Indian Journal of Experimental Biology
Vol. 63, October 2025, pp. 808-818
DOI: 10.56042/ijeb.v63i10.17369

Ashwagandha attenuates organ oxidative stress and inflammation in tramadol treated and withdrawal rats

Nadia M S Arafa[1]*, Shimaa S H Ahmed[2], Hoda G Hegazy[2] & Elham H A Ali[3]

[1]Physiology Department, Egyptian Drug Authority (EDA), Giza 12555, Egypt

[2]Zoology Department, Faculty of Science, Ain Shams University, Cairo 11566, Egypt

[3]Zoology Department, Faculty of Women for Arts, Science and Education, Ain Shams University, Cairo 11757, Egypt

Received 21 February 2025; revised 18 August 2025

This study investigated the ameliorative effects of Ashwagandha (*Withania somnifera*, ASH) on liver and kidney function, brain oxidative stress, and proinflammatory cytokines in rats subjected to tramadol (TRE) treatment and withdrawal regime. Two main rat groups: Group A (Treatment) were orally treated for 6 weeks, Control (0.5%CMC), ASH (200 mg/kg), TRE (40 mg/kg), and TRE+ASH groups. Group B (Withdrawal), were orally treated for ten weeks, Control, ASH (CMC six weeks followed by ASH four weeks), TRE (increasing doses, 40, 80 then 120 mg/kg/day for six weeks followed by CMC four weeks), and TRE+ASH (TRE increasing doses six weeks followed by ASH four weeks). The TRE+ASH groups showed a significant improvement compared to TRE treated and withdrawal groups. That includes reduction in AST, ALT, urea, and creatinine values indicating improved liver and kidney functions. Also, the brain cortex and brainstem tissues revealed enhanced antioxidant defence (CAT, GST, GPX, GSH and TAC) and reduced oxidative stress markers (NO and MDA). In addition, the proinflammatory cytokines (TNF-α and IL-1β) in cortex were significantly ($P \leq 0.05$) reduced by ASH supplementation. These results suggest ASH supplementation ameliorates the liver and kidney functions, oxidative brain damage and neuroinflammation during both treatment and withdrawal of TRE.

Keywords: Tramadol induced liver dysfunction, Tramadol induced kidney dysfunction, *Withania somnifera* supplementation, Oxidative stress markers, Brain cortex, Brainstem, Proinflammatory cytokines expression

Tramadol (TRE) is a synthetic constructed drug that works as a central opioid painkiller and is structurally related to morphine and codeine. Tramadol functions by inhibiting serotonin and norepinephrine reuptake and binding the μ opioid receptors. Persistent TRE usage increases its toxic potential through the accumulation of toxic metabolites in the body, resulting in lower clearance and different organ toxicities have been linked to its abuse[1]. Tramadol have been reviewed for neurotoxicity, hepatotoxicity, nephrotoxicity, lung and heart toxicity, and adrenal, as well as testicular and reproductive toxicity through oxidative stress[2]. Because of the increased incidence of TRE-related overdose and death, it has been classified as a controlled substance in several countries. In the United States, the Drug Enforcement Administration (2014) classified TRE into Schedule IV of the Controlled Substances Act, and the United Kingdom classified it as a Class C, Schedule 3 controlled substance and managing tramadol withdrawal considered a target[3]. Tramadol abuse considered as problematic issue in Egypt and Middle Eastern countries. Its usage in increasing doses has been linked to addiction associated with organ toxicity and increased oxidative stress[1] and the tramadol abuse presents a significant challenge in treatment. So searching and experimental evaluation of medicinal plants that could help manage detoxification of opioid addicts is a target.

Withania somnifera (L.) Dunal (WS, Ashwagandha, ASH) is an herbal plant planted in dry places in India. Additionally, it was grown in Iran, Sri Lanka, Nepal, and China. It has been recognised as an adaptogen in ayurvedic medicine for its ability to promote homeostasis of the whole body not only by one specific pharmacological mechanism. The plant contains more than thirty-five biologically active chemical substances, including alkaloids, flavonoids, saponins, and steroidal lactones (withanolides). *Withania somnifera* is consumed as an antioxidant, anti-inflammatory, antibacterial, aphrodisiac and liver tonic agent[4]. Standardised ashwagandha root extract

*Correspondence:
Phone: +201003712920
E mail: nadianeuro@yahoo.com

supplementation has been shown to have antioxidant and anti-inflammatory effects, leading to a radioprotective effect on the liver and spleen tissues of rats exposed to both acute and chronic doses of γ-radiation[5]. Ashwagandha extracts have been previously investigated for its impact on stress, anxiety, mood, and sleep disturbances in both humans and animal models through modulation of gamma aminobutyric acid A receptor (GABA-A) and serotonin reuptake inhibition pathways. Ashwagandha has shown potential in neuroinflammation, promote overall physiological balance by engaging multiple pathways, potentially improving cognitive function, mood, and resilience to illness [6].

Previous studies indicate that ASH may interfere with the central effects of addictive drugs, in addition to its antioxidant and anti-inflammatory properties. This suggests that ASH could be a potential treatment. To the best of our knowledge, no previous studies have explored its effect on TRE misuse[4-6].

Hence, the present study hypothesised an ameliorative role of Ashwagandha supplementation against the chronic TRE (therapeutic dose) treatment for 6 weeks or the withdrawal associated with its misuse by successive doses (4 weeks withdrawal after 6 weeks treatment with successive doses) in adult male rats on the induced perturbations in liver and kidney functions and brain oxidative stress and inflammation.

Materials and Methods

Tramadol hydrochloride (TRE) was obtained from the Memphis Company for pharmaceuticals and chemical industries under the supervision of the Egyptian Drug Authority (EDA). *Withania somnifera (*Ashwagandha, WS*,* ASH) was purchased from NOW FOODS as capsules (450 mg) from dry powdered roots and leaves (2.5% total withanoides-11 mg). Carboxymethylcellulose (CMC) was obtained from Sigma Aldrich as a white powder, and CMC (code: 21902 medium viscosity) was prepared by adding 5 g of CMC to 1000 mL of distilled water.

Experimental animals

The study was conducted with Wistar adult male rats (n=80). They were 6 months old and weighed 150 g and purchased from the UResearch Animal Facility (URAF) animal house, Egypt. The rats were group-housed in clear conventional cages containing rice strew wood bedding material. The housing room was controlled for temperature (25 ± 2 °C), humidity (48 ± 2%), and 12 hours. light/dark cycle and masking environment noise. The rats were given basic rat chow (R36; Lantmännen, Kimstad, Sweden) and water *ad libitum*. The experiments were authorised by the Institutional Animal Care and Use Committee (URAF-IACUC) according to the Cairo University Institutional Animal Care and Use Committee CU-IACUC with approval number 000611922.

Experimental design

The rats were divided into two main groups and left for 5 days for adaptation before the beginning of the experiments**.** The first group (A) is the main treatment group that was divided into four groups (n=10/group) and treated orally for 6 weeks as follows:

Control: Received (CMC, 0.5% as 0.5 mL/100 g bw); ASH (Ashwagandha): Administrated ashwagandha (200 mg/kg/day) according to a previous studied dose[7]; TRE (tramadol): TRE (40 mg/kg/day) was administered at a dose calculated equivalent to the maximum human therapeutic dose[8]; TRE +ASH: TRE (40 mg/kg/day) was administered first, and after 1 hour, the rats received ASH (200 mg/kg/day).

The second group (B) (withdrawal group) was divided into four groups and treated orally for ten weeks as follows:

Control: 0.5% CMC (0.5% as 0.5 mL/100 g bw); ASH: CMC was administered for the first 6 weeks, followed by ASH (200 mg/kg) for 4 weeks; TRE withdrawal group: TRE was administered for 6 weeks (with increasing doses from the therapeutic dose every two weeks in order of 40-80-120 mg/kg/day), followed by CMC administration for the remaining 4 weeks; TRE+ASH: TRE was administered in successive doses, for 6 weeks, followed by ASH (200 mg/kg) for 4 weeks.

At the end of each experiment, the rats in each group were suddenly decapitated by a technician with technical expertise with sharp blades, and their blood was collected for serum preparation to determine liver and kidney function parameters. Cortical and brainstem tissues were extracted from each rat brain, six from each tissue sample were homogenised in phosphate-buffered saline (PBS; pH 7.2) at 10% w/v and then centrifuged, and the supernatant was stored at -20 °C until use for oxidative stress markers estimation. The remaining four brains were preserved in neutral formalin (10%) for immunohistochemical investigation.

Biochemical investigations

Serum aminotransferases, AST and ALT were assayed by colourimetric endpoint, where oxaloacetate

formed reacts with 2, 4-dinitrophenylhydrazine to yield a coloured hydrazone for AST assay and pyruvate formed reacts with 2-4-dinitrophenyl hydrazine to yield a coloured hydrazone for ALT assay and both measured at 546 nm with sensitivity 7 U/L. Urea estimated by enzymatic colourimetric method, where urea is broken down into ammonia and carbon dioxide by urease. Ammonium reacts with salicylate and nitroferricyanide in alkaline solution of sodium hypochlorite forming green coloured compound with intensity related to urea present measured at 578 nm with sensitivity 1 mg/dL. Creatinine (Cr) was assayed by colourimetric endpoint method where creatinine reacts with picric acid in alkaline conditions forming yellow- orange coloured complex measured at 546 nm with sensitivity 1.0 mg/dL. The aforementioned parameters were assessed via kits purchased from Biomed Diagnostic Co., Egypt, according to the manufacturer′s instructions.

The supernatants of either the cortex or brainstem were used for spectrophotometric determination of the enzyme activities, glutathione peroxidase (GPx), by indirect measure of the cellular enzyme (c-GPx), as oxidized glutathione (GSSG) generated when c-GPx reduces an organic peroxide and GSSG then recycled to its reduced state GSH by the enzyme glutathione reductase and absorbance measured at 340 nm/min to obtain change of absorbance/min using GSH as reference standard. Glutathione-S-transferase (GST) assayed according to the conjugation of the enzyme with CDNB (1-chloro-2,4-dinitrobenzene) which measured at 340 nm. and catalase (CAT). The extinction coefficient of the CDNB-GSH conjugate at 340 nm, which is approximately 9.6×10^3 M^{-1} cm^{-1} and its mole calculated to determine the GSH activity (mM/min/g). In addition, the glutathione (GSH) content was assayed based on the reduction of 5,5` dithiobis (2-nitrobenzoic acid) (DTNB) with GSH producing a yellow compound directly proportional to GSH content which measured at 405 nm. Malondialdehyde (MDA) was detected through the reaction of thiobarbituric acid (TBA) with MDA in acidic medium at 95 °C for 30 min forming thiobarbituric acid resultant pink product that measured at 534 nm. The total antioxidant capacity (TAC) was performed by reaction of antioxidants in sample tissue homogenate with known amount of H2O2 and its residue is determined colorimetrically by enzymatic reaction to convert 3,5,dicloro-2-hydroxy benzensulphate to coloured product measured at 505 nm. Finally nitric oxide was determined as nitrite by the use of Griess reagent which converts nitrite into purple azo compound measured at 540 nm. The aforementioned parameters determined in tissue by a commercial biodiagnostic kit according to the manufacturer′s procedures. Every sample was measured in triplicate and the average was taken in calculation.

Immunohistochemical investigations

The sequences of the sections (5 μm) were obtained from a typical paraffin block. Primary antibodies against 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 PBS were used, after which the slides were inserted and incubated at 4 °C for 24 hours. after washing with PBS. A 3,3-N-diaminobenzidine tetrahydrochloride (DBA) solution was applied, the DBA developing Colour Kit was added to the antibody-identified slides, and the slides were subsequently incubated for 15 minutes and washed in water. Next, the sections were incubated with hematoxylin dye for 10 min. Then, dehydration was assessed with alcohol, and Canada balsam was added to the sections. The slides were examined at 400× with an Olympus BX43 microscope connected to a camera as previously described[9]. Six nonoverlapping fields were selected at random and examined from cortex tissue to assess TNF-α and IL-1β expression according to the brown colour density. The colourimetric analysis of the images revealed that the density of the brown colour was determined via the Trigit application[10].

Statistical analysis

A prior power analysis was conducted using G*Power 3.1 software to determine the appropriate sample size. we calculated an effect size of Cohen′s f = 0.25 for ANOVA. With α = 0.05 and desired power = 0.80. Based on this analysis, a minimum of 10 animals per group was found to be sufficient to detect statistically significant differences with adequate power. The recorded data were analyzed via the social sciences software package IBM (version 23). An analysis of variance (ANOVA) was performed, and the group comparisons were performed via the LSD post hoc test. Significant results were recorded at the 5% level. The percentage of the TRE group values changed from the corresponding control (TRE %) = ((TRE-control)/control) ×100. The percentage of

TRE+ASH values changes from the TRE corresponding group values (TRE+ASH%) = (((TRE+ASH)-TRE)/TRE) ×100.

Results

Effects of treatments on serum liver and kidney function biochemical parameters:

The data in Table 1 revealed no significant differences in the ALT, AST, urea and creatinine values with respect to ASH treatment compared with the control values. The TRE treatment (group A) and TRE withdrawal (group B) groups showed significantly increased liver and kidney function parameters, ALT, AST, creatinine, and urea values as compared with the control and ASH corresponding values. ASH supplementation compared to single tramadol treatments showed in TRE+ASH (group A and B) improved liver function enzymes by decreasing significantly ($P \leq 0.05$) their elevated values in TRE (group A and B) with the exception of the non-significant increased AST in withdrawal (group B). The TRE+ASH (group A and B) groups showed a significant decrease in the elevated TRE (A and B) levels of urea and creatinine. Meanwhile, TRE+ASH withdrawal group (B) exhibited a significant increase ($P \leq 0.05$) in ALT, AST and urea values as compared to those values in TRE+ASH group (A). While a non-significant change was shown in Cr in TRE+ASH group (A & B) in comparison with the corresponding control value. Moreover, the Cr in TRE+ASH group (A) was changed with not statistically difference ($P > 0.05$) when compared with ASH group.

Effect of treatments on brain cortex oxidative stress markers:

The results presented in Table 2 revealed no significant differences ($P > 0.05$) in the GPx, GST,

Table 1 — Serum liver and kidney functions in treatment (A) and withdrawal (B) groups

Item	Group	Control	ASH	TRE	TRE+ASH	TRE%	TRE+ASH%
ALT	A	81.03±1.16	76.02±0.96	147.13±6.02^{ab}	98.27±2.52^{abc}	81.57	-33.21
U/L	B	85.44±3.11	83.44±1.59	122.00±2.83^{ab*}	130.14±6.21^{abc*}	42.79	6.67
AST	A	212.16±4.95	182.92±5.70	355.73±22.35^{ab}	279.93±10.63^{abc}	67.67	-21.31
U/L	B	202.76±13.42	178.31±7.68	260.58±13.24^{ab*}	301.15±9.44^{b*}	28.52	15.57
Urea	A	8.97±0.55	7.62±0.39	19.87±0.49^{ab}	15.01±0.82^{abc}	121.52	-24.46
mg/dL	B	8.70±0.45	8.30±0.91	32.02±1.26^{ab*}	22.12±0.91^{abc*}	268.05	-30.92
Cr	A	102.78±5.51	103.70±5.86	147.22±7.42^{ab}	120.37±8.92^{c}	43.24	-18.24
mg/dL	B	109.26±8.32	97.22±4.70	169.44±9.16^{ab*}	126.85±9.67^{bc}	55.08	-25.14

Values are means ± SE (n=6), ASH, (Ashwagandha), TRE, (tramadol). [^{a}significant from control, ^{b}significant from ASH, and ^{c}significant from TRE, within the same row. *Significant from the treated corresponding group (A) $P \leq 0.05$. TRE %, is the percentage of TRE group values change from their corresponding control. TRE+ASH%, is the percentage of TRE+ASH values change from the TRE corresponding group values]

Table 2 — Cortex oxidative stress markers in treatment (A) and withdrawal (B) groups

Item	Group	Control	ASH	TRE	TRE+ASH	TRE%	TRE+ASH%
GPX	A	64.67±2.52	68.57±1.57	25.69±1.30^{ab}	47.23±1.28^{abc}	-60.28	83.85
mg/min/g	B	63.56±3.52	68.06±1.52	22.27±0.67^{ab}	42.91±1.25^{abc}	-64.96	92.68
GST	A	21.92±0.80	22.70±0.43	16.09±0.83^{ab}	24.23±1.67^{c}	-26.60	50.59
mM/min/g	B	21.80±0.70	23.93±1.56	17.17±0.76^{ab}	19.22±1.34^{b*}	-21.24	11.94
GSH	A	4.10±0.21	4.37±0.13	3.15±0.14^{ab}	3.52±0.28^{ab}	-23.17	11.75
mg/g	B	4.20±0.15	4.32±0.11	2.90±0.62^{ab}	3.60±0.14^{abc}	-30.95	24.14
CAT	A	30.06±1.50	29.97±1.19	17.14±1.16^{ab}	25.40±1.01^{abc}	-42.98	48.19
µM/min/g	B	30.88±1.53	29.40±1.22	21.91±0.73^{ab*}	24.54±0.81^{ab}	-29.05	12.00
NO	A	25.56±1.25	24.83±0.94	63.74±1.09^{ab}	48.66±1.51^{abc}	149.37	-23.66
µM/g	B	25.11±1.45	26.20±1.08	74.80±1.19^{ab*}	37.59±1.90^{abc*}	197.89	-49.75
MDA	A	35.68±0.84	32.77±1.36	68.11±2.41^{ab}	50.60±2.90 ^{abc}	90.89	-25.71
µM/g	B	35.12±1.04	33.68±1.27	48.43±1.76^{ab*}	34.15±13.0^{c*}	37.90	-29.49
TAC	A	77.77±2.70	80.67±1.83	38.30±1.73^{ab}	50.39±2.86^{abc}	-50.75	31.57
µM/g	B	77.38±3.56	74.71±0.91	17.13±0.90^{ab*}	30.95±3.1^{abc*}	-77.86	80.68

Values are means ± SE (n=6), ASH, (Ashwagandha), TRE, (tramadol). [^{a}significant from control, ^{b}significant from ASH, and ^{c}significant from TRE, within the same row. *Significant from the treated corresponding group (A) $P \leq 0.05$. TRE %, is the percentage of TRE group values change from their corresponding control. TRE+ASH%, is the percentage of TRE+ASH values change from the TRE corresponding group values]

and CAT activities or the GSH, NO, MDA and TAC values between the cortex tissues of the ASH-treated groups and those of the control group. The TRE-treated group (A) and TRE withdrawal group (B) presented significant reduction ($P \leq 0.05$) in cortex GPx, GST, GSH, CAT, and TAC and significant increase ($P \leq 0.05$) in the NO and MDA contents as compared with the control group corresponding values. With respect to the ASH treated groups, TRE+ASH group (A) and (B), the results revealed a significant increase in GPx, GST, CAT and TAC and a significant decrease ($P \leq 0.05$) in NO and MDA in the cortex, relative to the TRE group (A) and (B) values. That's with the exception of not statistically difference increase ($P > 0.05$) in the GSH contents in TRE+ASH group (A) as compared to that in TRE group (A) value, also the GST and CAT values in TRE+ASH group (B) as compared to that in TRE group (B). The results of the TRE+ASH group (B) data revealed a significant decrease ($P \leq 0.05$) in GST, NO, MDA and TAC as compared with those of the TRE+ASH group (B).

Effect of treatments on brainstem oxidative stress markers:

The results in Table 3 concerning brainstem tissue, with respect to ASH treatments, revealed no significant differences ($P > 0.05$) in the GPx, GST, or CAT activities or the GSH, NO, MDA and TAC contents compared with those of the corresponding controls. The results of the TRE-treated and withdrawal groups (A and B) revealed a significant reduction ($P \leq 0.05$) in brainstem GPx, GST, GSH, CAT, and TAC and a significant increase ($P \leq 0.05$) in NO and MDA from the control and ASH corresponding values. While results of ASH treated groups, TRE+ASH group (A) and (B), revealed significant increases ($P \leq 0.05$) in brainstem GPx, GST, CAT and TAC and significant decreases in NO and MDA as compared to TRE-treated and withdrawal groups corresponding values.

Effect of treatments on proinflammatory cytokines

The data from Fig. 1 revealed that the positive expression of TNF-α in the cortex was significantly greater in the TRE groups than in the control and ASH groups ($P \leq 0.05$). Moreover, the TRE+ASH groups presented a significant decrease in the positive expression of TNF-α compared with the corresponding TRE groups. The TRE+ASH withdrawal group (B) presented a significant increase in TNF-α expression compared with that in the TRE+ASH group (A).

The data from Fig. 2 revealed that the positive expression of IL-1β in the cortex was significantly elevated in the TRE groups compared with the control and ASH groups ($P \leq 0.05$). However, in the TRE+ASH groups, the positive expression of IL-1β was significantly alleviated compared with that in the corresponding TRE groups.

Discussion

Tramadol is a synthetic opioid analgesic, and its misuse is linked to the ongoing public health issue of overdose-related disorders worldwide. The mode of

Table 3 — Brainstem oxidative stress markers in treatment (A) and withdrawal (B) groups

Item	Group	Control	ASH	TRE	TRE+ASH	TRE%	TRE+ASH%
GPX	A	64.63±1.75	66.88±2.57	45.16±2.20^{ab}	58.19±2.70^{abc}	-30.13	28.85
mg/min/g	B	64.43±1.84	65.88±1.33	54.55±2.35^{ab*}	60.41±1.73^{abc}	-15.33	10.74
GST	A	8.15±0.46	8.94±0.39	6.57±0.34^{ab}	8.39±0.42^{c}	-19.39	27.70
mM/min/g	B	8.06±0.24	8.94±0.28	6.24±0.35^{ab}	7.95±0.31^{c}	-22.58	27.40
GSH	A	12.51±0.68	13.69±0.52	8.93±0.30^{ab}	9.46±0.16^{ab}	-28.62	5.94
mg/g	B	12.60±0.48	13.61±0.68	10.24±0.42^{ab}	12.60±0.67^{c}	-18.73	23.05
CAT	A	36.73±1.75	37.16±2.86	20.23±0.90^{ab}	35.88±0.89^{c}	-44.92	77.36
µM/min/g	B	36.47±1.17	37.21±2.34	20.52±2.01^{ab}	33.85±5.42^{c}	-43.73	64.96
NO	A	14.17±1.84	14.99±3.24	60.13±2.13^{ab}	21.19±0.70^{abc}	324.35	-64.76
µM/g	B	14.51±0.31	14.86±0.71	43.02±1.76^{ab*}	19.45±0.75^{ac}	196.49	-54.79
MDA	A	24.88±1.18	23.44±1.33	45.06±3.17^{ab}	26.05±1.15^{c}	81.11	-42.19
µM/g	B	24.59±1.40	23.23±0.60	41.29±2.19^{ab}	27.97±0.81^{c}	67.91	-32.26
TAC	A	14.13±0.77	14.92±0.82	7.73±0.39^{ab}	11.45±0.58^{c}	-45.29	48.12
µM/g	B	14.37±0.91	14.81±0.74	6.83±0.29^{ab}	15.08±0.74^{c*}	-52.47	120.79

Values are means ± SE (n=6), ASH, (Ashwagandha), TRE, (tramadol). [^{a}significant from control, ^{b}significant from ASH, and ^{c}significant from TRE, within the same row. *Significant from the treated corresponding group (A) $P \leq 0.05$. TRE %, is the percentage of TRE group values change from their corresponding control. TRE+ASH%, is the percentage of TRE+ASH values change from the TRE corresponding group values]

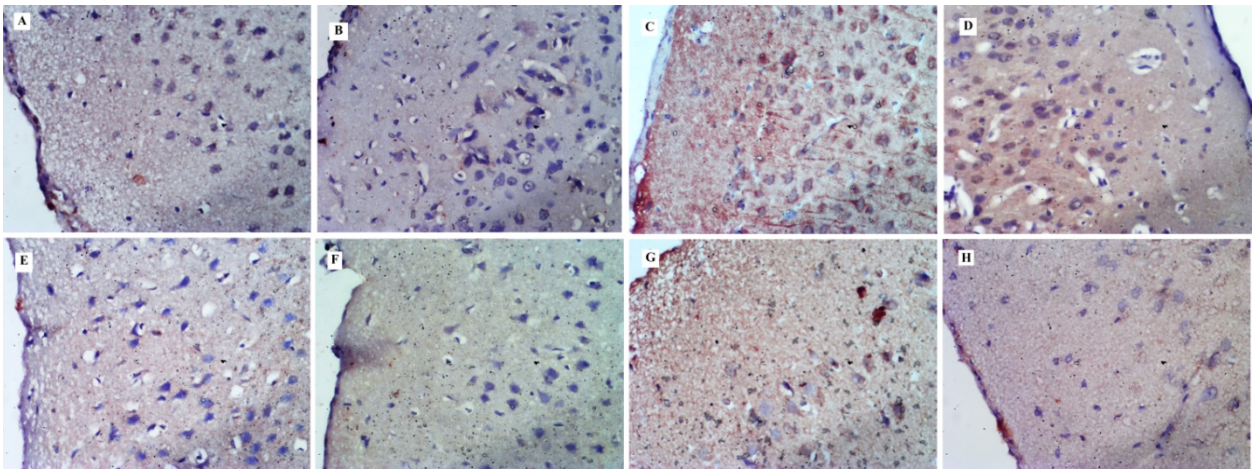

Fig. 1 — Photomicrograph of the immunohistochemistry stain of cortex tissue TNF-α contents in control group (A&E); Ashwagandha (ASH) groups (B&F); TRE, A group (C); TRE+ASH, A group (D); TRE, B group (G) and TRE+ ASH, B group (H). Histogram representing the density of brown colour of cortex TNF-α contents of different groups (I). [Values are means ± SE (n=6), Superscript, [a]significant from control, [b]significant from ASH, [c]significant from TRE and *significant from the treated corresponding group (A) at $P \leq 0.05$. (×=400)]

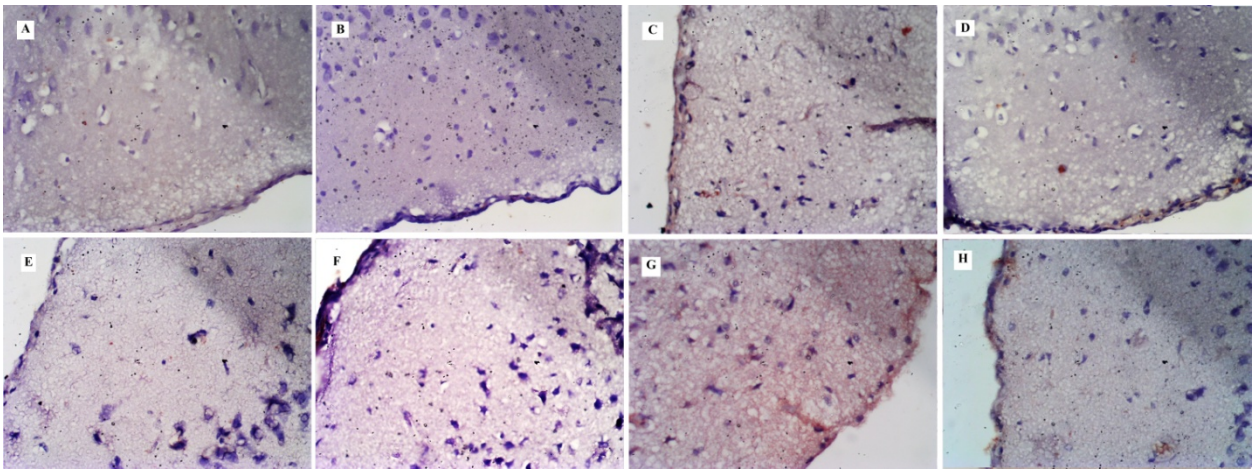

Fig. 2 — Photomicrograph of the immunohistochemistry stain of cortex tissue IL-1β contents in control group (A&E); Ashwagandha (ASH) groups (B&F); TRE, A group (C); TRE+ASH, A group (D); TRE, B group (G) and TRE+ ASH, B group (H). Histogram representing the density of brown colour of cortex IL-1β contents of different groups (I). [Values are means ± SE (n=6), Superscript, [a]significant from control, [b]significant from ASH, [c]significant from TRE and *significant from the treated corresponding group (A) at $P \leq 0.05$. (×=400)]

the analgesic effect of opioids involves their binding to opioid receptors in the brainstem, spinal cord, and gastrointestinal tract, leading to hyperpolarization of neurons and inhibition of neurotransmitter release, resulting in an analgesic effect. This effect is lost by prolonged usage, and the individual becomes tolerant to the opioid because the response to its action changes, and a higher dose is needed to restore the same potency[11]. Tramadol dependence, abuse by accumulated therapeutics conducted in rats or by a gradual increase in tramadol dose from the initial therapeutic-related dose, which is in line with the present study, is targeted for investigations as well as tramadol withdrawal. This finding is consistent with the results of various objective studies on the adverse effects of tramadol on different targeted organs in humans and experimental animals, which have been conducted for many years. Data from humans have revealed the risk of tramadol dependence due to tramadol abuse resulting from either long-term treatment or high-dose consumption, even for persons with no history of drug abuse[12,13].

The liver and kidneys are known to be the major organs involved in the metabolism and excretion of

TRE and are more strongly affected by the toxicity of TRE[14]. In the present study, the serum AST and ALT enzyme activities significantly increased after TRE consumption in both treatment and withdrawal groups compared with those in the control and ASH groups. Tramadol consumption in rodents has been previously reported by several studies to be responsible for elevated liver enzymes due to leakage from hepatocytes into the blood, resulting from enhanced hepatic cell lipid peroxidation associated with histopathological alterations[14,15].

In present study, the loss of kidney function in TRE-treated rats was implied by a significant increase in the serum urea and creatinine levels compared with those in the control groups. This elevation may be due to a reduced glomerular filtration rate or an increase in reactive oxygen species (ROS)[14]. Different studies have verified the adverse toxic effects of either therapeutic or additive doses of tramadol, which lead to its accumulation, which is reflected in hepatotoxicity (elevated blood AST and ALP) and nephrotoxicity (elevated blood urea and Cr) and elevated oxidative stress (hepatic and renal reduced SOD and CAT activities and GSH levels and elevated MDA levels)[14]. This finding is supported by a recent study suggesting that oxidative stress status of liver and kidney tissues is associated with TRE treatment in rats. The study reported significantly elevated MDA and NO contents, as well as significantly reduced SOD and CAT enzyme activities in liver and kidney tissues, thereby augmenting the link between TRE and oxidative stress with hepatotoxicity and nephrotoxicity[16]. On the other hand, a previous study suggested that mitochondrial dysfunction and oxidative stress are involved in the pathogenesis of TRE-induced renal damage[17]. This finding is in line with a recent study that revealed the protective therapeutic benefits of agents that acquired antioxidant and mitochondria-targeting activities against TRE nephrotoxicity[18].

Hence, the results of the present study in the ASH-treated groups could be explained by the therapeutic benefits of ASH due to its antioxidant activity according to its high levels of flavonoids, phenolic compounds and withanolide as active ingredients as well as its recorded activity in improving mitochondrial function in humans and animals[19]. Additionally, the present study confirmed ASH safety by not disturbing the liver and kidney determined markers when ASH was administered alone. Moreover, supplementation with TRE treatment or TRE withdrawal significantly ameliorated the increase in the measured parameters, revealing its ability to restore the activities of mitochondrial enzymes to near normal levels in the livers of rats with experimentally induced oxidative damage[19]. Ashwagandha root extract with less than 5% withanolide content was previously confirmed to be safe by repeated-dose toxicity studies in the range from 500 mg/kg to 2000 mg/kg for ninety days of administration in rats[20].

In contrast, liver toxicity was previously reported for ASH metabolites, withanone, which is a withanolide that is responsible for ASH liver toxicity. Withanone has the ability to form adducts with DNA, causing DNA lesions and leading to mutagenesis, apoptosis and cell death. This genotoxicity is related to the available contents of cellular GSH, which acts as a detoxifying agent for withanone by forming adducts with it. Additionally, a previous study reported the relationships between liver toxicity and the withanone concentration and available GSH[21].

The aforementioned studies concerning ASH safety may explain the present study results regarding the 200 mg/kg dose of ASH with minimum withanolide content, as used in the present study (2.5%), confirming the normality of the tested parameters detected in the ASH consumption group. Moreover, ASH treatment significantly improved the TRE-induced alterations in liver function in the TRE-treated and withdrawal groups. This occurred with the exception of liver function in the withdrawal groups, in which ASH after TRE withdrawal failed to overcome the elevated AST and ALT activities. These findings suggest that the beneficial effect of ASH supplementation in combination with TRE medication to overcome TRE-related liver and kidney toxicity rather than the effect of ASH on TRE withdrawal with respect to liver function. This could be explained by the liver cell damage resulting from the accumulated tramadol metabolites, the elevated lipid peroxidation and the reduced antioxidant defences that ASH failed to overcome[1]. The hepatoprotective effect of ASH root extract against silver nanoparticle-induced liver toxicity in mice was recently studied at a dose of 80 mg/kg for 14 days, and the results revealed improved liver function biomarkers[22]. Additionally, ASH treatment restored kidney function against mercuric chloride nephrotoxicity in rat groups that received daily doses of 250, 500 or 750 mg/kg for 30 days[23]. The aforementioned studies revealed the liver and kidney protective effects of ASH by the free

radical-scavenging mechanism and ASH detoxifying action. That′s in line with the anti-inflammatory activities related to its active phytochemical constituents that have the ability to inhibit the NF-κB and mitogen-activated protein kinase (MAPK) signaling pathways, as well as regulate cytokine expression[22,23].

With respect to the central nervous system, the TRE is known as the targeted site of action location. Therefore, one of the most common neurotoxic mechanisms following TRE abuse is oxidative stress in the brain [2]. Previous studies have indicated that chronic TRE administration alters neurotransmitter levels and induces oxidative stress in the brain[14]. Moreover, the hypothesis of a bidirectional interaction between opioids, such as TRE and oxidative stress, which are associated with particular brain diseases, is increasingly supported by evidence[24]. In this context, TRE chronically administered at a dose of 40 mg/kg for 4 weeks significantly increased the MDA content concomitant with a decrease in the GSH content in the cerebral cortex, which was associated with degenerative structural changes and neuronal apoptosis that support oxidative stress-dependent TRE neurotoxicity[25]. Additionally, rats treated with 40 mg/kg (twice a day) for three weeks presented elevated levels of the oxidative stress markers MDA and nitrites, decreased GSH contents, and decreased GST and CAT activities in brain tissue[26]. In tramadol-dependent mice, brain oxidative stress develops via decreased intracellular GSH levels and GPx activity with elevated brain MDA and NO production[27]. Similarly, TRE administration in rats for eight weeks at doses of 30 or 60 mg/kg resulted in elevated MDA and NO and decreased GSH contents and SOD and GPx enzyme activities in cerebral tissue in a dose-dependent manner, reflecting the induction of cerebral oxidative stress due to chronic TRE administration[14]. Another study used tramadol at doses of 42, 84 and 168 mg/kg/day as increasing doses for 30 days each for ten days and subjected adolescent rats to 4 weeks of withdrawal. The study demonstrated that the abuse of tramadol and withdrawal significantly elevated the lipid peroxidation marker MDA and reduced the activity of CAT, an antioxidant, supporting the brain oxidative stress induced by the abuse of tramadol[28].

With respect to the brainstem, tramadol affects the brainstem centers of ventilatory control. Tramadol analgesic actions are mediated via brainstem (locus coeruleus) monoaminergic pathways by the inhibition of norepinephrine and serotonin reuptake. Tramadol-related respiratory depression is reflected via the ventilatory control system by acting at the μ–opioid receptors in the respiratory integrating centers within the brainstem[29]. In tramadol-abused rats, tramadol was previously shown to induce derangements in the mitochondrial electron transport chain. The protein complexes in the respiratory chain are affected by tramadol. Complexes I, III and IV are inhibited by tramadol at high doses, and the inhibition of complex III consequently causes the generation of ROS, supporting the relationship between mitochondrial dysfunction and TRE-induced oxidative stress, cerebral dysfunction and neurons apoptosis[30].

Compared with those in the control and ASH groups, the expression of TNF-α and IL-1β in the cortex of the TRE groups was significantly greater. Chronic TRE intake leads to increased oxidative stress associated with the neuroinflammatory state. Reactive oxygen species are considered intense stimuli for the release of proinflammatory cytokines, including TNF-α, IL-1β, and IL-6. Moreover, the ability of IL-1β to initiate signaling forces subsequent extreme ROS. This finding was confirmed in a later study in which PC12 cells were exposed to tramadol *in vitro*, and elevated levels of ROS and annexin V (an apoptotic marker) were recorded and in the vivo study findings revealed that tramadol treatment for 3 weeks at a dose of 50 mg/kg provoked apoptosis, leading to neurodegeneration in the frontal cortex via increased gene expression of the proinflammatory cytokines TNF-α, IL-1β, and IL-6, augmenting the oxidative stress and inflammatory consequences of tramadol abuse[31]. Similarly, tramadol treatment for 3 weeks at a dose of 50 mg/kg triggered elevated levels of the proinflammatory cytokines TNF-α and IL-1β in the choroid plexus and significantly decreased GSH levels and SOD activity in the cerebrospinal fluid, with degeneration of the brush border of choroid plexus epithelial cells[24]. These findings are in line with previous studies on the use of tramadol for evaluating oxidative stress in the brain with elevated levels of inflammatory mediators in the cerebrum[14]. The increase in cerebral expression of TNF-α due to free radical-activating nuclear factor kappa B (NF-κB) indicates elevated production of TNF-α followed by tissue damage. NF-κB causes the transcription of inflammatory genes and produces a variety of inflammatory moderators. Microglial expansion in the cerebellar cortex may be related to this overproduction of inflammatory mediators[32].

The present study revealed a significant increase in CAT, GST, GPx, GSH, and TAC activities accompanied by an alleviation of NO and MDA contents in the cortex and brainstem in the TRE+ASH-treated and withdrawal groups compared with those in the TRE groups. The active constituents of ASH extracts, such as flavonoids and polyphenolic compounds, quench free radicals and acquire antioxidant activity[19]. Previous results have shown that the antioxidant activity may be due to withanolides, glycowithanolides and sitoindosides VII-X. Therefore, this study indicates that ASH could be a natural source of safe antioxidative and antistress agents, in line with the recently reviewed[33]. Additionally, withanolide-A and withaferin-A contents of ASH acquired antioxidant activities[34].

A study of a neuroinflammation and oxidative stress model induced by lipopolysaccharide in male rats[35] revealed an increase in the levels of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 and an increase in the levels of macrophage-microglial markers (Iba-1, ionized calcium-binding adaptor molecule 1). Where, Iba-1 is a neuroinflammatory marker of microgliosis. The study revealed that ASH pretreatment downregulated the elevated levels of serum cytokines as well as their expression, which was in line with the normalization of Iba-1 expression in the rat brain regions pyriform cortex, hippocampus and hypothalamus. The study also revealed the neuroprotective effect of ASH pretreatment in a lipopolysaccharide rat model of neurodegeneration caused by oxidative stress and apoptosis[35]. The neuroprotective effect of ASH supplementation was proven by its antioxidant potential for reducing the elevated MDA and NO contents and increasing GSH, SOD and CAT in brain areas, as recently discussed, in line with the improved antioxidant response genes of the brain cortex in ASH-supplemented mice, supporting the beneficial potential of ASH in neurodegenerative conditions[36].

The present data revealed a significant decrease in the levels of IL-1β and TNF-α in the cortex in the TRE+ASH group compared with those in the TRE group. In this respect, a previous study suggested that ASH root aquatic extract reduces the formation of proinflammatory cytokines via prevention of NF-κB activity in arthritic model rats[37]. Another study[38] reported that the serum TNF-α, IL-6, and IL-1β levels extensively decreased after ASH treatment in arthritic rats. In addition, ASH immunomodulatory activity was previously reviewed in humans and animals[39]. Also, ASH suggested previously to exert its effect by modulating elements of the proinflammatory cell signaling pathway[40]. A study revealed that ASH affect RNA stability, expression, and processing. These data suggest a mechanism of action involving the targeting of RNA-related routes, such as inflammation, which involves adjustments in gene expression. That′s in line with ASH Immunomodulatory activity in inflammatory complications as previously reviewed based on animal and human *in vitro* and *in vivo* studies[40]. The immune modulatory effect of ASH supplementation in TR groups related to its anti-anxiety and antidepressant activity recorded for animal and clinical studies[14]. That′s through the proposed mechanism neuroinflammation suppression primarily by downregulating proinflammatory cytokines and signaling pathways (p38 MAPK, NF-κB, and JNK), inhibiting reactive gliosis, and mitigating microglial and astrocyte activation, contributing to its neuroprotective and anxiolytic effects [35].

Conclusion

Ashwagandha supplementation in the context of tramadol misuse showed improvements in liver and kidney function, along with diminished antioxidant activity and inflammatory response in the brain cortex and brainstem. These findings indicate a potential supportive role for Ashwagandha in mitigating the effects of tramadol abuse; however, further studies are needed to confirm its clinical relevance.

The study was limited to examining the effects of a single standardised ASH extract dose in male rats on TRE (A & B) treated rats. More studies are needed using varying doses, both sexes, different time points, and different experimental protocols. Additionally, future studies should aim to isolate the active constituents of the extract and investigate their individual contributions to its overall mechanism of action. This would support the development of more targeted and effective therapeutic strategies for TRE addiction.

Ethical statement

The experiments and procedures were authorised by the Institutional Animal Care and Use Committee (URAF-IACUC) according to the Cairo University Institutional Animal Care and Use Committee CU-IACUC with approval number 000611922.

Conflict of interest

All the authors declare that they have no competing interest

References

1 Miotto K, Cho AK, Khalil MA, Blanco K, Sasaki JD, Rawson R. Trends in Tramadol: Pharmacology, Metabolism, and Misuse. *Anesth Analg*, 124 (2017) 44.

2 Mohammadnejad L & Soltaninejad K. Tramadol-induced organ toxicity via oxidative stress: a review study. *Int. J. Med. Toxicol. Forensic Med*, 12 (2022) 35430.

3 Srivastava AB, Mariani JJ & Levin FR. New directions in the treatment of opioid withdrawal. *The Lancet,* 395 (2020) 1938.

4 Sharifi-Rad J, Quispe C, Ayatollahi SA, Kobarfard F, Staniak M, Stępień A, Czopek K, Sen S, Acharya K, Matthews KR, Sener B, Devkota HP, Kırkın C, Özçelik B, Victoriano M, Martorell M, Suleria HAR, Alshehri MM, Chandran D, Kumar M, Cruz-Martins N & Cho WC. Chemical Composition, Biological Activity, and Health-Promoting Effects of Withania somnifera for Pharma-Food Industry Applications*. J. Food Qual*. 2021 (2021) 8985179.

5 Azab KS, Maarouf RE, Abdel-Rafei MK, El Bakary NM & Thabet NM. Withania somnifera (Ashwagandha) root extract counteract acute and chronic impact of γ-radiation on liver and spleen of rats. *Hum Exp Toxicol*, 41 (2022) 9603271221106344.

6 Speers AB, Cabey KA, Soumyanath A & Wright KM. Effects of Withania somnifera (Ashwagandha) on Stress and the Stress- Related Neuropsychiatric Disorders Anxiety, Depression, and Insomnia. *Curr Neuropharmacol*, 19 (2021) 1468.

7 Hashem HA, Nabil ZI, & El-Hak HNG. Ameliorative effect of ashwagandha (Withania somnifera) root extract on brain oxidative stress and depression of diabetic rats. *J Adv Vet Res*, 13 (2023) 508.

8 Reagan-Shaw S, Nihal M & Ahmad N. Dose translation from animal to human studies revisited. *FASEB J*, 22 (2008) 659.

9 Ma S, Liu X, Cheng B, Jia Z, Hua H & Xin Y. Chemical characterization of polysaccharides isolated from scrophularia ningpoensis and its protective effect on the cerebral ischemia/reperfusin injury in rat model. *Int J Biol Macromol*, 139 (2019) 955.

10 Tjandra AD, Heywood T & Chandrawati R. Trigit: A free web application for rapid colorimetric analysis of images. *Biosens. Bioelectron: X*, 14 (2023) 100361.

11 Holtman Jr & Joseph R. "Basic Science: Opioid Receptors." Anesthesiology In-Training Exam Review: Regional Anesthesia and Chronic Pain. Cham: *Springer International Publishing*, (2022) 191.

12 Manouchehri A, Nekoukar Z, Malakian A & Zakariaei Z. Tramadol poisoning and its management and complications: a scoping review. *Ann Med Surg*, 85 (2023) 3982.

13 Boun SS, Omonaiye O & Yaya S. Prevalence and health consequences of nonmedical use of tramadol in Africa: A systematic scoping review. *PLOS Glob Public Health*, 4 (2024) e0002784.

14 Mohamed HM, & Mahmoud AM. Chronic exposure to the opioid tramadol induces oxidative damage, inflammation and apoptosis, and alters cerebral monoamine neurotransmitters in rats. *Biomed Pharmacother*, 110 (2019) 239.

15 Adebajo OA, Adebajo PK, Ayoade OH, Akpan UU, Ojo JH & Akande F. Ameliorative Role of Virgin Coconut Oil on Tramadol-Induced Nephrotoxicity in Sprague–Dawley Rats. *Asian J. Res. Med. Med. Sci*, 6 (2024) 24.

16 Alowaid HFH, Hassan AA & Majeed MF. Functional and histological signs of early detection of liver and kidney stress under the influence of tramadol oral administration in male rats. *Adv. Anim. Vet. Sci*, 12 (2024) 1556.

17 Mousavi K, Manthari RK, Najibi A, Jia Z, Ommati MM & Heidari R. Mitochondrial dysfunction and oxidative stress are involved in the mechanism of tramadol-induced renal injury. *Curr Res Pharmacol Drug Discov*, 2 (2021) 100049.

18 Elmorsy EM, Al Doghaither HA & Al-Ghafari AB. Bioenergetics disruption, oxidative stress, and inflammation as underlying mechanisms of tramadol-induced nephrotoxicity. *J Biochem Mol Toxicol*, 38 (2024) e23777.

19 Mikulska P, Malinowska M, Ignacyk M, Szustowski P, Nowak J, Pesta K, Szeląg M, Szklanny D, Judasz E, Kaczmarek G, Ejiohuo OP, Paczkowska-Walendowska M, Gościniak A, Cielecka-Piontek J. Ashwagandha *(Withania somnifera)*-Current Research on the Health-Promoting Activities: A Narrative Review. Pharmaceutics. 15 (2023) 1057.

20 Wangikar P, Chaudhari P, Sharma E, Godse C, Vora A & Nair S. Acute and subchronic oral GLP toxicity of *Withania somnifera* root extract in Sprague Dawley rats. *Drug Metab Pers Ther*, 39 (2024) 145.

21 Siddiqui S, Ahmed N, Goswami M, Chakrabarty A & Chowdhury G. DNA damage by Withanone as a potential cause of liver toxicity observed for herbal products of Withania somnifera (Ashwagandha). *Curr Res Toxicol*, 2 (2021) 72.

22 Alghamdi AAA, Abdallah EAA & El-Refaei MF. Anti-inflammatory and anti-angiogenic effects of Withania somnifera extract on liver toxicity induced by silver nanoparticles in vivo. *J Med Life*, 17 (2024) 728.

23 Rabey HA El, Rezk SM, Abusaber A, Khlabi R, Alhawiti AH, M. Algorayed R & Bakry N. The Protective Activity of Withania somnifera Against Mercuric Chloride (HgCl2) -Induced Renal Toxicity in Male Rats. *Int J Nephrol,* 2024 (2024) 8023989.

24 Mehranpour M, Moghaddam MH, Abdollahifar MA, Salehi M & Aliaghaei A. Tramadol induces apoptosis, inflammation, and oxidative stress in rat choroid plexus. *Metab Brain Dis*, 38 (2023) 2679.

25 Albrakati A. Neuroprotective effect of physical exercise on neuronal apoptosis induced by tramadol in cerebral cortex of rats. Biointerface. *Res Appl Chem*, 10 (2020) 7209.

26 Taiwo A, Akinyinka A, Esther A, Ayotunde A, Abayomi A, Fasesan O & Olawale O. Neuro-protective effect of pre-treatment with Sorghum bicolor and vitamin C on tramadol induced brain oxidative stress and anxiety-like behaviour in male albino rats. *Pak J Pharm Sci,* 37(2024) 53.

27 Abdel-Zaher AO, Abdel-Rahman MS, & ELwasei FM. Protective effect of Nigella sativa oil against tramadol-induced tolerance and dependence in mice: Role of nitric oxide and oxidative stress. *NeuroToxicology*, 32 (2011) 725.

28 Nafea OE, ElKhishin IA, Awad OA & Mohamed DA. A study of the neurotoxic effects of tramadol and cannabis in adolescent male albino rats. *Int J Sci Rep*, 2 (2016) 143.

29 Greco MC, Navarra P & Tringali G. The analgesic agent tapentadol inhibits calcitonin gene-related peptide release from isolated rat brainstem via a serotonergic mechanism. *Life Sci*, 145 (2016) 161.

30 Mohamed TM, Ghaffar HM & El Husseiny RM. Effects of tramadol, clonazepam, and their combination on brain

mitochondrial complexes. *Toxicol Ind Health*, 31 (2015) 1325.

31 Aghajanpour F, Boroujeni ME, Jahanian A, Soltani R, Ezi S, Khatmi A, Abdollahifar MA, Mirbehbahani SH, Toreyhi H & Aliaghaei A. Tramadol: a potential neurotoxic agent affecting prefrontal cortices in adult male rats and PC-12 cell line. *Neurotox Res,* 38 (2020) 385.

32 Liu T, Zhang L, Joo D & Sun SC. NF-κB signaling in inflammation. *Signal Transduct Target Ther*, 2 (2017) 1.

33 Lerose V, Ponticelli M, Benedetto N, Carlucci V, Lela L, Tzvetkov NT & Milella L. Withania somnifera (L.) Dunal, a Potential Source of Phytochemicals for Treating Neurodegenerative Diseases: A Systematic Review. *Plants,* 13 (2024) 771.

34 Polumackanycz M, Petropoulos SA, Śledziński T, Goyke E, Konopacka A, Plenis A & Viapiana A. Withania somnifera L.: Phenolic Compounds Composition and Biological Activity of Commercial Samples and Its Aqueous and Hydromethanolic *Extracts. Antioxidants (Basel),* 12 (2023) 550.

35 Gupta M & Kaur G. Withania somnifera as a Potential Anxiolytic and Anti-inflammatory Candidate Against Systemic Lipopolysaccharide-Induced Neuroinflammation. *Neuromolecular Med,* 20 (2018) 343.

36 Gladen-Kolarsky N, Monestime O, Bollen M, Choi J, Yang L, Magaña AA, Maier CS, Soumyanath A & Gray NE. Withania somnifera (Ashwagandha) Improves Spatial Memory, Anxiety and Depressive-like Behavior in the 5xFAD Mouse Model of Alzheimer's Disease. *Antioxidants*, 13 (2024) 1164.

37 Khan M, Subramaneyaan M, Arora V, Banerjee B & Ahmed R. Effect of Withania somnifera (Ashwagandha) root extract on amelioration of oxidative stress and autoantibodies production in collagen-induced arthritic rats. *J. Complement. Integr. Med*, 12 (2015) 117.

38 Padigaru M, Morde A, Muz O, Orhan C, Erten F, Tuzcu M, Ozercan I, Sahin K & Rai D. Efficacy of a Novel Integrated Active Herbal Formulation in Experimentally Induced Osteoarthritis Rat Model. *Curr. Devel. Nutr,* 4 (2020)1762.

39 Alanazi HH & Elfaki E. The immunomodulatory role of withania somnifera (L.) dunal in inflammatory diseases. *Front. Pharmacol,* 14 (2023) 1084757.

40 Saha P, Ajgaonkar S, Maniar D, Sahare S, Mehta D & Nair S. Current insights into transcriptional role(s) for the nutraceutical Withania somnifera in inflammation and aging. *Front. Nutr,* 11 (2024) 1370951.