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Original Article

Evaluating the anticonvulsant potential of kaurenoic acid isolated from *annona senegalensis* leaves in pentylenetetrazole-induced seizures: behavioral, cognitive, and cellular insights in Wistar rats

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Abstract

BACKGROUND AND AIM: Understanding seizure behavior is crucial for accurate diagnosis, effective treatment, and improved patient quality of life. While many antiepileptic drugs (AEDs) can suppress seizures, they often do not address the underlying pathophysiological mechanisms. They may adversely affect memory, mood, and cognition, as seen with phenobarbital (PB). This study aimed to characterize pentylenetetrazole (PTZ)-induced seizures, assess locomotor and exploratory behavior, evaluate hippocampal neuronal impact, and compare the therapeutic effects of kaurenoic acid (KNA) to phenobarbital.

METHODOLOGY: Thirty adults male Wistar rats (150–250 g) were assigned to five groups (n = 6): Group 1 (vehicle control, water, 1 ml/kg, body weight), Group 2 (PTZ 40 mg/kg, body weight), Group 3 (PTZ 40 mg/kg, body weight + KNA 400 mg/kg, body weight), Group 4 (PTZ 40 mg/kg, body weight + KNA 800 mg/kg, body weight), and Group 5 (PTZ 40 mg/kg, body weight + PB 10 mg/kg, body weight). Rats' behavior was assessed and were anesthetized with Halothane, the whole brain was extracted and fixed with 10% formalin for histological analysis.

RESULTS: The results demonstrated a significant delay in the onset of the first neck muscle jerk ($F = 19.29$, $p < 0.0001$) and clonic-tonic seizures ($p < 0.0001$ and $p < 0.01$) in KNA- and PB-treated groups compared to PTZ-only controls. Both KNA and PB improved anxiety-related behavior, locomotor activity, and hippocampal cellular condition, although these improvements were not statistically significant ($p > 0.05$).

CONCLUSION: These findings suggest that KNA, like PB, can mitigate PTZ-induced behavioral and cellular disruptions, highlighting its potential as a therapeutic agent for seizure management.

Keywords:
Seizure behavior; rat-model; epilepsy; kaurenoic acid; hippocampus

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INTRODUCTION

Epilepsy is a chronic neurological disorder affecting approximately 50 million people worldwide, characterized by recurrent seizures. Sub-Saharan Africa accounts for a significant portion, with 9.39 cases per 1,000 individuals. Despite extensive research, the fundamental causes of epilepsy remain poorly understood, necessitating the development of reliable animal models to investigate the disease's underlying mechanisms and explore potential therapeutic approaches (Ba-Diop *et al*., 2014, Pitkänen *et al*., 2016, Chen *et al*., 2023). Seizures are temporary episodes of abnormal or excessive brain neuronal activity, manifesting in various forms, including motor, autonomic, sensory, or cognitive disturbances. Understanding seizure behavior is critical for accurate diagnosis, effective treatment, and improved quality of life for patients (Fisher *et al*., 2005).

Seizures result from an imbalance between excitatory and inhibitory neurotransmission in the brain, which may be triggered by genetic mutations, structural brain abnormalities, neurotransmitter imbalances (such as glutamate and GABA), ion channel dysfunction, or immune and metabolic factors. The behaviors associated with seizures can include sensory, motor, autonomic, cognitive, and emotional symptoms, such as fear and anxiety (Chen *et al*., 2023). Seizures are broadly categorized into focal (partial) and generalized seizures, including absence, myoclonic, tonic, tonic-clonic, and atonic seizures (Stafstrom & Carmant, 2015, Sirven, 2015). An epileptic seizure is considered transient, with a distinct beginning and end, though the termination may be obscured by

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postictal symptoms. In cases of prolonged or recurring seizures, known as status epilepticus, diagnosis typically requires clinical and EEG evaluation (Fisher *et al*., 2005).

Rats are widely used as animal models in seizure research due to their neuroanatomical similarities to humans, genetic consistency, and the ability to reliably induce seizures under controlled laboratory conditions (Löscher, 2011). Seizure behaviors in rats can range from mild motor activity changes to severe convulsive episodes (Sirven, 2015).

Various methods are employed to induce seizures in rat models, each with unique advantages and limitations. Chemical convulsants, such as pilocarpine, kainic acid, and pentylenetetrazole (PTZ), are commonly used to disrupt neurotransmitter systems, leading to heightened neuronal activity. PTZ kindling, a chronic epilepsy model, is characterized by sustained seizure susceptibility and associated molecular and cellular alterations, including oxidative stress and neurodegeneration in the hippocampus (Yusuf *et al*., 2012, Zhu *et al*., 2017). Antiepileptic drugs (AEDs) like phenobarbital, carbamazepine, valproate, and levetiracetam are the conventional means of managing seizures. However, these drugs primarily suppress seizure generation without addressing the underlying pathophysiological mechanisms and may have side effects, such as memory and cognitive impairments associated with phenobarbital (Eddy *et al*., 2011).

Traditional medicine (TM), often utilizing medicinal plants, plays a vital role in primary healthcare in many underdeveloped regions, including Africa, due to its accessibility, affordability, and cultural acceptance (Oyebode et al., 2016). The World Health Organization recognizes the importance of TM, supporting its integration into global health systems, as outlined in the Beijing Declaration of 2008, which advocates for the development of TM based on research and innovation. Annona senegalensis (AS), commonly found in African savannas, is widely used in traditional medicine to treat epileptic seizures. Studies have demonstrated its potential anticonvulsant effects in various animal seizure models (Okoli *et al*, 2010; Okoye *et al*, 2013, Dare *et al*., 2021). Kaurenoic acid, a bioactive compound isolated from AS, has shown sedative and anticonvulsant properties, but its precise mechanisms of action at the cellular and molecular levels remain unclear (Okoye *et al*, (2013).

This study characterized PTZ-induced seizures in a Wistar rat model, focusing on Racine scale 3 (neck jerks) and scale 6 (clonic, tonic-clonic seizures, and wild jumping), with corresponding EEG patterns of sharp spikes, spike-wave discharges, and high-amplitude polyspikes (Lüttjohann *et al*., 2009), (Van Erum *et al*., 2019). Additionally, we investigate the impact of PTZ-induced seizures on locomotor activity, exploratory behavior, and hippocampal neurons, comparing the therapeutic effects of kaurenoic acid with phenobarbital.

MATERIALS AND METHODS

Animals

Thirty adult male Wistar rats weighing between 150g to 250g were used for this study, which were procured and housed at the Kampala International University animal house. The animals were grouped into five groups of six animals per group in clean cages at average room temperature between 18 to 26^{0}C under a 12-hour light and 12-hour dark cycle. They were fed with pelletized rat feed and given water *ad libitum* for one month before the commencement of the experiment given two weeks to acclimatize to the environment.

Plant collection, preparation, crude extraction, and isolation of kaurenoic acid

A. senegalensis leaves were collected from the "Ishaka-Bushenyi" Municipality along Mbarara-Kasese Road, Western Uganda. Geographic coordinates are 0.5424° S and 30.1965° E. The Plant was identified/deposited at the Department of Biology at Mbarara University of Science and Technology, Uganda, and given a voucher No. Moses Odur 002.

The leaves removed from the stem were dried and subjected to an aqueous extraction method at the Department of Pharmacology Laboratory, Kampala International University Western Campus, Ishaka-Bushenyi, Uganda). Specifically, dry leaves were ground using a blender to obtain 2 kg of powder mixed with 10L of distilled water in a sterile conical flask. The mixture was placed on a shaker for 72 hours and then sieved to remove debris. The remaining liquid was filtered by gravity using Whatman no. 1 filter paper.

The filtrate was incubated at 35^{o}C for 1 week to evaporate the water and obtain an aqueous crude 100g dry powder. This was further subjected to solvent–solvent fractionation by separation funnel method with methanol, ethyl acetate, and n-hexane in order of increasing polarity to obtain the respective fractions. To isolate kaurenoic acid, the aqueous extract was transferred to a separation funnel, equal volume of n-hexane added and the funnel was shaken vigorously to partition non-polar compounds into the n-hexane layer. The funnel was allowed to settle then the upper (n-hexane) layer was separated from the lower (aqueous) layer. For ethyl acetate extraction, ethyl acetate was added to the remaining aqueous extract in the separation funnel, shaken vigorously to separate the semi-polar compounds including kaurenoic acid, which partitioned into the ethyl acetate phase and allowed to settle. The ethyl acetate layer was collected and evaporated under reduced pressure. Purification by column chromatography was done with silica gel column using n-hexane as the initial elution solvent. The sample was loaded onto the column by dissolving the concentrated ethyl acetate extract in a small volume of sovent (n-hexane acetate, 90:10). The ethyl acetate fraction was further eluted with gradient mixtures of n-hexane and ethyl acetate (90:10 to 20:80) to obtain a sub-fraction

(80:20) which was further characterized as kaurenoic acid based on the yield and potency (Okoye *et al*., 2013, Mtunzi *et al*., 2017).

Seizure Induction Behavioural Study

The animals were grouped into five groups for the experiment (n = 6). Sezures was induced with PTZ (Sigma–Aldrich, P6500, Lot No. MKCM4261) and seizure-behaviour was recorded in a square open-field standard box (100 x 100 x 50 cm) for 10 minutes with a PC Logitech B905 digital Webcam. The recorded videos were scored according to the Racine scale (scale 3 and 6 characterized by first neck jerks and clonic, tonic-clonic seizures respectively) (Lüttjohann *et al*., 2009; Van Erum *et al*., 2019). To investigate the anticonvulsant effect of kaurenoic acid (KNA) compared to phenobarbital (PB), Group 1 (negative control) received distilled water 1ml p.o, Group 2 (positive control) received PTZ (40mg/kg body weight, i.p), Group 3 received PTZ (40mg/kg body weight, i.p) and KNA (400mg/kg body weight, p.o.), Group 4 received PTZ (40mg/kg body weight, i.p) and KNA (800mg/kg body weight, p.o), and Group 5 received PTZ (40mg/kg body weight, i.p) and PB (10mg/kg body weight, i.p) (Yokoro *et al*., 2001, Okoye *et al*., 2013, Bora *et al*., 2021). The treatment groups (Groups 3, 4, and 5) received KNA (400mg/kg), KNA (800mg/kg), and PB (10mg/kg) thirty minutes after PTZ (40mg/kg i.p.). The treatment groups were compared with the negative and positive control groups that received only water (1ml p.o.) and PTZ (40mg/kg i.p) respectively. Immediately after the drug administration, the animals were recorded in an open field environment for ten minutes with a Logitec cameral connected to a laptop, and the time of first neck jerk and Clonic, tonic-clonic seizure (Racine scale 3 and scale 6) were determined. (Lüttjohann et al., 2009, Van Erum et al., 2019).

The Open Field Test and Thigmotaxis Assessment

The open field test (OFT) is a fundamental assay in epilepsy research, enabling the assessment of seizure behavior and the evaluation of potential anticonvulsant therapies. By providing quantitative measures of locomotor activity, exploratory behavior, and seizure-specific actions, the OFT contributes to a deeper understanding of seizure mechanisms and the development of effective treatments. The experimental device was a square open-field standard box (100 x 100 cm) surrounded by walls (50 cm high). The testing session lasted 10 min and was preceded by a 5 min period of habituation. Rats were tested individually. The apparatus was cleaned with 70% ethanol after each test. Thigmotaxis (i.e. walking close to the walls) was assessed by the percentage of time close to the walls over the total time in the test apparatus (Fig IV). Thigmotaxis is used to evaluate anxiolytic, anxiogenic, and even non-pharmacological treatments. It is a measure of the percent of the 10 min total test time that the subject remains adjacent to the outer wall of the maze which is indicative of anxiety-like behavior (Zhang *et al.*, 2023)

The OFT assay was carried out 24 hours after seizure induction and treatment to obtain a quantitative measure of explorative bahaviour, anxiety-related behavior, and locomotor activity of the animals. Each of the animals was placed in an open arena and its behavior was recorded with a Logitech camera connected to a computer. The videos were evaluated using ANY-maze software 7.3 and the explorative, anxiety-related behavior and locomotor activity were determined and compared. Time spent in the center versus the periphery of the arena can indicate anxiety levels. Animals with increased anxiety tend to spend more time near the walls (thigmotaxis) (Kraeuter *et al*., 2019, Leon, 2023).

Tissue Sample Preparation and Histological Assessment

Following the behavioural studies, the animals were anesthetized with Halothane (Piramal Pharma Limited, India), and the whole brain was extracted and fixed with 10% formalin in a tissue container for histological assessment (Aboghazleh et al., 2024). Each cerebral hemisphere was cut coronally into two halves to reach the site of the hippocampus and then the specimens were dehydrated in series of alcohol solutions with increasing concentrations to remove water and replace it with a medium that is compatible with embedding materials, cleared with xylene to remove the dehydrating agent to make the tissue transparent, and embedded in paraffin blocks. Serial coronal sections were cut into 5–7 µm thick and stained with hematoxylin and eosin (H&E) (El-Khair et al., 2014, Haggag et al., 2014)

Neuromorphology Photomicrography

Photomicrographs were obtained using a Nikon Wclipse-Ci Phase Contrast microscope (Nikon, Japan). The histoarchitectural images of the hippocampus were taken at 40x, 100x and 400x magnifications. The images were analyzed with a focus on the CA1 region using Image j software to estimate the number of neurons.

The histological image was uploaded in Image J and converted to an 8-bit grayscale image, which simplifies the thresholding and analysis. The threshold range was adjusted to highlight neuron bodies while minimizing background noise and the thresholded image was converted to a binary format which turn neurons into distinct regions for particle analysis and detailed measurement (Schneider *et al*., 2012).

Statistical Analysis

The data obtained was subjected to analysis of variance (ANOVA) followed by Tukey *post hoc* test using GraphPad (8.0.21) (Geoff *et al*., 2007), and the results are presented as mean ± SEM. Statistical significance was defined as p-value < 0.05.

RESULTS

Seizure Behaviour Test

The severity of seizure behavior was assessed by measuring the time to the first neck muscle jerk (TFMJ), revealing a statistically significant interaction between the control and experimental groups ($F = 19.29$, $p < 0.0001$). There was statistically significant short TFMJ observed in the PTZ-induced seizure group compared to the control group with no TFMJ ($p < 0.0001$). The treatment groups showed a significant increase in TFMJ compared to the

PTZ-induced seizure group, indicating delayed onset of seizure activity. Specifically, KNA (400 mg/kg), KNA (800 mg/kg), and PB (10 mg/kg) treatments significantly delayed the onset of the first neck muscle jerk ($p < 0.0001$, $p < 0.0001$, and $p < 0.01$, respectively). There was no statistically significant difference between KNA (400 mg/kg) and PB (100 mg/kg), but KNA (800 mg/kg) showed significantly better performance than PB (10 mg/kg) ($p < 0.01$). (Figure 1A)

The time of onset for clonic and tonic-clonic seizures (CTCS), characterized by lying on one side and wild jumping, also showed significant interactions between the groups ($F = 179.5$, $p < 0.0001$). PTZ-induced seizure group (G2) demonstrated a shorter time of onset of CTCS compared with the control group (G1) with no CTCS ($p < 0.0001$). A significant increase in CTCS onset time was observed in the treatment groups compared to the PTZ-induced seizure group, indicating a therapeutic effect. KNA (400 mg/kg), KNA (800 mg/kg), and PB (10mg/kg) significantly delayed the onset of CTCS ($p < 0.0001$, $p < 0.0001$, and $p < 0.0001$, respectively). However, there was no statistically significant difference in the performance between KNA (400 mg/kg), KNA (800 mg/kg), and PB (10 mg/kg) (Figure 1B)

Open Field Test

Thigmotaxis

There is no interaction in the thigmotaxis among the groups ($F = 1.29$, $p > 0.05$). There is a decrease in the thigmotaxis index in the PTZ group (Group 2) compared with the control (Group 1) and the treatment groups (Group 3, 4 and 5), however the differences observed were not statisticall significant across all the groups. (Figure II).

Locomotor Activity

The total ambulatory distance covered in the maze during the duration of the test in meters is an indication of the locomotor activity of the animals. Their respective tracks were combined and statistically analyzed to visualize any differences in ambulation. There is no interaction in the total distance covered between all the groups tested ($F = 2.19$, $p > 0.05$) (Figure III).

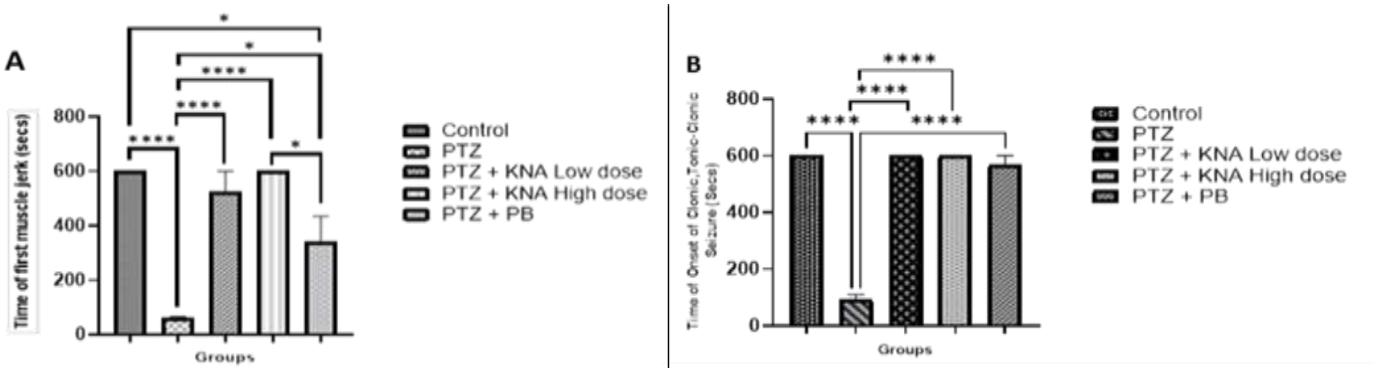

Figure I: (A) The mean time of the of first neck muscle jerk for the experimental groups. (B) The mean time of the onset of clonic, and tonic-clonic seizure for the experimental groups.

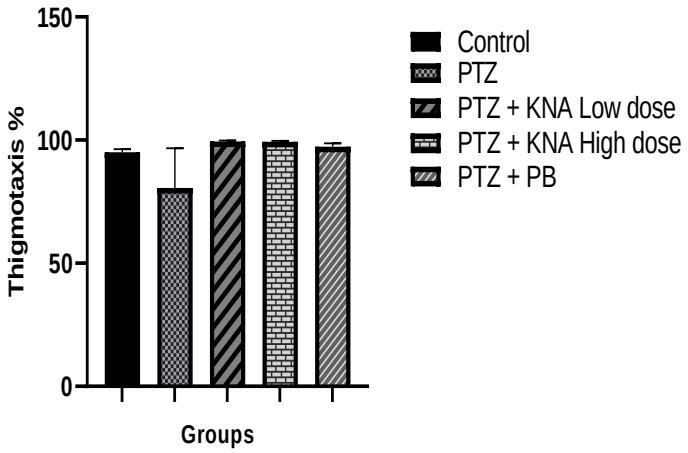

Figure II: The thigmotaxis percentage for the experimental groups. The results of the two way ANOVA shows no interaction ($F = 1.293$, $p < 0.3061$) in the mean ± SE thigmotaxis percentage across the groups.

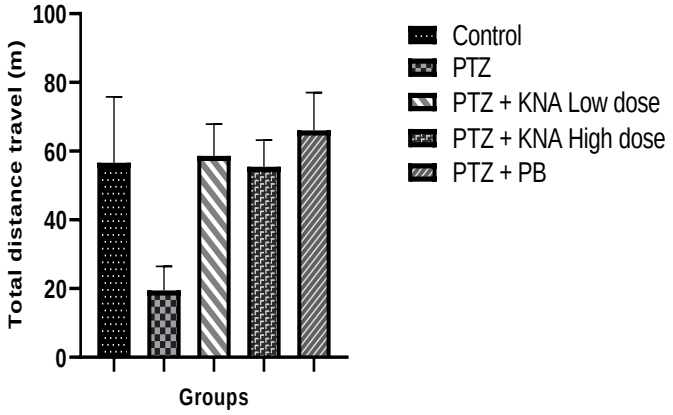

Figure III: The total distance traveled during the time of the test (m) for the experimental groups. The results of the two-way ANOVA showed no interaction ($F = 2.189$, $p < 0.1071$) in the mean ± SE total distance travel an indicator of the locomotor activities across the groups

Neuromorphological Study of the Hippocampus:

Histoarchitectural features of the CA1 region of the hippocampus to evaluate the morphology and determine differential cell number across the groups as shown in figures IV and V below.

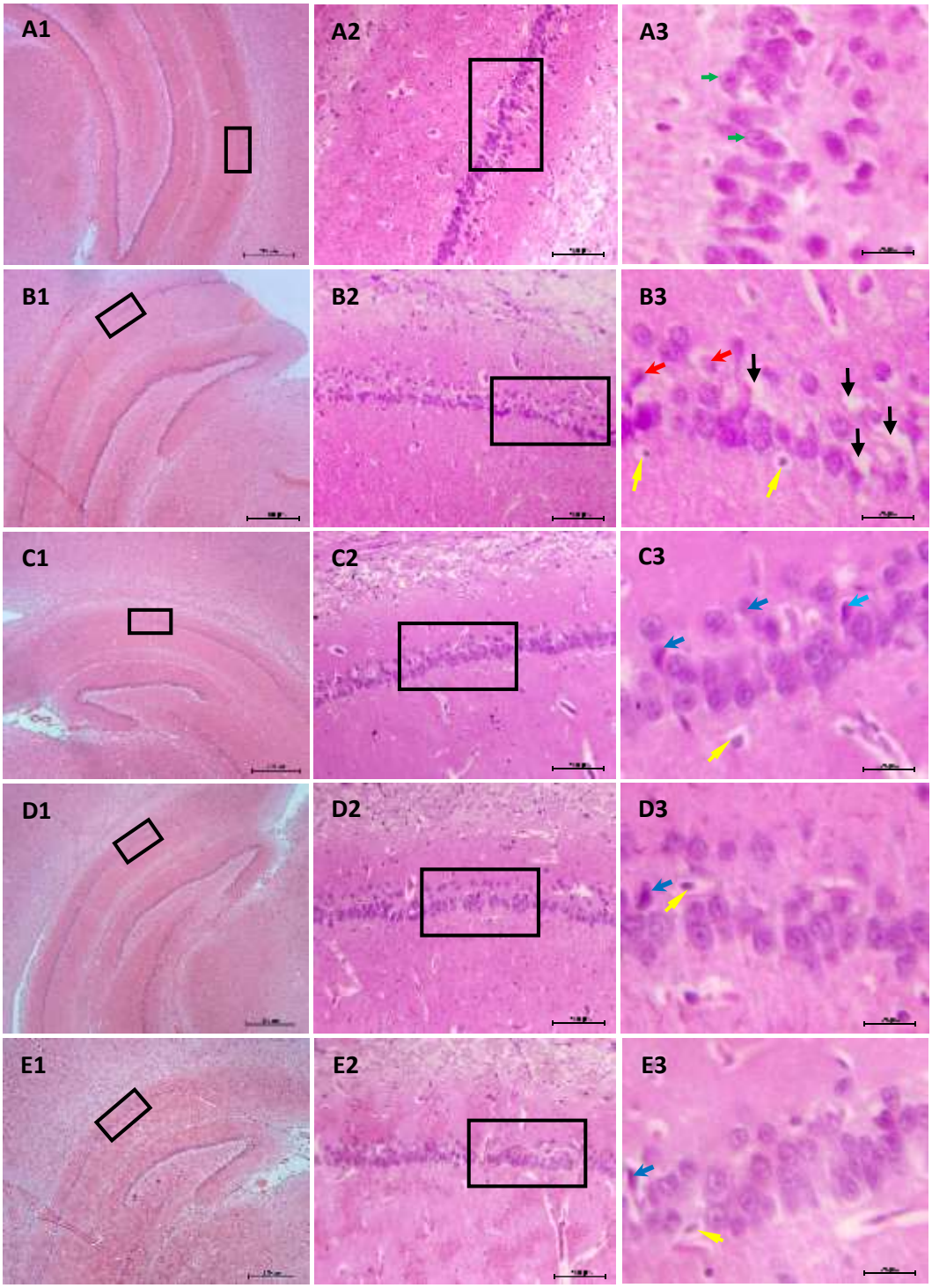

Figure IV: Photomicrographs of the hippocampus in (A1-3) Control, (B1-3) PTZ, (C1-3) PTZ+KNA Low dose, (D1-3) PTZ+KNA Low dose and (E1-3) PTZ+ PB. The peculiar features of the cells; Green arrow = typical healthy pyramidal cell; Red arrow = degenerating cell; Black arrow = vacuolation; Yellow arrow = pyknotic cell; Blue arrow = regenerating cell. Magnification of the panel A1-E1, 40x; scale bar = 200 µm, A2-E2, 100x; scale bar = 100 µm, A3-E3, 400x; scale bar = 25 µm.

The PTZ group (Group 2) showed a statistically significant reduction in the number of cell counts compared to the control group (Group 1) ($P < 0.05$). An increase in the number of cell count was observed in the KNA 400mg/kg (Group 3), KNA 800mg/kg (Group 4), and PB 10mg/kg (Group 5) compared with the PTZ 40mg/kg (Group 2), however, this was not statistically significant ($P > 0.05$), Figure V.

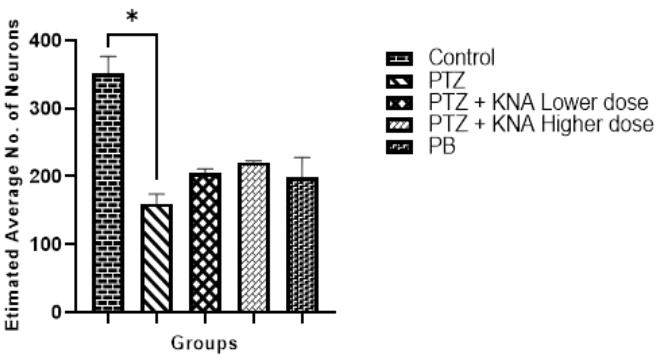

Figure V: Estimated average number of neurons for the experimental groups. The results of the two way ANOVA shows no interaction ($F = 3.006$, $p < 0.0525$) in the mean ± SE estimated number of neurons across the groups.

DISCUSSION

This study aimed to characterize seizure patterns in a PTZ-induced seizure model using Wistar rats, focusing on Racine scale 3 (neck jerks) and scale 6 (clonic-tonic seizures characterized by lying on one side and wild jumping). These behaviors correlate with distinct EEG patterns, including sharp spikes followed by spike-wave discharges and high-amplitude polyspikes, respectively (Van Erum et al., 2019). We investigated the therapeutic effects of kaurenoic acid (KNA) and phenobarbital (PB) on seizure behavior, anxiety-related behavior, and potential cellular changes in the hippocampus.

The study demonstrated that PTZ-induced seizures significantly impacted the CA1 region of the hippocampus, highlighting the structural changes associated with seizure activity. Both KNA and PB, known for their antiepileptic properties, significantly delayed the onset of neck muscle jerks and clonic-tonic seizures compared to the untreated PTZ group. This indicates a protective effect by delaying EEG correlates associated with seizure severity and reducing the impact on cellular activity within the brain.

Anxiety-related behavior and locomotor activity were assessed using the Open Field Test (OFT). Animals in all groups displayed more time spent in the peripheral zone compared to the central zone, indicating heightened anxiety. The thigmotaxis index, which measures anxiety-related behavior, was decreased in the PTZ group but improved with KNA and PB treatment, although differences were not statistically significant. This finding contrasts with previous studies by Simon et al. (1994), which reported an increase in thigmotaxis with PTZ and a decrease with phenobarbital, suggesting variability in the behavioral response to PTZ and treatment regimens. Additionally, the total ambulatory distance, an indicator of locomotor activity, was reduced in the PTZ group but was significantly improved with KNA and PB, aligning with previous findings.

Histological analysis of CA1 neurons in the hippocampus revealed a significant reduction in cell count in the PTZ group compared to controls, consistent with the neurotoxic effects of PTZ, which disrupts GABAA receptor function and enhances glutamatergic activity, leading to oxidative stress and neuronal damage (Ahmed et al., 2012; Zhu et al., 2017). Treatment with KNA and PB showed a partial restoration of neuronal cell counts, suggesting their potential neuroprotective effects against PTZ-induced damage, although these changes were not statistically significant.

Overall, these results indicate that KNA and PB can mitigate some of the cellular and behavioral impacts of PTZ-induced seizures. The observed neuroprotective effects suggest that KNA, in particular, may counteract seizure-induced cellular disruption, highlighting its potential as a therapeutic agent.

Conclusion: Our findings demonstrate that kaurenoic acid (KNA), isolated from *Annona senegalensis*, exhibits significant antiepileptic potential in PTZ-induced seizure models, effectively delaying the onset of seizures and reducing seizure severity better than phenobarbital (PB), a conventional antiepileptic drug. Both KNA and PB improved anxiety-related behavior and locomotor activity affected by PTZ-induced seizures and provided neuroprotective effects in the hippocampus, suggesting a positive impact on cognitive functions. These results underscore the potential of KNA as a promising antiepileptic candidate that warrants further development and clinical consideration.

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