. 2, H). (Fig. 2, I): Histogram representing the density of brown color of cortex TNF-α contents of different groups Values are means ± SE, Superscript, [a] significant from control, [b] significant from ASH, and [c] significant from TRE, and [*] significant from the treated corresponding group (A) at p ≤0.05.

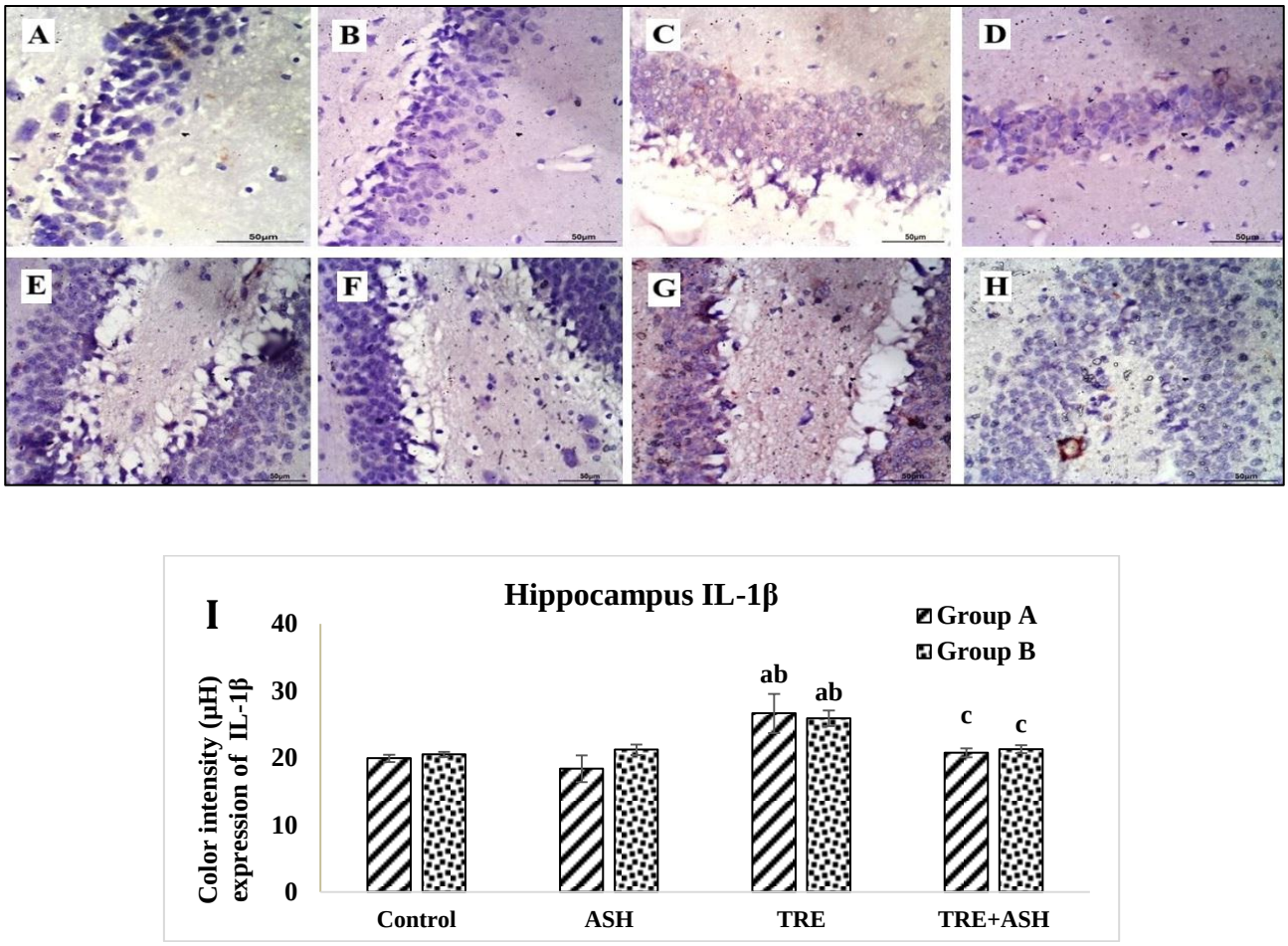


Fig. 3 The photomicrograph of the immunohistochemistry stain of hippocampus tissue IL- 1β contents in control group (Fig. 3, A& E); Ashwagandha (ASH) groups (Fig. 3, B&F); Tramadol (TRE, A) group (Fig. 3, C); TRE+ASH, A group (Fig. 3, D); TRE, B group (Fig., G) and TRE+ ASH, B group (Fig. 3, H). (Fig. 3, I): Histogram representing the density of brown color of cortex IL- 1β contents of different groups Values are means ± SE, Superscript, [a] significant from control, [b] significant from ASH, and [c] significant from TRE, and [*] significant from the treated corresponding group (A) at $p \leq 0.05$.

Behavior results

The number of crossing lines in the open field is depicted in Fig. (4, A). The TRE-treated and withdrawal groups showed a noteworthy lower number of crossing lines than the control by -19.3% and -33.5%, respectively, and ASH groups, but a significant decrease was noticed when comparing the TRE withdrawal group with the treated one. Whereas the TRE+ASH treated, and withdrawal groups showed a substantial elevation in the number of crossing lines when compared with TRE treated corresponding groups by 32.3% and 36%, respectively. While the TRE+ASH withdrawal group showed lower crossing lines than the control, and ASH corresponding groups.

The mobility time in the open field is shown in Fig. (4, B). The TRE-treated and withdrawal groups showed a substantial lower mobility time than the control by -31.8% and -26.8% respectively, or ASH corresponding groups at $p \leq 0.05$. Moreover, the TRE+ASH treated, and withdrawal group showed a considerablly higher mobility time than the TRE groups by 35.6% and 32.4%, respectively.

The ASH-treated and withdrawal groups illustrated a meaningful lower rearing number than the control group at $p \leq 0.05$ as shown in Fig. (4, C). Moreover, the TRE-treated group showed a substantially higher rearing than the control by 16% and ASH groups. But also, the TRE withdrawal group showed considerable alleviation related to control by -31%, ASH withdrawal and treated groups. While the TRE+ASH groups showed a significant increase when compared with ASH groups. The TRE+ASH withdrawal group illustrated a noteworthy elevation than the withdrawal groups of the control, ASH, and TRE by 85.3%, corresponding groups. Also, a significant increase was noticed when compared with the treated one.

The TRE-treated and withdrawal groups displayed a markedly higher grooming number than the control by 68.7% and 20%, respectively and ASH groups at $p \leq 0.05$. Also, the TRE withdrawal group revealed a lower grooming number than the TRE-treated one. Whereas the TRE+ASH treated group showed a substantial elevation related to the control and ASH groups. While TRE+ASH withdrawal group showed a

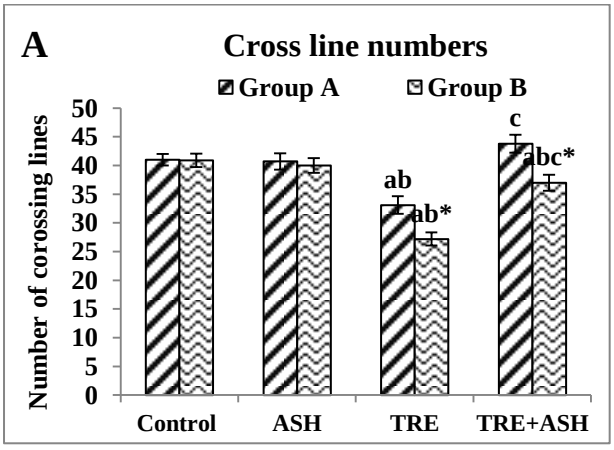
significant decrease when compared with TRE withdrawal group by -38.5% and the TRE-treated corresponding group as illustrated in Fig. (4, D).

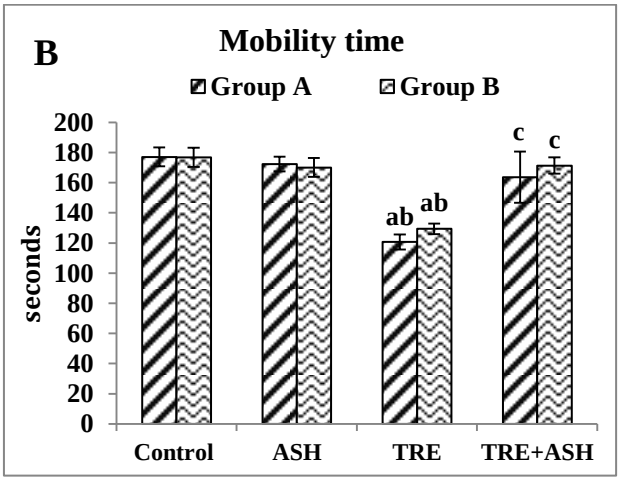
The immobility time in the open field showed a marked elevation in TRE-treated and withdrawal groups by 45.9% and 38.5%, respectively, related to the control corresponding groups at $p \leq 0.05$ as revealed in Fig. (4, E). Moreover, the TRE-treated group showed a meaningful higher immobility time than the ASH-treated group. While the TRE+ASH treated and withdrawal showed a lower immobility time by -24.0% and -24.6 %, respectively, than the TRE corresponding groups.

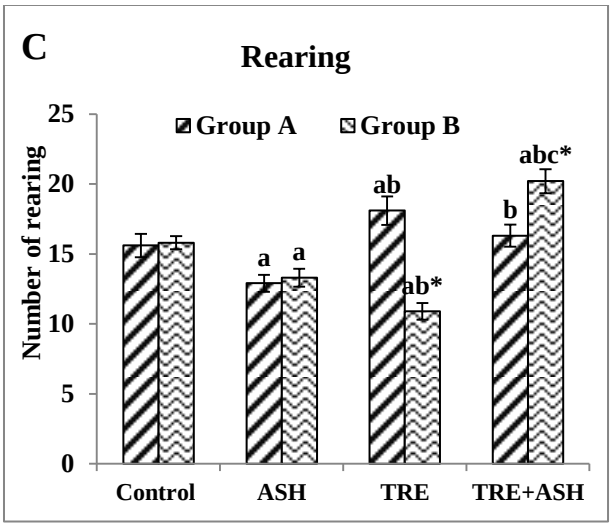
The number of entries in Y-Maze in TRE treated and withdrawal groups showed a substantial lower number of entries than the control by -45.6% and -49.5% respectively, and ASH corresponding groups at $p \leq 0.05$ (Fig. 5, A). Moreover, TRE+ASH treated, and withdrawal groups illustrated a meaningful lower number of entries than the control and ASH groups. However, a significant increase was noticed when compared with TRE groups by 55.4% and 46.9%, respectively. Moreover, the TRE+ASH withdrawal group showed a marked lower number of entries than the corresponding treated group.

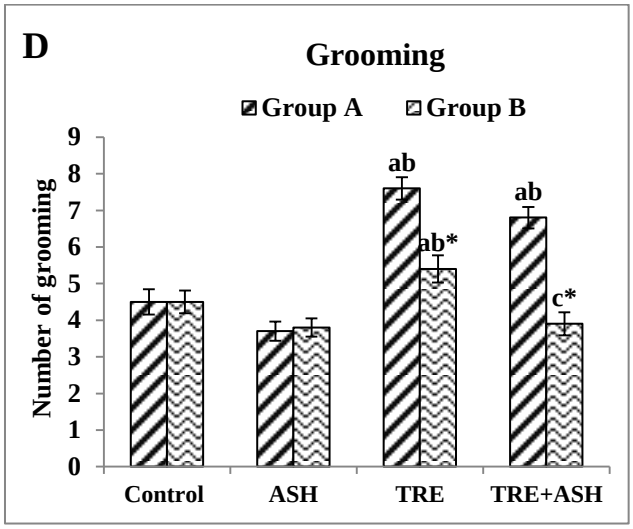
The percentage of alternation in the Y-maze is depicted in Fig. (5, B). The TRE groups showed a substantial alleviation in the percentage of alternation by -18.3% and -20.2% respectively, related to the control and ASH corresponding groups at $p \leq 0.05$. Moreover, TRE+ASH groups revealed a significantly higher percentage of alternation than the TRE corresponding groups by 13.3% and 19.4%, respectively.

The percentage of correct alternation in TRE treated, and withdrawal groups showed a meaningful incline in comparison with the control by -47.1% and -51% respectively and ASH corresponding groups at $p \leq 0.05$ as shown in Fig. (5, C). While the TRE+ASH groups revealed a substantially higher percentage of correct alternation than the TRE corresponding groups by 84.3% and 99%, respectively.









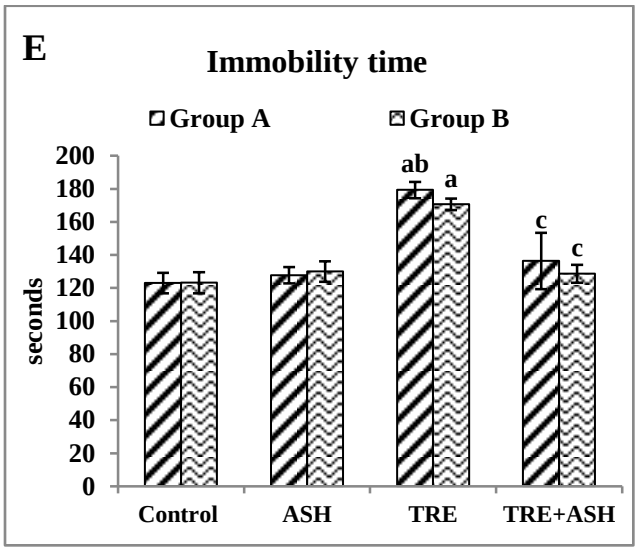
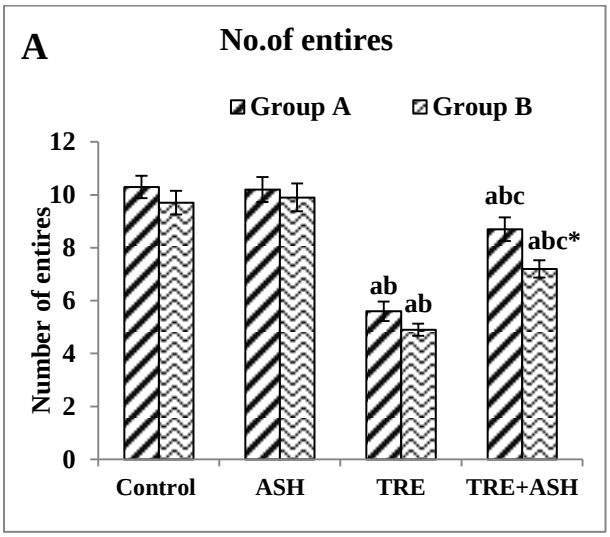
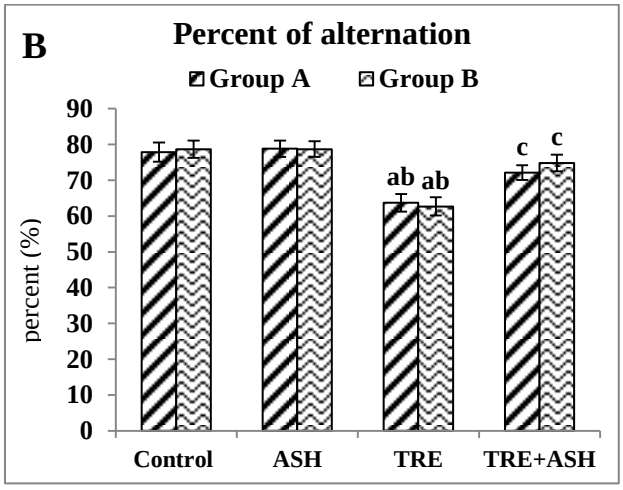


Fig. 4 Cross lines numbers (Fig. 4, A), mobility time (Fig. 4, B), rearing (Fig. 4, C), grooming numbers (Fig. 4, D) and immobility time (Fig. 4, E) in the open field of treated and withdrawal groups. Values are means± SE (n=10), ASH= (Ashwagandha), TRE (tramadol), [a] significant from control, [b] significant from ASH and [c] significant from TRE, and * significant from the same treatment group at $p \leq 0.05$.





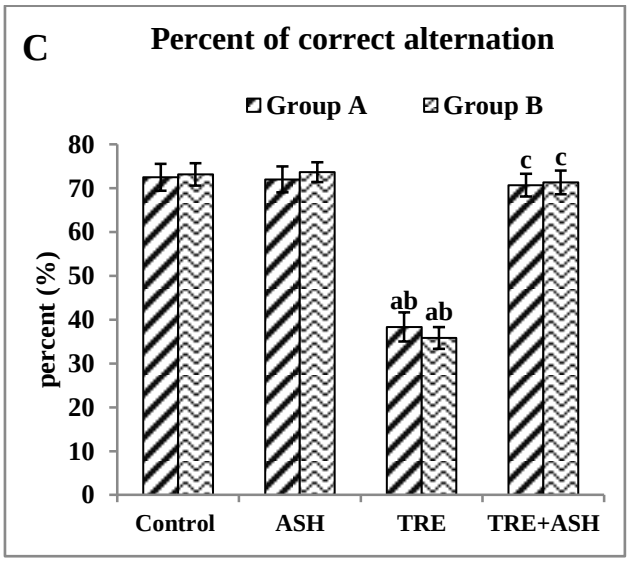


Fig. 5 Number of entries (Fig. 5, A), % alternation (Fig. 5, B) and % Correct alternation (Fig. 5, C) in Y-maze treated and withdrawal groups. Values are means± SE (n=10), ASH (Ashwagandha), TRE (tramadol), [a] significant from control, [b] significant from ASH and [c] significant from TRE, and * significant from the same treatment group at $p \leq 0.05$.

Discussion

The current study showed that cortex and brainstem glutamate (GLU) and aspartate levels significantly increased in TRE groups. In contrast, GABA and glycine contents significantly decreased compared with control and ASH groups. Earlier works revealed that opioids can damage the neurotransmitter balance as a result of elevated glutamate contents, while they have a repressive impact on GABA levels [22, 23].

Besides TRE owns NMDA antagonistic and GABA agonistic properties which explain the elevation of ASP [25]. TRE inhibits NMDA transmission which prohibits depression outcomes [24-26] and restrains the GABAA transmission at high concentrations [26]. Thus, the GABA and glycine (GLY) neurotransmitters are rapidly inhibitory in the mature brain and spinal cord. Moreover, GABA and GLY neurotransmitter inhibition affords the control of proper central pain and the similar presynaptic terminals are regularly co-released GLY and GABA [27].

On the other hand, long-term TRE abuse can result in oxidative damage, inflammation, and disruption of the GABA neurotransmitter system, which will help to elucidate the toxicology of TRE abuse. The changes in neurotransmitters at low and high doses reveal disruption of the GABAergic system (glutamine, succinate semialdehyde, and gamma-hydroxybutyric acid at high doses, and decreases of succinic semialdehyde low doses) [28].

In the present work, TRE significantly reduced NE, DA, 5-HT, and β-endorphins (β-END) in the cortex and brainstem. The decrease of 5-HT and DA refers to the ability of TRE to prevent 5-HT uptake (by binding to transporter hSERT) [29] and inhibit receptors D2 (by binding to transporter hNET) [5, 30] resulting decrease in their levels in both brain areas. Bameri et al. [31] introduced proof that the DA-ergic system is intricate in oxidative impairment induced by TRE. TRE causes painlessness through 2 mechanisms: trigger opioid receptors and boost the NE and 5-HT systems [32]. Hosseini-Sharifabad and coworkers [33] explained the inhibitory influences of TRE on several neurotransmitters and receptors like muscarinic, NMDA and AMPA or its stimulatory impact on the opioid, DA, 5-HT, or GABA in the brain. Also, the opioid receptors mediate the regulation of neurotransmission across different brain regions [34]. The augmentation of releasing DA in the striatum because of opioids unintended impact inducing tegmental DAergic neurons liberation by preventing GABA interneurons or GABA ends comprising opioid receptors in the ventral tegmental area or through the direct stimulation of opioid receptors on accumbal acetylcholine or GABA neurons [35].

In the present study, TRE showed a substantial reduction in β-endorphins in relation to control and ASH groups. β-Endorphin, an effective endogenic morphine created in the anterior pituitary gland has a function in pain reorganization. The medicinal approach of β-END as anti-inflammation and antioxidation therapy is still not investigated although its pain reliever impacts [36]**.** TRE works as a μ-opioid receptor agonist and as a 5-HT and NE reuptake blockade [37]**.** Bipolar subjects showed an increased expression of the μ-opioid receptor gene and decreased β-END levels [38]. So, regarding these facts, the μ-opioid receptor agonist (TRE) reduced β-END levels.

In the current study, TRE-treated rats showed a considerablly higher AChE activity compared to the control ones. These results were agreed with [39]. They displayed that the exposure of each TRE or morphine for 7, 14 and 21 days via ip at assorted doses (repetitive medicinal or inclined doses) gave a meaningful elevation in the brain AChE activity. Also, [40] reported that TRE increases AChE activities in the cerebral cortex and modulates neurotransmitter signaling in the central nervous system of rats.

In the present work, the TRE treatment or the withdrawal groups showed a substantial increase in hippocampus TNF-α and IL-1β. Mohamed and Mahmoud [41] attributed the detected rat brain inflammatory effect caused by TRE to the NF-κB stimulation. Their results confirmed a considerable and dose-dependent rise of the p65 subunit of NF-κB, connected with elevated brain IL-6 and TNF-α mRNA quantities in rats administered TRE for 8 weeks via oral route. Also, they recommended that these rises in the oxidation and inflammation levels after the TRE treatment could stimulate and enhance apoptosis in the CNS. Moreover, Gholami and coworkers [42] showed that treatment with doses of TRE (25, 50, 75, 100, or 150 mg/kg ip for 21 days) induced elevation of the hippocampus TNF-α and IL-1β levels by dose-dependently as a step in the activated apoptosis and autophagy. Also, [43] reported that TRE treated animals' group showed a remarkable increase in the hippocampus TNF-α, and IL-1β contents accompanied by high reactive oxidative species (ROS). They proposed that ROS may initiate inflammatory markers by triggering the redox-sensitive transcription factor NF-κB, Jun N-terminal kinase (JNK), and inducing the manufacture of several inflammatory markers. In addition, [44] proposed that the TRE neuro-poisonousness influences may be facilitated by affecting the phosphoinositide 3-kinase

(PI3K)/Akt/mTOR signaling routes and its downriver inflammation, programed cell death, and autophagy associated flows.

The current investigation found that ASH treatment had no significant effect on the cortex and brainstem neurotransmitter levels compared to the control. A prior investigation found that the ASH extract did not affect GABAA receptor binding or NMDA glutamate receptor subtypes in the cortex or subcortex zones. The information suggests that the ASH extract favorably influences actions in the cortex and basal forebrain cholinergic transmission [45]. This explains the non-significant results when comparing the ASH-treated and withdrawal groups to the control groups. Previous article summarized the therapeutic advantages of ASH alone notably in cancer and neurodegenerative disorders such as "Alzheimer's disease, Parkinson's disease, Cerebral ischemia, Huntington's disease, and Epilepsy" Available data suggested that ASH is effective in controlling disease progression and could be a potential therapeutic target to benefit human health status [46].

The current results showed that the ASH +TRE (A & B) groups showed a reduction in the cortex and brainstem aspartate and glutamate contents compared with the TRE groups except the brainstem ASP was increased by 13.3% than the TRE group. Whereas the cortex and brainstem glycine and GABA contents were significantly elevated in the ASH+TRE treated, and the withdrawal groups compared with TRE groups. These effects were due to ASH co-administration with TRE. Mikulska et al. [47] reported the neuroprotective, anti-inflammatory, sedative and adaptogenic properties and influences on sleep of ASH treatment. The GABA-mimetic activity of ASH root extract has been shown for several decades. Ashwagandha roots have GABA-mimetic activity in adult rats, NMDA potentiating and GABAergic activity [48]. Withaferin A and withanolide A did not activate GABAA or GABAAρ1 receptors, recommending that further component(s) in ASH may be dependable for GABAA receptor-facilitated responses [49]. Furthermore, [50] recorded that the adjustment of GABAergic and 5-HTergic paths by ASH may trigger its capability to lower depression, worry, and stress concurrently. Another study showed the beneficial effects of ASH on brain neurotransmitter contents (elevated GABA and alleviated DA) in alcohol-dependent rats in the midbrain, striatum, and cortex of the brain [51].

In the present results, treatment with TRE+ASH in withdrawal group (B) did not show improvement in GLU and ASP neurotransmitters as revealed by the percentage of change from TRE, in comparison with the corresponding group (A) or even not-improved as in brainstem ASP contents. This result may be explained by the neurodegeneration effect of TRE especially in group (B). Long-term TRE use is associated with brain degeneration recorded by decreased thickness of the retinal nerve fiber layers which can be a potential marker and an early sign of degeneration [52].

In the present study, the ASH+TRE withdrawal group showed a significant increase in DA, 5-HT and NE in the brainstem and DA in the cortex compared with TRE withdrawal groups. In support of the present findings, the study of Bashir et al. [53] revealed that withanolide A lessens the NMDA receptor signaling, which is important for memory failure in epilepsy rat model. Ashwagandha improves the binding affinity of dopaminergic D2 receptor and tyrosine hydroxylase level. An increase in the catecholamine levels after ASH treatment suggests that ASH induces catecholamines in the Parkinson disease mouse corpus striatum [54]. Moreover, ASH proved a neuroprotective impact in sleep deprivation affects brain neurotransmitters in male rats and is a powerful stressor by normalizing and regulating neurotransmitters and restoring antioxidants in the cortex [55]. Moreover, ASH with 2.5% withanolides can successfully enhance stress and anxiety by lowering cortisol and elevating 5-HT in normal people with mild to moderate signs [56]. Moreover, ASH having withanolide A shows noteworthy neuroprotection and anti-depressing impacts [57]. The ASH antidepressive influences are linked to the upregulation of BDNF-TrkB signals and the prevention of ROS through the Nrf2-HO-1 route. They proposed that the beneficial perspective of ASH as an herb therapy enhances persistent stress and depressive state.

In the present study, the ASH+TRE groups (A&B) showed a noteworthy increase in β-endorphins expression in the brainstem compared with TRE-treated and withdrawal groups. In the current work, we suggested that ASH has the same action of β-endorphin in acting as an antistress. Specified the well-recognized link between neuropsychiatric illnesses and stress, it is expected that the anti-stress action of ASH shows a central function in its ability to strength advantages for anxiety, depression, and insomnia [50]. Ashwagandha may also increase the synthesis of β-endorphin. The proopiomelanocortin (POMC) gene expression was implicated in β-END production [58]. They investigated this gene in different Asian herbs and

supplements and one of the investigated samples contained ASH as one of its components.

On the other hand, the ASH and TRE groups had lower cortical and brainstem AChE activity than the TRE groups through that ASH root extract. Idrees et al. [59] reported ASH's ability to reduce the levels of AChE. Withanolide-A is essential for correcting the inclination in cholinergic indicators like choline acetyl transferase and acetylcholine [60]. Bashir et al. [53] reported that withanolide-A might be a prospective medicine for the treatment of Alzheimer's illness by preventing the brain AChE. Another study [61] revealed that the cholinergic effects of ASH leaf-derived extract, rich in withanone, on scopolamine-induced cholinergic deficits and using ASH leaves to protect and increase brain processes implicated in cholinergic nerve system-associated cognitive responses The extract administration generated notable alterations in the acetylcholine and Arc levels (Arc is a protein that acts as a good marker for individual neurons and synapses experiencing activity-dependent modifications) in control and amnesic animals. This interprets the significant decrease of AChE activity in the cortex and brain stem in our study's ASH plus TRE-treated groups.

One of the ASH components separated from its root is vitanon. When administered to rats p.o, it causes considerable enhancements in cognition. This enhancement was explained by the prevention of amyloid β-42, and a drop in TNF-α, IL-1β, IL-6, MCP-1, nitric oxide, and MDA [62]. Previous work on neuroinflammation, induced by lipopolysaccharide (LPS) in animals, treatment with ASH displayed prevention of gliosis reaction; TNF-α, IL-1β, and IL-6 formation; and nitro-oxidative markers activity. The ASH anti-inflammation includes the prevention of lipopolysaccharide triggered NF-κB, P38 and JNK/SAPK MAPK paths. These results recommended the possible effect of ASH in conquering neuro-inflammation related to several neurological illnesses [63]. Withaferin-A has been displayed to prevent the NF-κB expression by inhibiting NF-κB phosphorylation through preventing IκB kinase activation. It also prevented the NF-κB expression by connecting the IκB kinase catalytic location and preventing neuroinflammatory impacts [64]. Moreover, Abomosallam et al. [65] concluded that ASH extract nono-emulsion markedly boosted the plant extract ingredients permeability via the blood brain barrier to enhance its neuroprotection against neuro-poisonousness persuaded by Penconazole. They revealed that treatment with ASH leave extract nono-emulsion meaningfully reduced the cytokines levels which might also linked to the glycowithanolides contents and the greater antioxidant action of the nanoemulsion than the extract induced stoppage of neuroinflammation and programmed cell death.

Behavior

The present study revealed a noteworthy elevation in rearing, grooming and immobility time but a significant decrease was noticed in cross lines number and mobility time in OFT in TRE-treated and withdrawal groups when compared with ASH and control groups. Repetitive therapy of TRE (50 mg/kg, oral, for 21 days) induced substantial influence on rat's locomotive action by reducing spontaneous locomotive movement [6, 66]. TRE abuse can produce learning and memory damage and also prolonged brain injury [40]. These findings related to TRE administration resulted in the rat's behavior alterations like anxiety, irritability, and aggression [21**,** 67]. Tramadol abuse might be a danger reason for damaged emotional and mental well-being accelerated through changed neurotransmission and inflammation [41]. The TRE induced increase in rearing and grooming could be explained by the monoamine's reuptake inhibition. The pain killing dose of TRE improved ventral hippocampal extracellular 5-HT and NE contents in the rats although it decreased cortex and brainstem monoamine levels in the present study was explained above by increasing neurotransmitter turnover [68,69].

The present study revealed a substantial reduction in the number of entries, percentage of alternation and percentage of correct alternations in YMT of TRE-treated groups. These findings were like the earlier study which revealed that rats treated with TRE showed a drop in short-term memory. Which was denoted by the meaningful reduction in the free alternation percentage in YMT [70]. Former studies revealed that TRE reduces the cholinergic action and increases the expression of AChE which relates to memory damage [39, 40]. Excessive AChE is associated with memory loss, Alzheimer's, dementia, Parkinson's disorder, and several mental and neurodegeneration conditions [71, 72]**.**

In the current study, the ASH groups showed no significant change in grooming, number of cross lines, mobility time and immobility time, and a substantially lower rearing was noticed than the control groups. This results in OFT due to the modulatory effect of ASH which is noticed in monoamines and neurotransmitters. The results in the current work showed a noteworthy incline in cross lines number and mobility time but a significant decrease was noticed in rearing, grooming and immobility time in ASH+TRE treated and withdrawal groups when compared

with TRE-treated and withdrawal groups. This result agrees with other positive behavioral and physiological effects of ASH. Gladen-Kolarsky et al. [73] supported the therapeutic ability for ASH to enhance cognition and lower anxiety and depression in Alzheimer's illness. This improvement may be because of the impact of ASH on GABA and 5-HTergic routes. It is noted that GABA stimulation reduces the activity of hypo-pituitary adrenal axis [74]. Ashwagandha modulated N-methyl-D-aspartate (NMDA) receptor density, a receptor of glutamate, boosted motor learning faults by modifying AMPA (α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor role. AMPA distinguish the GLU receptors that facilitate the transport of signals among the hippocampus neural circles [75]. Ashwagandha had several phyto-ingredients such as withanolides and further alkaloids that are pharmaceutically and therapeutically valuable in neural illnesses involving stress and depressive action. Ashwagandha modulates brain oxidative and inflammatory markers like TNF-α, IL-6, lipid peroxidation, GSH, and SOD [47]. Interestingly, withanolide-free fractions confirm anti-anxiety and anti-depression impacts in mice, indicating a responsibility for further components.

The present results showed that the number of entries, the percentage of alternation and the percentage of correct alternations in YMT were improved in ASH+TRE treated and withdrawal groups in comparison with TRE groups. In agreement with the present results, [76,77] displayed that ASH adjusted memory deficiencies in mice Alzheimer's model produced by scopolamine. The AChE prevention of ASH may clarify the anti-amnestic influences detected. The protective effect against memory impairment (assessed by YMT) and neuroinflammatory impact in mice treated with thioacetamide caused hepatic encephalopathy were demonstrated for ASH treatment (200 or 400 mg/kg, for 29 days) [78]. Furthermore, ASH offers safety from oxidative injury and memory loss inducible by Bisphenol A [79]. A conventional root extract of ASH showed antidepressant efficacy [50].

Conclusion

The results presented that the ASH treatment with TRE in group (A) was more effective than the treatment with TRE in group (B) as revealed by the percentage of change recorded in different groups, and this effect could be due to the degradation of brain cells occurring by TRE misuse doses which were recorded previously.

The results of this study support that four weeks of dietary supplementation of ASH can improve the harmful effects of TRE through modulating neurotransmitter levels and its anti-inflammatory effect. No effect was recorded for ASH treatment alone. While these results are encouraging, extra study is affirmed to estimate the prospective nootropic impacts of ASH at different treatment policies and prolonged treatment periods. Future studies should be performed to evaluate the ASH in different doses and the molecular effect of both TRE and ASH treatment. Also, different sex and different experimental periods should be taken into consideration in the upcoming works.

Conflicts of interest

The authors declare there are no conflicts of interest.

Funds

The authors declare there are no funds or any financial money received regarding this work.

Authors' contributions

SSHA performed the experiment and the analysis of parameters and wrote the draft. NMSA suggested the idea and reviewed the manuscript. HGSH suggested the idea and authored the article. EHAA suggested the idea, performed data analysis, and reviewed the manuscript and all the authors approved the final article.

Ethical considerations

The approval of the animal protocol was obtained from the URAF committee (URAF-IACUC) approval number 000611922.

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