

RETRACTED ARTICLE: 1,3,4, Oxadiazole Compound A3 Provides Robust Protection Against PTZ-Induced Neuroinflammation and Oxidative Stress by Regulating *Nrf2*-Pathway

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Background: Epilepsy is a common neurological disorder that is characterized by recurrent episodes of seizures. Various studies have demonstrated a direct association between oxidative stress and inflammation in several neurological disorders including epilepsy. This study aimed to investigate the neuroprotective effects of a synthetic 1,3,4, oxadiazole compound A3 against pentylenetetrazole (PTZ)-induced kindling and seizure model.

Methodology: PTZ was administered in a sub-convulsive dose of 40 mg/kg for 15 days, at 48-hour intervals to male Swiss-Animo mice until animals were fully kindled. Two different doses of A3 (10 mg/kg and 30 mg/kg) were administered to find out the effective dose of A3 and to further demonstrate the relative role of nuclear factor- κ B-related factor (*Nrf2*) in the PTZ-induced kindled model.

Results: Our results demonstrated a compromised antioxidant capacity associated with a low level of catalase (CAT), superoxide dismutase (SOD), glutathione (GST), and glutathione S-transferase (GSH) in the kindled group. However, the PTZ-induced group demonstrated an elevated level of lipid peroxidation (LPO) level parallel to pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α), mediators as cyclooxygenase (COX-2), and nuclear factor kappa B (NF κ B). Furthermore, the A3 treatment reversed these changes and overexpressed the antioxidant *Nrf2* gene and its downstream *HO-1*. To further investigate the involvement of *Nrf2*, we employed an *Nrf2*-inhibitor, i.e., all-trans retinoic acid (ATRA), that further aggravated the PTZ toxicity. Moreover, vascular endothelial growth factor (VEGF) expression was evaluated to assess the extent of NF κ B disruption.

Conclusion: The findings of this study suggest that A3 could mediate neuroprotection possibly by activating *Nrf2* dependent downregulation of inflammatory cascades.

Keywords: epilepsy, PTZ, oxidative stress, *Nrf2*, *HO-1*, neuroinflammation

Introduction

Epilepsy is one of the most prevalent neurological disorders which affects people of all ages.¹ It causes cognitive and motor deficits along with electroencephalographic changes including recurrent unprovoked seizures. Though the exact etiological causes of seizures episodes are not known, neuronal hyperexcitability, cortical stimulation, oxidative stress, genetic factors, and psychiatric comorbidities are among the leading pathogenetic factors.²⁻⁴ Numerous antiepileptic drugs are in practice, however, the frequent adverse effects including vital organs toxicity have handicapped the frequent usage of these medications.⁵⁻⁷

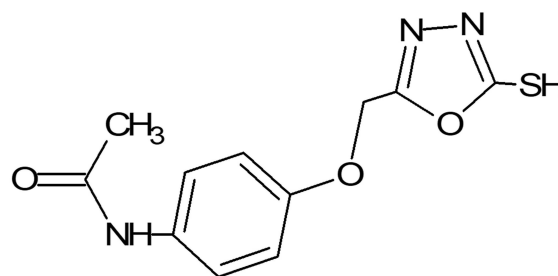
Neuroinflammation is considered the hallmark for neurodegeneration, though it's a natural response to preserve innate homeostasis.^{8,9} Accumulating evidence

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from clinical and experimental studies indicates that brain inflammation might be a cause or a consequence of epilepsy.¹⁰ However, an intensified neuroinflammatory response leads to neuronal and cellular dysfunction, as proinflammatory cytokines such as IL-1 β , IL-6, and TNF- α are increased in the brains of epileptic animals.^{11,12} Similarly, the level of these proinflammatory cytokines is also increased in the serum or cerebrospinal fluid of patients with epilepsy.^{13,14} Consistent studies reiterated the role of inflammatory mediators in seizure pathophysiology,^{10,15,16} and these mediators can provoke leukocyte infiltration that can trigger biochemical and functional anomalies including BBB disruption, lipid peroxidation (LPO), and angiogenesis.^{17,18} Furthermore, the activated resident glial cells trigger the release of proinflammatory cytokines which compromises the prognosis of epilepsy.^{10,19} Multiple studies suggested the use of pentylenetetrazole (PTZ) as a replicated epileptic model in rodents as its pathology mimics the human absence seizures.^{20–22} PTZ is a non-competitive antagonist of GABA-A receptor and several studies demonstrated a reduced antioxidant enzyme level associated with elevated nitric oxide after PTZ-treatment.^{23,24} Hence, it is very prudent to maintain a low level of oxidative stress markers to suppress the subsequent neuroinflammation.²⁵

Nuclear factor erythroid 2-related factor 2 (*Nrf2*, or NFE2L2) is a ubiquitously expressed antioxidant that governs the expression of various other antioxidant proteins and enzymes and hence plays an important role in the cellular defense system of the body.²⁶ Once activated, *Nrf2*, which is otherwise localized in the cytoplasm, enters the nucleus and activates multiple inducible antioxidant enzymes such as NAD(P)H quinone oxidoreductase, inducible heme-oxygenase (*HO-1*), superoxide dismutase (SOD), and glutathione peroxidase (GPx).²⁷ Consistent reports validated the correlation between *Nrf2* and NF- κ B, suggesting that *Nrf2* can abrogate the inflammatory cascade in addition to halt oxidative stress.^{28–31} Furthermore, the *Nrf2* neuroprotective role is established not only in laboratory stroke and depression models but also in human brain samples.^{32–37}

Numerous synthetic products have been tested in the past for their biological activities and therapeutic potential. Five-membered heterocyclic compounds oxadiazoles have been previously researched for antioxidant and anti-inflammatory potential.^{38–40} This research was carried out using a novel 1,3,4 oxadiazole derivative, (N-{4-[(5-sulfanyl-1,3,4-oxadiazol-2-yl) methoxy] phenyl} acetamide



N-(4-[(5-sulfanyl-1,3,4-oxadiazol-2-yl)methoxy]phenyl)acetamide

Figure 1 Structure of 1,3,4 oxadiazole compound A3.

(named as A3), which has reported antioxidant and anti-inflammatory activities⁴¹ (Figure 1). Its potential safety and neuroprotective properties were demonstrated previously as it alleviated infarcted area in the animal model of stroke.^{36,37} Furthermore, these oxadiazole derivatives were recently screened for inhibition of voltage-activated (T-type) channels.⁴¹ Many lines of evidence support a major role for T-type Ca²⁺ channels in the etiology of epilepsy. Several studies demonstrated an increased T-type Ca²⁺ channel expression and increase in T-type currents during the onset of epilepsy.^{42,43} Thus, T-type calcium channel is a good target for the treatment of epilepsy. These previous findings served as a basis for our study and we extrapolated these findings to further explore the neuroprotective ability of A3 in a mouse model of PTZ via the *Nrf2*-dependent pathway.

Materials and Methods

Chemicals and Reagents

3-diaminobenzidine tetrahydrochloride hydrate (DAB) (#D5637) was purchased from Sigma-Aldrich (St. Louis, MO). Mouse monoclonal anti-p-NF- κ B (SC-271908), mouse monoclonal anti-*HO-1* (SC-136960), mouse monoclonal anti-TNF- α (SC-52B83), rabbit polyclonal anti-*Nrf2* (SC-722), mouse monoclonal anti-VEGF (SC-7269), and ABC Elite kit (SC-516216) were procured from Santa Cruz Biotechnology (Dallas, TX). ELISA kits p-NF- κ B (SU-B28069) and TNF- α ELISA kit (SU-B3098) were procured from Shanghai Yuchun Biotechnology (Shanghai, China), while COX-2 (E-EL-M0959) was purchased from Elabscience Biotechnology Inc. (Houston, TX). The horseradish peroxidase-conjugated secondary antibody (ab-6789) was purchased from Abcam

(Cambridge, UK). Proteinase K was obtained from MP Bio USA. All other reagents, such as 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), DPX Mounting media, trichloroacetic acid (TCA), and N-(1-naphthyl) ethylenediamine dihydrochloride, were obtained from Sigma-Aldrich.

Animals and Ethics Approval

Adult male Swiss-Albino mice (weight 25–30 g) were habituated under laboratory conditions at 25°C for 7 days, with 12-hour alternating light and dark cycles, and provided a standard commercial diet and water ad libitum. All experimental procedures were carried out following the ARRIVE guidelines and Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD). Experimental protocols were approved by the Research and Ethics Committee (REC) of the Riphah Institute of Pharmaceutical Sciences (Approval ID: Ref. No. REC/RIPS/2018/14; date of approval: November 15, 2018).

Acute Toxicity Evaluation

For acute toxicity, nulliparous, non-pregnant female rats were divided into the control and treatment groups (n=5/group). Following OECD 425 guidelines, only one rat was administered an oral dose of 2,000 mg/kg of synthetic compound A3 after overnight starvation.⁴⁴ After careful observation and survival for 24 hours, other rats were subjected to the same protocol. The animals were closely monitored for any signs of distress and mortality for 48 hours; and then daily for 14 days for other signs, such as squinted eyes, writhing tremors, salivation, loss of fur, convulsions, overall behavioral changes, stress, and mortality. Blood samples were collected on the 15th day via cardiac puncture for various biochemical analyses, like hematological profiles, renal function tests, and liver function tests. The animals were eventually sacrificed under anesthesia and vital organs were processed for histopathological screening.

Seizure Induction Using Pentylentetrazole

Seizures were induced using PTZ as an inducing agent, using a previously described protocol, with slight modifications.^{46,47} Briefly, a subconvulsive dose of 40 mg/kg PTZ was dissolved in normal saline and injected intraperitoneally (IP) into the PTZ-kindled group at 48-hour intervals for 15 days unless they showed full kindling

and stage 5 or 6 of the Racine scale was reached, upon three consecutive injections. Only successfully kindled animals were included in the study.

Study Design and Drug Treatment

Animals were randomly divided into seven groups (n=10/group) as follows: group 1 (control group): the animal in this group received saline (containing 5% DMSO) every alternate day for 15 days; group 2 (PTZ control group): the animal in this group received 40 mg/kg PTZ at 48-hour intervals for 15 days until stage 5 convulsions were reached, and a total eight doses were administered; group 3/4 (treated group): mice received neuroprotective doses of A3 10 (10mg/kg) and A3 30 (30mg/kg), which were injected intraperitoneally 30 minutes before PTZ; group 5 (ATRA+PTZ group): mice were administered 5 mg/kg dose of all-trans retinoic acid (ATRA) 30 minutes before PTZ; group 6 (ATRA+PTZ+A3): animals were first administered ATRA, followed by A3 and PTZ; group 7 (standard group): mice received 2 mg/kg of diazepam 30 minutes before PTZ administration. ATRA, PTZ, A3, and diazepam were all dissolved in normal saline containing 5% DMSO and subsequently administered every alternate day for 15 days (Figure 2). Notably, A3 dose was selected through a previous study using a neurodegenerative model already established in our lab.³⁶

Behavioral Evaluation Racine's Scale

To evaluate behavioral characteristics, modified Racine's scale was used where seizure activity was observed for 30 minutes following PTZ administration. Behavioral characteristics such as latency time, seizure intensity, and stages of convulsion were recorded for 30 minutes after each PTZ dose observing following parameters from Racine's Scale:⁴⁸ stage 0=no response; stage 1=vibrissae twitching, hyperactivity, and restlessness; stage 2=head clonus, head nodding, and myoclonic jerks; stage 3=unilateral or bilateral limb clonus; stage 4=forelimb clonic seizures; stage 5=generalized clonic seizures with falling on one side; stage 6=hind limb extensor; and stage 7=death (Table 1). Mean seizure intensity was calculated by taking means of all animal's individual seizure scores divided by the total number of animals and plotted them against treatment duration. Seizure latency is the time duration between the PTZ dose administration and emergence of the first clonic jerk or a sudden twitch. Seizure frequency was calculated by counting the number of seizures the animal

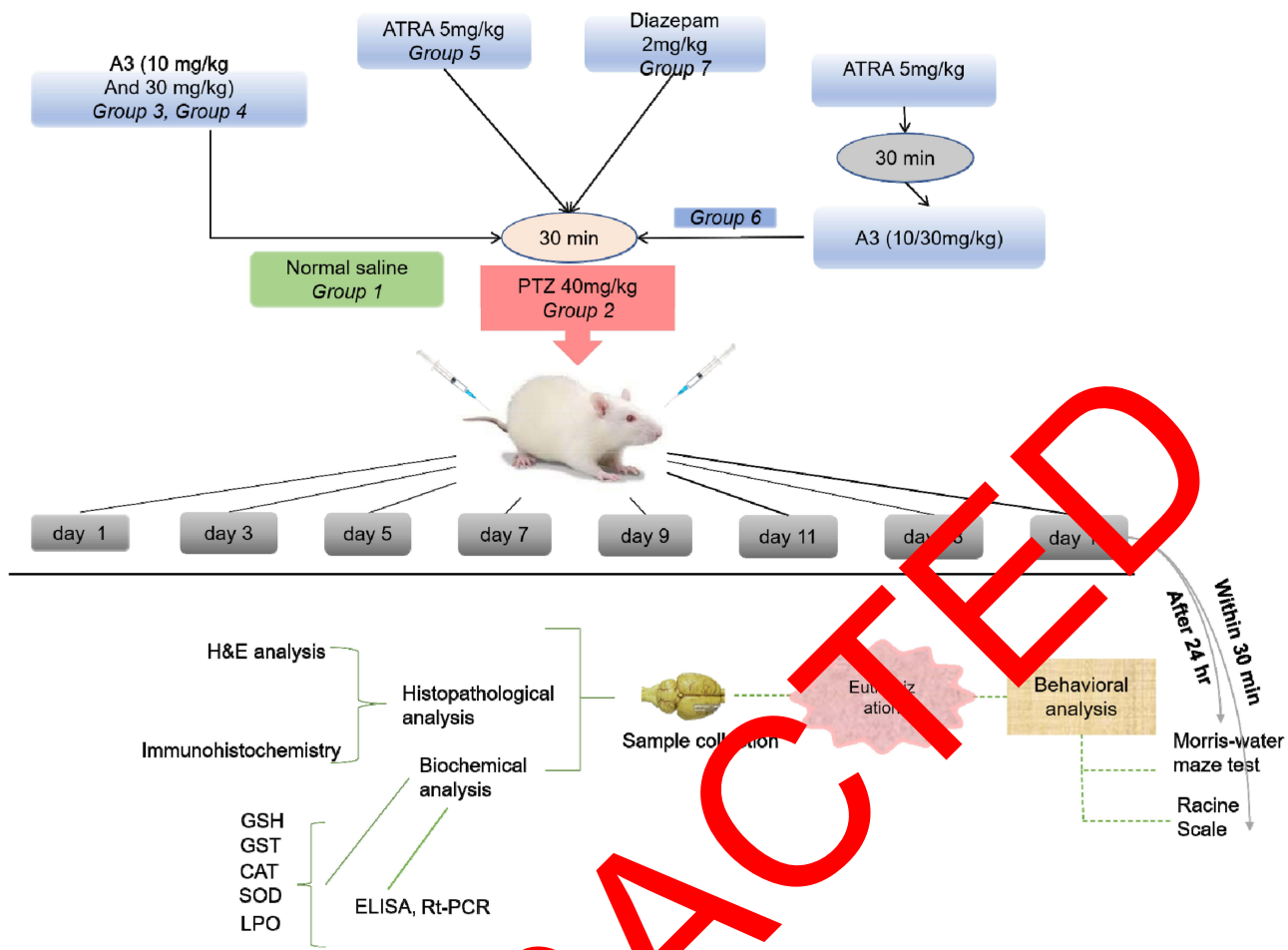


Figure 2 Diagrammatic illustration of the experimental protocol. The treatment protocol was performed for 15 days. In all these groups, a loading dose of PTZ (40 mg/kg, IP) was injected 30 minutes after drug treatment (ATRA, A3, diazepam) except in the saline group. Brain tissues were collected after 24 hours of the last dose for further analysis.

experienced, within 30 minutes of PTZ administration, regardless of the seizure stage. Animals were confirmed

as kindled when they reached stage 5 or 6 of the Racine’s scale after three consecutive PTZ injections at 48 hour intervals. The investigator performing behavioral evaluation was blinded to the group allocations in order to avoid any bias.

Table 1 Modified Racine’s Scale

Stages	Seizure Intensity
0	No seizure
1	Hyperactivity, restlessness, and vibrissae twitching
2	Head nodding, head clonus, and myoclonic jerks
3	Unilateral or bilateral limb clonus
4	Forelimb clonic seizures
5	Generalized clonic seizures with falling
6	Hind limb extensor
7	Death

Notes: Adapted from: Alvi AM, Al Kury LT, Alattar A, et al. Carveol Attenuates Seizure Severity and Neuroinflammation in Pentylentetrazole-Kindled Epileptic Rats by Regulating the Nrf2 Signaling Pathway. *Oxid Med Cell Longev*. 2021;2021:9966663. doi:10.1155/2021/9966663.⁸⁰ Copyright © 2021 Arooj Mohsin Alvi et al. Creative Commons Attribution 4.0 International (CC BY 4.0; <https://creativecommons.org/licenses/by/4.0/legalcode>).

Morris Water Maze (MWM) Test

To assess cognitive deficits inflicted by PTZ and the spatial learning ability of the mice, MWM test was performed as described previously.³⁷ The apparatus comprises a circular pool with a diameter of 120 cm and a height of 50 cm. The pool was divided hypothetically into four equal quadrants with respect to the target quadrant. Target quadrant contained the probe or elevated platform placed almost 1 cm below the water surface. The position of the probe was fixed, while each time the animal was dropped in a different quadrant. Water temperature was maintained at 25°C±1°C and a blind observer noted the escape latency: time taken by animal to locate and

climb on the raised platform. The experiment continued for 4 days with training sessions where animals were trained to locate and climb the probe with a stay time of 5–7 seconds. The observer recorded the time at which the animal was released into the water and the time it took to locate and climb the platform, and if it failed to locate the platform within 90 seconds, the observer would guide the animal to the platform himself. The training sessions were carried out twice a day, with 25 minute intervals and the escape latency for each animal was recorded during 3 days of testing sessions. Decreased escape latency indicated neurodegeneration. On the final day of the experiment, a probe test was conducted to test spatial memory of the animals in which the probe was removed and the animals were dropped in opposite quadrants and the time spent by the animals in each quadrant was noted for 60 seconds. Time spent in the target quadrant was considered as a measure of the extent of memory function and impairment.

Tissue Collection and Preparation

Brain tissues were collected at 24 hours after the last dose of PTZ was administered. Animals were quickly decapitated under anesthesia and their brains were isolated on an ice-cold glass plate. After separating the areas of interest (ie, the cortex and hippocampus), half of the samples were frozen at -80°C for biochemical evaluation, while the other half were preserved in 4% formalin solution for staining analyses. To proceed for biochemical analyses, the brain tissues were first homogenized in 0.1 M sodium phosphate buffer (pH 7.4) using phenylmethylsulfonyl fluoride (PMSF) as a protease inhibitor and then centrifuged at 4°C for 10 minutes at $4000\times g$. Supernatant was separated and processed for various biochemical assays.

Antioxidant Assays

Reduced Glutathione (GSH) Activity

GSH activity was determined as a previously mentioned protocol.³⁶ A sample (0.2 mL) of the tissue supernatant was mixed with 2 mL of DTNB mixture and 0.2 M phosphate buffer making a final volume of 3 mL. The mixture was allowed to stand for 10 minutes and the absorbance was measured at 412 nm, using phosphate buffer solution without the tissue supernatant and DTNB solution as a control and blank, respectively. Final GSH activity was calculated by subtracting the absorbance of control from that of tissue lysate, and expressed as $\mu\text{mol}/\text{mg}$ of protein.

Glutathione-s-Transferase (GST) Activity

For the estimation of GST activity, CDNB (1 mM) and GSH solution (5 mM) was freshly prepared in 0.1 M phosphate buffer. Then 1.2 mL reaction mixture was kept in three glass vials and 60 μL of tissue homogenate was added to each, with blank containing only water. Aliquots (210 μL) from this reaction mixture were pipetted out in a microtiter plate and absorbance at 340 nm was measured using an ELISA plate reader (BioTek ELx808, Winooski, VT) for 5 minutes (23°C). GST activity was expressed as μmol of CDNB conjugate/min/mg of protein.^{49,50}

Superoxide Dismutase (SOD) Activity

SOD activity was estimated by mixing 0.1 mL tissue homogenate with 0.8 mL potassium phosphate buffer solution (0.1 M, pH 7.4) and 0.1 mL pyrogallol solution (1 M), yielding a total reaction mixture of 3 mL. Absorbance of this mixture was measured at 312 nm and SOD activity was expressed as μg of tissue.⁵¹

Catalase (CAT) Activity

Estimation of CAT activity was carried out using previously established protocols with minor modifications.⁵¹ To 0.05 mL of tissue homogenate, we added 1.95 mL of 50 mM phosphate buffer solution (pH 7) and 1 L of 30 mM H_2O_2 solution. The absorbance of this reaction mixture was measured at 240 nm and CAT activity was calculated using the formula:

$$\text{CAT} = \delta\text{O.D} \div E \times \text{Volume of sample (mL)} \times \text{protein (mg)}$$

where $\delta\text{O.D}$ represents the change in absorbance per minute and E is the extinction coefficient of H_2O_2 having a value of 0.071 mmol/cm. Protein levels were measured using Lowery method. CAT activity was expressed in units of μmol of $\text{H}_2\text{O}_2/\text{min}/\text{mg}$ of protein.

Determination of Lipid Peroxidation (LPO)

Detection of thiobarbituric acid reactive substances (TBARS) was used as an estimation of LPO. Previously established protocols were used to detect TBARS levels.⁵² An assay mixture containing 200 μL of supernatant, 200 μL of 100 mM ascorbic acid, 20 μL of ferric chloride, and 580 μL of 0.1 M phosphate buffer (pH 7.4) was prepared. It was incubated in a water bath for 60 minutes, at a temperature of 37°C . Next, 1,000 μL each of 0.66% thiobarbituric acid and 10% trichloroacetic acid was added to the reaction mixture to stop the reaction and the tubes were again kept in a water bath for 20 minutes, then cooled in

an ice bath, and centrifuged at $3,000\times g$ for 10 minutes and supernatant was collected. Absorbance of the collected supernatant was measured at 535 nm and expressed in units of TBARS-nmol/mg of proteins.

Histological Preparation

Following brain extraction, tissues fixed in 4% paraformaldehyde solution were washed and sliced into 3 mm thin coronal sections with a sharp blade. These coronal sections were then fixed in paraffin blocks and 4 μm thin slices were prepared using a microtome and processed for the following staining techniques.⁵³

Hematoxylin and Eosin Staining (H&E Staining)

H&E staining was carried out using our previously established lab protocols.⁵⁴ Paraffinized tissues were deparaffinized using xylene and graded alcohol and immersed in hematoxylin solution for as long as the nucleus retains the dye. Slides were then treated with HCl (1%) and ammonia water (1%) and stained with eosin solution and air-dried. Next, slides were dehydrated using graded ethanol and xylene and coverslipped and observed under an Olympus light microscope (Olympus, Japan). Five images were taken from each slide, and analyzed using imageJ software while focusing on nuclear size, shape, number, morphology, and edema.

Immunohistochemical Analysis

Immunohistochemical analysis was performed using our previously established lab protocols.⁵⁵ After rehydrating tissue using xylene, graded ethanol, and water, slides were treated with proteinase K for antigen retrieval. Next, slides were first washed with PBS and kept in 3% H_2O_2 solution for 5 minutes and blocking serum was then applied at room temperature to block undesired antigenic areas. After 1 hour, anti-mouse VEGF antibody, anti-rabbit *Nrf2* antibody, anti-mouse p-NF- κB antibody, anti-mouse *HO-1* antibody, and anti-mouse TNF- α antibody (dilution 1:100, Santa Cruz Biotechnology, Dallas, TX) were applied and kept overnight at 4°C. The next morning, after washing slides with PBS, secondary antibody was applied to each slide to enhance signal detection and ABC staining kit for 1 hour each. Slides were finally stained with DAB solution for 5 minutes, and dehydrated using xylene and 100% ethanol solution, coverslipped, and air-dried. Images were obtained using an Olympus

microscope and observed at 10x and 40x magnifications. Five images per slide were chosen to calculate the number of cortical and hippocampal stained cells and analyzed using imageJ software. Means were plotted against the groups.

ELISA (Enzyme-Linked Immunosorbent Assay)

ELISA kits were used to quantify the expression of TNF- α , COX-2, and p-NF- κB , according to manufacturer's instructions. Then 2,500 μL of PBS was added to 50 mg of brain tissue and a small quantity of PMSE was also added to the solution as a protease inhibitor.⁵⁶ The mixture was first homogenized at 10,000 rpm and then centrifuged at $4,000\times g$ for 10 minutes and the supernatant was collected. BCA method (Bioassay) was used to calculate the protein concentration in the supernatant of each group and an equal quantity of protein was loaded to quantify protein concentration of TNF- α , COX-2, and p-NF- κB using an ELISA plate reader (BioTek EL \times 808). The protein concentration obtained (pg/mL) was then normalized to the total protein content (pg/mg total proteins).

Real-Time Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from isolated brain samples using TRIzol reagent as described previously.⁵⁷ A NanoDrop plate (Skanit RE 4.1, Thermo Scientific) was used to assess both the quality as well as quantity of RNA, while a viva cDNA synthesis kit (Vivantis cDSK01-050) was used to convert RNA to cDNA. Polymerase chain reaction was carried out using a Galaxy XP Thermal Cycler (BIOER, PRC) and 2X Amplifyme Universal qPCR mix (Blirt, Germany), according to manufacturer's manual.

The sequences of forward and reverse primers were as follows:

mice *Nrf2*_Forward: CGAGATATACGCAGGAGAG
GTAAGA

mice *Nrf2*_Reverse: GCTCGACAATGTTCTCCA
GCTT

mice *HO-1*-Forward: CCTTCCCGAACATCGACAG
CC and

mice *HO-1*-Reverse: GCAGCTCCTCAAACAGCT
CAA

mice *GAPDH*-Forward: CGCTCTCTGCTCCTCC
TGTT and

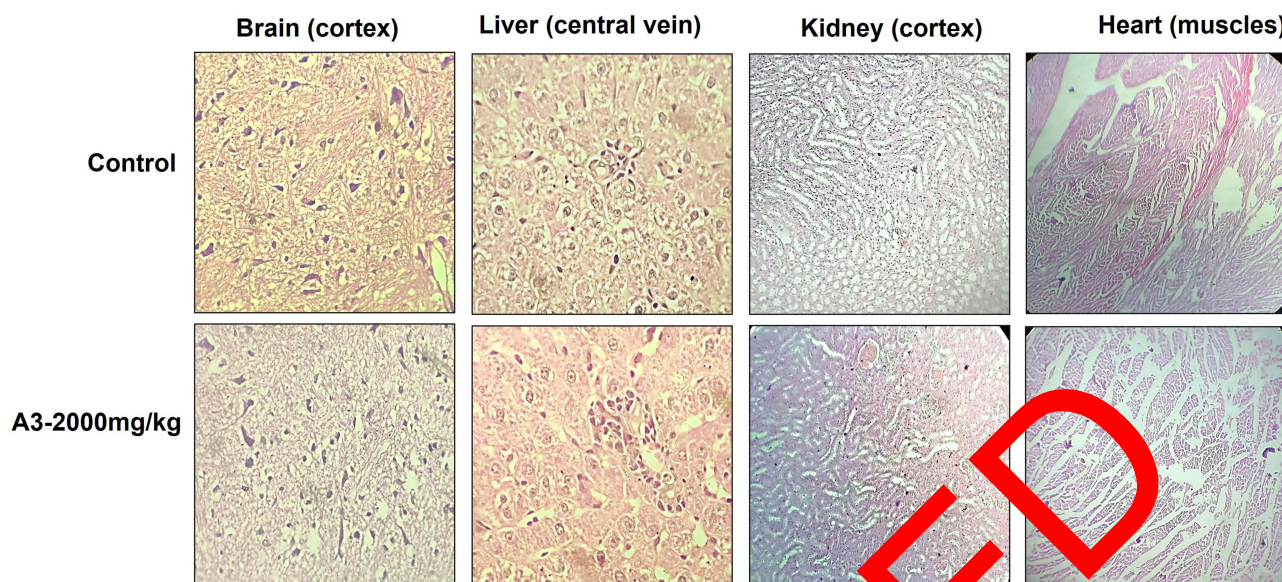


Figure 3 Histopathology of control and A3-treated groups at a limited dose (2,000 mg/kg).

mice_GAPDH-Reverse: CCATGGTGTCTGAGCG
TGT

The relative gene expression of both *Nrf2* and *HO-1* was calculated using the $2^{-\Delta\Delta CT}$ method for real-time quantitative PCR.

Statistical Analysis

Graph pad prism-8 software was used to carry out all statistical analyses. $p < 0.05$ was set as statistically significant and * or # represent statistical differences relative to saline and disease group, respectively. * or # indicates $p < 0.05$, ** or ## indicate $p < 0.01$, while *** or ### indicates $p < 0.001$ significance levels. All data is expressed as mean \pm SEM. One-way ANOVA was used to analyze behavioral analysis and oxidative data, while the rest of the data was analyzed using two-way ANOVA. A post-hoc Bonferroni test was used for multiple comparisons. ImageJ software was used to analyse the morphological and histological data.

Results

Acute Oral Toxicity Testing of A3

Acute oral toxicity assessment of A3 was carried out according to OECD guidelines 425. Skin, fur, urine color, fecal consistency, sleep pattern, respiration, and other physical parameters were observed for 14 days after administration of 2,000 mg/kg of A3. No abrupt variations and signs of distress and convulsions were observed. All animals survived and the weight progression

of all organs seemed normal. Histopathology of the brain, liver, kidney, and heart revealed no vacuolation, dystrophy, and/or atrophy (Figure 3). Assessment of liver function tests, kidney functions, and antioxidant profile of all vital organs along with hematological profile was normal as compared to the saline group (Supplementary Figures S1 and S2). A detailed toxic profile of A3 indicated its safety for up to as high a dose as 2,000 mg/kg.

Anticonvulsant Effect of A3 on PTZ-Induced Seizure-Like Behavior

Animals undergoing PTZ-treatment exhibited significant generalized tonic-clonic seizures corresponding to stage 5 or 6 of the modified Racine scale (Table 1), which is depicted clearly by significantly higher mean seizure intensity score as compared to the saline group (Figure 4A, *** $p < 0.001$). Similarly, PTZ-treatment resulted in a significantly shorter latency period as opposed to saline, indicating an immediate onset of seizures after successive PTZ administration (Figure 4B). These immediate onsets of severe seizures thus result in a survival rate of merely 60% (Figure 4C). Upon treatment with a lower dose of A3 (10 mg/kg), animals exhibited a significant improvement in mean seizure severity (Figure 4A, # $p < 0.05$), along with a significant prolongation in latency period of seizure initiation (Figure 4B, # $p < 0.05$). This also resulted in a significant improvement in survival percentage to nearly 85% compared to PTZ (Figure 4C). A dose of 30 mg/kg of A3 showed a similar

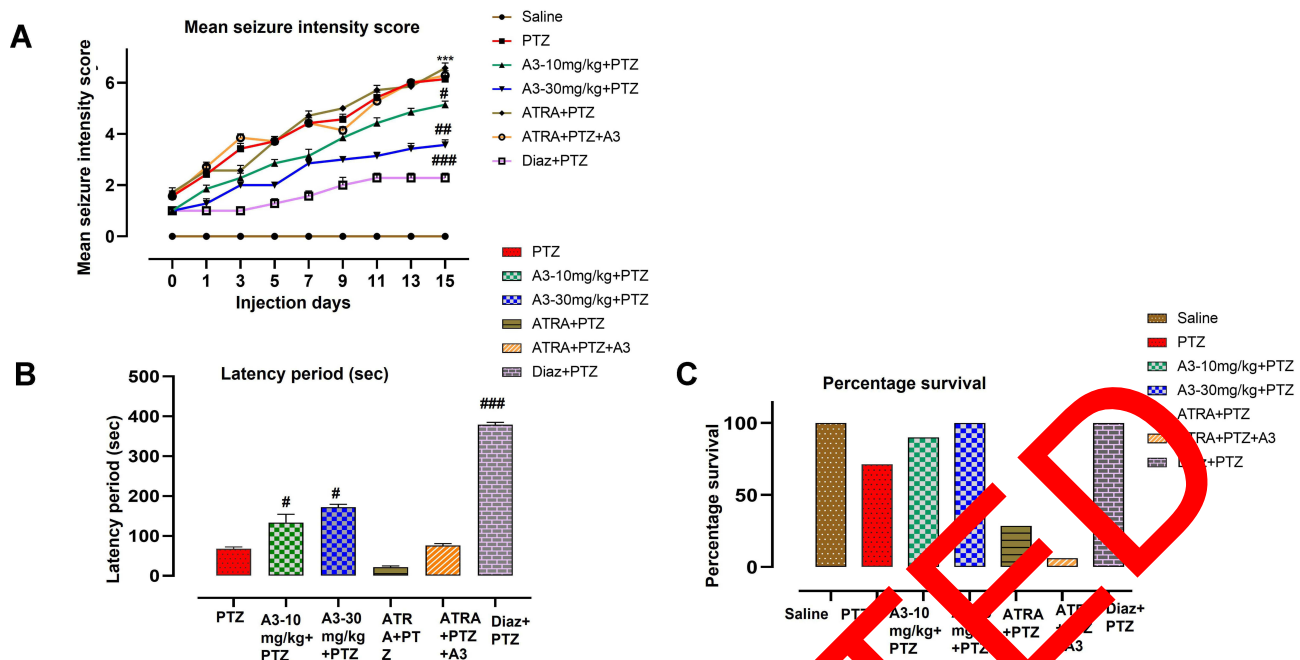


Figure 4 Effect of A3 on PTZ-induced seizure-like behavior. **(A)** Mean seizure intensity score was recorded after each injection of PTZ. Each criterion was scored from 1–7. A3 caused a significant reduction in the mean seizure score as compared to PTZ-kindled animals. **(B)** Latency period was measured as the time duration after PTZ administration and appearance of first clonic seizure. A3 displayed a delayed latency period as compared to PTZ. **(C)** Percentage survival of saline versus PTZ-kindled group showed a high mortality rate whereas A3-30 mg/kg displayed an improved survival of the treated animals. All data, except percentage survival, were expressed as mean±SEM (n=10/group). # $p<0.05$ or ## $p<0.01$ or ### $p<0.001$ denotes a significant difference from the PTZ-kindled group, while *** $p<0.001$ denotes a significant difference compared to the saline group.

pattern of protection where no animal exhibited a higher seizure score, thus shortening the time to reach the mean seizure score above 5 during the whole kindling process (Figure 4A, ## $p<0.01$) accompanied by a significantly extended period of latency to initiate seizure compared to PTZ-treatment (Figure 4B, # $p<0.05$). None of the animals receiving 30 mg/kg of A3 died, thereby exhibiting a 100% survival score (Figure 4C). Diazepam was employed as a standard drug that displayed significant neuroprotection as projected by its mean seizure score, latency period, and survival rate. Moreover, upon co-treatment with the *Nrf2*-inhibitor ATRA, PTZ-treated groups displayed aggravation in neurological indices which could not be mitigated by A3 treatment, thus ensuring termination of A3-induced neuroprotection by ATRA administration.

A3 Attenuated Memory and Cognitive Impairment in Epileptic Mice

To determine the effect of A3 on memory and cognitive impairment, the Morris water maze test was conducted. PTZ-treated animals exhibited significant memory and cognitive impairment as displayed by higher latency time as opposed to the saline group (Figure 5A, *** $p<0.001$). A3-treatment dose-dependently improved memory deficits to a

loaden platform (Figure 5A, ## $p<0.01$, ### $p<0.001$). Twenty-four hours after the last acquisition period, a probe trial was conducted to assess reference memory. Time spent by each animal was noted and increased time spent in any quadrant other than the target quadrant was a measure of impaired spatial learning as demonstrated clearly by the PTZ-treated animals. Animals spent only 20% of their time in the target quadrant, thus manifesting significant memory impairment as compared to the control group (Figure 5B and C, *** $p<0.001$). A3 treatment imparted significant improvement in neurological impairment at both doses (Figure 5B and C; ## $p<0.01$, # $p<0.05$, ## $p<0.01$). Besides, A3 treatment presented little to no improvement in the ATRA-treated group confirming cessation of action of A3 upon ATRA administration.

A3 Improves Neuronal Survival in PTZ-Induced Degenerated Neurons

To study the morphological damage induced by PTZ, H&E staining was performed. The microscopic images of the frontal, cortical and hippocampus region in the saline group projected well-demarcated, rounded, and intact cells without any nuclear condensation or cellular distortion

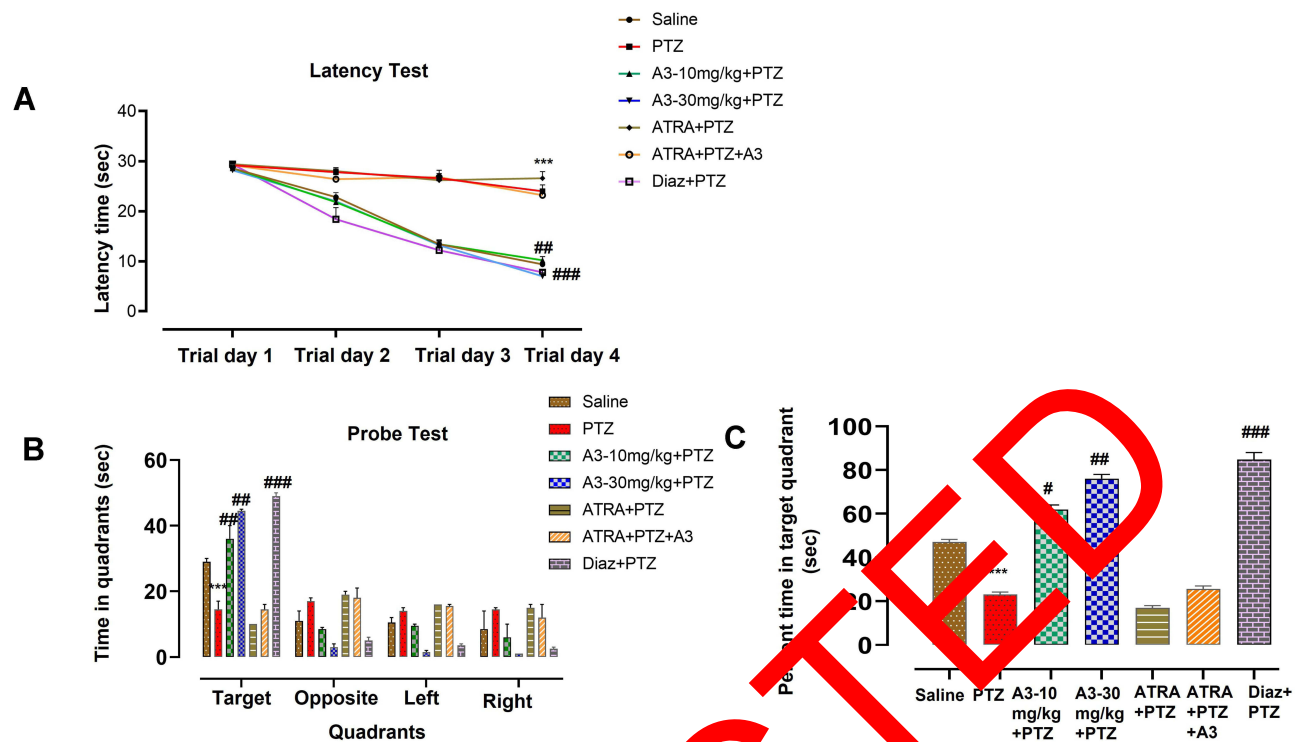


Figure 5 Effect of A3 on PTZ-induced memory impairment. (A) Latency time of mice to the hidden platform. (B) Time spent by PTZ and treated mice in each quadrant in probe test on 4th day. (C) Percentage time spent by animals in the target quadrant. All data were expressed as mean±SEM (n=10/group). *** $p < 0.001$ denotes a significant difference from the saline group. #### $p < 0.001$, ### $p < 0.01$, or # $p < 0.05$ denotes a significant difference from the PTZ-kindled group.

(Figure 6). PTZ-treated animals displayed significant morphological alterations and atypical features as karyotic, atrophied, pyknotic, and swollen nuclei (Figure 6; cortex: * $p < 0.05$, CA1: *** $p < 0.001$, CA3 and DG: ** $p < 0.01$). On further examination, the photomicrographs confirmed that histopathological damage was significantly ameliorated by A3 treatment as observed by greater number of surviving neurons (Figure 6, ## $p < 0.01$). Moreover, the ATRA-treated

PTZ-group aggravated the morphological damage and remained refractory to A3 treatment.

A3 Enhances the Antioxidant Capacity of the Brain Through Nrf2 and Inducible HO-1
 PTZ-induced free-radical overload, which induced *Nrf2* expression as determined by RT-PCR (Figure 7A, * $p < 0.05$). This was further confirmed by

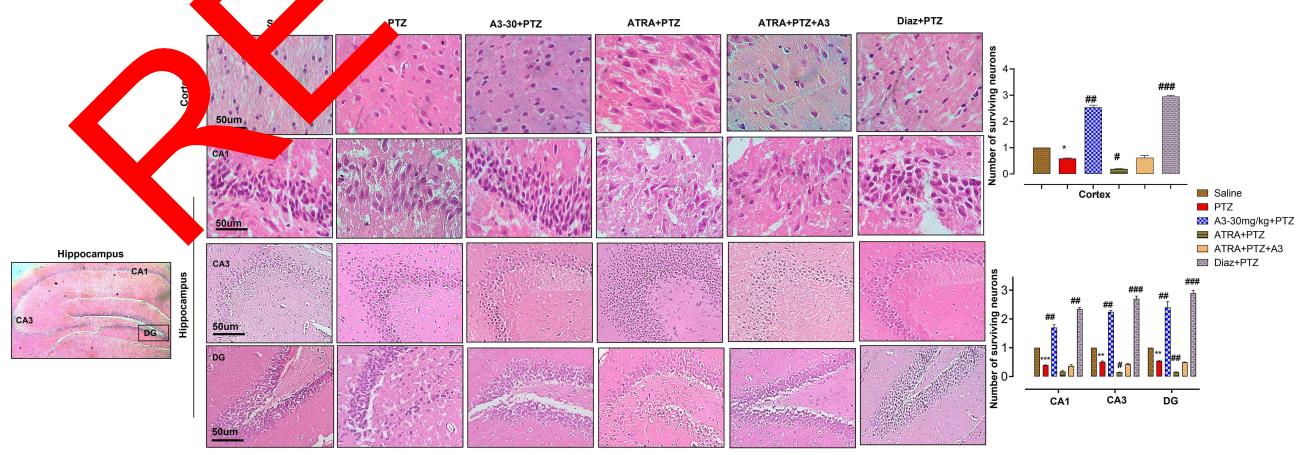


Figure 6 Representative photomicrographs of H&E-stained cortex and hippocampal tissue indicating the presence of karyotic and atrophied nuclei in PTZ-kindled animals while only a few cells with degenerative signs in the A3-30 mg/kg group (40×, scale bar= 50 μm). All data are expressed as mean±SEM (n=5/group). * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$ denotes a significant difference from the saline group. # $p < 0.05$ or ## $p < 0.01$ or ### $p < 0.001$ denotes a significant difference from the PTZ-kindled group.

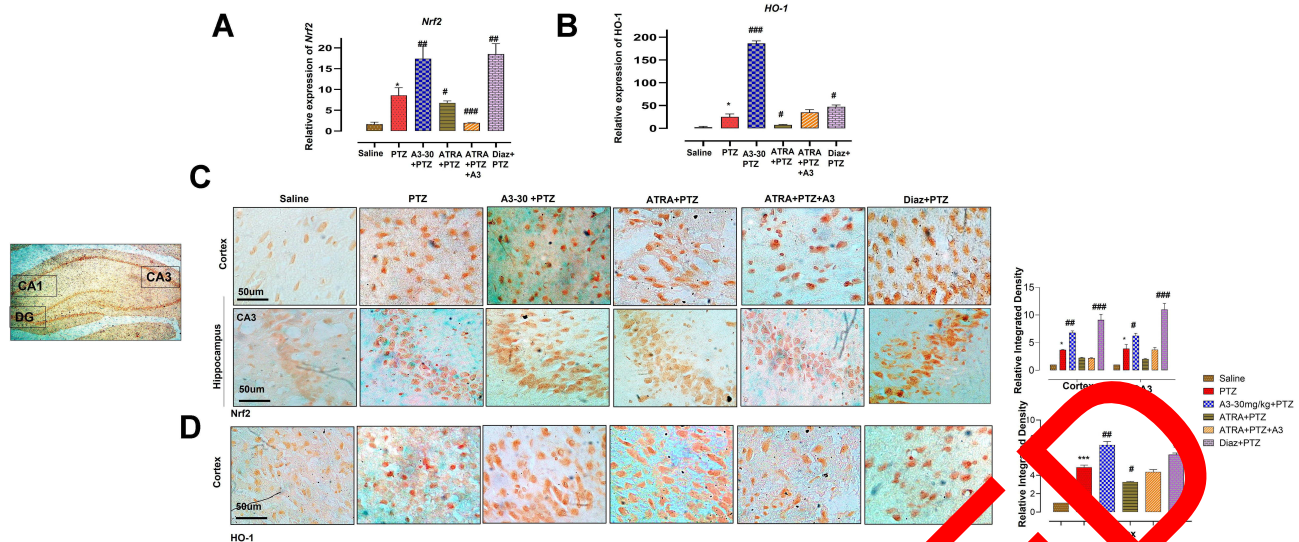


Figure 7 A3 augments the antioxidant capacity of the brain via *Nrf2/HO-1* signaling pathway. **(A)** The gene expression of *Nrf2* was quantified by RT-PCR. **(B)** The gene expression of *HO-1* as quantified by RT-PCR. **(C)** Immunohistochemistry results for *Nrf2* in the cortex and hippocampal tissues of the brain. Histograms exhibit higher *Nrf2* nuclear localization in treated brain tissues. **(D)** Immunohistochemistry results for *HO-1* in the cortical tissues of the brain. Histograms represent higher *HO-1* nuclear localization in treated brain tissues. Bar 50 μm , magnification 40 \times ($n=5/\text{group}$). * $p<0.05$ or *** $p<0.001$ indicates significant difference relative to saline, while # $p<0.05$ or ### $p<0.001$ shows a significant difference relative to the PTZ group. All the data is presented as mean \pm SEM.

immunohistochemical analysis where a significant upregulation in *Nrf2* protein was observed compared with the saline group (Figure 7C, * $p<0.05$). In line with *Nrf2* upregulation, downstream inducible *HO-1* protein expression also enhanced significantly as confirmed by RT-PCR (Figure 7B, * $p<0.05$) and immunohistochemistry (Figure 7D, cortex: *** $p<0.001$). A3 treatment of the PTZ-kindled animals further enhanced the body's antioxidant ability, thus further upregulating the expression of *Nrf2* and subsequently *HO-1* relative to PTZ (Figure 7A–D). ATRA-treatment, however, abrogated A3-mediated upregulation of *Nrf2* and *HO-1*, depicting an obvious involvement of the *Nrf2/HO-1* pathway in the antioxidant ability of A3.

A3 Ameliorates the Inflammatory Mediators via the *Nrf2* Signaling Pathway

To further elaborate on the role of A3 in neuroinflammation, we studied various inflammatory mediators and proinflammatory cytokines. p-NF- κ B, TNF- α , and COX-2 expression were determined through ELISA and immunohistochemistry. The PTZ-treated group presented an exaggerated level of the proinflammatory cytokine TNF- α , p-NF- κ B, and COX-2 as quantified by ELISA both in the cortex and hippocampus (Figure 8A and B, ** $p<0.01$, Figure 8C, * $p<0.05$, *** $p<0.001$). Immunohistochemical results also

produced the same results and validated the ELISA findings (Figure 8D and E, cortex: ** $p<0.01$; CA3: ** $p<0.01$, * $p<0.05$). A3 attenuated both pro-inflammatory and inflammatory markers both in cortical and hippocampal tissues, indicating the neuroprotective potential of A3 (Figure 8A–E). Furthermore, co-treatment with ATRA and PTZ aggravated the neuroinflammatory markers and A3-treatment failed to revert the deleterious effects of PTZ in the ATRA-treated group.

A3 Improves BBB Disruption Through VEGF

Following epileptiform activity in the brain of PTZ-treated animals, VEGF is induced as a consequence of BBB disruption. Likewise, our study also reported an abrupt induction of VEGF following PTZ-injections compared to the saline group, which is clearly demonstrated through immunohistochemistry pictograms (Figure 9, *** $p<0.001$). A3 treatment ameliorated the intensified VEGF expression in both cortical and hippocampal regions to a significant extent, restoring BBB's permeability to a huge extent (Figure 9, cortex, CA3: ### $p<0.001$). ATRA-treatment, on the other hand, induced significant angiogenic factor VEGF, resulting in BBB dysfunction and diminished A3's restorative potential in ATRA-treated animals.

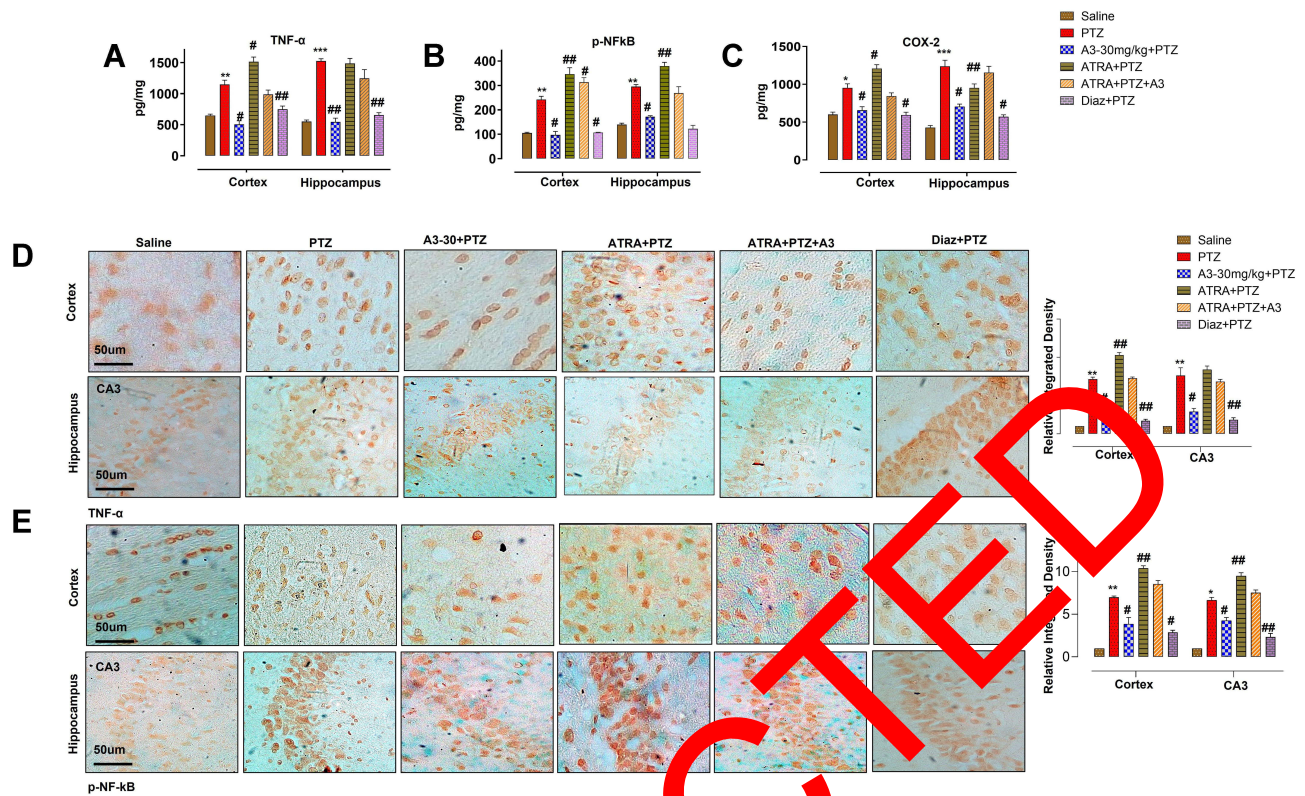


Figure 8 Effect of A3 on outcomes of PTZ-induced inflammatory mediators. (A) The protein expression of TNF- α is quantified by ELISA. (B) The protein expression of p-NF-kB is quantified by ELISA. (C) The protein expression of COX-2 is quantified by ELISA. (D) Immunohistochemistry results for TNF- α . (E) Immunohistochemistry results for p-NF-kB in the cortex and hippocampal tissues of the brain. PTZ inhibited nucleus localization in the treated tissue while TNF- α exhibited cytoplasmic localization. The data were expressed as the mean \pm SEM, n=5/group. * $p < 0.05$ or ** $p < 0.01$ or *** $p < 0.001$ shows difference relative to saline, while ## $p < 0.01$ or # $p < 0.05$ shows a significant difference relative to PTZ. Bar 50 μ m, magnification 40 \times .

Effect of A3 on PTZ-Induced Oxidative Stress Markers and Lipid Peroxidation

Our study depicts the involvement of the *Nrf2/HO-1* pathway in the neuroprotective nature of A3 and *Nrf2* can reverse the oxidative damage caused by A3 which is explored by investigating oxidative stress markers and outcomes of lipid peroxidation. We measured the levels

of major antioxidants like SOD, CAT, GST, GSH, and thiobarbituric acid reactive substances (TBARS) both in the cortex and hippocampus (Figure 10). PTZ treatment-induced oxidative stress is evident by reduced levels of innate antioxidants SOD, CAT, GST, and GSH, and up-regulated products of lipid peroxidation as TBARS (Figure 10A–E, *** $p < 0.001$). A3 treatment successfully

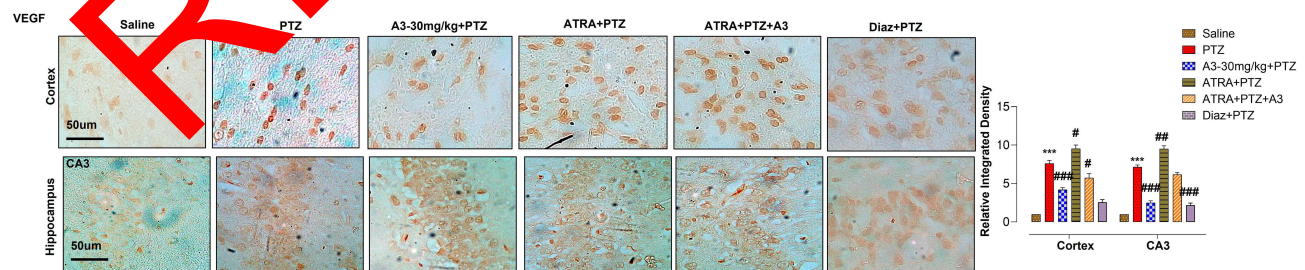


Figure 9 A3 improves BBB disruption through VEGF. Immunohistochemistry results for VEGF in the cortex and hippocampal tissues of the brain. VEGF exhibited cytoplasmic localization in both tissues of the brain. The data were expressed as the mean \pm SEM (n=5/group). *** $p < 0.001$ shows difference relative to saline, while # $p < 0.05$ or ## $p < 0.01$ or ### $p < 0.001$ shows a significant difference relative to PTZ. Bar 50 μ m, magnification 40 \times .

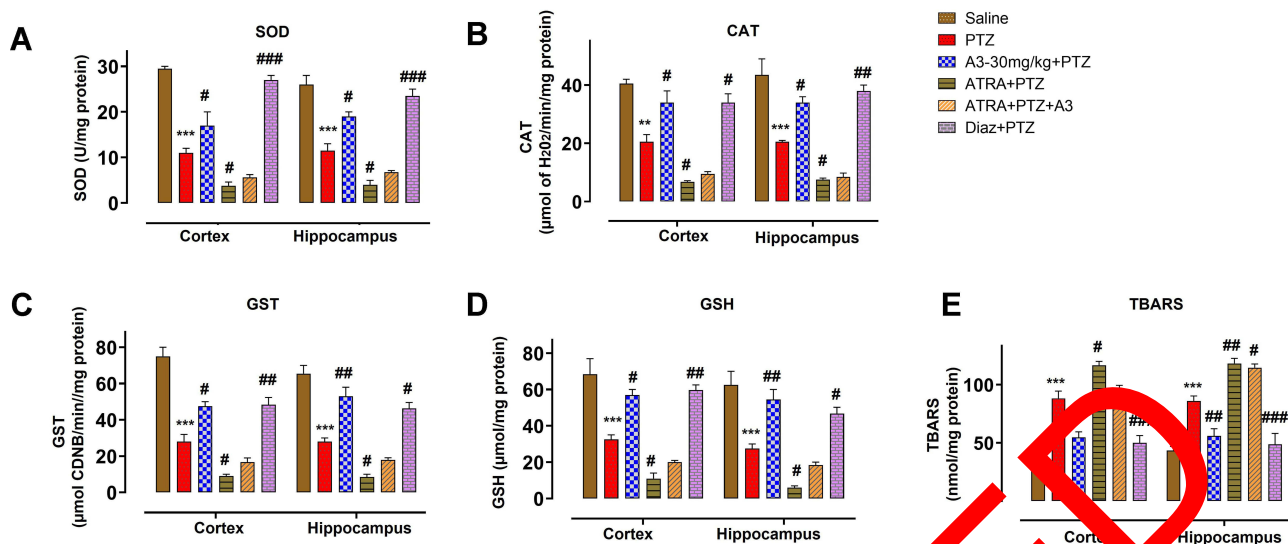


Figure 10 Effect of A3 on oxidative enzymes (SOD, CAT, GST, GSH, and LPO) in the cortex and hippocampus of mice's brain. (A) SOD level, (B) CAT level, (C) GST level, (D) GSH level, (E) LPO level. *** $p < 0.001$ or ** $p < 0.01$ denotes a significant difference from the saline group, # $p < 0.05$ or ## $p < 0.01$ or ### $p < 0.001$ denotes significant differences from the PTZ group ($n=5/\text{group}$). The data are expressed as mean \pm SEM.

mitigated these effects and induced the levels of SOD, CAT, GST, and GSH in both the cortex and hippocampus (Figure 10A, # $p < 0.05$; Figure 10B, # $p < 0.05$; Figure 10C, cortex: # $p < 0.05$; hippocampus: ## $p < 0.01$; Figure 10D, cortex: # $p < 0.05$, hippocampus: ## $p < 0.01$). Conversely, the levels of TBARS were noticeably reduced after A3 treatment in both cortex and hippocampus as compared to the PTZ-group (Figure 10E, ## $p < 0.01$). Furthermore, A3 treatment in ATRA-groups demonstrated a minimum antioxidant effect indicating its cessation of antioxidant potential (Figure 10), which is in harmony with our previous findings (Figure 7).

Discussion

The findings of the current study demonstrated that pretreatment with 1,3,4-oxadiazole compound A3 significantly attenuated the behavioral and biochemical alterations caused by PTZ-kindling. A3 not only upregulated the levels of innate antioxidants SOD, CAT, GST, and GSH but also alleviated the PTZ-induced LPO levels along with various inflammatory mediators as TNF- α , COX-2, and NF- κ B which forms the basis of neuroinflammation. Moreover, the present study also aimed at investigating the involvement of the *Nrf2*-pathway in the neuroprotective potential of A3. The outcomes of this study will provide an insight into the use of *Nrf2* as a therapeutic target in epilepsy and will also help us in elucidating the cascading mechanisms involved.

Plant-derived natural compounds have been continuously employed as treatment options against numerous disorders because of their minimal side-effects, improved therapeutic potential, and reduced toxicity profile. Along with natural substances, synthetic moieties are consistently employed in various research models as a part of various treatment protocols. 1,3,4-oxadiazole (A3), which originated from oxadiazoles and has demonstrated potent antioxidant properties. Previous studies have shown that A3 possesses robust antioxidant, anti-inflammatory, and protective properties in various degenerative models.³⁶ However, no direct studies suggesting antiepileptic potential of A3 have been reported yet. The present study was designed to investigate the neuroprotective potential of A3 in ameliorating behavioral and cognitive deficits, oxidative stress, and neuroinflammation in the PTZ-induced chronic model of epilepsy. The results demonstrated that A3 had significant potential in regressing seizures and accompanying progressive changes during PTZ-kindling and thereby provides a robust protection to the brain tissues undergoing stress-induced neuroinflammation by augmenting the endogenous *Nrf2* antioxidant pathway.

In this study, a sub-convulsive PTZ dose was utilized to induce seizure symptoms, possibly by disturbing neurotransmitter regulation and hemostasis.^{21,58,59} Our results showed that A3 not only attenuated the episodes but also decreased seizure intensity and frequency, and delayed its onset. These recurrent seizures generate neuronal excitability in hippocampal neurons, causing numerous

cognitive and memory deficits in the brain.⁶⁰ These behavioral anomalies were demonstrated by a significant delay in escape latency and the inability of the animal to locate the target quadrant in the probe test, which are consistent with the previously established research.⁶¹ A3, however, significantly improved these behavioral outcomes by shortening latency time and modifying behavior in the probe test.

The involvement of ROS in the pathophysiology of various neurological disorders including epilepsy has been extensively researched.^{17,62} Because of the brain's limited antioxidant capacity and high lipid content, it is more susceptible to oxidative damage.⁶³ Furthermore, the severity of oxidative damage is proportional to the frequency of epileptic episodes.⁶⁴ Enhanced production of free radicals including hydroxyl radicals has been observed in the rat's cortex following PTZ-evoked seizures, whereas a substantial rise in lipid peroxidation accompanied by a decrease in antioxidants has also been observed in various studies, implying the role of oxidative stress in the pathophysiology of epilepsy.⁶⁵ Furthermore, PTZ can ubiquitously raise the level of nitric oxide throughout the brain.⁶⁶ Consistently, our findings are similar to these previous reports. This notion is further supported by the fact that certain clinically used antiepileptic medications (AEDs) reduced ROS in seizure,⁶⁷ while many others increased oxidative damage.^{68,69} Hence, it can be implied that adjunct therapy with antioxidants in combination with AEDs can be beneficial in the management of epilepsy, as demonstrated previously.⁷⁰ Our study revealed diminished levels of CA, SOD, GSH, and GST, which is in line with various studies recommending PTZ administration induced oxidative stress.^{71,72} Furthermore, A3 treatment resulted in the augmentation of these antioxidants which may account in part, for its neuroprotective ability. Excessive PTZ administration resulted in considerable oxidative stress, which exhausted the first line antioxidant defense ability of the body. This led to the activation of another important endogenous antioxidant pathway which is governed by *Nrf2*, and excessively stimulates the release of cytoprotective enzymes and ROS scavengers in both glial cells as well as neurons.^{30,73–76} The *Nrf2*-ARE pathway has been extensively researched in protecting the brain from PTZ-mediated neuronal damages by employing *Nrf2*-knockout mice^{77,78} and exaggerated *Nrf2* mRNA levels have been observed in the hippocampal region of the mice's brain that initiated unprovoked seizures.⁷⁹ Additionally, direct ARE activation and nuclear translocation of *Nrf2* have been suggested to be involved in seizure pathology.⁸⁰ Another study has reported a direct correlation of inducible *HO-1* expression and ARE

activation.⁸¹ Hence, *Nrf2* has been well researched in promoting neuroprotection after oxidative insult. The findings of this study also corroborate well with the previous findings and we documented an exaggerated *Nrf2* activation along with downstream inducible *HO-1* cytoprotective protein in the PTZ-kindled animals. Upon A3 administration, an enhanced upregulation of these cytoprotective proteins was observed which were abolished after *Nrf2* inhibition through ATRA, indicating a substantial involvement of the *Nrf2* pathway in the cytoprotective potential of A3.

Neuroinflammatory cascade results in an increased influx of pro-inflammatory cytokines which further induce hyperexcitability by N-methyl-D-aspartate (NMDA) receptor and Toll-like receptor 4 (TLR4) and seizures.⁶⁰ Furthermore, increasing evidence considered inflammatory processes as a biomarker of epileptogenesis.^{82,83} Anti-inflammatory treatments can drastically reduce spontaneous seizure frequency and the severity of the disease.⁸⁴ Furthermore, the release of these cytokines further activates NF- κ B and generates cellular stress response.⁸⁵ Hence, the involvement of NF- κ B in the pathophysiology of several neurological diseases including epilepsy has been well-documented.^{85–87} In addition, cyclooxygenase-2 (COX-2) is expressed at a low level in hippocampal neurons but it is markedly increased within an hour after a seizure.⁸⁸ Furthermore, previous studies have demonstrated increased TNF-levels in different cerebral areas in epileptic models associated with neurodegeneration.^{89,90} Likely, IL-6 is also involved in the propagation of epileptic patients and its level proportionally rises in recurrent seizures.⁹¹ We previously demonstrated that A3 counteracted elevated expression of COX-2, TNF- α , and p-NF κ B in ischemic brain injury.³⁶ Furthermore, a growing body of evidence suggests that enhanced *Nrf2* pathway activation, however, downregulates the NF- κ B pathway and its associated neuroinflammatory cascades.^{28,29} ATRA treatment exacerbated the expression of these neuroinflammatory markers and negatively modulated the neuroprotection provided by A3, thus confirming our hypothesis that A3 modulates the *Nrf2*/*HO-1* pathway and exerts its anti-inflammatory effect.

Several studies demonstrated the detrimental effect of seizures on blood-brain barrier (BBB) disintegration.⁹² Recently, seizure severity is linked directly to BBB disruption, and it not only contributes to epileptogenesis but is also involved in the recurrence of seizures.⁹³ A large number of protein factors act on vasculature including fibroblast growth factors, neuropeptides as calcitonin gene-related peptide (CGRP), and substance-P (SP), vascular endothelial growth factors (VEGF), platelet-derived growth factor, and

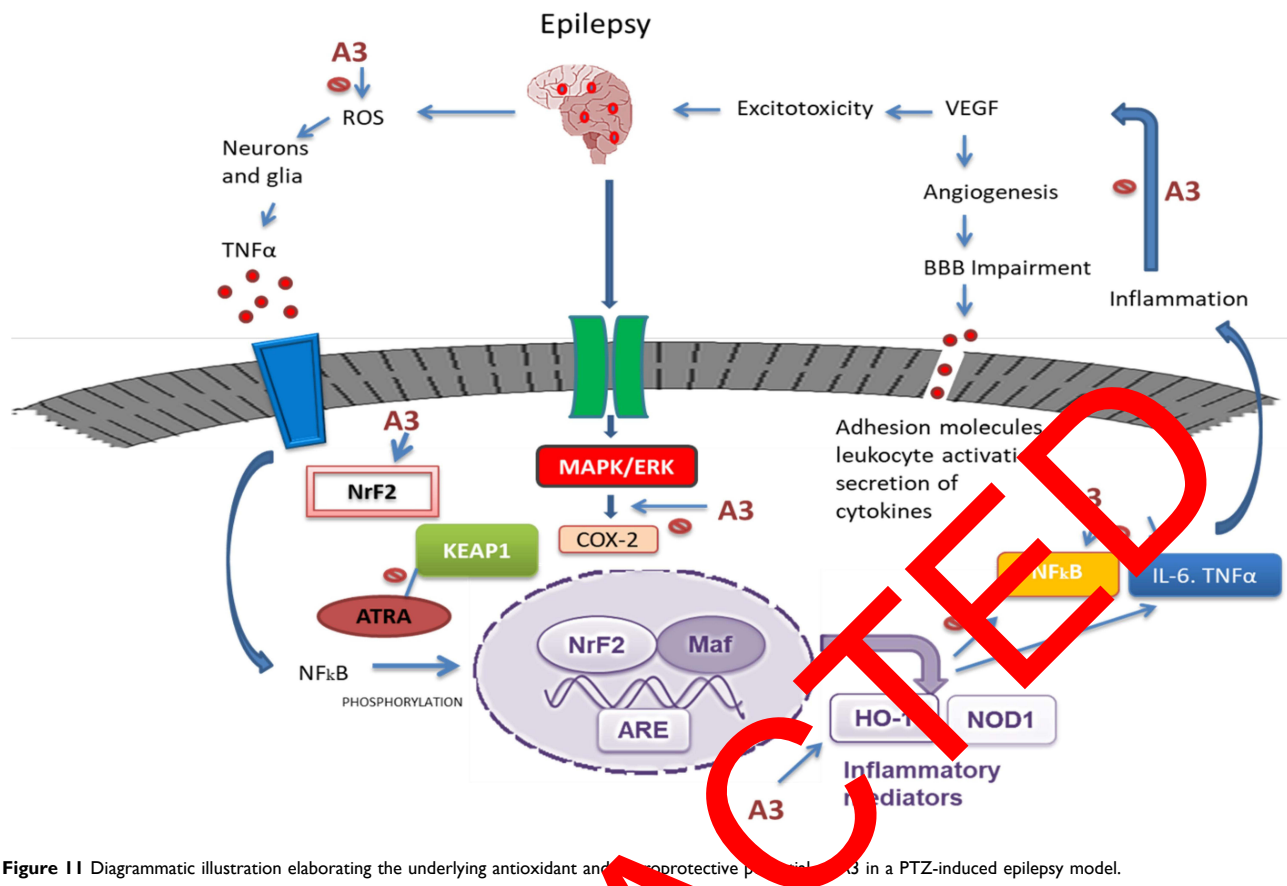


Figure 11 Diagrammatic illustration elaborating the underlying antioxidant and neuroprotective potential of A3 in a PTZ-induced epilepsy model.

angiopietins.⁹⁴ Proteins of the VEGF family possess potent vascular effects increasing the permeability of the BBB via the NO-synthase pathway⁹⁵ and inducing excessive angiogenesis as evident in patients with temporal lobe epilepsy.⁹⁶ Receptors for VEGF have been localized in neurons, vascular endothelium, and glia.⁹⁷ In response to seizures, VEGF is upregulated in the brain, however, recently, it has been implicated as an inflammatory mediator and thus contributes to the inflammatory response in epilepsy.⁹⁸ Additionally, our experimental data suggested an upregulated VEGF expression in the PTZ-group, which was mitigated after A3 administration, indicating an improved angiogenesis.

Conclusion

In conclusion, our study revealed that A3 could be a potent antioxidant and anti-inflammatory therapeutic candidate that can protect animals against PTZ-induced epilepsy. We also demonstrated that A3 has a relative safety profile since no impairment was found in the kidneys, heart, liver, or brain, which was supported by biochemical analysis. Furthermore, we found that the *Nrf2*-pathway is involved

in A3's neuroprotective action (Figure 11). Additionally, A3 can inhibit inflammatory mediators; however, further experimentation is still required to unveil the underlying mechanism in epileptic seizures.

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Disclosure

The authors report no conflicts of interest in this work.

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