

Kaurenoic Acid from *Annona senegalensis* Attenuates Hippocampal NMDA Receptor Overexpression and Enhances Cognitive Function in a Rat Model of PTZ-Induced Seizures

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Abstract

Background:

Overactivation of N-methyl-D-aspartate receptors (NMDARs) is implicated in seizure-induced excitotoxicity and neurodegeneration. Kaurenoic acid, a diterpenoid from *Annona senegalensis*, has shown anticonvulsant potential, but its modulatory effect on NMDARs in epilepsy remains unclear. This study investigates the neuroprotective and cognitive effects of kaurenoic acid and its impact on hippocampal NMDAR expression in a PTZ-induced seizure model in rats.

Methods:

Kaurenoic acid (KNA) was isolated from the ethyl acetate fraction of *A. senegalensis* leaves. Thirty male Wistar rats were divided into five groups (n=6): control, PTZ only, KNA (400 or 800 mg/kg, p.o) + PTZ, and phenobarbital (PB, 10 mg/kg, i.p) + PTZ. Seizure activity was scored using Racine's scale. Cognitive performance was assessed via the Open Field and Novel Object Recognition tests. Hippocampal NMDAR levels were measured using ELISA. Data were analyzed with ANOVA and Tukey's post hoc test.

Results:

KNA significantly increased seizure thresholds in myoclonic (173 ± 33 s) and tonic-clonic seizures (444 ± 41 s), comparable to PB (233 ± 43 s and 534 ± 42 s), $p < 0.0001$. KNA improved thigmotaxis (98%) and preserved cognitive function with a positive discrimination index ($44 \pm 17\%$), whereas PB impaired cognition ($-61 \pm 18\%$). Locomotor activity decreased with both KNA and PB. PTZ elevated NMDAR expression (27 ± 1 μmol) compared to control (22.8 ± 0.2 μmol), while both KNA and PB reduced NMDAR levels (17 ± 3 and 23 ± 1 μmol , respectively).

Conclusion:

Kaurenoic acid demonstrated strong anticonvulsant and neuroprotective effects, effectively lowering hippocampal NMDAR expression and preserving cognitive function better than phenobarbital. These findings support its therapeutic potential as a safer alternative for seizure management.

INTRODUCTION

N-methyl-D-aspartate receptors (NMDARs) overexcitation has been linked to neurological conditions that induce neuronal death, including epilepsy, stroke, Alzheimer's disease (AD), and Parkinson's disease, according to related research (Chen et al., 2022; Essiz et al., 2021). The receptor is composed of 4 subunits derived from 3 gene families: GluN1-3. Each receptor is a tetramer composed of 2 GluN1 subunits and either 2 GluN2 or 2 GluN3 subunits. The NMDAR's pharmacological regulation is determined by the different subunit binding site combinations. The NR2 subunit contains the glutamate binding site, while the NR1 subunit has the glycine binding site. Different neuroanatomic manifestations are seen for these binding sites (Kapur, 2018; Sivakumar et al., 2022).

In epilepsy, NMDAR regulation has drawn a lot of attention due to its role in the brain's neuroexcitatory and functional plasticity. NMDARs' functions and mechanisms in epilepsy are yet unknown, but they

have been shown to contribute to seizures. During seizures in temporal lobe epilepsy (TLE), glutamate levels in the extracellular fluid increase which can directly activate NMDARs and result in neuroexcitatory injury. Cognitive impairment may be exacerbated by impaired NMDAR function. It should come as no surprise that NMDARs are a very desirable therapeutic target. Autoantibodies against NMDARs cause a distinctive condition that highlights the vital role these receptors play in behavior, cognition, motor, respiratory, and autonomic regulation (Benarroch, 2011).

Using a range of techniques, including immunoblotting, binding affinities, and evaluation of subunit gene expression, some researchers have assessed the potential changes of NMDARs in epilepsy, given that NMDAR activity is crucial for neuronal excitation in the central nervous system. The primary excitatory input to the hippocampus is glutamatergic impulses from the entorhinal cortex, and a change in glutamate-mediated excitability may play a role in the pathophysiology of epileptic discharges (Carter et al., 2011). Furthermore, as NMDAR antagonists offer defense against such damage, NMDARs may be in charge of the selective excitotoxic cell death of certain hippocampus neuronal populations brought on by seizures (Meldrum, 1993). Research has demonstrated that certain AEDs influence glutamatergic synapses via NMDA receptors, which are typically excitatory amino acid (glutamate) receptors.

By blocking these excitatory receptors, these AEDs lessen impulse conduction (Das et al., 2002; Howard et al., 2011). Numerous studies have examined the role of NMDARs in epileptic seizures and kaurenoic acid, a diterpenoids isolated from *Annona senegalensis* root bark extract has shown an anticonvulsant potential and phenobarbital with notable effects (Chen et al., 2022; Okoye et al., 2013); however the relationship between kaurenoic acid activities and NMDARs with respect to epileptic seizure is unclear. This study is aimed to investigate the prophylactic potential of kaurenoic acid isolated from *Annona senegalensis* leaves on the level of expression of NMDARs in the hippocampus of PTZ-induced seizure in Wistar rats.

MATERIALS AND METHODS

Plant Material, Extraction and Isolation of Kaurenoic Acid

Annona 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 harvested and processed to obtain 100g dry powder of the aqueous crude according to Dare et al, 2024.

To isolate kaurenoic acid, solvent–solvent fractionation by separation funnel method with methanol, ethyl acetate, and n-hexane in the increasing polarity order was employed to obtain the respective fractions. To carry out this process, the dried crude 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 layer of ethyl acetate was gathered and allowed to evaporate at a lower pressure. A silica gel column was used for purification by column chromatography, with n-hexane serving as the initial elution solvent. By dissolving the concentrated ethyl acetate extract in a tiny amount of solvent (n-hexane acetate, 90:10), the sample was put onto the column. To obtain a sub-fraction (80:20), the ethyl acetate fraction was further eluted using gradient mixes of n-hexane and ethyl acetate (90:10 to 20:80). Based on the yield and potency, this sub-fraction was further identified as kaurenoic acid (Okoye et al., 2013; Mtunzi et al., 2017).

HPLC Characterization of Kaurenoic Acid

Methanol, ethyl acetate, and n-hexane extract of *Annona senegalensis* were subjected to HPLC analysis using a JASCO LC-4000 Series, Japan, HPLC system. The system contained employed an asymmetric twin-piston solvent delivery system SSQD (Slow Suction, Quick Delivery) which provides significantly better flow and pressure profiles and UV-Visible Detectors (UV-4070/4075). Reverse phase column chromatographic separation was performed using Octadecylsilane, Size: 4.6mm X 150–250mm column with ethyl acetate mobile phase. For sample preparation, filtration was carried out to remove any particulates that could clog the column and the sample was diluted to an appropriate concentration suitable for analysis (50µm). For solvent selection, HPLC-graded solvent which are free from impurities (water, 99.8% methanol, 99.8% acetonitrile, 99.8% isopropanol, 99.8% ethanol and 99.9% ethyl acetate) were used. Samples were stored in sterile samples and refrigerated to maintain stability. The standard concentrations used for analytical applications ranged from 10–70 µg/mL, 1–2% of the total column volume and 1 µg/µL concentration of the extract.

Data collection and processing were done using ChromNAV Chromatography Data System (CDS) software and the detection wavelength was between 200 and 400 nm. The injection volume of samples and reference solutions was 3–5µL, and the flow rate was 3.0 mL/min. By monitoring retention time and examining UV spectra, the peaks were found at 20 and 400nm Retention Time (5min) a little quantity of the reference standards.

Experimental Animals and Design

For this investigation, thirty (30) adult male wistar rats were acquired and kept in the animal house in hygienic cages with a 12-hour light and 12-hour dark cycle at an average ambient temperature between 18⁰C and 26⁰C, fed with pelletized rat diet and given unlimited water. Two weeks to the start of the experiment, the animals were weighed (150–250 grams) and grouped into five (5) groups at random (n = 6) with identification marks and allowed to become familiar with their new environment. Group 1

(received distilled water as negative control) and Group 2 (received PTZ as positive control) while Group 3 (received low dose of KNA), Group 4 (received high dose of KNA) and Group 5 (received phenobarbital) before PTZ.

Seizure Activity and Behavioural Study

PTZ (Sigma–Aldrich, P6500, Lot No. MKCM4261) was used to induce seizures and seizure-behaviour of individual animal was recorded with a PC Logitech B905 digital Webcam connected to a laptop in a square open-field standard box (100 x 100 x 50 cm) for 10 minutes. The seizure behavioural activities of the animals were scored according to Racine scale 3 and 6 characterized by myoclonic neck jerks with sharp spikes followed by spike-wave discharges EEG correlates and clonic, tonic-clonic seizures, and wild jumping with high amplitude, spike-wave discharges EEG correlate respectively. To investigate the prophylactic potential of KNA and PB against PTZ-induced seizure, 400mg/kg and 800mg/kg of KNA were administered orally to group 3 and group 4 respectively while group 5 received 10mg/kg of phenobarbital (PB) thirty minutes before administration of PTZ (40mg/kg), and these were compared with group 1 and group 2 which received 1m/kg orally and 40mg/kg PTZ intraperitoneally.

Thigmotaxis, Locomotor Behaviour and Discriminative Index

To determine the thigmotaxtic index, a metric used to assess anxiogenic and anxiolytic behaviour, the percentage of the 10-minute test duration that the individual stays next to the maze's outer wall, a sign of anxiety-like behavior was measured (Zhang et al., 2023) while the total ambulatory distance covered during the open field test (OFT), measured as an indicator of locomotor activity. To ascertain the non-spatial or recognition, novel object recognition test (NORT) was employed. The test was conducted 20 to 24 hours after the medicine was administered in a 100 × 100 cm square open-field standard box with walls 50 cm high. A Logitech B905 2MP Portable Webcam was mounted on top of the box and linked to a laptop to capture the animals' exploratory activities.

The test process was divided into three stages: recognition, familiarization, and habituation. During the habituation phase, each animal was carefully put in the object-free box and given five minutes to explore. The initial exploration session of the habituation phase was followed by the familiarization phase 24 hours later. Each animal was given ten minutes to investigate after two identical plastic items (A and B) were positioned in the box's neighboring corners, about ten centimeters from the walls. After fifteen minutes, item B was used to start the recognition phase. It was then swapped out for a new object C that was different in color and form but had a comparable size, texture, and weight. For ten minutes, each animal was left alone in the box, and the amount of time it spent exploring the objects was noted (Fig. 1)

The videos recorded were analyzed using ANY-maze 7.3 software to track the object exploration time which was used to calculate the percentage novel object preference index $[TC / (TC + TA) \times 100]$ and discrimination ratio $[(TC - TA) / (TC + TA)]$, where TA and TC signify time spent exploring object A and C

respectively. The object exploration time was calculated by making two virtual zones around each object (A and C) using the software and the time spent in each zone was calculated (Antunes and Biala, 2012; Mazumder et al., 2017).

Level of Expression of NMDA Receptor Using ELISA

Rat N-Methyl-d-aspartate (NMDA) Receptor 2 ELISA Kit, Bioassay Technology Laboratory, Zhejiang, China (Dhamad and Abdal Rhida, 2020; Tajima et al., 2023) was used to investigate the level of the expression of NMDA Receptor 2 in the hippocampus of the animals. Following the behavioural tests, the animals were anaesthetized with halothane (Piramal Pharma Limited, India), brain was extracted and the hippocampus removed by micro-dissection. The hippocampal tissue was homogenized with buffer following established techniques or the manufacturer's instructions, centrifuged and the supernatant collected. To carry out the assay, all the reagents, samples and standards were prepared brought to room temperature before use according to the user instruction. The sample and ELISA reagent were added into each well and incubate for 1 hour at 37°C. The plates were washed 5 times and the substrate solution A and B were added and incubated in the dark for 10 minutes at 37°C. Afterwards the stop solution was added and the blue color changed into yellow immediately. The optical density (OD value) of each well was determined immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

Statistical Analysis

GraphPad (8.0.2) was used to perform an analysis of variance (ANOVA) and a Tukey post hoc test on the collected data (Geoff et al., 2007). The findings are shown as mean $\pm$ SEM and p-value of less than 0.05 was considered statistically significant.

Ethical Consideration

Ethical Clearance was obtained from OOU Research Ethics Committee (OOU-REC) with reference: OOU/SCIENG/EC/0008/240924. Animals used were properly handled and cared for, with strict adherence to the standard protocol and institutional guidelines.

RESULTS

Seizure Severity Assessment

KNA and phenobarbital demonstrated significant anticonvulsant effects in PTZ-induced seizure models, delaying the onset of seizure activities at different stages. Both compounds delayed the initial occurrence of first neck jerks (Fig. 1) and clonic, tonic-clonic seizure (lying on the side) (Fig. 2), corresponding to Racine scale 3 and 6 characterized by sharp spikes followed by spike-wave discharges and high-amplitude polyspikes spike-wave discharges EEG correlate respectively.

Assessment of Thigmotaxis, Locomotor Activities and Discriminative Index

The thigmotaxis index was reduced in the PTZ group compared to the control group. This reduction was ameliorated in the groups receiving low and high doses of KNA and PB. While the control, KNA, and PB groups spent more time near the walls, indicating a preference for the periphery, there was no statistically significant difference among the groups (Fig. 3)

The total ambulatory distance covered during the OFT, measured as an indicator of locomotor activity, revealed no statistically significant differences among the groups. The PTZ, KNA, and PB groups showed a reduction in the distance traveled compared to the control (Fig. 4). The discriminative index, a measures learning and memory, was used to assess the animals' cognitive ability using the novel object recognition test (NORT). Except for the PB group, which had a statistically significant negative discriminative index ($p < 0.05$), all the groups displayed a positive discriminative index. In contrast to the other groups, the high dosage of KNA showed a comparatively higher discriminative index, indicating enhanced cognitive function (Fig. 5)

Determination of the Level of Expression of NMDA Receptor in the Hippocampus

The level of NMDA receptor expression was upregulated in the PTZ group compared to the control but was relatively downregulated in the KNA and PB groups. The effect of the KNA high dose was statistically significant ($p < 0.05$) compared to the PTZ group (Fig. 6)

DISCUSSION

The prophylactic potential of KNA and PB against PTZ-induced seizure in rats investigated in this study presented a delayed onset of myoclonic and clonic, tonic-clonic seizure with KNA showing a better performance than PB. The observed anticonvulsant activity of KNA is consistent with previous findings. Okoye et al. (2013) reported that KNA, isolated from the root bark of *Annona senegalensis*, significantly delayed the onset of myoclonic spasms and tonic-clonic phases in PTZ-induced seizures in mice, demonstrating a dose-dependent effect. Similarly, Lima et al. (2007) found that kaurenoic acid inhibited tonic hind-limb extension in spinal seizures induced by sudden cooling, with an effective dose (ED₅₀) of 2.5 mg/kg. In PTZ-induced seizures, KNA increased the latency to seizure onset and provided 45% and 65% protection at doses of 0.625 and 1.25 mg/kg, respectively.

The thigmotaxis index was reduced insignificantly in the PTZ group compared to the control group which was ameliorated in the treatment groups receiving low and high doses of KNA and PB. The control, KNA, and PB groups spent more time near the walls, indicating a preference for the periphery and positive thigmotaxis while the PTZ group had a negative thigmotaxis showing less anxiety-related behaviour. A high thigmotaxis index indicates high anxiety-like behaviour while low thigmotaxis index indicates a low

anxiety-like behaviour (Kraeuter et al., 2019, Leon, 2023). In this study study, PTZ demonstrated an anxiolytic effect which is agreement with the report of Kajita and Mushiake (2024) stating that anxiety-like behaviors decreased compared with those of the control group in the open field, light/dark transition, and elevated plus-maze tests after 10 PTZ injections.

As for the locomotive behaviour, the total ambulatory distance covered by the animals during exploration period, a measurement of voluntary movement have been shown to be sensitive to drug treatment and disease progression (Gibbs and Crosbie-Watson, 2017). The decreased in the total ambulatory distance covered observed in the PTZ group compared with the control which was improved by KNA and PB suggests sedative and/or motor impairment effects of PTZ on the animals. In this study, although PTZ did not significantly raise the amount of NMDA receptor expression in the PTZ group as compared to the control group statistically, nevertheless, the upregulation observed in this finding is in line with other research showing that PTZ raises NMDA receptor subunit expression (Rahimi-Madiseh et al., 2022).

Compared to PTZ, kaurenoic acid and phenobarbital decrease the amount of NMDA receptor subunit expression, which this study demonstrated to be statistically significant in the prophylactic trial with a greater dosage of KNA. This agrees with previous studies by (Kapur, 2018; Prasad et al., 2002) which reported that NMDA receptor antagonist could play a crucial role in seizure reduction and/or termination when combined with benzodiazepines and neuroprotection against status epilepticus-induced cell loss in the hippocampus (Mazarati and Wasterlain, 1999; Niquet et al., 2017; Kapur, 2018).

In conclusion, kaurenoic acid outperformed phenobarbital in its ability to preserve cognitive function and successfully reduce hippocampus NMDAR expression. It also showed excellent anticonvulsant and neuroprotective effects. These results lend credence to its potential as a safer treatment option for seizure control.

Declarations

Data Availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

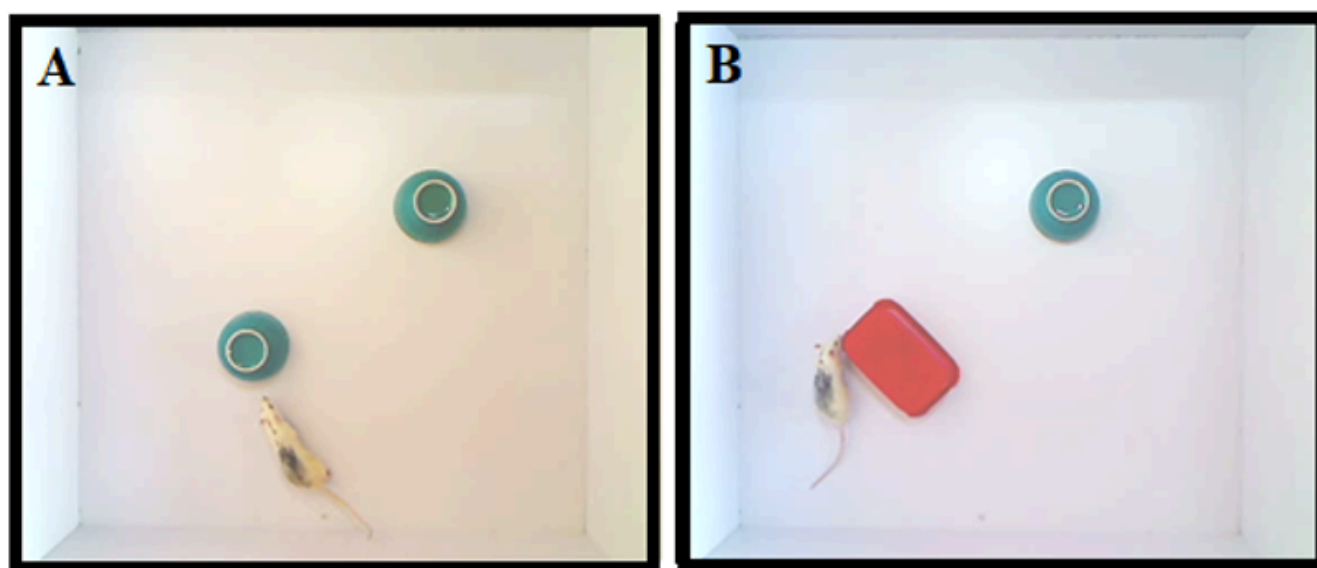


Figure 1

Figure 3.5: The Novel Object Recognition Test Arena: A = Familiar phase with two familiar objects (green). B = Discriminative phase with one familiar object (green) and one novel object (red).

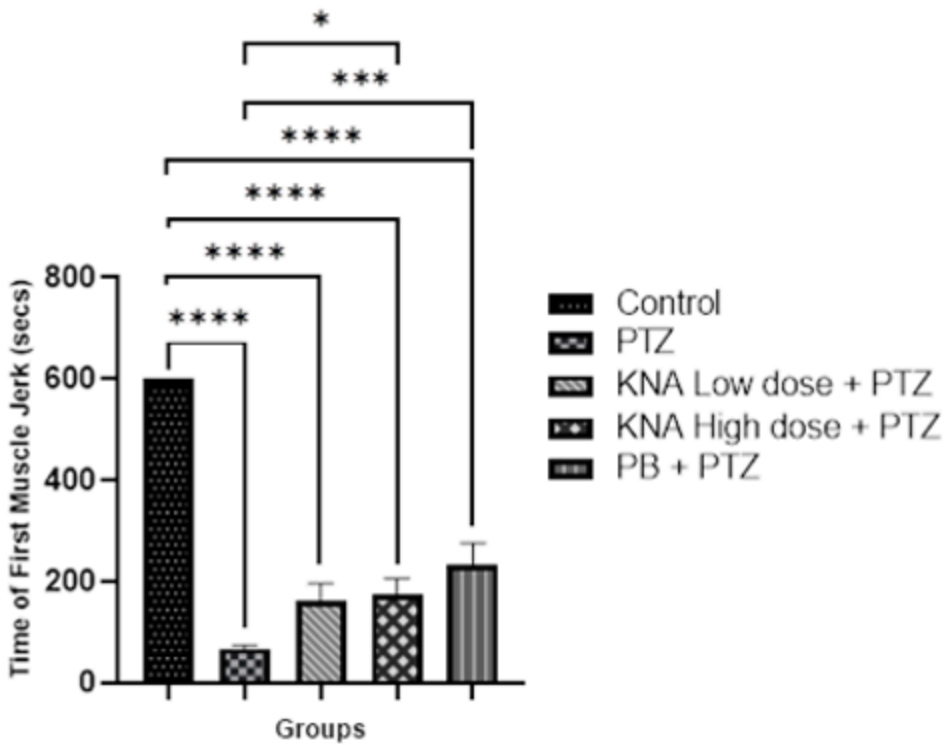


Figure 1: The mean time of first neck jerks for the prophylactic experimental groups was analyzed using two way ANOVA with Tukey's post hoc test adjustment. Data presented as mean $\pm$ SEM. Statistical significance is noted as follows: $P > 0.05$ (ns), * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$.

Figure 2

See image above for figure legend.

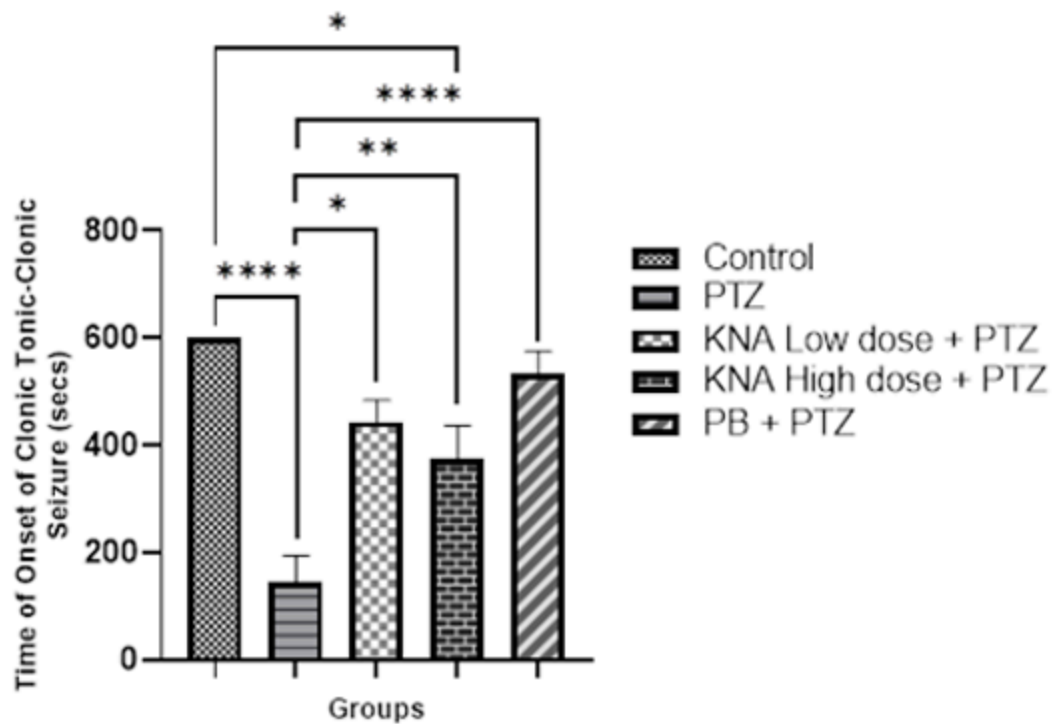


Figure 2: The mean time of the onset of clonic, tonic-clonic seizure for the prophylactic experimental groups was analyzed using two way ANOVA with Tukey's post hoc test adjustment. Data presented as mean $\pm$ SEM. Statistical significance is noted as follows: $P > 0.05$ (ns), * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$.

Figure 3

See image above for figure legend.

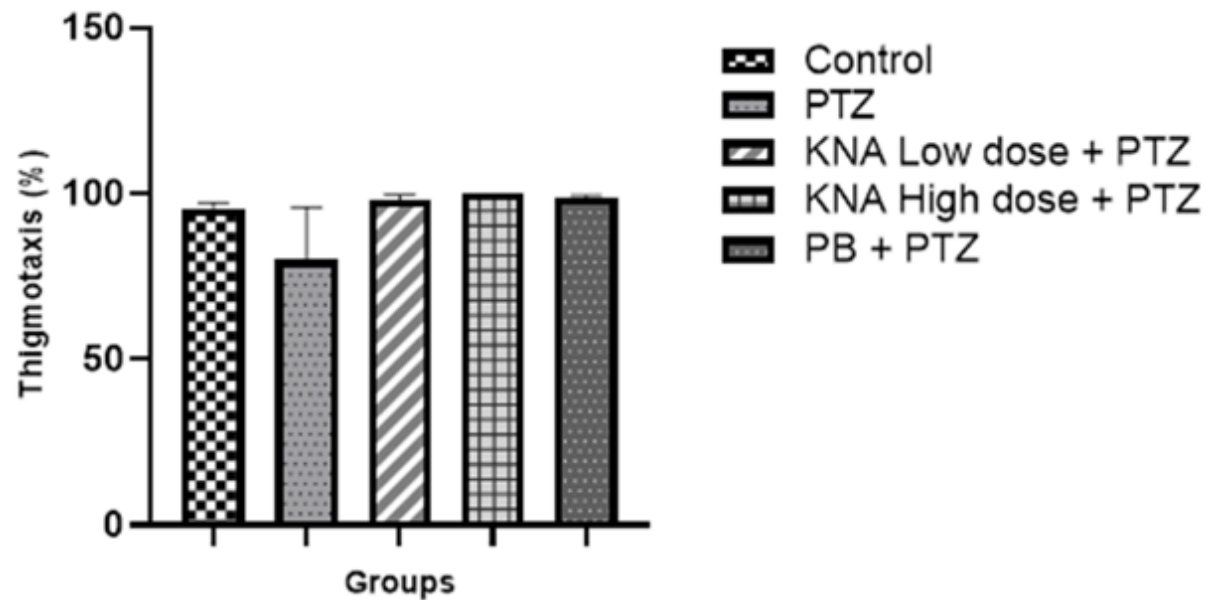


Figure 3: The thigmotaxis index for the prophylactic experimental groups was analyzed using two way ANOVA with Tukey's post hoc test adjustment. Data presented as mean $\pm$ SEM. Statistical significance is noted as follows: $P > 0.05$ (ns), * $P < 0.05$.

Figure 4

See image above for figure legend.

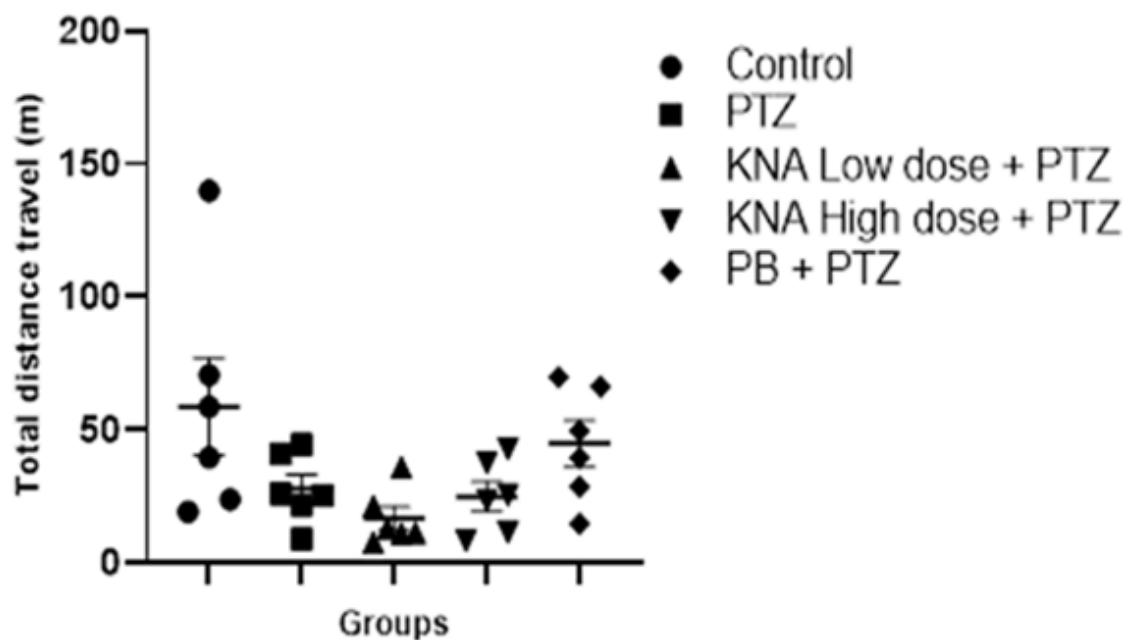


Figure 4: The total distance travel during the time of the test (m) for the prophylactic experimental groups was analyzed using two way ANOVA with Tukey's post hoc test adjustment. Data presented as mean $\pm$ SEM. Statistical significance is noted as follows: $P > 0.05$ (ns), * $P < 0.05$.

Figure 5

See image above for figure legend.

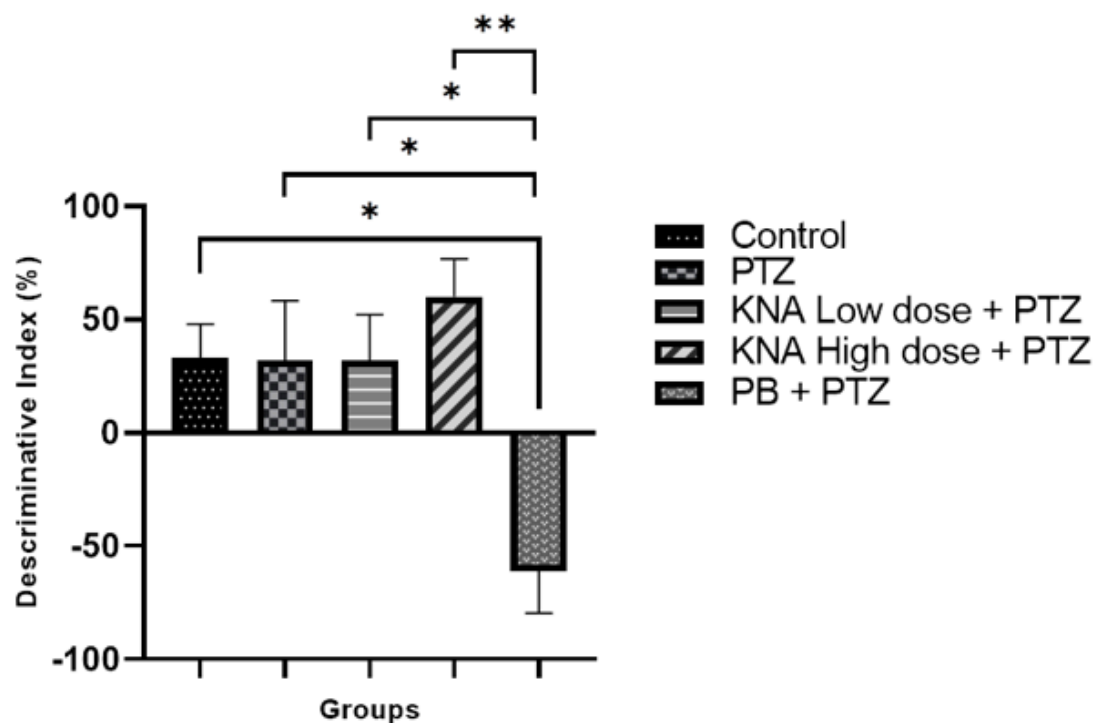


Figure 5: The discriminative index (%) for the prophylactic experimental groups was analyzed using two way ANOVA with Tukey's post hoc test adjustment. Data presented as mean $\pm$ SEM. Statistical significance is noted as follows: $P > 0.05$ (ns), * $P < 0.05$, ** $P < 0.01$.

Figure 6

See image above for figure legend.

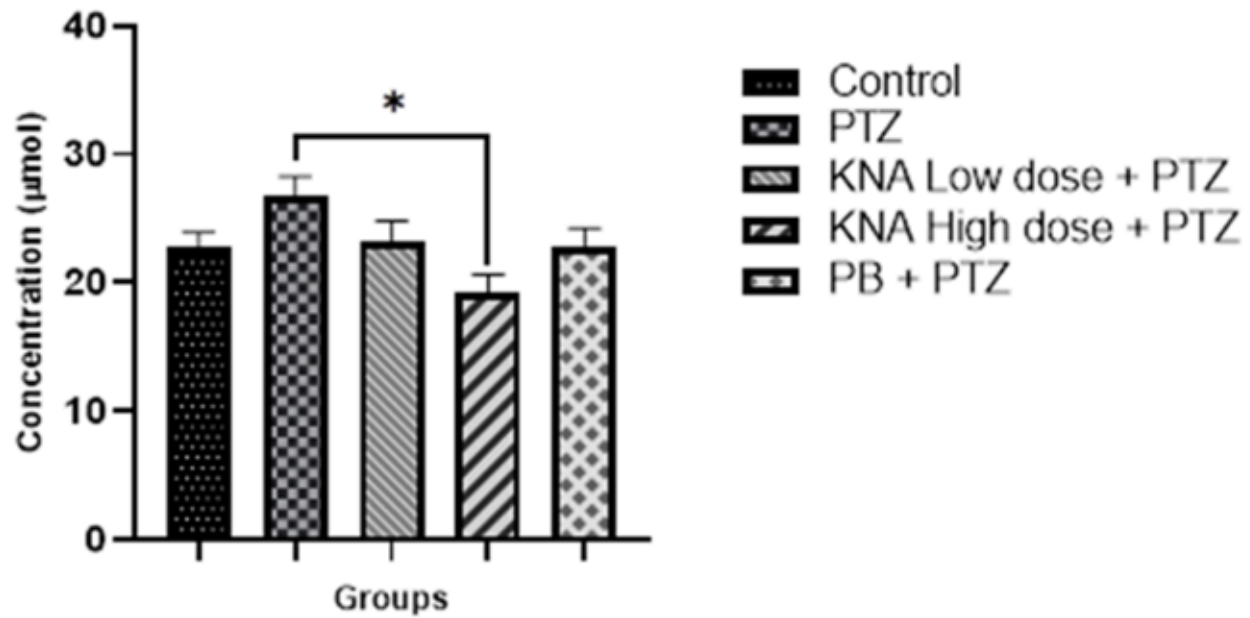


Figure 6: Level of expression of NMDA receptor in the hippocampus of the prophylactic experimental groups was analyzed using two way ANOVA with Tukey's post hoc test adjustment. Data presented as mean $\pm$ SEM. Statistical significance is noted as follows: $P > 0.05$ (ns), * $P < 0.05$,

Figure 7

See image above for figure legend.