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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.

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Abstract: The study focused on the neuroprotective role of *Sorghum bicolor* and vitamin C in the amelioration of oxidative stress and anxiety-like behavoiur induced by tramadol in male albino rats. The study design involved 7 groups and a control group with 5 male albino rats in each group. Tramadol (40 mg/kg) treatment was administered for 21 days. Tramadol 40mg/kg was administered in all groups. Pretreatment with varying doses of Sorghum bicolor and Vitamin C was done in three of the groups. Behavioral assessment of anxiety and locomotors actions of the groups were compared using Elevated Plus Maze (EPM) and Open Field Test (OFT). In conclusion, Sorghum bicolor and Vitamin C tramadol ameliorated oxidative stress and anxiety-like behaviour induced by tramadol. Pretreatment with Sorghum bicolor or vitamin C (100mg) can also reduced anxiogenic responses in male albino rats that are induced by chronic tramadol use.

Keywords: Tramadol, Sorghum bicolor, vitamin C, oxidative stress, anxiety.

INTRODUCTION

Tramadol has been widely used as an analgesic for many years (Ogemudia *et al*, 2022). The drug was first launched in Germany in the 1970's. Over the years, the drug has gained worldwide popularity due to its potency in the management of mild to moderately severe pain. It has of comparable efficacy to many narcotic-like medications (Engelhardt *et al*., 2003). It is a synthetic opioid with a codeine-like structure (4 aminopyridine core) and has a weak affinity for the morphine opioid receptor (MOP), via which it mediates its major analgesic action (Dayer *et al*, 1994).

Tramadol's antinociceptive action has also been partially connected to its blockage of norepinephrine and serotonin reuptake which contributes to the antinociceptive effect and anxiety reliving effect in pain management (Tsai and Chu, 2001). In addition, the analgesic effect of tramadol has also been linked to its action on N-methyl-D-aspartate (NMDA) receptor and on α adrenoceptor (Minami, Uezono and Ueta, 2007). It is due to the multimodal effect that some authors have classified tramadol as an atypical opioid (Bravo, Mico and Berrocoso, 2017).

Although it was primarily considered a safe and dependent free alternative to narcotic-like analgesic medication, abusive use of tramadol has now become a top healthcare priority in many countries in Africa, Asia and a couple of countries round the world (Abel-Hammid *et al*, 2016, International Narcotics Control Board. 2005). Contrary to the initial manufacturing claims, tramadol dependence has been widely reported in several studies (Abel-Hammid *et al*, 2016). Further, due to its multiple mechanism of action and consequent effects, the abusive use of tramadol has become very commonplace. Tramadol has been used off prescription, for libido enhancement, premature ejaculation and to induce euphoria (Martyn-St James *et al*, 2015, Peprah *et al*, 2020). Of particular concern, are the increasing numbers of youths who abuse tramadol. A propelling reason among many youths is the use of tramadol to improve sexual performance (Peprah *et al*, 2020). In most cases large doses of tramadol are consumed to achieve sexual potency with attendant side effects, such as seizures, respiratory depression, loss of consciousness, suicide and development of tramadol dependency (Shadnia *et al*, 2008, Ahmed *et al*, 2018).

Another major consequence is the multiple organ toxicity that has been associated with abusive use of tramadol (Ali *et al*., 2020). A more frequently reported organ toxicity is neurotoxicity (Mohammadnejad and Soltaninejad, 2022). Neurotoxicity is a very common organ toxicity associated with chronic and abusive use of tramadol. Several mechanisms are involved, including mitochondrial dysfunction, apoptosis, inhibition of neurogenesis and oxidative stress (Mohammadnejad and Soltaninejad,

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2022). The induction of oxidative stress has been the most frequently reported underlying pathology associated with tramadol induced neurotoxicity. Oxidative stress refers to an excess production of very reactive oxygen species (ROS). These ROS radicals nave the potential to cause cell damage. The overproduction of these ROS is far in excess of the innate capacity of the body mechanisms to control or clear away. These excess production leads to cell damage, DNA fragmentation, polyunsaturated fatty acid oxidation and amino acid oxidation (Mohammadnejad and Soltaninejad, 2022). Tramadol affects mitochondrial electron transport, increases lipid peroxidation and releases of highly reactive oxygen species (ROS) causing neuronal cell damage. It mops up antioxidants in neuronal tissues and leads to increases in indicators of oxidative damage. Studies in rodents have shown that abusive use of tramadol is linked to increases in brain malondehyde (MDA), reductions in gluthaione, superoxide dismutase (SOD), catalase enzyme, (CAT) and increased nitric oxide production (Abdel-Zaher *et al*, 2011). The oxidative damage due to tramadol toxicity has been demonstrated in experiments in rodent species even at therapeutic dose range when used for a prolonged period (Mohammadnejad and Soltaninejad, 2022).

The induction of oxidative stress with consequent neurotoxicity has been postulated to be responsible for tramadol dependency and abuse. A number of studies have linked oxidative stress induction as a possible pathophysiology in tramadol dependency (Abdel-Zaher *et al*, 2011). The mechanisms may involve a complex but potentially synergistic coupling with oxidative stress damage of the cerebrum (Mohamed and Mahmoud, 2019). Potentially, the use of antioxidants in the management of tramadol dependence, or abuse can ameliorate the symptoms (Abdel-Zaher *et al*, 2011, Adelakun *et al*, 2022)

Sorghum bicolor (Popularly marketed as Jobelyn) is a popular herbal and dietary supplement in Nigeria. (Asehinde *et al*, 2018). The constituents of sorghum bicolor contains many phytochemicals which include, luteolin, apigenin and narrington (Umukoro *et al*, 2013). Several studies have reported about the antioxidant properties of sorghum bicolor in many pathological conditions (Makanjuola *et al*, 2017, Omorogbe *et al*, 2018). The rich antioxidant properties of *sorghum bicolor* has enabled the use of this herbal preparation in a variety of disease conditions which are due to the induction of oxidative stress. Previous investigations have reported that the active ingredients of this *Sorghum* extract have anti-inflammatory actions and have been reported to lead to reduction in neuronal cell death as demonstrated in several studies (Umukoro *et al.*, 2013, Benson *et al.*, 2013). Sorghum bicolor has been reported to have immune modulating activities and proved to be effective in several other disease conditions that are due to oxidative stress damage and chronic inflammatory conditions (Umukoro *et al.*, 2013).

Vitamin C is a common antioxidant and is important in a variety of roles in the human body (Bendich *et al., 1986)*. It consists mostly of monosaccharides. Humans cannot synthesize vitamin C and hence dietary sources are the primary source for humans (Kader *et al*, 2020). Vitamin C functions as an electron donor and an enzyme cofactor in redox reactions (Schlueter & Johnston, 2011). This properties enables vitamin C to act as an antioxidant, enzyme inducer and enhancer of immune response (Schlueter and Johnston, 2011). This active antioxidant works via dual mechanisms. It reacts with aqueous peroxyl radicals and aids or enhances the antioxidant properties of fat-soluble vitamin E (Bendich *et al*., 1986). Earlier studies showed that vitamin C promotes antioxidant activities that has a neuroprotective effect and anti-apoptotic activity in tramadol induced neurotoxicity by limiting oxidative stress through suppression of lipid peroxidation and down-regulation of p53 (Kader *et al*., 2020).

The aim of our study were threefold. We aimed (1) to establish the induction of oxidative stress with the use of tramadol by measuring non enzymatic and enzymatic markers of oxidative stress, (2) to compare pretreatment of sorghum bicolor antioxidant properties with 100mg of vitamin C in ameliorating oxidative stress induced by repeated doses of tramadol in the cerebral cortex of male albino rats (3), to evaluate the neuro-protective role of pretreatment with sorghum bicolor and compared it with vitamin C in ameliorating anxiety-like behavior symptoms induced by tramadol use.

MATERIALS AND METHODS

Drugs and chemicals

A brand of sorghum bicolor (JB), marketed by Health Forever Products Ltd, Lagos, Nigeria was obtained from Mex Pharmaceuticals, Ogun State, Nigeria. The caplet formulations of 250 mg of *Sorghum bicolor* leaf powder extract with National Agency for Food and Drug Administration and Control (NAFDAC) registration number A7 1873L with batch number JX1/100/0707/34 was used.

Tramadol hydrochloride manufactured by Richly Gold International, Nigeria Limited was used in this study. Tramadol Hydrochloride in capsules containing 50 mg of tramadol hydrochloride with NAFDAC registration number; A4-0490, batch number; TRC2002, was used in the investigation. The drug was obtained following due authorization from the Pharmaceutical Store and Letter of approval for the study. Both drugs excluding the excipients were dissolved in a measured volume of distilled water.

Vitamin C (ascorbic acid) 100mg was obtained in tablet form from Mex Pharmaceuticals, Ogun State, Nigeria. All of the solutions and preparations were made on the day of the test.

Animals

Male albino rats (with weights between 100-170 g) were bought from the Central Animal House, Lagos. The rats were placed under supervised standard environmental conditions in the laboratory (12hr, light /dark cycle). They were fed with standardized pelletized ratchow (Ladokun Feed, Ibadan) and given water *and libitum*. Acclimatization was done within 14 days. The study was carried out by following the ethical guidelines of the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1996). Ethical approval was obtained from the Ethical Committee of the University of Lagos with a registration consent approval number; CMUL/ACUREC/11/21/972.

Study design/experimental design

The study focused on the neuroprotective effect of *sorghum bicolor* on amelioration of oxidative stress and anxiety-like behaviour induced by twice-a-day tramadol administration in rats. The dose of tramadol was modified based on observations from similar studies by Rafati *et al*, 2006 and Aghajanpour *et al*., 2020, respectively. *sorghum bicolor* doses were selected by extrapolating 10 % of the acute toxicity dose tested at 2000 mg/kg as reported by Omorogbe *et al*., 2018. The animals were treated orally daily for 21 days and thereafter subjected to behavioural tests for anxiety and locomotor impairment using an elevated plus maze and video-recorded open field test. All drugs were adminsitered orally via orogastric tube. The rats were divided into 7 groups with 5 male albino rats in each group. The arrangements of the groups were as follows:

In group 1 (control), the rats in this group were given distilled water at 0, 1 and 12h later.

Group 2 (tramadol-control), rats in this group received distilled water (10mL/kg, p.o) at 0h and tramadol (40mg/kg) after 1h and 12h, respectively daily for 21 days.

Group 3 (JB 50 + Tramadol 40mgJBg (Tra 40), received *sorghum bicolor* (50 mg/kg, p.o) at 0 h and tramadol (40 mg/kg) after 1 h and 12 h, respectively daily for 21 days.

Group 4 JB 100 + Tra 40), received sorghum bicolor (100mg/kg. p.o) at 0h and tramadol (40 mg/kg) after 1h and 12h, respectively daily for 21 days.

Group 5 (JB 200 + Tra 40), received Sorghum bicolor (200 mg/kg p.o) at 0 h and tramadol (40 mg/kg) after 1h and 12h, respectively daily for 21 days.

Group 6 (Vit C 100 + Tra 40), received Vitamin C (100 mg/kg, p.o) at 0h and tramadol (40mg/kg) after 1h and 12h, respectively daily for 21 days.

Group 7 (JB 200), the rats were pretreated with sorghum bicolor (200 mg/kg) at 0 h and distilled water (10 mL/kg) after 1 h and 12 h, respectively daily for 21 days.

Elevated plus-maze (epm) test for anxiety

The EPM test was used to assess the effect of treatment with tramadol and withdrawal on anxiety-like behavior in rats. Each rat was placed at the centre of the maze with its head facing an open arm and the frequencies and duration of arm entries were recorded for five minutes (5 min). An entry was recorded and scored if all the four paws of the animals were completely inside any one arm of the EPM (Pellow, 1985). Ethanol (70 %) was always used to clean the plus-maze after each test. The results were expressed as time spent in arms and the percentage of the number of entries in arms. The index of open arm avoidance (IOAA) was calculated.

IOAA = 100 - (%duration of time spent in open arms + % entries into open arms)/2

Open field test (OFT)

Assessment of locomotor function impairment and anxiety-like behaviours in rats was performed in the open field test (Walsh and Cummins, 1976; Stanford, 2007). A wooden cubic box measuring 40 cm in three dimensions ($40\times40\times40cm^3$) was divided horizontally into 16 squares, each measuring 10 centimeters in the length, and breadth (10cm X $10cm^2$) the central 4 squares ($20\times20cm^2$) are considered the center and the surrounding 4 sides ($10\times15cm^2$) and 4 corners ($10\times15cm^2$) are considered as the surrounding. In each test sequence, an albino rat was placed at the center of the box and allowed to acclimatize to the box for 1 minute, then allowed to explore the box for 5 minutes which was recorded using a video camera and analyzed. The following behavioural items (Total distance travelled, average speed, number of entries in the center area and time spent in the center area) were analyzed from the videos with an Automated behavioural testing video tracker (AnyMaze Software version 6.19).

Animal Sacrifice

Twenty-four (24hrs) after the last treatment and behavioral assessments, animals were weighed and later anaesthetized with intraperitoneal administration of Ketamine (100 mg/kg). They were then cut open and perfused intracardially with cold normal saline. The brain was isolated, weighed and rinsed with cold Tris-KCl buffer (0.15 M, pH 7.4) and kept on ice. The brain tissues were individually homogenized in 10% w/v sodium phosphate buffer (0.1M, pH 7.4) using a mechanical grinder and were centrifuged at 4°C in a refrigerated centrifuge at 10,000 rpm for 10 min. Aliquots of the supernatants were stored at -20°C for biochemical assessment.

Measurement of oxidative stress parameters

Estimation of lipid peroxidation level

Estimation of tissue malondialdehyde, which is a parameter that can be used as an index of lipid peroxidation. Estimation was done by taking assays of thiobarbituric reacting substances (TBARs) (Nagababu *et al*., 2010). Measurement of 100µL of supernatant was done, it was diluted ten times in 0.15M Tris-KCl buffer and deproteinized with 500µL trichloroacetic acid (30%). A benchtop centrifuge was used to centrifuge the mixture at 4000 rpm for 600 seconds at room temperature, After centrifuging, 200 µL of the supernatant was put into Eppendorf tubes, followed by the addition of 200µL thiobarbituric acid (1%) and the mixture obtained was heated at 80°C for 1h. Cooling of the the tubes were achieved by placing them on ice. We removed 200µL and put it into a micro-titer plate and absorbance was measured at 532nm. The result was calculated using an index of absorption for MDA (molar extinction coefficient 1.56x105/M/cm). The concentrations of TBARS in the tissues were expressed as ηmol MDA/mg protein.

Estimation of nitrite levels

The nitrite concentration was estimated using a method that involved the use of Griess reagent as described in a study (Green *et al*., 1982) We used Griess reagent, as the indicator of nitric oxide release. Measurement of 100 µL of Griess reagent (1:1 solution of 1% sulfanilamide in 5 % phosphoric acid and 0.1% of N-1-naphthyl ethylenediamine dihydrochloride) was done and added to 100 µL of the supernatant. Absorbance was measured at 540 nm in LT-4500 microplate reader (Labtech, UK). The nitrite concentrations were then estimated from a standard curve obtained from sodium nitrite (0-100µM). It was expressed in µmoles /mg protein.

Estimation of reduced glutathione levels (GSH)

Reduced glutathione is an antioxidant marker. It is a grouped as a non -enzymatic marker. The method described by Jollow *et al*., 1980 was used to measure glithathione. About 100 µL of supernatant was diluted ten times in 0.15M Tris-KCl buffer and deproteinized with 500 µL trichloroacetic acid (30 %). We used a benchtop centrifuge to centrifuge resultant mixture at 4000 rpm for 10 min at room temperature. We measured 100 µL of the deproteinized supernatant and mixed it with 100 µL of 51, 51 -Dithios-nitrobenzoic acid (DTNB, 0.0006 M) in a microplate. The absorbance was read within 5 min at 405 nm in an LT-4500 microplate reader (Labtech, UK). Glutathione concentration was extrapolated from the standard curve of glutathione (0-200µM). It was expressed as a µmoles GSH/ mg protein.

Estimation of catalase enzyme assay

Catalase enzyme (CAT) activity was estimated using the method described by Goth *et al*,, 1991. Catalase enyme was done using the colorimetric assay based on observations of the yellow complex with molybdate and H2O2. We measured 50µL of (2X) diluted supernatant and added it into a micro-titer plate, subsequently we added 50µL of a reaction mixture containing 65 mmol/mL of H2O2 in sodium-potassium phosphate buffer (60mM, pH 7.4). The enzymatic reaction was incubated for 3 min and stopped with 100µL of ammonium molybdate (64.8mM) in sulfuric acid. The absorbance at 405 nm was measured in an LT-4500 microplate reader (Labtech, UK). The unit for catalase enzyme activity was U/ mg protein.

Determination of super oxide dismutase (SOD)

The level of SOD activity was determined using the method described by Misra and Fridovich (1972). Superoxide dismutase activity is determined based on observations of its ability to inhibit the autoxidation of adrenaline in sodium carbonate buffer (pH 10.7), Measures of 50µL of 2X diluted supernatant was added into a micro-titer plate containing 150µL of carbonate buffer. The consequent reaction that was observed was due to the addition of 30µL of freshly prepared 0.3mM adrenaline to the mixture. Blank swere prepared using 50 µL of distilled water. The increase in absorbance at 495 nm was monitored every 60 seconds for 300 seconds in the LT-4500 microplate reader (Labtech, UK). The SOD activity was expressed as U/mg protein.

Assay for glutathione-S-transferase {GST)

The assay for GST activity in tissue samples was performed according to Habig and Jakoby (1974). Briefly, 50 µL of 2X diluted supernatant was added into a 96-well plate, then 150µL of reaction mix was added. The reaction mixture was prepared using potassium phosphate (0.1M, pH 7.4), GSH (20mM in 0.1M KH2PO4 buffer) and CDNB (20mM in 95 % ethanol) in a ratio of 16:1:1. The absorbance was read at 405 nm for 5 min in a micro plate reader (LT4500, UK). The unit of the enzyme was expressed as U/mg protein.

Measurement of neurotransmitter-related brain enzymes

Measurement of Acetylcholinesterase (AChE) in the Brain

AChE was evaluated using a method described by Ellman *et al*., (1961). About, 50 µL of phosphate buffer (0.1 M, pH 7.4) was used to dilute 50µL aliquots of brain supernatants. It was then followed by the addition of 50µL of DTNB (0.0001M) in a 96-well plate. The first measurement of the initial absorbance was first measured after 5min of incubation with DTNB. The second measurement of the absorbance was measured at 405 nm in a microplate reader (LT4500, UK) after 50 µL of acetylthiocholine iodide (0.028M). The rate of acetyl-cholinesterase activity (µmol/min/mg tissue) was calculated using the for:

$R = 5.74 \times 10^{-4} \times A/Co$

Where

R = Rate in moles of substrate hydrolyzed/min/g tissue
A = Change in absorbance/min,
Co = Original concentration of the tissue

Assay for glutamic acid decarboxylase (GAD)

The assay for GAD activity in the Brain supernatant was done using a method described by Yu *et al*., 2011 Measurements of 50µL aliquots of 2X diluted brain tissue supernatant were incubated with a reaction mixture containing 20 mM sodium acetate, 70µM bromocresol, 10 mM pyridoxal-5-phosphate (PLP) and 2µL glutamate (from a 1M stock in acetate buffer). The increase in absorbance at 630 nm for 5min was read in a microplate reader (LT4500, UK). The unit of the enzyme was expressed as µmoles min -1mg protein-1 using the extinction coefficient 23700 M-1cm-1.

Estimation of total protein concentration

The Biuret method using bovine serum albumin was used to determine the protein concentrations of the various samples following the procedures and standards described by Gornall *et al*, 1949. The dilution of the were done to appropriate 1: 4 ration with sodium phosphate buffer, thereafter, about 50µL of diluted supernatant was added to the microtitre plate and also added 200µL of Biuret reagent. We incubated the at room temperature for 30 minutes after the absorbance was read at 540nm in LT-4500 microplate reader (Labtech, UK). We then extrapolated the protein concentration from BSA standard curve.

STATISTICAL ANALYSIS

The results were presented using mean ± (standard Error of Mean) SEM. We analysed the data using One-way (Analysis of Variance)ANOVA and Two-way ANOVA followed by Tukey's *post hoc* multiple comparison test using Graphpad Prism (GraphPad software, version 8.0).

RESULTS

Effect of pretreatment on body and brain weight in a twice-daily 21 days repeated administration of Tramadol in rats

Tramadol administered twice daily for 21 days significantly (P<0.05) reduced body weight in rats when compared with the control rats (fig. 1A). However, pre-treatment with SB (100 and 200 mg/kg) caused a further significant (p<0.05) reduction in body weight in tramadol twice-daily treated rats (fig. 1B). Vit C (100mg/kg) was able to significantly prevent loss in body weight in twice-daily tramadol-treated rats. Administration of SB (200 mg/kg) alone showed a significant reduction in body weight when compared with control animals. There was no significant difference across treatments on the relative brain weight except in JB (100mg/kg) + Tram 40 (x2) which showed a significantly (P<0.05) increased value (fig. 1C).

Brain oxidative stress markers in a twice-daily 21 days of tramadol in rats pretreated with sorghum bicolor

Twice-daily 21 days repeated administration of tramadol significantly increased levels of MDA and nitrites in rat brains (fig. 2A and B). However, pretreatment with SB (50, 1,00 and 200mg/kg) significantly (P<0.05) reduced MDA levels but not nitrites levels. Similarly, Vit C (100 mg/kg) significantly reduced MDA but not nitrite levels. Sorghum bicolor (200mg/kg) alone significantly increased brain MDA levels when compared with control, but no significant change in nitrites.

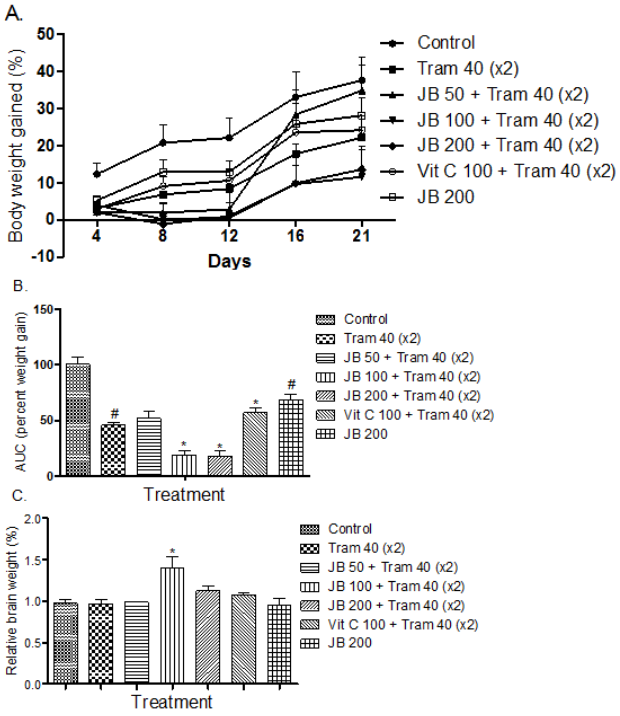

Fig. 1 A-C: Effect of pretreatment with *SB* (50, 100 and 200mg/kg) on body weight in twice-daily 21 days repeated administration of tramadol in rats. A. Percent body weight gained (B) Area under the curve (percent weight gained), (C) Relative brain weight. Bars represent Mean ± S.E (n=5). # P<0.05 vs control and *P<0.05 vs Tram 40 using one way ANOVA followed by Tukey's *post hoc* test for multiple comparisons. Tram-Tramadol, JB- Sorghum bicolor, Vit - Vitamin. C.

Brain antioxidant markers in a once-daily 21 days repeated administration of tramadol in rats pretreated with sorghum bicolor The twice daily treatment of Tramadol significantly reduced markers of enzymatic antioxidants level, The levels of A) Reduced glutathione (B) Glutathione-S-transferase (GST), (C) Catalase and (D), Superoxide dismutase (SOD) Superoxide dismutase (SOD) as shown in fig. 3 A-D.

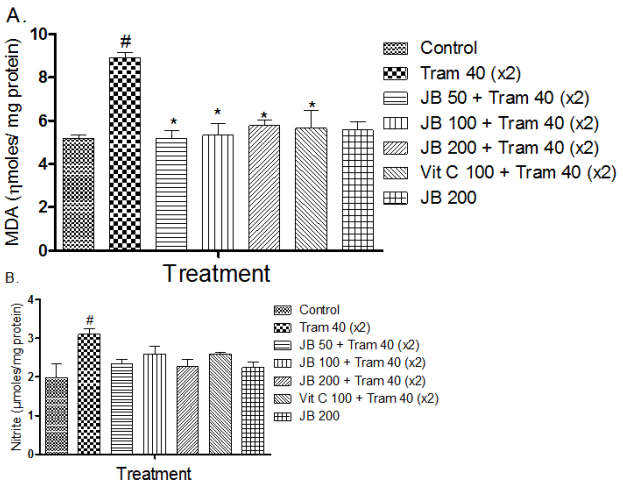

Fig. 2 A-B: Effect of pretreatment with *JB (Sorghum Bicolor)* (50, 100 and 200 mg/kg) on brain oxidative stress levels in twice-daily 21 days repeated administration of tramadol in rats. (A) Malondialdehyde (MDA) and (B) nitrites. Bars represents Mean ± S.E (n=5). #P<0.05 vs control and *P<0.05 vs Tram 40 using one way ANOVA followed by Tukey's *post hoc* test for multiple comparisons. Tram-Tramadol, SB- Sorghum bicolor, Vit - Vitamin C.

Brain neurotransmitter-related enzymes activity in a once-daily 21 days repeated administration of tramadol in rats pretreated with Sorghum bicolor

Acetylcholinesterase (AChE) activity as shown in fig. 4A was significantly increased in twice-daily repeated administration of tramadol in rats when compared with the control group. However, pretreatment with SB (50, 100 and 200mg/kg) significantly caused a reduction in the activity of AChE in twice-daily tramadol-treated rats. Vit C (100mg/kg) also significantly reduced AChE activity.

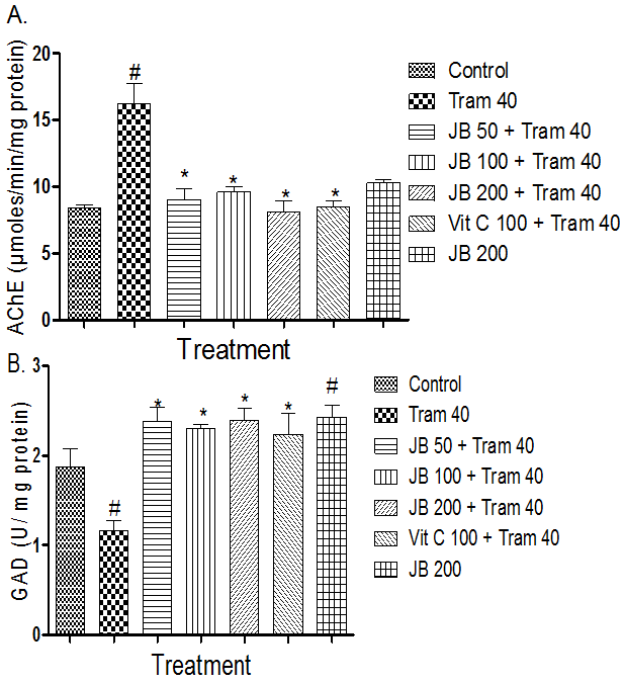

Fig. 4 A-B: Effect of pretreatment with SB (50, 100 and 200 mg/kg) on neurotransmitter-related enzymes in twice-daily 21 days repeated administration of tramadol in rats. (A) Acetylcholinesterase (AChE), and (B) Glutamic acid decarboxylase (GAD). Bars represents Mean ± S.E (n=5). #P<0.05 vs control and *P<0.05 vs Tram 40 using one

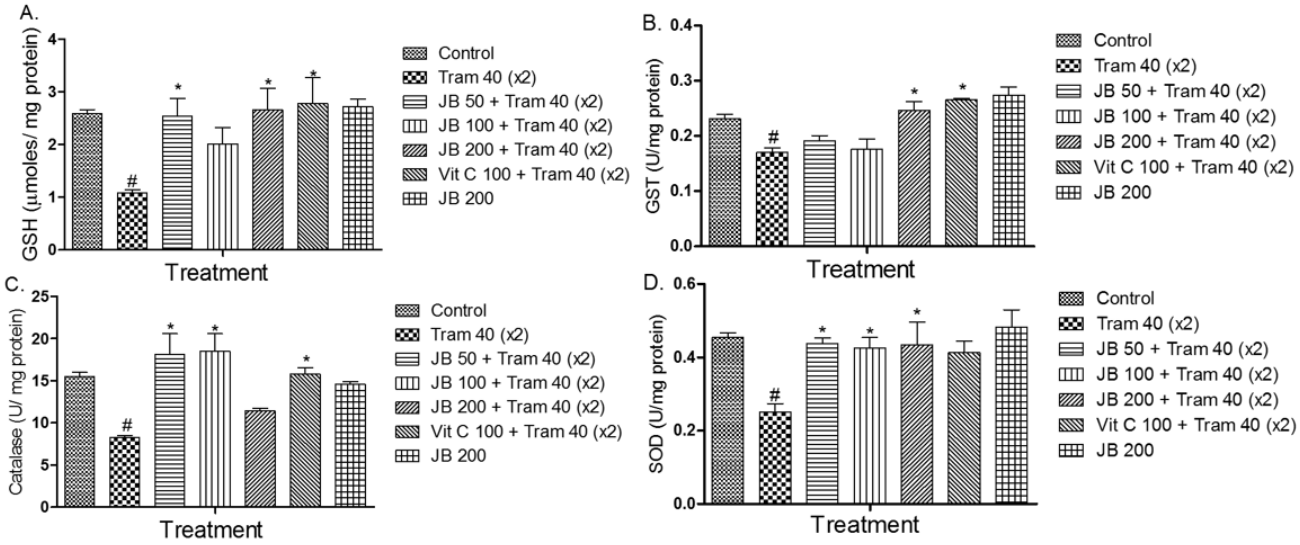

Fig. 3A-D: Effect of pretreatment with JB (50, 100 and 200mg/kg) on brain antioxidant in twice-daily 21 days repeated administration of tramadol in rats. (A) Reduced glutathione (GSH), (B) Glutathione-S-transferase (GST), (C) Catalase, and (D) Superoxide dismutase (SOD). Bars represents Mean ± S.E (n=5). # P<0.05 vs control and *P<0.05 vs Tram 40 using one way ANOVA llowed by Tukey's *post hoc* test for multiple comparisons. Tram-Tramadol, JB- Sorghum bicolor , Vit - Vitamin.

way ANOVA followed by Tukey's *post hoc* test for multiple comparisons. Tram-Tramadol, SB- Sorghum bicolor, Vit - Vitamin C.

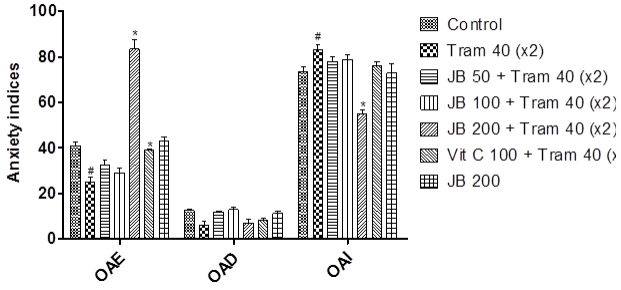

Fig. 5: Effect of pretreatment with SB (50, 100 and 200 mg/kg) on anxiety indices measured using the EPM in twice-daily 21 days repeated administration of tramadol-treated rats. Bars represents Mean ± S.E (n=5). # P<0.05 vs control and * P<0.05 vs Tram 40 using one way ANOVA followed by Tukey's post hoc test for multiple comparisons. Tram-Tramadol, SBSorghum bicolor, Vit – Vitamin, OAE-Open ARM Entries, OAD- Open Arm Duration and OAI-Open Arm Avoidance Index.

GAD activity was significantly reduced in the brains of twice-daily repeated treatment with tramadol rats when compared with the control (fig. 4B). SB (50, 100 and 200 mg/kg) and Vit C (100mg/kg) significantly increased the GAD levels in brains of twice-daily repeated treatment with tramadol rats. SB (200mg/kg) alone did not cause any change in AChE activity but significantly increased GAD activity in rats.

EPM measures of anxiety indices in daily 21 days administration of tramadol in rats pretreated with sorghum bicolor

Administration of tramadol induced anxiogenic response (fig. 5) in rats when compared with control rats as measured by the reduced frequency of open arm entries (24.96±2.40 vs 40.87±1.88; P<0.05), time spent in the open arm area (6.27±0.1.33 vs 12.67±0.30; P>0.05) and increased open arm avoidance index (83.18±2.33±73.23± 2.53; P<0.05). However, only pretreatment with SB (200 mg/kg) was able to significantly reduced anxiogenic responses on the EPM. Pretreatment with Vit C (100 mg/kg) when compared with tramadol-control showed significant reduction in anxiety indices by increasing frequency of visit to open arm. The effect of SB (200 mg/kg) administered alone when compared with control group was not significantly different on the EPM measurement (fig. 5).

Locomotory activity and anxiety measures in 21 days administration of tramadol in rats pretreated with Sorghum bicolor

Administration of tramadol significantly (P<0.05) caused

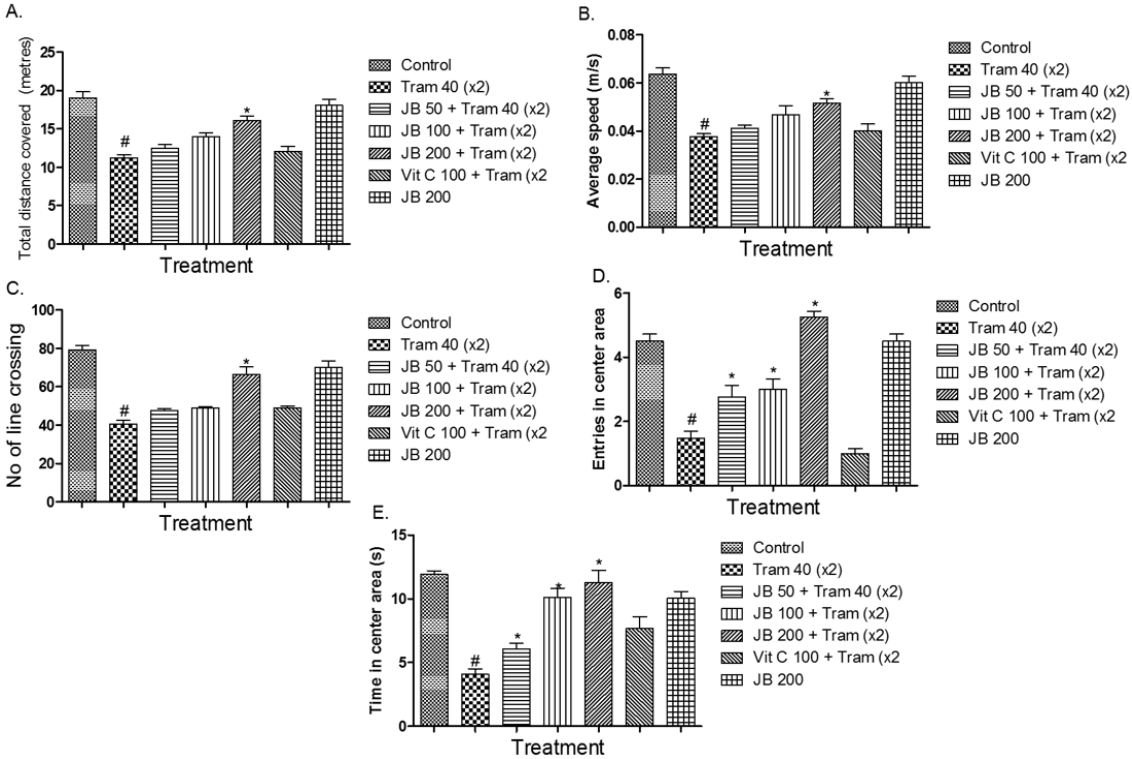

Fig. 6 A-E: Effect of pretreatment with JB (50, 100 and 200mg/kg) on locomotory and anxiety measures in the OFT in daily 21 days administration of tramadol-treated rats. Bars represents Mean ± S.E (n=5). # P<0.05 vs control and *P<0.05 vs Tram 40 using one way ANOVA followed by Tukey's post hoc test for multiple comparisons. Tram-Tramadol, JB- Sorghum bicolor , Vit - Vitamin C

impairment in locomotor activity as measured by reduction in total distance travelled (fig. 6 A), average speed (fig. 6B) and the number of the lines crossed (fig. 6C) in the open field test (OFT). Similarly, the anxiogenic response was measured in twice-daily tramadol-treated rats as seen in the decreased entries into the centre area (fig. 6D) and time spent in the centre area (fig. 6E) of the open field box. Only pretreatment with JB (200 mg/kg) was able to significantly reverse twice-daily tramadol-induced impairment in locomotor activity, while anxiety-related behaviour was prevented in rats treated with the three doses of SB. Vit C (100mg/kg) could not significantly prevent locomotor activity impairment and anxiogenic response in twice-daily tramadol-treated rats ($P<0.05$).

DISCUSSION

The study compared the neuroprotective effects of jobelyn, a brand of *Sorghum bicolor* with 100mg dose of Vitamin C, in the amelioration of oxidative stress and anxiety -like Behavoiur in male albino rats. The aim was to investigate the neuroprotective effect on tramadol induced oxidative stress and amelioration of tramadol induced anxiety.

The effect on body and brain weights were compared across the groups with controls. The results showed that tramadol treated rats had comparatively higher reductions in body weights when compared to controls. Pretreatments with the varying dose range of SB also caused significant reductions in body weights. There was no significant body weight loss among rats pretreated with vitamin C and tramadol (group 6: Vit C +Tra 40mg/kg). Generally, there were no marked brain weights differences across all treatment groups in comparison to controls. Body and brain weights were not significantly affected in tramadol use.

Our study showed that tramadol treatment induced elevations in oxidative stress markers (MDA and Nitrites) and caused significant depletions in nonenzymatic and enzymatic antioxidants (Gluthaione, GSH, Catalase and SOD). Similar findings have been reported in several studies on tramadol toxicity (Abdel-Zaher *et al*, 2011, Rafiat *et al*, Kader *et al*, 2020, Mohammadnejad and Soltaninejad., 2022). The susceptibility of the brain to oxidative stress damage is due to the high rate of lipid peroxidation as a result of the high concentrations of polyunsaturated fat in the brain and high utilization of oxygen (Mohammadnejad and Soltaninejad, 2022, Abdel-Zaher *et al*, 2020). Additionally, the redox reaction initiates apoptotic mechanisms in nervous tissues (Awadalla & Salah-Edun, 2016). Elevations of MDA and Nitric Oxide induced by tramadol in chronic use is due to inhibition of mitochondrial electron transport complexes (I, II, III). MDA is a common marker of Polyunsaturated Fat, MDA is produced by free radicals (Gawel *et al*, 2004). Tramadol also increases glutamate production and NO production. Glutamate production is a competitive inhibitor of cysteine, which increases glutamate in cells leading to the depletion of GSH, a major reductant that hydrolyses lipid hydroperoxide and hydrogen peroxide to water. (Parola and Robino, 2001, Abdel- Zaher *et al*,. 2011). As consistently reported in literature, tramadol treated rats had lower levels of nonenzymatic and enzymatic oxidative makers (Mohammadnejad and Soltaninejad, 2022). The principal pathophysiology is due to the affinity of tramadol for oxygen reaction leading to a plethora of reactions producing reactive oxygen species and free radicals, such as superoxide, hydrogen peroxide, hydroxyl and peroxynitrite which leads to lipid peroxidation of neural tissues and reduce antioxidant enzymes in the brain and different tissues (Doostmohammadi M, Rahimi HR 2020). The induction of reactive oxygen species has been implicated in causation of various chronic neurologic disorders, seizures, dependence, tolerance and neuronal death (Rashid, Sinh, Sil, 2013).

Further, in tramadol treated rats there was a significantly higher level of acetylcholinesterase activity relative to controls. Tramadol treatment lead to increased levels of acetylcholinesterase, an enzyme that hydrolyses the breakdown of acetylcholine to choline and acetic acid (Trang & Khandhar 2021). Previous studies have reported on tramadol depletion of cholinergic activity and its possible relationship with memory loss (Zebedee *et al*, 2022, Hosseini-Sharifabad *et al*, 2016). Acetylcholinesterase activity is implicated in dementia, Parkinson's disease, memory impairment and various cognitive and neurodegenerative disorders (Trang & Kandhar, 2021, Bisset, Sobhi & Bensouici, 2022). The neurotransmitter usually found at postsynaptic neuromuscular junction, nerves and muscles. serves to terminate neuronal transmission.

Significant reductions in acetylcholinesterase were seen in rats that were pretreated with varying doses of SB and 100mg of Vitamin C. SB and Vitamin C were attenuated the effect of chronic tramadol use.

Pretreatment with Sorghum bicolor in tramadol treated rats ranging from 50mg to 200mg/kg significantly reduced MDA , increased GSH, GST, catalase, SOD levels, glutamic acid decarboxylase levels when compared with tramadol only treatments and controls, demonstrating the antioxidant activity of SB. SB also significantly reduced AChE in the cortex of rat brains. Pretreatment with SB has a neuro-protective effect on rats treated with tramadol.

The potential role of SB in ameliorating oxidative stress and providing neuroprotective effect can be useful in a

number of conditions due to chronic tramadol use such as in tramadol dependency and tramadol abuse. A number of studies have documented SB in alcohol use and stress induced memory deficits (Oyinbo *et al*., 2018, Umukoro *et al*, 2015). SB neuroprotective role is related to the content. SB contain high constituents of flavonoids which confers it its high oxygen absorbance capacity (Oyinbo *et al*, 2018). A major constituent of SB that confers most of the antioxidant properties is the high content of 3-deoxyanthocyanidins which is stable in very diverse environment and lacks oxygen in the C-3 moiety which makes it have a high oxygen absorbance. This is coupled with rich contents of various other bioactive naturally occurring antioxidants (Okubena *et al*, 2017). Our study findings suggest that SB also showed similar antioxidant efficacy to vitamin C in attenuation of tramadol induced oxidative stress. SB comparable antioxidant activity to vitamin C potentially opens up therapeutic options in the management of tramadol induced oxidative stress.

Our study demonstrated that tramadol use induces anxiety -like behavoiur in rats using the EPM and OFT. The EPM assesses anxiety like behavoiur of avoiding open spaces and heights and tendency to prefer closed spaces and avoidance of danger. While the OFT assesses locomotory actions. It utilizes the fact that rats when in fearful or anxious state, will have reduced locomotor exploratory action to the center square. There were significantly noticeable anxiety -like behaviours in male rats pretreated with tramadol Male rats treated with tramadol had lesser frequency of open arm entries, time spent and increased avoidance of the open arm area. However in male rats pretreated with SB or Vit C showed significantly marked reductions in anxiety-like behaviour in comparison with male rats treated with tramadol only. Similar results were obtained in the OFT in male rats treated with only tramadol. In male rats treated with only tramadol there were impairments, reductions in locomotor action and avoidance of open spaces when compared with controls and with rats treated with SB 200mg/kg. Anxiety –like behaviors (center area entries) were prevented with the dose of SB. There were no significant differences that were observed with male rats pretreated with Vitamin C.

The neuronal cell death, gliosis formation, accompanying tramadol usage was demonstrated on histology. These are due to oxidative stress induction in the cortex of rats, Pretreatment of tramadol with SB and Vit C prevented neuronal cell death and resulting loss. As earlier reported free radicals production ultimately lead to DNA damage and apoptosis (Mowaad, El-Shamarka and Khadrawy, 2022).

The study results seem to differ from some previous studies which have reported that tramadol has anti anxiety actions because of its norepinephrine and serotonin reuptake blockage due to its analgesic action (Caspani *et al*, 2014, Gholami *et al*, 2014). Our study findings showed that chronic tramadol use is associated with oxidative stress and anxiety-like behaviours in rats.

In rodents that were pretreated with 100mg of Vitamin C before tramadol treatment significantly had lower anxiety. Comparatively, similar reduction of anxiety and increased locomotory action was only observed at a pretreatment dosage of 200mg of SB. The observation seems to suggest that chronic or heavy dosage of tramadol use induces subtle changes in behaviour in rats. Several studies in tramadol use in nociceptive indications have been reported to have an anxiety reliving effect in some studies. As also observed, it seems that there also may be a role for SB and Vitamin C in the management of oxidative stress and anxiety like behaviour in the abusive use of tramadol. Pre treatment with SB significantly reduced anxiety-like behavior which was not noticed with 100mg of vitamin C. It suggests that the antioxidant properties may be linked to amelioration of anxiogenic responses. It may be postulated that oxidative stress may be a major pathophysiologic mechanism in dependency on tramadol (Zaher, Abdel-Rahman, ELwasei, 2011). Notably, tramadol in repeated administration as seen in dependency induces increases in brain MDA level and NO production and further depletes intracellular GSH level and GSH-Px activity in the brain which may potentiate anxiety symptoms as seen in tramadol dependence (Zaher, Abdel-Rahman, ELwasei, 2011). The study suggests that the antioxidant properties of SB in high doses was able to ameliorate anxiety induced in tramadol abuse or dependence. Our study showed that pretreatment with SB has significant effect in the amelioration of oxidative stress induced by tramadol use or chronic abuse similar to 100mg of Vitamin C.

CONCLUSION

Tramadol use, abuse and withdrawal is associated with behavioral deficits as evidenced by increased anxiety responses and consequent increases in enzymatic and non enzymatic stress markers. Preventive treatment with Sorghum bicolor improved behavioural deficits and ameliorated oxidative stress induced by tramadol Similar responses were observed in treatment with Vitamin C.

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