Original Article

Zingiber officinale Ameliorates Tramadol-induced Histopathological Distortions in CA1 and CA3 of the Hippocampus of Adult Wistar Rats

Ubong Udeme Ekpo, Uduak Emmanuel Umana, Abubakar Adamu Sadeeq

Department of Human Anatomy, Faculty of Basic Medical Sciences, College of Medical Sciences, Ahmadu Bello University, Zaria, Nigeria

Abstract

Background: Tramadol has a high potential for misuse resulting in cognitive impairment. *Zingiber officinale*, however, possesses neuroprotective qualities. **Objective:** Microscopically assessed hippocampal CA1 and CA3 following *Z. officinale* and tramadol treatment. **Materials and Methods:** Two milliliters/kilogram of distilled water was given to Group 1, Groups 2–5 were administered 50 mg/kg of tramadol while Group 3 was also administered 12.5 mg/kg of naltrexone, and Groups 4 and 5 were also administered 500, and 1000 mg/kg ethanol extract of *Z. officinale* (EEZO), respectively, orally for 21 days. The rats were euthanized and their brains were collected, fixed in 10% formal saline, and processed routinely using crystal fast violet (CFV) stain for the demonstration of Nissl substance, glial fibrillary acidic protein (GFAP) for the demonstration of astrocytes, and Hematoxylin and Eosin for general histoarchitecture and estimation of cell number and volume using physical dissector and Cavalieri estimator, respectively. **Results:** CFV stain revealed alterations in regions of CA1 and CA3 of the hippocampus presenting as indistinct staining intensity and peripheral Nissl substance accumulation in the tramadol-treated group. GFAP revealed numerous reactive astrocyte processes. The area of reactive astrocytes remarkably increased ($P < 0.05$) and the intensity of the Nissl substance remarkably reduced in the tramadol-exposed group. When compared to the control, the tramadol-exposed group's hippocampal volume considerably ($P < 0.05$) decreased (coefficient of error [CE] =0.050). The tramadol treatment group (CE = 0.090) relative to the control group (CE = 0.060) showed a striking decrease ($P < 0.05$) in the number of pyramidal cells in the CA3 region. The tramadol treatment group (CE = 0.090) compared to the control group (CE = 0.060) showed a striking decrease ($P < 0.05$) in the number of pyramidal cells in the CA3 region. Tramadol toxicity was attenuated in the groups treated with EEZO in a dose-dependent manner. **Conclusion:** *Z. officinale* possesses a potential neuroprotective effect against tramadol-induced neurotoxicity.

Keywords: *Cornu ammonis, histochemical, immunohistochemical, stereology*

Introduction

Tramadol is a prescription pain reliever mostly used to cure modest to mildly rigorous pain.[1] In addition, it is a preferred analgesic both during and after surgery, with a dose tailored to the patient's level of sensitivity and discomfort.[2,3] There are signs that the prescription opioid tramadol is addictive and prone to abuse.[4-7] Like other opiates, tramadol can cause both mental and physical dependence.[8] Tramadol has the ability to hinder the reuptake of serotonin and norepinephrine neurotransmitters, which can subsequently cause an increase in their levels in the brain. This may lead to oxidative stress and harm to neurons, particularly in the hippocampal region of the brain that is responsible for memory and learning.[9] In addition, tramadol has been discovered to stimulate the N-methyl-D-aspartate receptor, which can result in neuronal damage and excitotoxicity.[10] Moreover, tramadol can activate microglia, immune cells in the brain that produce pro-inflammatory cytokines, contributing to neuroinflammation.[11] Constant tramadol administration may cause the body to accumulate toxic metabolites, raise the contingency of pharmacokinetic interactions, and/or lower the clearance of tramadol, all of which raise the drug's possibility for toxicity.[12] The hippocampus is an outgrowth of the cerebral cortex's

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Orcid
Ubong Udeme Ekpo {ORCID: 0000-0002-9454-2526}
Uduak Emmanuel Umana {ORCID: 0000-0003-2096-7238}
Abubakar Adamu Sadeeq {ORCID: 0000-0001-5883-3425}

Address for correspondence:
Dr. Ubong Udeme Ekpo, Department of Human Anatomy, Faculty of Basic Medical Sciences, College of Medical Sciences, Ahmadu Bello University, Zaria, Nigeria.
E-mail: ubongekpo023@gmail.com

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temporal region.[13] The hippocampus plays a significant part in memory and learning. It is a malleable and susceptible structure that is injured by many stimuli. Studies have revealed that it is also impacted by several neurological and psychiatric illnesses.[14]

Zingiber officinale has been reported to show neuroprotective effects due to the phenolic and flavonoid compounds present in it.[15] According to published evidence, *Z. officinale* accelerates the brain's antioxidant defense mechanisms and restores normal levels of melonlialdehyde (MDA) in rat models when exerting its neuroprotective effects.[16] Research has been done so far on the detrimental effects of tramadol on the hippocampus's neurotoxicity. Therefore, the purpose of this study was to investigate the effects of tramadol and *Z. officinale* on astrocytes, hippocampal volume, pyramidal cell number, and Nissl substances in adult Wistar rats.

Materials and Methods

This study's ethical endorsement was given by the Ethics Committee on Animal Use and Care, Ahmadu Bello University (ABU), with consent code ABUCAUC/2022/031.

Materials

Plant collection and identification

Locally grown ginger rhizomes were purchased in Samaru, Zaria, Nigeria. These rhizomes were recognized and verified at the ABU, Zaria, Herbarium unit of the Botany Department, Faculty of Life Sciences, where a voucher number (V/N) of ABU02261 was issued.

Preparation of plant extract

Z. officinale's ethanol extract was made in the Pharmacognosy and Drug Development Department, ABU, Zaria. Fresh ginger rhizomes were meticulously rinsed with fresh water to get rid of dirt, and then 2500 g of them were grinded and cold-macerated in 2 L of 70% ethanol while being meticulously agitated on occasion. For the aim of utterly dissolving the active ingredients, the mixture was left alone for 48 h. The extracted material was concentrated using a rotary evaporator and vaporized to dryness on a water bath at 50°C after being initially sieved through mesh cloth and then suction-filtered using Whatman no. 1 filter paper.[17] Before further experimentation and investigation, the dried ginger extracts were appropriately kept in a container.

Phytochemical assessment of ethanol extract of Zingiber officinale (ginger) rhizome

The Pharmacognosy and Drug Development Department, Faculty of Pharmaceutical Sciences, ABU, Kaduna, Nigeria, carried out a phytochemical screening of ethanol extract of *Z. officinale* (EEZO) rhizome. The phytochemical screening approach proposed by Evans[18], Sofowora[19] and Trease and Evans[20] was used.

Acute toxicity study (LD_{50}) of Zingiber officinale

The acute toxicity assessment for EEZO rhizome was carried out using the method of Lorke.[21]

Experimental animals

From the Animal House facility of the Faculty of Pharmaceutical Sciences at ABU, Zaria, Kaduna, Nigeria, 30 male Wistar rats (150–190 g) were collected. Before experimentation, they were moved and kept in wired cages at the Human Anatomy Department, Faculty of Basic Medical Sciences, ABU, Zaria, where they were given 2 weeks to acclimatize. The rats were given pelletized feed and water *ad libitum*.

Drugs

Tramadol

Tramadol hydrochloride (50 mg capsules) is produced by VADIS PHARM. LTD. Plot RD-14-Trans-Ekulu, Enugu State, Nigeria, was bought and used for the study.

Naltrexone

The standard medication for tramadol toxicity in this study was naltrexone (50 mg tablets), which was acquired from Healing Pharma India Pvt. Ltd. Shop No. 01, Plot No.: 25-B, Dev Industrial Estate, Gorwa, Vadodara-16.

Ketamine

For the experiment, ketamine (50 mg/mL ketamine hydrochloride injection USP) was utilized as the anesthetic. It was procured from Swiss Parenterals PVT Ltd., Gujarat, India.

Drugs administration

The dosage of the medication was determined by animal weight and then given.

Experimental design and treatment of animals

For this study, 30 male Wistar rats (150–190 g) were employed. They were shared into 5 groups, each containing 6 rats. As the control, Group 1 received 2 mL/kg of distilled water, while Group 2 received 50 mg/kg of tramadol.[22] Animals in Group 3 were administered tramadol (50 mg/kg) with 12.5 mg/kg of naltrexone. Group 4 animals were given tramadol (50 mg/kg) and 10% LD_{50} of *Z. officinale* (500 mg/kg) and Group 5 administered with tramadol (50 mg/kg) and 20% LD_{50} of *Z. officinale* (1000 mg/kg) orally consecutively for 21 days. The animals were euthanized 24 h after the last dose.

Animal sacrifice

The animals were anesthetized with ketamine and perfused transcardiacally with normal saline and 10% formal saline. After perfusion, the brains were carefully taken out from the skull and rinsed with normal saline. After the brains

were harvested, they were fixed in 10% formal saline for 48 h for proper fixation.

Histopathological studies

Cresyl fast violet

The method of Carson[23] was adopted. Hippocampal sections of the brain were deparaffinized and dehydrated to distilled water. The slides were quickly rinsed in one change of distilled water after being incubated in Cresyl Violet Stain Solution (0.1%) for 2 min. Following a brief dehydration in alcohol (absolute), the sections were immediately cleaned in xylene, mounted using synthetic resin, and cover-slipped with a DPX Mountant.

Glial fibrillary protein analysis

Hsu *et al*.[24] modified avidin-biotin-peroxidase complex method as stated by Yoshida *et al*.[25] was employed in this study. Hippocampal sections were cut into 5 µm-thick slices and preserved in 10% phosphate-buffered formalin for 6 h. The sections were treated with 1/100 anti-glial fibrillary acidic protein (GFAP) (Abcam, UK) primary antibodies, for 90 min after endogenous peroxidase had been blocked. The section was then treated with Mouse/Rabbit HRP (BioSB, Spain) for 45 min. The immunostained slices were dried, cleaned, and mounted in DPX after being mildly counterstained for 1 min with Mayer and Hematoxylin (Dako, Glostrup, Denmark). To perform the sections of the control for the immunohistochemistry of GFAP, the primary antibody was removed, and a nonimmune serum was substituted.

Quantification of Nissl substance distribution and astrocytes reactivity

Cresyl fast violet (CFV), a superb neuronal (cell body specific) stain that helps demonstrate Nissl substance in neurons, was used to assess the amount of Nissl substance in hippocampus pyramidal neurons.[26,27] The staining intensity from the CFV-stained micrographs was measured to quantify the reactivity of Nissl substances (digital micrograph imaging) as specified by the manufacturer.[28] Astrocyte reactivity was also assessed using a computer running image analysis program following instructions as recommended by the manufacturer (Image J, NIH, US).[28] Prejudice values arising from nonidentical picture quality (image acquisition setting) were reduced using the imageJ region of interest management device for an assessment of certain parts of the micrographs.

Stereological studies

Pyramidal cell number

To determine the pyramidal cell number, the hippocampus of the Wistar rat per group was isolated, processed, and sectioned at 5 µm after a pilot study on how many slices could be derived. Tissue sections of the hippocampus were designated using a systematic uniform random sampling method. The sections derived were stained using Hematoxylin and Eosin stain (H and E). Using the physical fractionator method described by Yurt *et al*.,[29] an unbiased estimate of the number of pyramidal cells in the regions of CA1 and CA3 of the Wistar rat was obtained. A transparent counting frame with an acceptance and rejection region was applied to the two succeeding sections to tally the quantity of pyramidal cells. The "look up" section was obtained from one of the section planes, while the "reference" section was taken from the other. Pyramidal cells were selected for the reference part, and counting was done in the look-up section. Pyramidal cells in the reference area but not in the lookup area were counted as the presence of the particle.[30-32] The sum-up of number of pyramidal cells was subsequently estimated using the formula:

$$N = Nv.\ V\ (ref)$$

$$\text{Where } Nv = \frac{\sum Q}{10.\ V\ (dis)}$$

$$V\ (dis) = \frac{\text{T. area of frame} \sum Q}{400.400\ (\text{Final Magnification})^{[32]}}$$

$$= \text{total number of cells counted}$$

$$\text{Final Magnification} = 400$$

Reference volume V (ref) was derived from the estimated volume calculated.

The coefficient of error (CE) was calculated as follows:

$$CE = \frac{SEM}{MEAN}$$

Where standard error of the mean (SEM) = $\frac{\sqrt{\text{Total variance}}}{\sum p}$

n = 10 (number of hippocampal sections).

Volume estimation

The absolute volume of the hippocampus was estimated using the Cavalieri estimator for volume estimation following the technique suggested by West *et al*.[33] Following a pilot study on the number of slices that could be derived and the number of slices that will result in the lowest CE, the hippocampus of the rats was isolated, processed, and sectioned serially using a microtome (Biobase Biodustry, Shandong, China), as stated by Gundersen and Jensen.[34] A method of systematic uniform random sampling was used to choose tissue samples from the hippocampus. H and E was used to stain the sections that were produced. Each hippocampus slice has a clear counting grid randomly placed on its surface. It was recorded how many points struck the hippocampus. Using Cavalieri's method,[35] the volume was calculated as follows:

$V = T.(a/p).\Sigma p$

Where ": =" indicates that the result is the estimated value rather than the true value, "V" is the total volume of the hippocampus, "T" = 0.05 mm is the average slice thickness, "a/p" ¼ is the area associated with each point in the counting grid (4 mm^2), and "Σp" is the total number of points hippocampus.

The CE was calculated as follows:

$$CE = \frac{\sqrt{\text{Total variance}}}{\sum p}$$

Statistical analysis

The data's standard error of the mean was displayed. The differences between and within the groups were assessed using the Kruskal–Wallis test and Dunn's *post hoc* test, respectively. In addition, analysis of variance and the Tukey *post hoc* test were used. The statistical significance was determined at $P \geq 0.05$. Utilizing statistical tools and services (IBM SPSS 26, Chicago and incorporated in Delaware), the data were evaluated.

Results

Acute toxicity analysis

The percentage yield of EEZO was calculated to be 1.72%. The acute toxicity assessment (LD_{50}) of EEZO was calculated to be above 5000 mg/kg bw. No visible behavioral changes were observed.

Phytochemical analysis

Phytochemical analysis of EEZO revealed positive and negative reactions for primary and some secondary metabolites: cardiac glycoside, flavonoids, saponins, tannins, tripenoids, carbohydrates, alkaloids, steroids, phenolic compounds were present, while anthraquinone was not seen.

Histochemical studies

Effects of ethanol extract of Zingiber officinale, naltrexone, and tramadol on Nissl substance

A histochemical study using CFV stain revealed the normal appearance of distinct intensely stained CA1 and CA3 regions in the control group. Regions of CA1 and CA3 of Group II treated with only tramadol (50 mg/kg) showed indistinct staining intensity with pathological changes such as karyolysis, cytoplasmic vacuolation, perineural vacuolation, dark neurons, and chromatolysis [Figures 1 and 2]. Group III (tramadol + naltrexone 12.5 mg/kg) revealed the reduced staining intensity of CA1 and CA3 with mild distortions in the histochemistry of the hippocampus presenting as chromatolysis [Figures 1 and 2]. Group IV treated with tramadol + 500 mg/kg of EEZO showed reduced indistinct staining intensity of the hippocampi regions (CA1 and CA3) with mild distortions in the histochemistry of the hippocampus presenting as karyolysis and chromatolysis while Group V (tramadol + 1000 mg/kg of EEZO) revealed reduced indistinct staining intensity of the hippocampi regions (CA1 and CA3) with mild distortions in the histochemistry of the hippocampus presenting as chromatolysis [Figures 1 and 2].

Effects of ethanol extract of Zingiber officinale, naltrexone, and tramadol on Nissl substance quantification

There was a remarkable decrease ($P < 0.05$) in the staining intensity of Nissl substance in Groups II (50 mg/kg of tramadol), III (tramadol + 12.5 0f naltrexone), and IV (tramadol + 500 mg/kg of EEZO) relative to the control. A notable increase ($P < 0.05$) in the intensity of Nissl substance was observed in Group V (tramadol + 1000 mg/kg of EEZO) in relation to the tramadol-treated group [Figure 3].

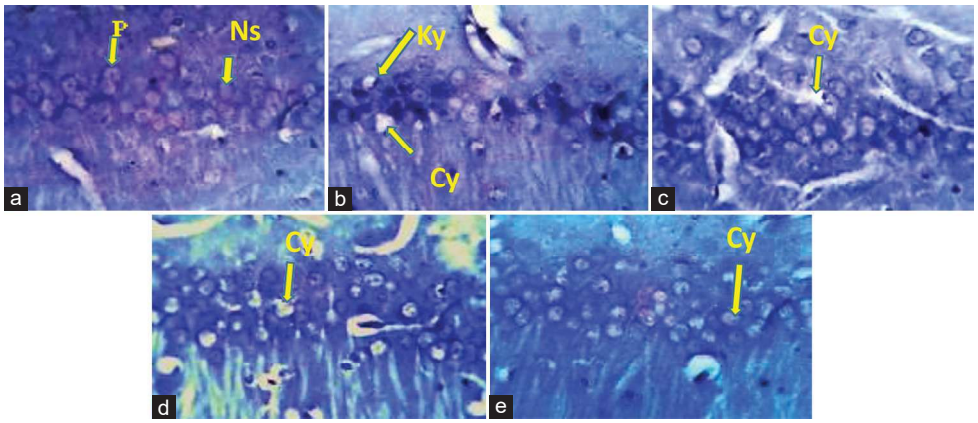

Figure 1: Photomicrograph of CA1 region of the hippocampal section of Wistar rat (CFV × 250). (a) Group I (2 mL/kg of distilled water) with normal appearance of distinct intensely stained CA1 and CA3 regions. Pyramidal cells (P); Nissl substance (Ns). (b) Group II (50 mg/kg of tramadol) showing indistinct staining intensity of CA1 region. Pyramidal cells (P); Karyolysis (Ly); Chromatolysis (Cy). (c) Group III (50 mg/kg of Tm + 12.5 mg/kg of Nalt) showing reduced staining intensity of CA1 region. Chromatolysis (Cy). (d) Group IV (50 mg/kg of Tm + 500 mg/kg of EZO) with reduced indistinct staining intensity of CA1 region. Chromatolysis (Cy). (e) Group V (50 mg/kg of Tm + 1000 mg/kg of EZO) showing reduced indistinct staining intensity of CA1 region. Chromatolysis (Cy). CFV: Crysl fast violet

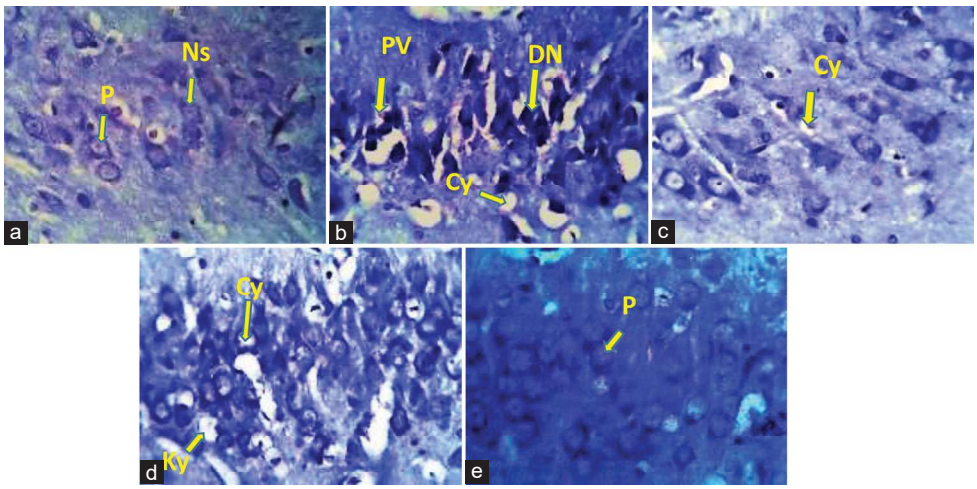

Figure 2: Photomicrograph of CA3 region of the hippocampal section of Wistar rat (CFV × 250). (a) Group I (2 mL/kg of distilled water) with normal appearance of distinct intensely stained CA3 region. Pyramidal cells (P); Nissl substance (Ns). (b) Group II (50 mg/kg of tramadol) showing the indistinct staining intensity of CA3 region. Cytoplasmic vacuolation (CV); Dark Neuron (DN); Perineural Vacuolation (PV). (c) Group III (50 mg/kg of Tm + 12.5 mg/kg of Nalt) showing reduced staining intensity of CA3 region. Chromatolysis (Cy). (d) Group IV (50 mg/kg of Tm + 500 mg/kg of EZO) with reduced indistinct staining intensity of CA3 region. Karyolysis (Ky); Chromatolysis (Cy). (e) Group V (50 mg/kg of Tm + 1000 mg/kg of EZO) showing reduced indistinct staining intensity of CA3 regions. Pyramidal cells (P). CFV: Crysl fast violet, EZO: Ethanol extract of *Zingiber officinale*

Immunochemical studies

Effects of ethanol extract of Zingiber officinale, naltrexone, and tramadol on reactive astrocytes

The control group displayed a confined presence of GFAP-immunoreactive astrocytes that had a regular spatial layout, size, and dark brown cytoplasmic fibers, constituting an orderly network arrangement in the neuropil, as observed during the astrocyte immunohistochemical demonstration using the GFAP stain. These astrocytes also had normal astrocyte processes that did not overlap. The majority of the astrocytes in the CA1 and CA3 areas of the hippocampal region do not exhibit measurable levels of GFAP expression. Group II treated with only tramadol (50 mg/kg) revealed numerous reactive astrocyte processes; extensive overlapping and interdigitation of astrocytes processes; astrocytes proliferation; and astrocyte cell bodies hypertrophy and thickening of astrocytes processes. Group III treated with tramadol and naltrexone (12.5 mg/kg) revealed moderately reactive astrocytes processes; some astrocytes processes overlapping and moderate astrocytes hypertrophy. Group IV treated with tramadol + 500 mg/kg of EEZO revealed less expression of detectable levels of astrocytes and astrocytes processes which do not overlap while Group V treated with tramadol 50 mg/kg + 1000 mg/kg of EEZO revealed moderate reactive astrocytes processes and moderate astrocytes hypertrophy [Figures 4 and 5].

Effects of ethanol extract of Zingiber officinale, naltrexone, and tramadol on quantification of reactive astrocytes

There was a remarkable increase ($P < 0.05$) in the area of reactive astrocytes in the tramadol-exposed group in relation to the control. While remarkable decrease ($P < 0.05$) in the area of reactive astrocytes was detected in groups IV (tramadol + 500 mg/kg of EEZO) and V (tramadol + 1000 mg/kg of EEZO) in relation to the tramadol exposed group [Figure 6].

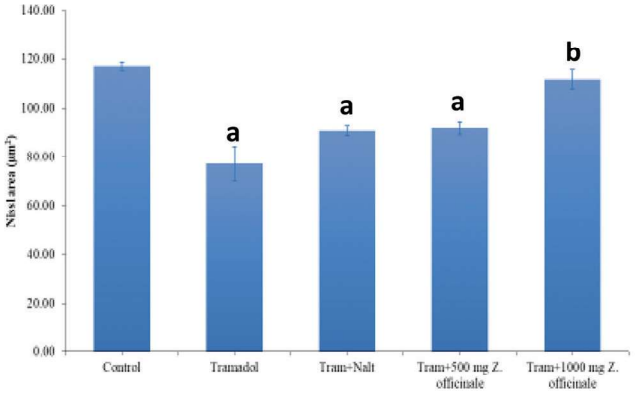

Figure 3: Quantification of Nissl substance reactivity of Wistar rats following oral administration of tramadol and *Zingiber officinale*. *n* = 6; mean ± SEM, one-way ANOVA, *Tukey post hoc test*, a = *P* < 0.05 when compared to control, and b = *P* < 0.05 when compared to tramadol treated group. Control = 2 mL/kg of H_2O, Tram = 50 mg/kg of Tramadol, Nalt = 12.5 mg/kg of Naltrexone, EZO: Ethanol extract of *Zingiber officinale*, SEM: Standard error of the mean, ANOVA: Analysis of variance

Effects of ethanol extract of Zingiber officinale, naltrexone, and tramadol on hippocampal volume

The volume of the hippocampus remarkably decreased ($P < 0.05$) in Groups II (50 mg/kg of tramadol) (CE = 0.050), Group III (tramadol + 12.5 mg/kg of naltrexone) (CE = 0.053), Group IV (tramadol + 500 mg/kg of EEZO) (CE = 0.051), and Group V (tramadol + 500 mg/kg of EEZO) (CE = 0.059) in relation to the control. Furthermore, there was a remarkable decrease in the hippocampal volume of Group III (CE = 0.053) and Group V (CE = 0.059) in relation to tramadol exposed group [Figure 7].

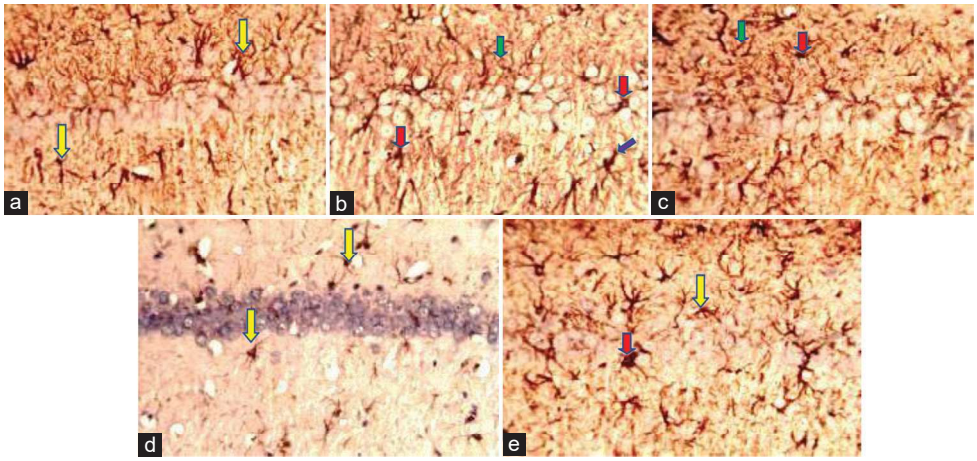

Figure 4: Photomicrograph of CA1 region of the hippocampal section of Wistar rat (GFAP × 250). (a) Group I (2 mL/kg of distilled water) with few expressions of detectable levels of astrocytes (arrows); no overlapping of astrocytes processes. (b) Group II (50 mg/kg of tramadol) with numerous reactive astrocytes processes; extensive overlapping and interdigitation of astrocytes processes (green arrow); astrocytes proliferation; astrocytes cell body hypertrophy (red arrows); thickening of astrocytes processes (purple arrow). (c) Group III (50 mg/kg of Tm + 12.5 mg/kg of Nalt) with moderately reactive astrocytes processes; some astrocytes processes overlapping (green arrow); astrocytes cell bodies hypertrophy (red arrow). (d) Group IV (50 mg/kg of Tm + 500 mg/kg of EZO) with less expression of detectable levels of astrocytes; astrocytes processes are not overlapping. (e) Group V (50 mg/kg of Tm + 1000 mg/kg of EZO) with moderate reactive astrocytes processes (yellow); some astrocytes hypertrophy (red). GFAP: Glial fibrillary acidic protein, EZO: Ethanol extract of *Zingiber officinale*

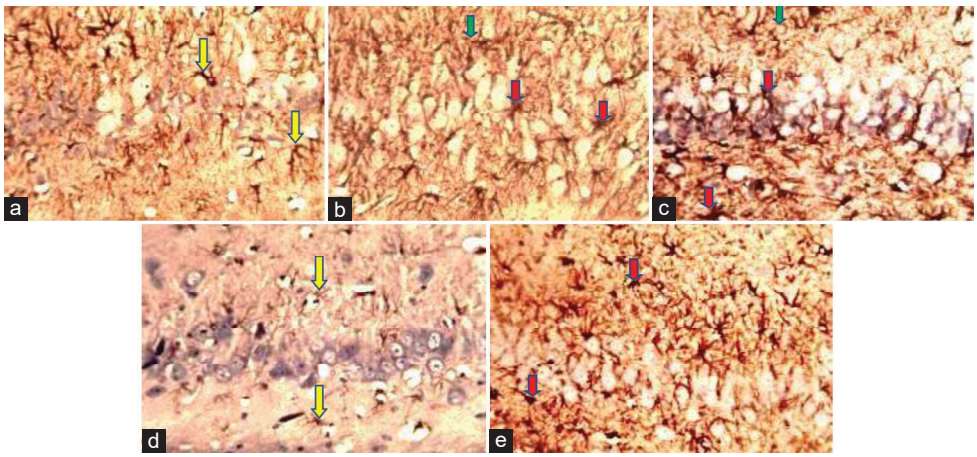

Figure 5: Photomicrograph of CA3 region of the hippocampal section of Wistar rat (GFAP × 250). (a) Group I (2 mL/kg of distilled water) with few expressions of detectable levels of astrocytes (arrows); no overlapping of astrocytes processes. (b) Group II (50 mg/kg of tramadol) with numerous reactive astrocytes processes; extensive overlapping and interdigitation of astrocytes processes (green arrow); astrocytes proliferation; astrocytes cell body hypertrophy (red arrows). (c) Group III (50 mg/kg of Tm + 12.5 mg/kg of Nalt) with moderately reactive astrocytes processes; some astrocytes processes overlapping (green arrow); astrocytes cell bodies hypertrophy (red arrows). (d) Group IV (50 mg/kg of Tm + 500 mg/kg of EZO) with less expression of detectable levels of astrocytes; astrocytes processes are not overlapping. (e) Group V (50 mg/kg of Tm + 1000 mg/kg of EZO) with moderate reactive astrocytes processes (yellow); some astrocytes hypertrophy (red). EZO: Ethanol extract of Zingiber officinale, GFAP: Glial fibrillary acidic protein

Effects of ethanol extract of Zingiber officinale, naltrexone, and tramadol on pyramidal cells number

There was a remarkable decrease ($P < 0.05$) in the pyramidal cells number in the CA1 region of the hippocampus of Group III (tramadol + 12.5 mg/kg of naltrexone) (CE = 0.043) and Group V (tramadol + 1000 mg/kg EEZO) (CE = 0.074) in relation to the control. A remarkable increase in the cells number in the CA1 region was detected in Group IV (tramadol + 500 mg/kg EEZO) (CE = 0.048) when compared to Group III and a remarkable decrease in Group V (tramadol + 1000 mg/kg EEZO) (CE = 0.074) in relation to Group IV [Figure 8]. In the CA3, a remarkable decrease ($P < 0.05$) in the number of pyramidal cells was detected in the tramadol-exposed group (CE = 0.090) in relation to the control (CE = 0.060) [Figures 8 and 9].

Discussion

The central nervous system's tissue and activities are impacted by tramadol, which readily penetrates the blood-brain barrier.[35,36] Given that it uses a lot of oxygen, elevated quantities of polyunsaturated fatty acids, and antioxidant concentrations that are not too high, the brain is predominantly prone to oxidative damage.[37] The evidence in favor of *Z. officinale's* neuroprotective properties points

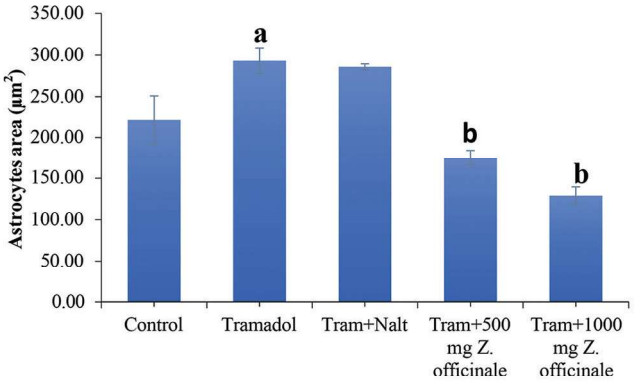

Figure 6: Area of reactive astrocytes of Wistar rats following oral administration of tramadol and *Zingiber officinale*. *n* = 6; Kruskal–Wallis, *Dunn's post hoc test*, a = *P* < 0.05 when compared to control, and b = *P* < 0.05 when compared to tramadol group. Control = 2 ml/kg of H_2O, Tram = 50 mg/kg of Tramadol, Nalt = 12.5 mg/kg of Naltrexone, EZO: Ethanol extract of *Zingiber officinale*

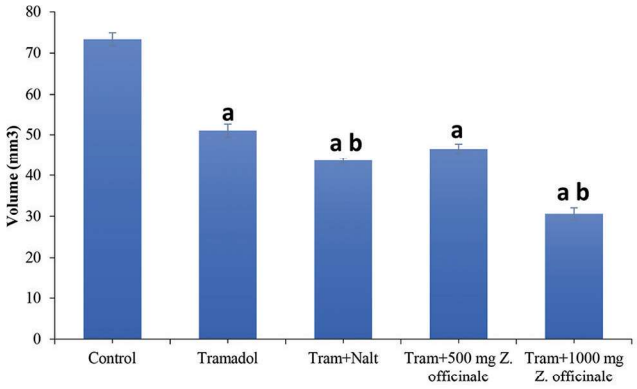

Figure 7: Hippocampal volume of Wistar rats following oral administration of tramadol and *Zingiber officinale*. *n* = 6; mean ± SEM, one-way ANOVA, *Tukey post hoc test*, a = *P* < 0.05 when compared to control, and b = *P* < 0.05 when compared to tramadol group. Control = 2 mL/kg of H_2O, Tram = 50 mg/kg of Tramadol, Nalt = 12.5 mg/kg of Naltrexone, EZO: Ethanol extract of *Zingiber officinale*, SEM: Standard error of the mean, ANOVA: Analysis of variance

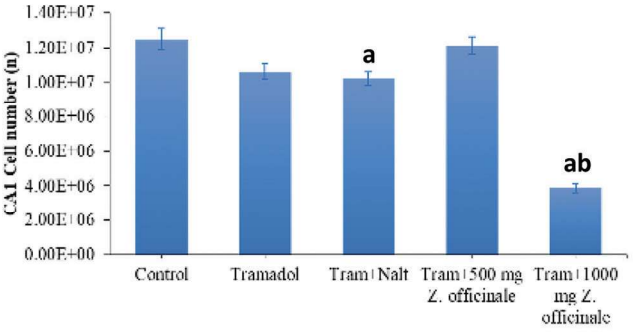

Figure 8: CA1 pyramidal cell number of Wistar rats following oral administration of tramadol and *Zingiber officinale*. *n* = 6; mean ± SEM, one-way ANOVA, *Tukey post hoc test*, a = *P* < 0.05 when compared to control, and b = *P* < 0.05 when compared to tramadol group. SEM: Standard error of the mean, ANOVA: Analysis of variance

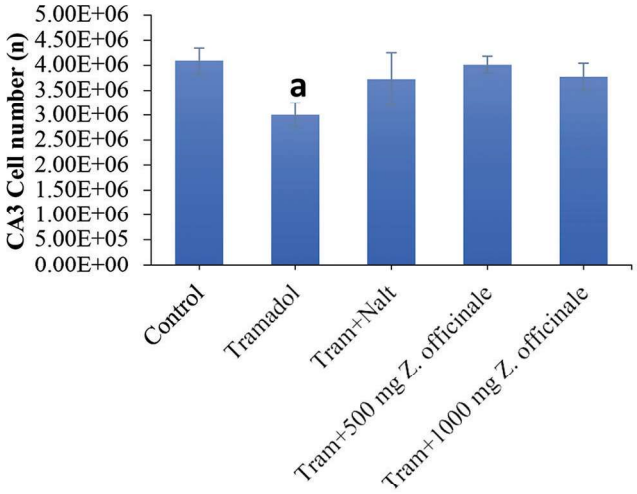

Figure 9: CA3 pyramidal cell number of Wistar rats following oral administration of tramadol and *Zingiber officinale*. *n* = 6; Kruskal–Wallis, *Dunn's post hoc test*, a = *P* < 0.05 when compared to control group. Control = 2 mL/kg of H_2O, Tram = 50 mg/kg of Tramadol, Nalt = 12.5 mg/kg of Naltrexone, EZO: Ethanol extract of *Zingiber officinale*

to the possibility that this plant accelerates the brain's natural anti-oxidant defenses while bringing MDA levels in rat models back to normal.[16] This study investigated the protective effects of EEZO on Nissl substance, reactive astrocytes, the quantity of pyramidal cells in the regions of CA1 and CA3 of the hippocampus, and the hippocampal volume in adult Wistar rats given tramadol.

Phytochemical screening of EEZO revealed that it contains tannins, flavonoids, saponins, steroids, cardiac glycoside, carbohydrates, alkanoids, tripenoids, phenolic compounds, and the absence of anthraquinone. This result is similar to several other studies which revealed positive and negative reactions for the above secondary metabolites.[38-42] *Z. officinale* and its components are crucial neuroprotectants. *Z. officinale*'s precise mode of operation in this view is not entirely understood. Nonetheless, it is believed to have neuroprotective properties as a result of the phenolic and flavonoid components.[43] Flavonoids are hypothesized to improve cognitive performance by increasing neuronal function, inducing neurogenesis, and having neuroprotective qualities.[44,45] They are capable of crossing the blood–brain barrier and have been detected in areas of the rat brain linked with memory and learning soon after being orally administered.[46,47] Numerous epidemiological studies indicate that consuming foods or supplements high in plant-derived polyphenols may halt the development and progression of neurodegenerative diseases.[48,49] Another discovery that supports *Z. officinale's* ability to act as a neuroprotective agent reveals that it has this ability by boosting the anti-oxidant defense mechanisms of the brain and bringing MDA levels back to standard in rat models.[16] Gingerol and derivatives of *Z. officinale* are reported to be effective at scavenging hydroxyl and superoxide radicals.[50-52] The protective impact of Z. officinale on rat models was observed by Sharma and Singh,[53] who reported that it diminishes LPO while increasing QR, SOD, GPx, GSH, GST, GR, CAT, and protein levels.

Histochemical assessment of the hippocampus (CA1 and CA3 regions) using CFV stain in this study, revealed histopathological changes such as karyolysis, cytoplasmic vacuolation, dark neuron, perineural vacuolation, chromatolysis and indistinct staining intensity of the hippocampi in tramadol-treated group. However, these histopathological changes were attenuated in the extract-treated groups in a dose-dependent manner. The loss of Nissl bodies and resulting decreased staining intensity of the Nissl bodies in the tramadol group, which are both signs of neuronal degeneration, could be attributed to the detrimental effects of tramadol administration, which suggests that opioids may be involved in neurodegeneration.

Neuronal degeneration leads to a decrease in Nissl granules.[54] The Nissl substance is affected by both chemical and toxic agents, which interfere with its metabolic function.[55] Awadalla and Salah-Eldin[56] reported alterations in Nissl granules in the cerebral cortex and hippocampus, where most neurons showed pale blue-colored granules at the rims of cytoplasm, indicating a decrease in Nissl substance. A study on the administration of both tramadol and alcohol in the medial prefrontal cortex of young male rats revealed alterations in the distribution of Nissl substance, suggesting either neuronal degeneration or impaired metabolism.[57] *Z. officinale* has the potential to increase the number of intracellular Nissl granules and neurons in the hippocampus, according to Jesudoss *et al*.[58] In a study on protection against Alzheimer's disease-induced behavioral impairment in rats, *Z. officinale* root extract improved Nissl granules in the rats' brains.[59]

According to Yang and Wang,[63] GFAP, a significant protein in glial filaments within astrocytes, which is responsible for supporting the cytoskeleton, can be a potential indicator of brain damage. Studies show that these protein biomarkers play a key role in observing brain injury and prognoses in individuals with traumatic brain injury or concussion.[61-63]

Hence, this report also studies the effect of *Z. officinale* extract on the expression of astrocytes using a GFAP stain. Exposure of rats to 50 mg/kg of tramadol resulted in numerous reactive astrocytes processes; extensive overlapping and interdigitation of astrocytes processes; astrocytes proliferation; astrocytes cell body hypertrophy and thickening of astrocytes processes. In addition, there was a notable upsurge ($P < 0.05$) in the area of reactive astrocytes in this group. The groups that were treated with the extract exhibited a reduction in the pathological changes brought on by tramadol toxicity. This discovery corresponds with the outcomes of the study performed by Adekomi *et al*.,[67] which evaluated the impact of concurrent treatment with alcohol and tramadol on the mental abilities and neuroinflammatory responses of male rats' medial prefrontal cortex in their early stages of development. Giving young rats 60 mg/kg twice daily for 30 days resulted in numerous reactive astrocyte processes and significant losses of neurons. Barbosa *et al*.[64] detected an upsurge in the expression GFAP in the cerebral cortex following varying doses of opioids. In another study, astrocytes showed enlarged cell bodies and thickened processes after intraperitoneally administering 50 mg/kg/day of tramadol for 4 weeks, with an increase in GFAP expression.[65]

GFAP overexpression is considered to be a factor in opioid tolerance and dependence, as supported by numerous studies.[66-68] Chronic drug misuse is believed to cause astrogliosis, which is a distinctive immune response to neurotoxicity and brain injury that can alter synaptogenesis and neurogenesis, or result in apoptosis and/or necrosis.[69,70] The increased GFAP expression found in this study is consistent with the histopathological evidence of glial proliferation and hypertrophy, which are themselves a response to opioid-induced damage. El-Akabawy and El-Kholy[70] demonstrated in their study on the neuroprotective effects of *Z. officinale* on the brains of streptozotocin-induced diabetic rats that ginger's therapeutic effects were facilitated by controlling the astroglial response to injury, bringing down AChE expression, and enhancing neurogenesis. Torkzadeh-Mahani *et al*.[68] found that *Z. officinale* extract significantly reduced GFAP levels to control levels following morphine administration. Another study reported a significant improvement in GFAP expression induced by nicotine following treatment with both ginger and cinnamon oil,[71] which is similar to the current study. The results of a study stipulated that the combined aqueous extract of ginger and garlic resulted in a reduction in GFAP expression after the administration of lead acetate.[72] Mabrouk *et al*.[73] observed moderately immune-reactive astrocytes in mice administered with Topamax and Ginger oil in sections from the animals.

Nowadays, in medical sciences, estimation of volume and cell number aids in diagnosis and therapy by studying tissues, cells, organelles, and the spatial placement of cells during the disease and treatment with advanced microscopic calculations. Also, it added to the advancement of science in the field of histology during health and disease through the study of, surface, length, volume, and number of biological objects.[32] In this present study, we estimated hippocampal volume and pyramidal cells number following treatment with tramadol and *Z. officinale*. Findings from this study showed a notable reduction ($P < 0.05$) in hippocampal volume and number of pyramidal cells in the region of CA3 in tramadol exposed group in relation to the control. Results from this study indicate that the pyramidal cell number in the hippocampus was moderately compensated by *Z. officinale*. The loss of pyramidal cells seen in this study may be attributed to apoptosis, which results in cellular death.[74] Programmed cell death, also known as

apoptosis, is an active process of natural cell death that takes place during development as well as a response to the cytotoxic effects of certain neurotoxins.[75] Long-term opioid use may cause structural changes and neuronal death.[71] Numerous investigations have demonstrated a connection between prolonged opioid use and elevated reactive oxygen species (ROS) generation.[76-81]

Oxidative stress is a result of an imbalance between the body's ability to produce ROS and its capacity to eliminate them. Large doses of reactive intermediary cause the breakdown of cellular constituents and the production of dangerous compounds. Large amounts of the reactive intermediate lead to the breakdown of cellular constituents and the production of subsequent dangerous compounds. Nuclear factor-kappaB (NF-B), a redox-sensitive transcription factor, can be activated by ROS. NF-B then causes the transcription of inflammatory genes and the production of several inflammatory mediators.[54] In addition to producing ROS, exposure to opioid receptor agonists has been related to stimulate apoptotic processes.[82]

The findings from this present study are similar to that of Ezi *et al*.[83] who compared the experimental group to the control group and observed a notable reduction in the amount of white matter, Purkinje neurons, molecular layer, and granular layer in the cerebellum. Thus, the finding from the study revealed that tramadol treatment resulted in cerebellar atrophy in rats. Another study also reported that the tubular diameter and epithelial height mean values significantly decreased after using tramadol for 30 days.[84] Rafati *et al*.[85] observed that the motor prefrontal cortex's volume and its divisions were found to have dropped by about 15%, its total number of neurons by about 44%, and its whole number of oligodendrocytes by about 41% in the morphine-treated group compared to that in the saline group (P = 0.05). Adekomi *et al*.[60] also reported a remarkable decrease in the number of normal neurons and the number of degenerating neurons in the motor prefrontal cortex was raised.

In vitro and *in vivo* research has demonstrated the powerful antioxidant effects of *Z. officinale*. With its high antioxidant activity, it has a strong impact on scavenging DPPH radicals and regulating the peroxidation of lipids.[86] Bordbar *et al*.[87] reported that *Z. officinale* enlarged the volumes of the seminiferous tubule in the 100 mg/kg treatment group relative to the control group, based on stereotypical studies on the herb's impact on the testicles in rats with busulfan-induced infertility.

Conclusion

The findings of this investigation showed that *Z. officinale* has the propensity to preserve the structures and cells of the nervous system. To manage and cure opiate-induced neurodegeneration, it may be employed.

Patient informed consent

There is no need for patient informed consent.

Ethics committee approval

The Ahmadu Bello University Ethics Committee on Animal Use and Care approved this work in accordance with ethical standards, with approval number ABUCAUC/2022/031.

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Conflict of Interest

There is no conflict of interest to declare.

Author Contributions subject and rate

- Ekpo Ubong Udeme (40%): Design the research, data collection, and analyses.
- Umana Uduak Emmanuel (30%): Supervision and research organization.
- Abubakar Addamu Sadeeq (30%): Supervision and research organization.

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