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ORIGINAL RESEARCH article

Jobelyn® ameliorates anxiety response and oxido-inflammatory markers induced by tramadol use and discontinuation in rats

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Abstract: Jobelyn® is a multi-functional natural dietary supplement made from Sorghum bicolour with very high anti-oxidant and anti-inflammatory capacities. The study investigated the role of Jobelyn® in the attenuation of oxido-inflammatory markers induced by tramadol use, abuse and discontinuation over 17 days in rats. The experimental observational study was carried out using male adult albino rats weighing between 100 and 170 g. The experimental design involved five groups. Rats were randomly divided into groups of five, consisting of group 1 (normal control rats), and group 2 (rats treated with tramadol at 40 mg/kg/day) were administered for 10 days and discontinued for seven days. Group 3 administered incremental doses of tramadol from 40 mg/kg/day to 100 mg/kg/day over 10 days and discontinued for seven days. A similar treatment protocol was administered for group 4 and group 5 but were treated with Jobelyn® at a dose of 200 mg/kg/day at the discontinuation phases for seven days. Behavioral assessments (elevated plus maze model of anxiety and open field model of locomotor activity) and biomarkers of oxido-inflammatory stress were assessed. Tramadol-treated groups had significant anxiety responses and locomotory deficits in comparison to the control group. Tramadol-treated groups had significant elevations of nitrites and malondehyde and reduced enzymatic markers such as catalase, glutathione, reduced glutathione, superoxide dismutase, G-s-transferase, glutamic acid decarboxylase and increased activity of acetylcholinesterase when compared to control group. Administration of Jobelyn® attenuated the responses and ameliorated the oxido-inflammatory biomarkers similar to levels in control group. Tramadol induces oxido-inflammatory stress markers in the prefrontal, striatum and hippocampus in rats. Anxiety and locomotory behavioral actions on tramadol treatment were elevated despite discontinuation for seven days. Thus, Jobelyn® at 200 mg/kg/day ameliorated oxido-inflammatory markers induced by tramadol and decreased anxiety responses in albino rats.

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Introduction

Abusive use of tramadol has been reported to have significant morbidity and mortality in humans [1]. Tramadol has proved to be an effective analgesia in the treatment of moderate, chronic and severe pains. The major mechanism of action responsible for the analgesic properties is the affinity for morphine opioid receptor (MOP) or μ receptor [2]. Tramadol has become a popular drug not for its analgesic properties, but due to its off-the-label uses. In many countries, the abusive use of tramadol has become a major social problem [3]. The increasing epidemic of tramadol abuse has become a cause for concern and laws have been enacted in many countries to regulate the use [3]. The epidemic is largely due to other atypical uses of tramadol [3, 4]. Atypical use of tramadol is quite prevalent, tramadol has been used to treat premature ejaculation, and libido enhancement and has been reported to have euphoric and anti-anxiety properties [5, 6]. The anti-anxiety properties are as a result of the receptor blockage of 5-HT [7-9]. Similar to the mechanism of action of selective serotonin receptor inhibitors, receptor blockage of 5-HT is instrumental in reliving anxiety [9]. This intrinsic mechanism of tramadol confers on it the anti-anxiety properties which may potentially help relieve depression [7-9]. However, regardless of the beneficial effect, tramadol abuse has varied deleterious effects due to its potential to have opioid-like consequences in abuse and dependence. More often, symptoms such as seizures, respiratory depression, coma and death are reported [7]. The majority of other symptoms are due to organ toxicity. Organ toxicity is due to the induction of oxide-inflammatory stress in tramadol abuse [10]. A major concern is the effect on the central nervous system. Neurotoxicity was well documented in chronic tramadol use and abuse and was adduced to some of the symptoms in abuse [10, 11]. The brain is particularly vulnerable due to the high utilization of oxygen, high lipid content and low number of antioxidants [11]. Several studies have focused on the chronic use of tramadol at various doses and few have experimented with the effect on the oxido-inflammatory markers when tramadol is discontinued. Oxido-inflammatory stress may underlie many of the other side effects or symptoms that are reported in tramadol abuse [11]. These cascades may persist when discontinued. Strengthening the buffer capacity of the brain through the use of antioxidants is a way of mitigating the effect of tramadol abuse. Particularly, neurotoxicity can affect behavioral responses and cognition usually seen in dependence. Attenuation of tramadol-induced oxido-inflammatory cascade may alleviate the behavioral and oxido-inflammatory correlates seen in tramadol abuse. A common herbal antioxidant that was investigated in many chronic inflammatory and oxidative stress conditions is Jobelyn® (JB). This common antioxidant JB, is made from sorghum bicolor and contains a variety of naturally occurring antioxidants which have wide beneficial effects in many chronic inflammation and disease conditions [12]. We investigated the role of JB in attenuating anxiety behavior and oxido-inflammatory stress in use, abuse and sudden discontinuation of tramadol at a fixed dose and incremental doses using rats.

Material and methods

Animals: Male adult albino rats weighing between 100 g and 170 g, were obtained from the Central Animal House, Sagamu, Ogun State, Nigeria were used. Rats were placed under standard environmental conditions with 12/12 hrs, light/dark cycle). Rats were fed with standardized pelletized rat chow (Ladokun Feed, Ibadan) with a free access to water ad libitum. Acclimatization was for 14 days. Ethical approval was obtained from the Ethical Committee of the University of Lagos and registered under CMUL/ACUREC/11/21/972.

Acute toxicity test of Jobelyn®: Acute toxicity test was done following the method described by the Organization of Economic Cooperation and Development 423 guideline (OECD) [13]. Rats were divided into two groups, each group contained three rats. The first group was administered 200 mg/kg of JB extract, the extract was prepared by dissolving the contents of the capsules in equal volumes of water and the second group was administered

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distilled water as a control. JB manufactured by Health Forever Products Limited, Lagos, Nigeria was used. Rats were monitored for signs of acute toxicity within the first four hours and then every day for 14 days. The rats were sacrificed on the 14th day and the organs were weighed and monitored macroscopically.

Experimental design: The study design aimed at assessing the effect of use, abuse and subsequent discontinuation of tramadol and the ameliorative effect of JB. The doses of tramadol were based on the previous studies [14, 15]. JB (200 mg/kg/day) was used as the recommended highest dose based on earlier research on the attenuation of oxidative stress in neuronal toxicity [16]. The rats were randomly divided into five groups of five rats per group. The administration of the test substances involved only water, tramadol and JB. In group 1 which served as a control, water was administered throughout the 17 days at 10 ml/kg. In group 2, tramadol was administered at a constant dosage of 40 mg/kg for 10 days and then discontinued and served with water for the remaining seven days. In group 3, incremental doses of tramadol from 40 to 100 mg/kg, which was spread out over 10 days and stopped and then served with water for the remaining seven days. In group 4, tramadol was administered at a dose of 40 mg/kg for 10 days and stopped, followed by JB for the remaining seven days. In group 5, tramadol dosage was gradually increased from 40 to 100 mg/kg over 10 days, and JB was then administered after the stoppage of tramadol for another seven days.

Elevated plus-maze test (EPM): This test was used to evaluate the effect of tramadol treatment and discontinuation on anxiety-like behavior. Experiments were conducted on day 17th, a week after the stoppage of tramadol. Each rat was placed at the center of the maze with its head facing an open arm and the frequencies and duration of arm entries were recorded for five minutes. An entry was scored when the four paws of the rats were all on one arm. 70.0% of ethanol was used to clean the EPM after each test [17]. Data were expressed as time spent on arms and the percentage of the number of entries into arms. The index of open arm avoidance (IOAA) was calculated as

$$\text{IOAA} = 100 - \frac{\%\text{ duration of time spent in open arms} + \%\text{ entries into open arms}}{2}$$

Open field test: Assessment of locomotory function impairments and anxiety-like behavior in rats was performed in the open field test at the end of day 17, a week after the discontinuation of tramadol. A wooden cubic box measuring 40 cm in three dimensions (40×40×40 cm^3) was horizontally divided into 16 squares of equal size, measuring 10 cm in length, and breadth (10x10 cm^2) the central four squares (20×20 cm^2) are considered the center and the surrounding four sides (10×15 cm^2) and four corners (10×15 cm^2) are considered as surrounding, the rat was placed at the center of the box and allowed to acclimatize for one minute, then allowed to explore the box for five minutes which was recorded by a video camera and analyzed [17]. The following behavioral items (total distance traveled, average speed, number of entries in the center area, and time spent in the center area) were analyzed with an automated behavioral testing video tracker (AnyMaze Software version 6.19).

Measurement of oxidative stress parameters: 20 hrs after the behavioral tests, blood was collected and the serum was stored at - 40^{o}C. Rats were euthanized and the rats were euthanized by dislocation of the cervical using ether anesthesia. Homogenization of the whole brain was done using 10.0% phosphate buffer. The supernatant was used for biochemical analysis.

Lipid peroxidation level: Tissue malondialdehyde was measured as an index of lipid peroxidation using the assay of thiobarbituric reacting substances [18]. 100 µL of supernatant was diluted ten times in 0.15 M Tris-KCl buffer and deproteinized with 500 µL trichloroacetic acid (30.0%). The mixture was then centrifuged in a benchtop centrifuge at 4000 rpm for 10 min at room temperature, 200 µL of the supernatant was removed into Eppendorf tubes, followed by the addition of 200 µL thiobarbituric acid (1.0%), and the mixture was heated at 80°C for one

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hour. The tubes were cooled by placing them on ice, 200 µL was then removed into a micro-titer plate, and absorbance was measured at 532 nm. Data was calculated using an index of absorption for MDA (molar extinction coefficient 1.56 x 105 /M/cm). TBARS concentrations in the tissues were expressed as ηmole MDA/ mg protein.

Nitrite levels: Brain nitrite concentration was estimated by Griess reagent [19], which serves as an indicator of nitric oxide production. 100 µL of Griess reagent (1:1 solution of 1.0% sulfanilamide in 5.0% phosphoric acid and 0.1% of N-1-naphthyl ethylenediamine dihydrochloride) was added to 100 µL of the supernatant and the absorbance was measured at 540 nm in LT-4500 microplate reader (Labtech). The nitrite concentrations were then estimated from a standard curve obtained from sodium nitrite (0.0-100 µM) and expressed as µmoles/mg protein.

Reduced glutathione (GSH*) levels:* GSH as a non-enzymatic antioxidant maker was measured in the supernatant following the method described by Jollow and others [20]. 100 µL of supernatant was diluted 10 times in 0.15 M Tris-KCl buffer, and deproteinized with 500 µL trichloroacetic acid (30%). The mixture was centrifuged in a benchtop centrifuge at 4000 rpm for 10 min at room temperature. 100 µL of the deproteinized supernatant was mixed with 100 µL of 51-di-nitrobenzoic acid (DTNB, 0.0006 M) in microplate. The absorbance was read within five minutes at 405 nm in an LT-4500 the microplate reader (Labtech, UK). The glutathione concentration was extrapolated from the standard curve of glutathione (0.0-200 µM) and expressed as µmole GSH/ mg protein.

Catalase enzyme assay: Catalase activity in the supernatants of the brain sections was determined using the colorimetric assay based on the yellow complex with molybdate and H_2O_2 which was described by Goth et al. [21]. 50 µL of 2X diluted supernatant was added to a microtiter plate, and then 50 µL of a reaction mixture containing 65.0 mmol/mL of H_2O_2 in sodium-potassium phosphate buffer (60 mM, pH 7.4) was added. The enzymatic reaction was incubated for three minutes and stopped with 100 µL of ammonium molybdate (64.8 mM) in sulfuric acid. The absorbance at 405 nm was measured in LT-4500 microplate reader (Labtech). The catalase enzyme activity unit was expressed as U/mg protein.

Determination of superoxide dismutase (SOD): The level of SOD was determined by the method of Misra and Fridovich [18]. Superoxide dismutase activity is determined based on its ability to inhibit the autoxidation of adrenaline in sodium carbonate buffer (pH 10.7). 50 µL of 2X diluted supernatant was added into a micro-titer plate containing 150 µL of carbonate buffer. The reaction started with the addition of 30 µL of freshly prepared 0.3 mM adrenaline to the mixture. Blank was prepared using 50 µL of distilled water. The increase in absorbance at 495 nm was monitored every 60 sec for 300 sec in the LT-4500 microplate reader (Labtech). The SOD activity was expressed as U/mg protein.

Assay for glutamic acid decarboxylase (GAD): The procedure described by Yu et al. [22] was used to estimate GAD enzyme activity in the supernatant of the brain tissues. Briefly, 50 µL aliquots of 2X diluted brain tissue supernatant were incubated with a reaction mixture containing 20 mM sodium acetate, 70 µM bromocresol, 10 mM pyridoxal-5-phosphate, and 2.0 µL glutamate (from 1.0 M stock in acetate buffer). The increase in absorbance at 630 nm for five minutes was read in a microplate reader (LT4500). The unit of the enzyme was expressed as µmoles per min. mg protein using the extinction coefficient 23700 M^{-1} x cm^{-1}.

Determination of brain acetylcholinesterase (AChE): The procedure described by Ellman et al. [23] was used to estimate AChE activity in the supernatant of the brain tissues. Briefly, 50 µL aliquots of brain supernatant were diluted with 50 µL of phosphate buffer followed by the addition of 50 µL of DTNB (0.0001 M) in a 96-well plate. The initial absorbance was first measured after five minutes of incubation with DTNB. Thereafter, 50 µL of acetylthiocholine iodide (0.028 M) was added to the mixture for three minutes and the absorbance was again measured at 405 nm in a microplate reader (LT4500). The rate of AChE activity (µmol/min/mg tissue) was

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calculated as R = 5.74 x 10-4x A/Co Where: R rate in moles of substrate hydrolyzed per min per g tissue, A change in absorbance/min, and Co = original concentration of the tissue.

Estimation of total protein: Protein concentrations of the various samples were determined by the Biuret method using bovine serum albumin (BSA) as standard [24]. The samples were diluted to make up 1: 4 with sodium phosphate buffer. 50 µL of diluted supernatant was added to the microtiter plate, and 200 µL of Biuret reagent was added. The mixture was incubated at room temperature for 30 min after the absorbance was read at 540 nm in LT-4500 microplate reader (Labtech). The protein concentration was extrapolated from BSA standard curve.

Estimation of TNF-α and IL-6: TNF-α and IL-6 concentrations were determined using supernatants that were obtained from the prefrontal cortex, hippocampus and striatum. This procedure followed the manufacturer's instructions. Specific mouse TNF-α was measured using TNF-α and IL-6 mouse and IL-6 (BioLegend ELISA MAX™ Deluxe kit, USA). We adapted the sensitivity limit of 4.0 pg/mL and were done at room temperature. We use a microplate reader with 450 nm filter. Estimation of the TNF-α and IL-6 from the tissues was extrapolated from the standard curve of TNF-α and IL-6 standards included in the assay kits. Concentrations of TNF-α and IL6 in the different brain regions were measured as pg/g tissues.

Statistical analysis: Data were analyzed using one-way ANOVA and two-way ANOVA followed by Tukey's *post-hoc* multiple comparison test using Graphpad Prism (Graphpad software, version 8.0).

Results

Acute toxicity test: Administration of a single oral dose JB (200 mg/kg) did not show any sign of immediate toxicity in rats when compared with the control group within the four hours post-administration. Daily observation of rats for 14 days for delayed toxic effect did not show any loss of appetite, diarrhea, weight loss or change in skin coloration of the rat. The change in weight is shown in **Table 1**. Thus, there was no delayed effect on body weight or organ weights of rats that received JB (200 mg/kg) when compared with control rats at the end of the 14-day observation period.

Table 1: Acute toxicity test in rats using control and Jobelyn® treated group

	Control	JB (200 mg/kg)
Body weight in gram (g)		
Initial body weight	144.33±8.29	150.33±2.03
Final body weight	170.00±10.00	175.67±1.86
Weight gained	25.67±2.33	25.33±1.53
Relative organ weight (mean±S.E.M.)		
Brain	0.75±0.12	0.89±0.01
Liver	3.72±0.11	3.00±0.17
Heart	0.35±0.02	0.31±0.03
Kidneys	0.72±0.05	0.64±0.17
Spleen	0.41±0.08	0.37±0.03

Data of the behavioral assessments are detailed accordingly, thus, the anxiety indices on the EPM are shown in **Figure 1**. Thus, the open arm entries and duration time on the open arms of the EPM are significantly increased upon administration of 40 mg/kg tramadol and 100 or 200 mg/kg Jobelyn® together.

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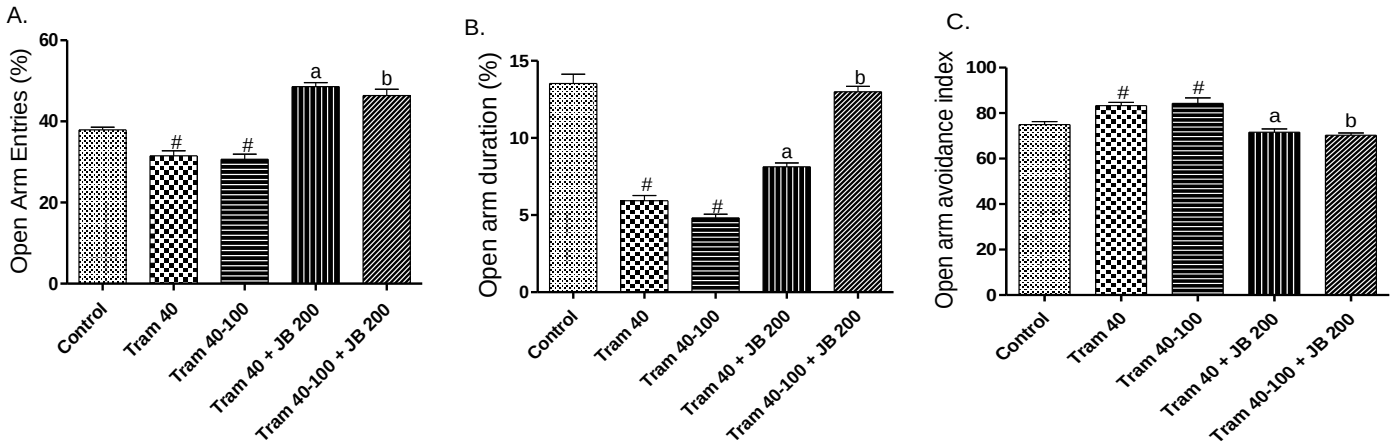
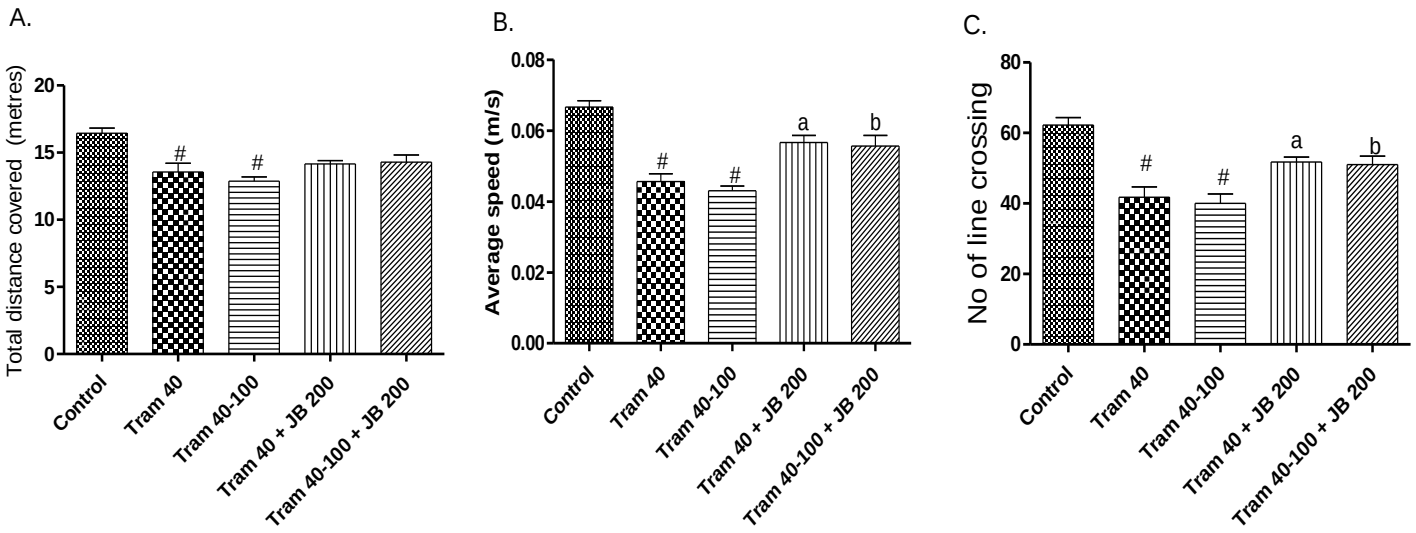
Figure 1: Anxiety indices of tramadol and Jobelyn® by elevated plus-maze test



Bars represent mean±S.E., (n=5). # P < 0.05 vs control using one-way ANOVA followed by Tukey's *post hoc* test.
Tram: tramadol, JB: Jobelyn®

In **Figure 2**, findings of the behavioral assessments of open filed model of locomotor motion are detailed accordingly, the, motor activities in the OFT are shown. Data of the ambulatory activities are significantly increased in the rats after administration of 40 mg/kg tramadol and 100 or 200 mg/kg Jobelyn® together (after tramadol stoppage).

Figure 2: Locomotor activity of the rats upon tramadol and Jobelyn® intake by open field test



Bars represent mean±S.E., (n=5). # P < 0.05 vs control using one-way ANOVA followed by Tukey's *post hoc* test.
Tram: tramadol, JB: Jobelyn®

In **Figure 3**, data of the behavioral assessments of the open-filed model of the locomotor motion of rats are detailed accordingly. Thus, the locomotion activities in the model are shown. Data of the freezing activities are significantly decreased in the rats after administration of 40 mg/kg tramadol and 100 or 200 mg/kg Jobelyn® together (after tramadol stoppage).

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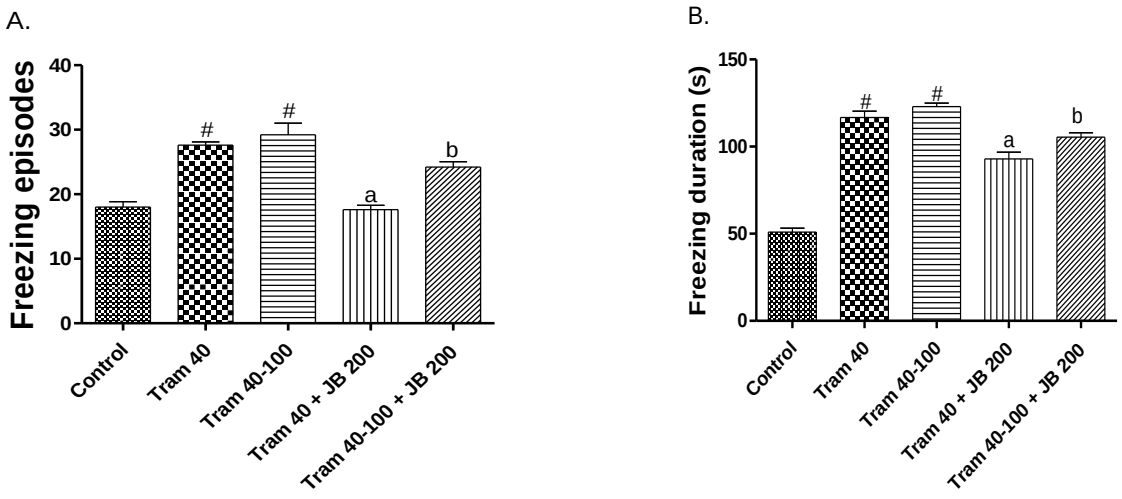
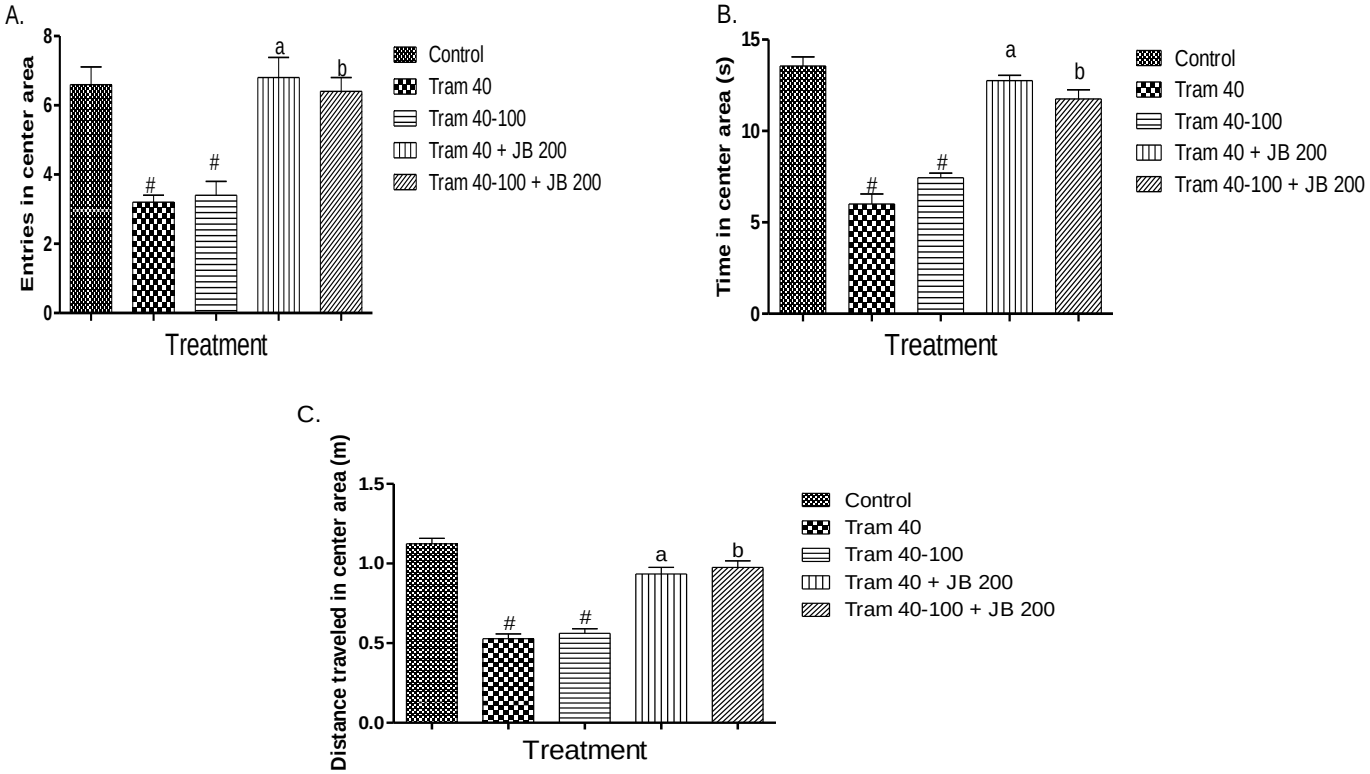
Figure 2: Freezing activity of the rats upon tramadol and Jobelyn® intake by using open field test



Bars represent mean±S.E., (n=5). # P < 0.05 vs control using one-way ANOVA followed by Tukey's *post hoc* test.
Tram: tramadol, JB: Jobelyn®

In **Figure 4**, the findings of the behavioral assessments of open filed model of locomotor motion in the central area are detailed accordingly, the, central activities in the OFT are shown. Data of the central activities are significantly increased in the rats after administration of 40 mg/kg tramadol and 100 or 200 mg/kg Jobelyn® together.

Figure 4: Central area activities of the rats upon tramadol and Jobelyn® intake by open field test



Bars represent mean±S.E., (n=5). # P < 0.05 vs control using one-way ANOVA followed by Tukey's *post hoc* test.
Tram: tramadol, JB: Jobelyn®

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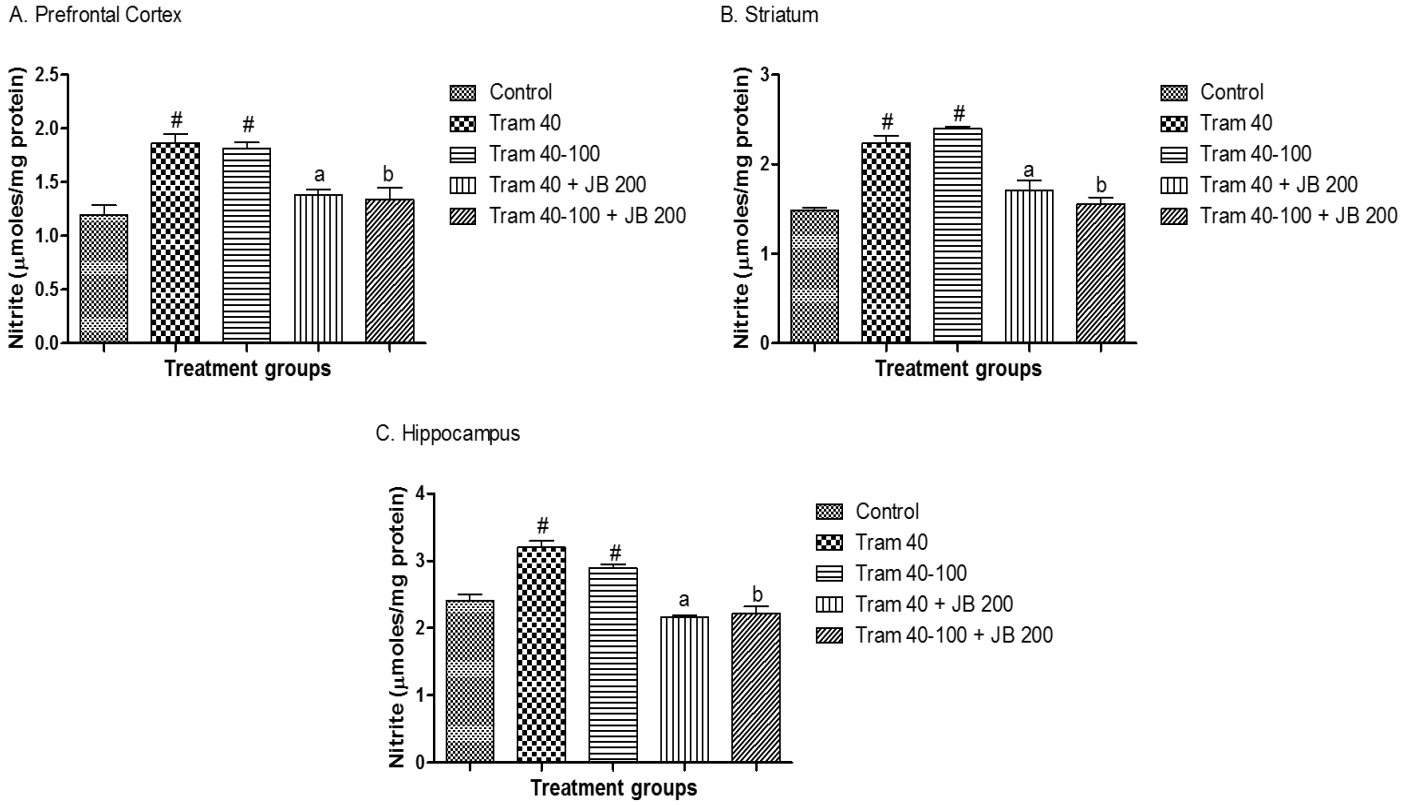
Amelioration of nitric oxide (NO) in tramadol discontinuation with JB 200 mg/kg: Nitrite level in the prefrontal, striatum and hippocampus were measured as a maker of NO production and indirect measure of inflammation and oxidative stress. Tramadol at a fixed dose of 40 mg/kg, for 10 days and tramadol at incremental doses of 40-100 mg/kg over 10 days significantly increased NO production compared to the controls. Administration of JB reduced nitric oxide production and there was no significant difference between elevations of NO production and controls as shown in **Figure 5**.

Figure 5: Amelioration of nitrite in the prefrontal, striatum and hippocampus in control post-discontinuation of tramadol administration and Jobelyn® treatment



Bars represent mean±S.E., (n=5). # $P < 0.05$ vs control using one-way ANOVA followed by Tukey's *post hoc* test.
Tram: tramadol, JB: Jobelyn®

Amelioration of malondehyde (MDA) in tramadol discontinuation on JB 200 mg/kg treatment: MDA measurements in the prefrontal, striatum and hippocampus are shown in **Figure 6**. Significant differences in MDA levels as measured in the tramadol-only groups were observed in comparison to controls. Administration of JB 200 mg/kg reduced the elevations of MDA in the tramadol 40 mg/kg fixed dosing and tramadol 40-100 mg/kg groups when compared with the controls.

Restoration of enzymatic oxidative stress markers in tramadol induced dependence on the administration of JB 200 mg/kg: Measurements of the levels of gluthaione-s-hydroenase (GSH), superoxide dismutase (SOD), catalase gluthathione-S-transferase (GST) and glutamic acid decarboxylase (GAD) were significantly depleted in tramadol only treated groups when compared to the controls. However, on the administration of JB 200 mg/kg, levels of the enzymatic markers were not significantly different from the controls (**Figure 7**).

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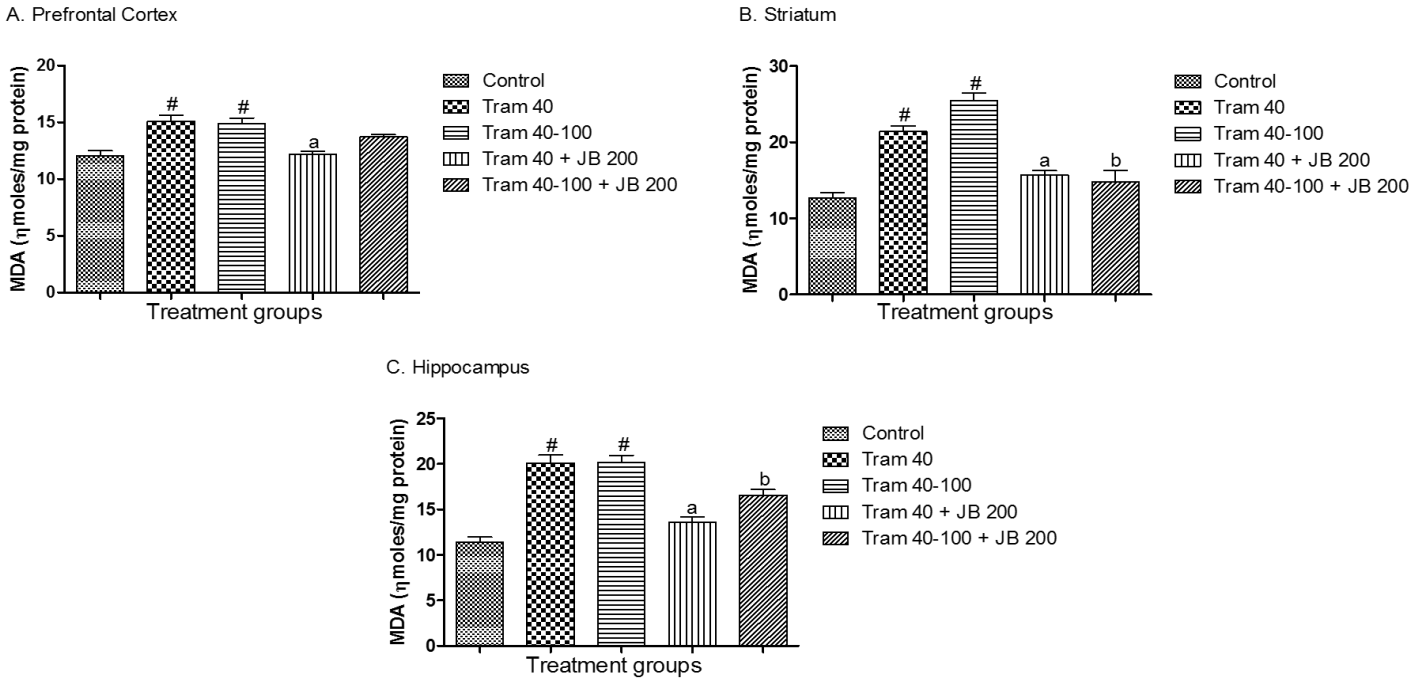
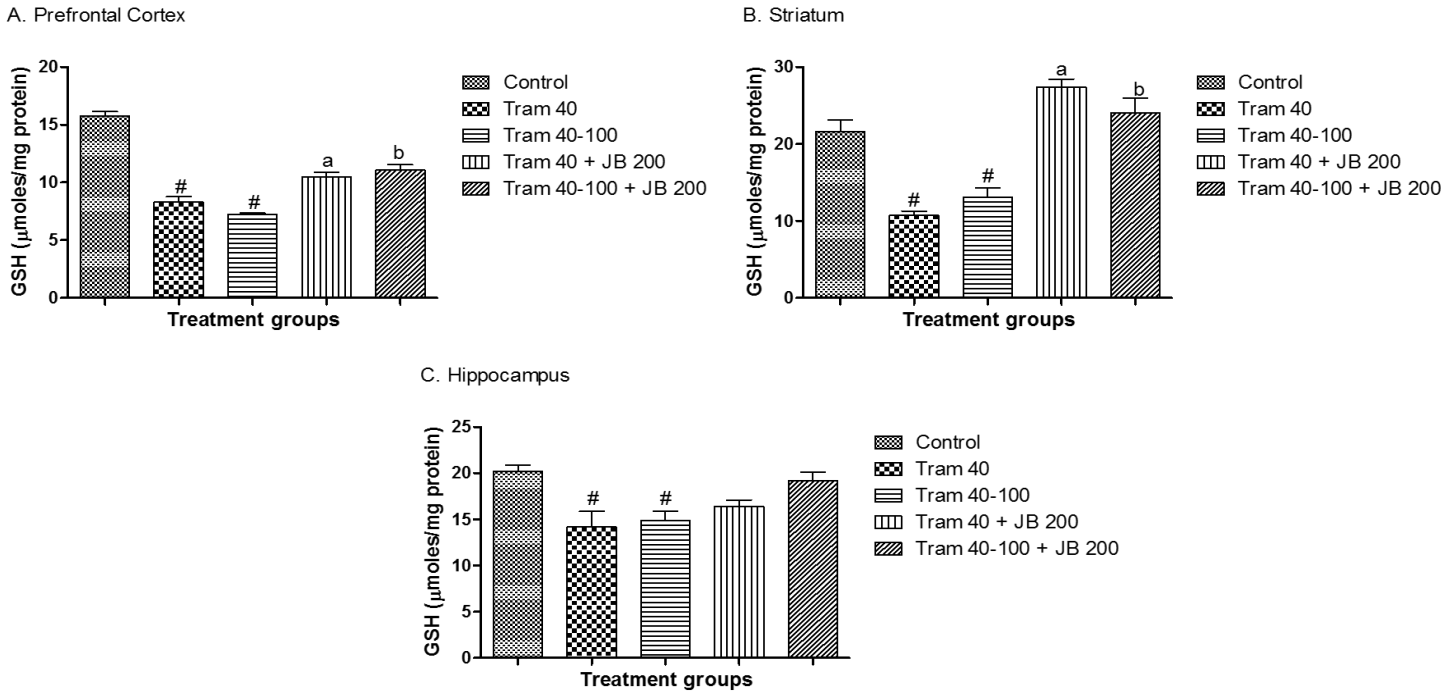
Figure 6: Amelioration of gluthaione-s-hydroenase activity in tramadol dependence on Jobelyn® intake



Bars represent mean±S.E., (n=5). # P < 0.05 P=0.028 vs control using one-way ANOVA followed by Tukey's *post hoc* test.
Tram: tramadol, JB: Jobelyn®

Figure 7: Glutathione level as oxidative markers in treatment groups



Bars represent Mean±S.E., (n=5). # P < 0.05, P = 0.034 vs control using one way ANOVA followed by Tukey's *post hoc* test.
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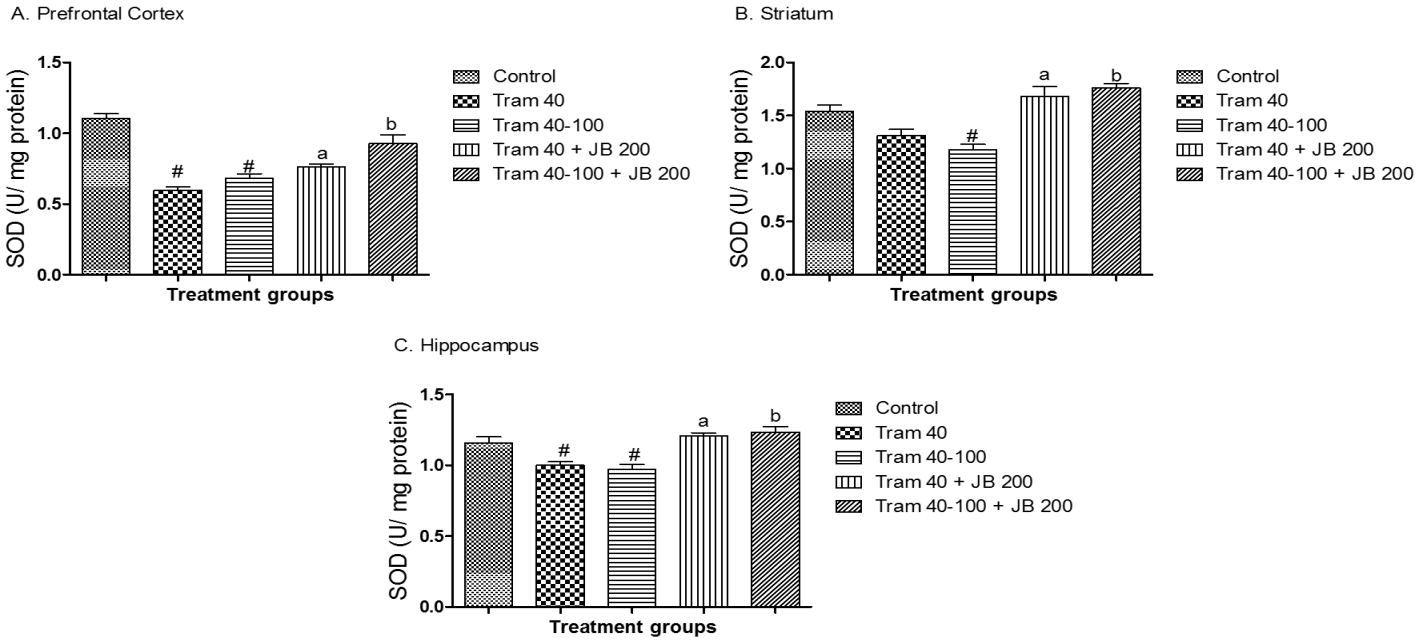
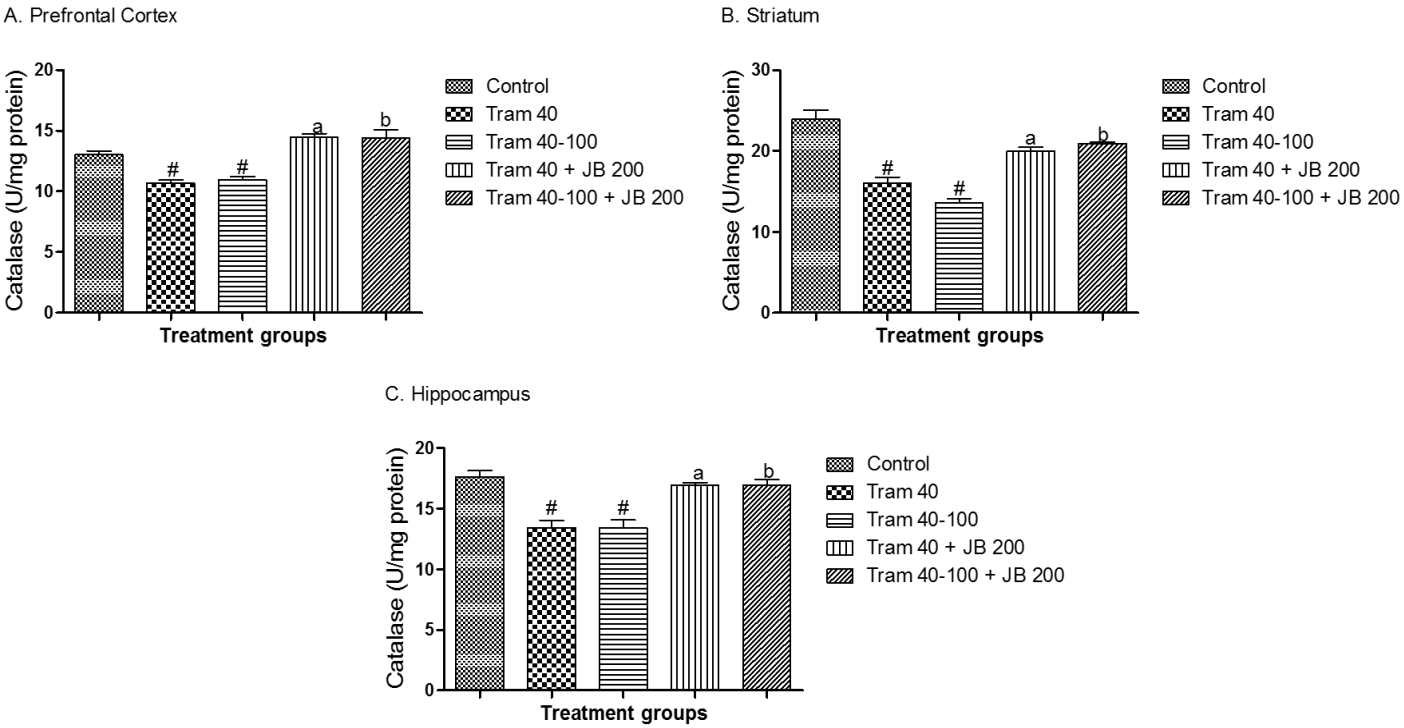
Figure 8: Superoxide dismutase as enzymatic oxidative markers in treatment groups



Bars represent mean±S.E., (n=5). # P < 0.05, P=0.04 vs control using one-way ANOVA followed by Tukey's *post hoc* test.
Tram: tramadol, JB: Jobelyn®

Figure 9: Catalase level as enzymatic oxidative markers in the treatment group



Bars represent mean±S.E., (n=5). # P < 0.05, P= 0.032 vs control using one-way ANOVA followed by Tukey's *post hoc* test.
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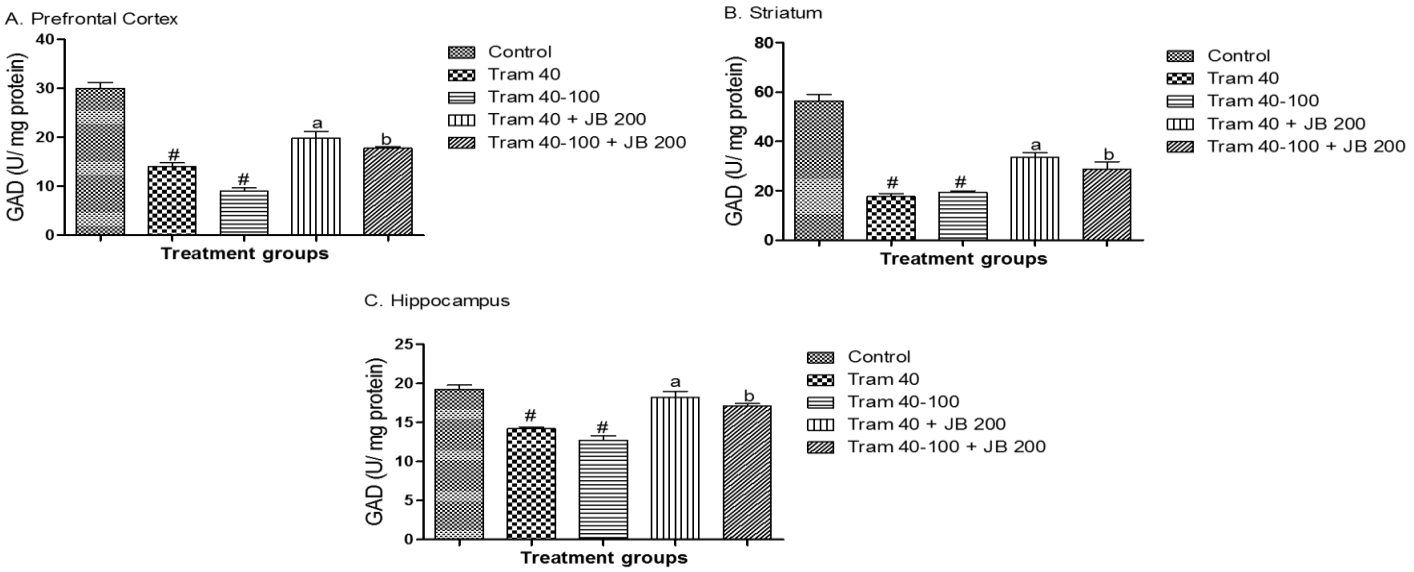
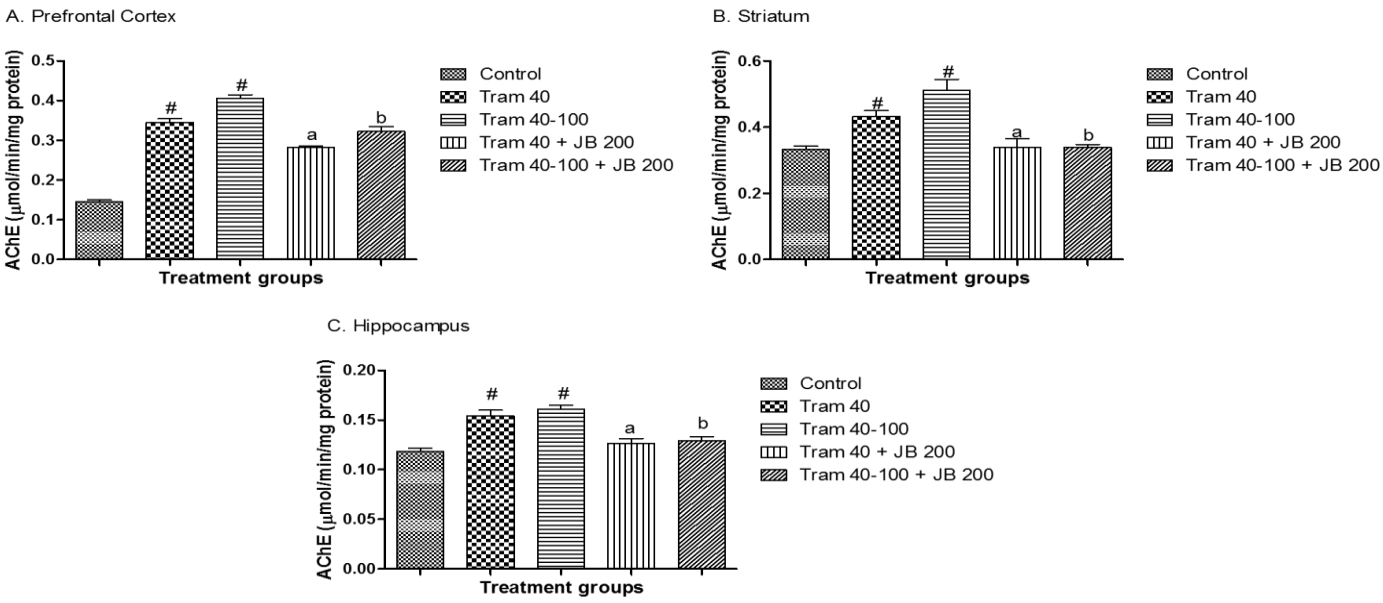
Figure 10: Glutamic acid decarboxylase as enzymatic oxidative markers in treatment groups



Bars represent mean±S.E., (n=5). # $P < 0.05$, $P = 0.025$, vs control using one-way ANOVA followed by Tukey's *post hoc* test.
Tram: tramadol, JB: Jobelyn®

Brain neurotransmitter-related enzyme activity in tramadol treated with JB: AChE activity as shown in **Figure 11** was significantly increased in the administration of tramadol in rats to induce dependence when compared with the control group. However, administration of JB (200 mg/kg) caused a reduction in the activity of AChE to levels that were not significantly different from controls.

Figure 11: Acetylcholinesterase a levels as enzymatic oxidative markers in treatment groups



Bars represent Mean±S.E., (n=5). # $P < 0.05$, $P = 0.038$ vs control using one-way ANOVA followed by Tukey's *post hoc* test.
Tram: tramadol, JB: Jobelyn®

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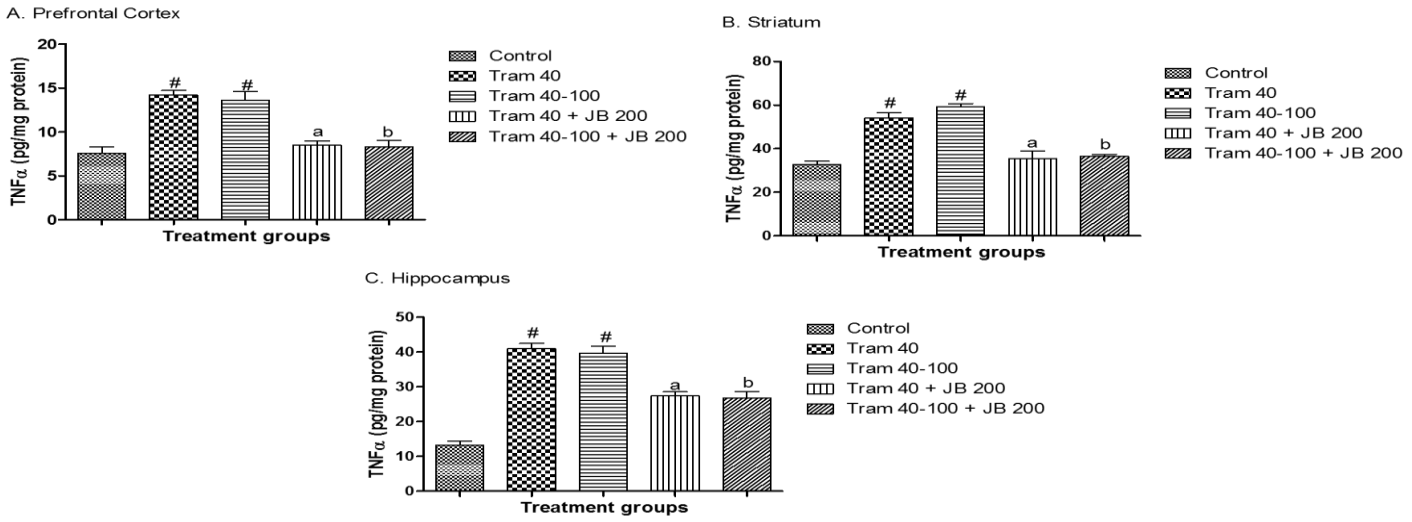
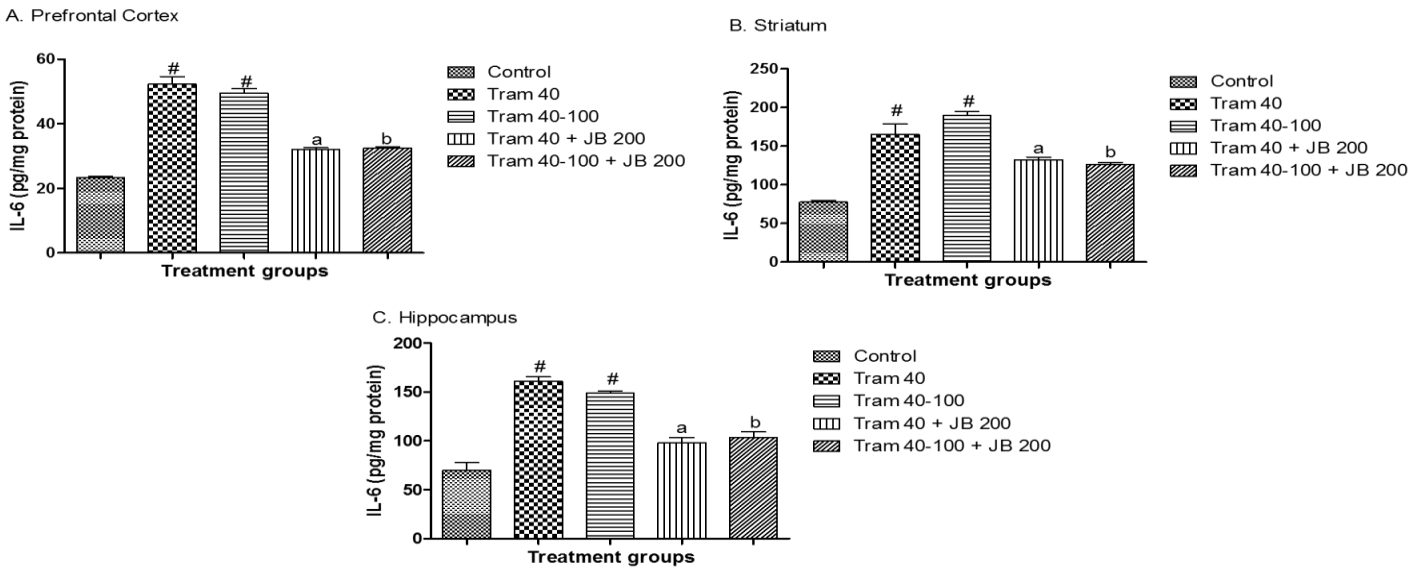
Oxido-inflammatory markers in tramadol induced dependence and on administration of JB 200 mg/kg: Assays of tumor necrosis factor (TNF) and interleukin-6 as markers of neuro-inflammation in controls and in tramadol dependence were compared with treatment with varying doses of JB assays of TNF-α and interleukin-6 (IL-6) were evaluated in controls, on tramadol administration and treatment with JB. The results showed significant elevations in TNF and IL-6 in rats in groups treated with tramadol 40 mg and varying doses of tramadol 40 mg to 100 mg (**Figures 12** and **13**).

Figure 12: Assay of TNF-α in treatment groups and controls of rats



Mean±S.E., (n=5). # P < 0.05, P = 0.036 vs control using one-way ANOVA followed by Tukey's *post hoc* test.
Tram: tramadol, JB- Jobelyn®

Figure 13: Assay of IL-6 in treatment groups and controls of rats



Mean±S.E., (n=5). # P < 0.05, P = 0.035 vs control using one-way ANOVA followed by Tukey's *post hoc* test.
Tram: tramadol, JB: Jobelyn®

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Discussion

This study focused on the induction of oxido-inflammatory stress induced by short-term use of tramadol and sudden discontinuation in rats. Administration of tramadol was done at constant dosing and incremental doses. JB was used to attenuate the oxido-inflammatory stress in rats. The current study focused on the prefrontal, hippocampus and striatum which are areas that are mostly affected in behavioral models in abuse [25-29]. The prefrontal cortex largely plays a major role in executive functioning, reward appreciation and mediates purposive action, behavior and self-control due to the numerous projections to many parts of the brain [30]. The behavioral deficits seen in dependence have been adduced to the affectation of this principal part of the brain [26]. The hippocampus mediates memory which plays a vital part in response to cues and situational memory. The glutaminergic plasticity of the hippocampus is disrupted, further perpetuating addiction [31]. We first assessed behavioral correlates of tramadol use and compared the behavior with other groups that have been administered JB after discontinuation of tramadol. The outcome was anxiety response and locomotory observations in controls, we aimed to observe the responses post-discontinuation and explore the role of oxido-inflammatory stress and amelioration by JB. Anxiety is known to be associated with tramadol abuse and dependence in humans [32]. Conversely, tramadol administration in experimental animals has been reported to reduce anxiety due to its serotonergic reuptake inhibition [9]. We compared five groups, after which tramadol was discontinued and feeding was the same with normal controls for the remaining seven days, on day 17. The 4th and 5th groups were treated with tramadol at similar doses respectively and discontinued after 10 days, then, JB at 200 mg/kg for seven days was instituted, respectively. Anxiety responses were assessed on day 17. The tramadol-only treatment groups when compared with controls, and those on JB treatment post-discontinuation of tramadol. The present results indicated that tramadol-treated groups had significantly higher indices of anxiety responses using the EPM and OFT. Rats treated with tramadol at 40 mg/kg daily and those treated with graded incremental doses from 40 mg/kg to 100 mg/kg, respectively, had significantly higher frequencies of open-arm avoidance. Shorter duration on the open arm and lower number of open arm entries about controls. The addition of JB to tramadol treatment (4th & 5th groups) reduced anxiety responses with improved duration of open arm entries and lower observations of open arm avoidance and closely approximated the frequency of observation in controls. The results indicated that abusive use of tramadol even on middle term basis was associated with increased anxiety indices in rats. The results are similar to the findings that have been reported on tramadol abuse and possible anxiety in some reports of human subject's post-discontinuation of tramadol [33]. As noted, some studies have studied oxido-inflammatory stress and behavioral deficits post tramadol use in animal models. The reference study in humans showed that stoppage of tramadol showed almost normal levels of oxidative stress after three months unlike, the comparison with morphine [33]. The anxiolytic and anti-depressant effect of tramadol seems to be overshadowed. However, it seems that prolonged and abusive use of tramadol or tramadol dependence even after discontinuation tended to increase anxiety responses rather than reducing it as our study findings show.

Regarding locomotion in rats, we observed that similar to the anxiety responses, similar patterns were observed. In rats treated with tramadol, average speed, number of line crossings and distance covered were significantly reduced when compared to controls. However, for the other groups on JB treatment, there were marked improvements in average speed, distance covered, and number of line crossings with no significant differences observed with controls. Duration of stay in the center area followed a similar pattern, with the tramadol-treated only group having a higher number of observations of a lower duration of stay in the center area in OFT. There were no significant differences frequency of duration of stay when compared with normal controls. It seems in these groups, with the addition of JB, there were improvements in locomotion and reduced anxiety responses. JB

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administration seemed to ameliorate anxiety about tramadol discontinuation. In the assessment of oxido-inflammatory stress, we focused on three major areas of the brain that are particularly affected by dependence and abusive use of tramadol [30]. Tramadol use on long -term basis has been reported to have a neurotoxic effect and can lead to neurodegeneration of the prefrontal cortex through induction of reactive oxygen species [14]. Similarly, induction of neuroinflammation by long term use of tramadol was significantly associated with atrophy and microgliosis of the striatum [31]. Studies have also shown that tramadol use can lead to degeneration in the hippocampus with resultant memory loss [32]. These areas have also been implicated in dependence due to the affectation of cognitive processes and the decision making [30]. Particularly, the prefrontal cortex was implicated in the emotional and behavioral alterations involved in drug addictions [33]. Chromic exposure to tramadol was demonstrated to have a degenerative effect on the cerebral cortex, cerebellum and striatum via neuroinflammation causing microgliosis ultimately causing behavioral alterations, and poor executive functioning similar to the effects of opioids in the prefrontal cortex in rats [26]. However, the acute stoppage of tramadol at fixed doses and increasing can lead to the induction of reactive oxidative studies and inflammatory cascade affecting these three regions of the brain. The study demonstrated that in tramadol-treated groups, significant elevations in non-enzymatic and enzymatic oxidative markers were observed. Further, increased neurotransmitter degradation and upsurge in interleukin-6 and tumor necrosis factor were also demonstrated.

Our findings show that even in moderate tramadol use and discontinuation, significant increases in oxidative stress markers and inflammatory markers in the prefrontal, striatum and hippocampus are observed. The brain is particularly susceptible to oxidative stress due to high lipid content, low levels of antioxidants and high consumption of oxygen [34]. Nitrites are seen as a byproduct of reactive oxygen species [35]. MDA is a major oxidative stress marker from lipid peroxidation formed by reactive oxygen species [36]. Thus, tramadol elevated MDA and nitrites in three regions of the brain studied when compared to normal controls. Similarly, there were significant reductions in enzymatic oxidative markers (GSH, SOD, Catalase & GAD), in all the regions when compared to normal controls. The highest evaluations are indicative of oxidative stress and their role has been well documented [37]. Further, AChE activity was elevated with treatments with tramadol and regardless of the discontinuation, measures of AChE were significantly increased in comparison to normal controls. The enzyme is responsible for the degradation of ACh and its increased activity in the hippocampus has been associated with memory loss [38]. The tramadol-treated group also had increased elevations in TNFα, and interleukin-6 which are markers of inflammatory cascade, despite discontinuation. In comparison to controls, the tramadol-treated group had significantly higher elevations of TNF-α and interleukin-6 over controls. However, when treated with JB after tramadol discontinuation, there was a reduction in TNF-α and Il-6, to near the levels measured among controls. The elevation was most elevated in the striatum. In the prefrontal, striatum, and hippocampus, TNF- α and Il-6 are major markers of acute inflammation and it can be induced by reactive oxygen species [39]. Our study showed that in fixed moderate dosing use and subsequent discontinuation, tramadol causes a cascade of pro-inflammation regardless of discontinuation. A previous study observed that tramadol use and withdrawal cause elevation of TNF and Interleukin-1 [40]. On administration of JB, the proinflammatory markers were attenuated and there was no significant difference when compared with normal controls. In general, our study demonstrates the ameliorative effect of JB on oxido-inflammatory cascade and anxiety responses induced by tramadol use and subsequent discontinuation in the prefrontal, hippocampus and striatum. The ameliorative effect is probably due to the rich content of antioxidants as documented by several studies [20, 41]. JB attenuated anxiety responses, and oxido-inflammatory stress markers induced by tramadol abuse and subsequent discontinuation.

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Conclusion: Tramadol induced oxido-inflammatory stress markers in the prefrontal, striatum and hippocampus in rats. Anxiety and locomotory actions on tramadol treatment were elevated despite discontinuation for seven days. Jobelyn® ameliorated oxido-inflammatory markers induced by tramadol and decreased anxiety response in rats.

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Author declarations: The authors confirm that all relevant ethical guidelines have been followed and any necessary IRB and/or ethics committee approvals have been obtained.